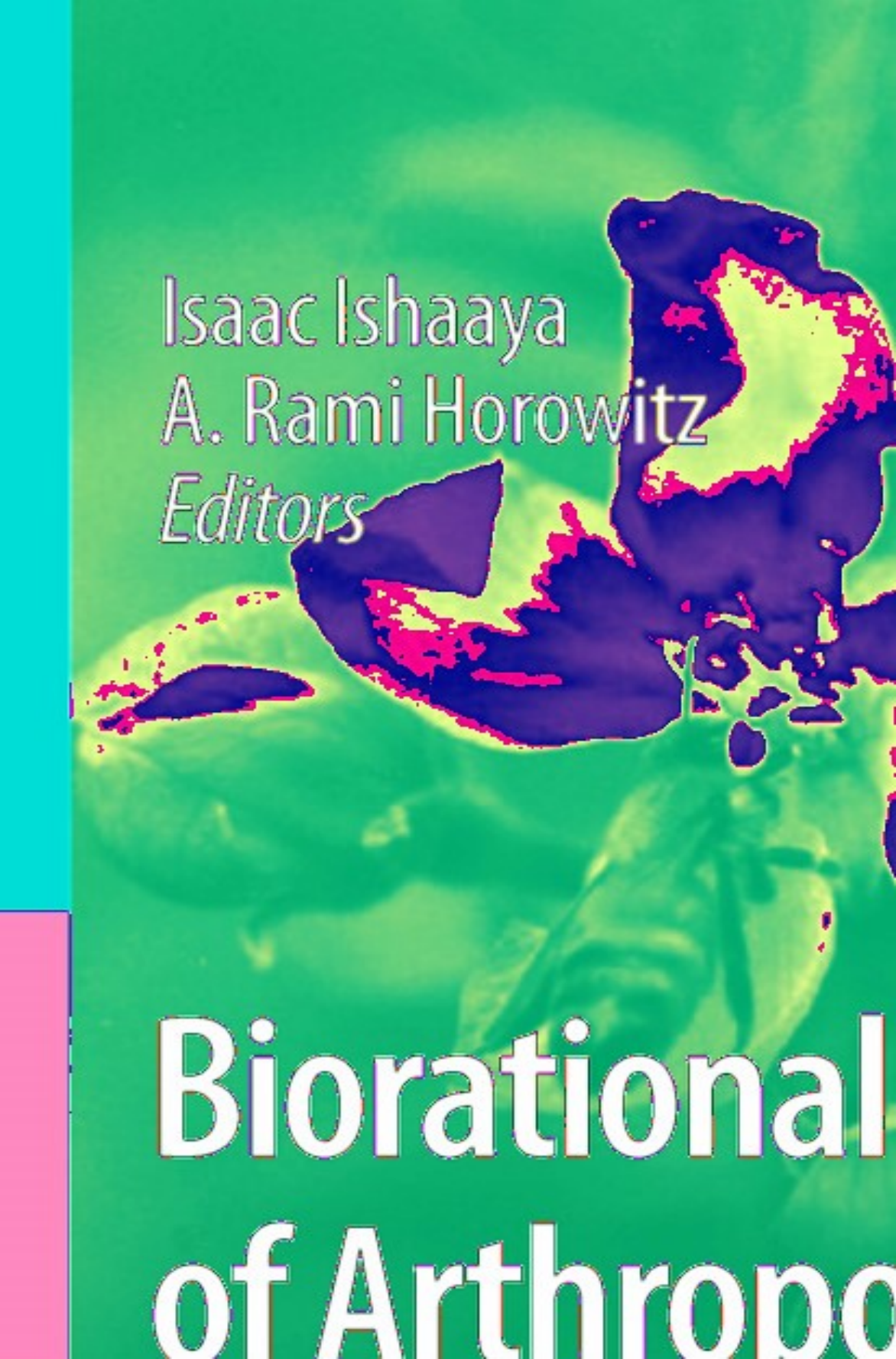
Isaac Ishaaya
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Editors
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Biorational Control of Arthropod Pests

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Editors

Biorational Control of Arthropod Pests

Application and Resistance Management

Springer

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Preface

For nearly 50 years, pest control was mostly based on broad-spectrum conventional insecticides such as organochlorines, organophosphates, carbamates and pyrethroids. However, the severe adverse effects of pesticides on the environment, problems of resistance reaching crisis proportions and public protests led to stricter regulations and legislation aimed at reducing their use. Ways to reduce the use of synthetic pesticides in plant protection and to use more alternative and novel methods for pest control or biorational control are the challenges of pest control for the twenty-first century.

The term biorational (biological + rational) pesticides can be defined as the use of specific and selective chemicals, often with a unique modes of action, that are compatible with natural enemies and the environment, with minimal effect on non-target organisms. Biorational control is based on a diversity of chemical, biological and physical approaches for controlling insect pests which results in minimum risk to man and the environment.

Among the highlights of this book are the use of selective control agents acting on specific biochemical sites such as neuropeptides, ecdysteroids and juvenile hormone analogs; GABA, ACh, ryanodine and octopamine receptors; pheromone and insect communication disruption along with plant constituents for selectively controlling arthropod pests. Novel biotechnology strategies that exploit genetically modified plants, insects, and symbionts for the management of insect pests and disease-borne vectors are presented. Furthermore, physical control techniques can serve as important tools to protect our crops from arthropod pests. Finally, countermeasures for resistance to biorational control agents using advanced biological and biochemical approaches are also discussed.

The authors of the various chapters are world expert in fields related to biorational control, have reviewed and collated the widespread literature in a concise and consolidated form. The book is intended to serve as a text for researchers, university professors, graduate students, agricultural organizations and chemical industries.

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Biorational Pest Control – An Overview

A. Rami Horowitz, Peter C. Ellsworth, and Isaac Ishaaya

1 Introduction

Fifty years ago, Stern et al. (1959) introduced the concept of "Integrated Control" during a time when insect pest control was mostly based on broad-spectrum, conventional insecticides such as organochlorines, organophosphates (OPs), and carbamates, all neurotoxic. Their work on economic thresholds and economic injury levels implemented within an ecological framework where chemical and biological controls could thrive together is the basis for the modern day Integrated Pest Management (IPM) concept. However, along the way, IPM's overdependence on these broad-spectrum insecticides led to criticism that IPM was nothing more than Integrated Pesticide Management (e.g. Ehler 2006). Severe adverse effects of pesticides on the environment, problems of resistance reaching crisis proportions, and public protests have driven demand for alternative pest control tactics. With advances in the development of biorational pesticides and other selective chemistries, there is now real opportunity to realize the "Integrated Control" concept that Stern and colleagues (1959) pioneered. Today, more than ever, tools of physiology, toxicology, and biotechnology can help us realize the vision of more holistically harmonizing biological and chemical controls.

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The US Federal definition of IPM underwent a recent paradigm shift towards "risk reduction" as a way of placing focus on the consequences and impacts of pests and pest management tactics rather than just pesticides, per se:

> Integrated Pest Management, or IPM, is a long-standing, science-based, decision-making process that identifies and reduces risks from pests and pest management related strategies. It coordinates the use of pest biology, environmental information, and available technology to prevent unacceptable levels of pest damage by the most economical means, while posing the least possible risk to people, property, resources, and the environment. IPM provides an effective strategy for managing pests in all arenas from developed agricultural, residential, and public areas to wild lands. IPM serves as an umbrella to provide an effective, all encompassing, low-risk approach to protect resources and people from pests. Anonymous (2004)

Reducing the risks associated with pest management tactics like pesticides in plant protection is the challenge of pest control for the twenty-first century. Biorational agents and approaches will be the key for elevating our IPM strategies to meet the societal challenges before us (Ishaaya 2003; Horowitz and Ishaaya 2004; Ishaaya et al. 2005).

This volume was written to address the demand for safer, environmentally friendly approaches to pest management, and to describe new strategies to reduce resistance problems. The biological control of pests is not dealt with, but rather control-methods that are compatible with beneficial organisms. This overview is based on the different chapters of this book, which deals with biorational approaches for insect (and mite) pest control. One such approach is based on disrupting the activity of specific biochemical sites such as neuropeptides, ecdysone and juvenile hormones, tyramine, octopamine and GABA receptors. Another is the use of natural products obtained from tropical plants and other biological systems for pest control. The use of semiochemicals and of insect signaling still needs to be improved and commercialized. Novel biotechnology control strategies ("the genetic approach") exploit genetically modified-plants, -insects, and -symbionts in the combat against insect pests and disease-borne vectors. Countermeasures for resistance to biorational control agents using advanced biological and biochemical approaches are also discussed.

2 The Term 'Biorational'

Precise use of terminology is very important in communicating concepts to fellow scientists, students of various pest management disciplines, and end-users of pest management technology. However, precise usage of the term "biorational" is difficult, mainly because of its inconsistent use through history. Before presenting an overview of this volume which is dedicated to biorational pest control, a short review of the underlying concepts that have given rise to a plethora of pest control terminology is needed and should be considered along with other thoughtful and authoritative treatments of this and related subject areas (e.g., Crump et al. 1999; Eilenberg et al. 2001; Ware and Whitacre 2004).

Classification systems for pesticides have been proposed along themes of chemical structure (e.g., carbamate, chlorinated hydrocarbon), mode of action, route of entry (e.g., feeding, contact), relative toxicity (e.g., to humans, mammals, vertebrates, fish, or invertebrates), source of origin (e.g., 'natural', botanical, analog, or synthetic), process (design model used) and functional role. It is no wonder that attempts to unify these competing and overlapping systems have often met with frustration. However, a continuing push has been made by scientific and public interests to incorporate additional terminology in the development of safer and more sustainable alternative pest control technologies (e.g., ATTRA-National Agricultural Information Service).

Because of their widespread impact on industry and consumers, two major entities, the Internet and US-EPA (2008), influence any discussion about pesticides and pest control technologies even when at odds with more technically precise or scientifically developed terminology. Popular sources associate the term "biorational" as being substances of microbial or plant origin, or having certain physical or contact routes of entry. Some erroneously view biorationals as fully interchangeable with organic pesticides as defined by USDA standards. Others identify biorationals with pesticides of natural origin or that are synthesized identically or as analogs to naturally occurring plant or insect chemicals.

US-EPA does not explicitly define or identify biorationals within their classification system. Ware and Whitacre (2004) attributed erroneously to EPA the view that biorationals are inherently different from conventional pesticides and have lower risks associated with their use. However, this view is what is ascribed by EPA to "biopesticides", a common but inappropriate synonym for the term biorational (for a review of biopesticides, see Rosell et al. 2008). EPA divides biopesticides, which are "derived from natural materials", into three groups: "microbials", plant-incorporated protectants (PIPs), and biochemicals. Microbial pesticides consist of a microorganism such as viruses, bacteria, fungi or protozoa. More broadly, microbial pesticides are generally considered a form of biological control, as they entail the usage of living organisms to induce mortality of target pests (Eilenberg et al. 2001). In addition, some microbials act as antagonists and may not kill the target outright and therefore are better referred to as biopestistats (Crump et al. 1999).

PIPs have come into public consciousness as products of genetic-engineering. EPA regulates the genetic material and its protein products in the plant. The scientific community is divided on how PIPs should be viewed, but many suggest this as a specialized case of host-plant resistance in pest management terminology. EPA's definition of "biochemical pesticides" is problematic as well under the description that they "…are naturally occurring substances that control pests by non-toxic mechanisms." In other words they interfere or otherwise alter pest behavior or function, but do not kill the pest outright. They include such things as pheromones and other semiochemicals. As such, these are not pesticides *sensu* Crump et al. (1999).

Crump and colleague's classification system (1999) is helpful in the organization of many pesticides. However, the major dichotomies in it are whether the substance is "biological" (or living) or "chemical", and then whether the substance kills the

target or not. While logical, this system is insufficient to accommodate the more subtle functional distinctions that are possible. Crump et al. (1999) also concluded that the diversity of definitions applied to "biorational" leads to confusion. They therefore concluded that the term was "inherently defective" and should be avoided. Yet, given its widespread usage and being the subject of this volume, a more concerted effort is needed to identify and define the concept of biorational and related terms.

A practical desire to avoid prior issues with chemical pesticides, i.e. environmental persistence, food safety, resistance, secondary pest outbreaks, and pest resurgences have deep roots in our pest management culture. Fifty years ago, Stern et al. (1959) dedicated a section in their landmark paper to terminology. They defined "*Selective insecticide*" as:

> An insecticide which while killing the pest individuals spares much or most of the other fauna, including beneficial species, either through differential toxic action or through the manner in which the insecticide is utilized (formulation, dosage, timing, etc.)

This extraordinary insight was based in recognition of six key drawbacks of insecticides: (1) arthropod resistance, (2) secondary pest outbreaks resulting from interference of the insecticide with biological control or through its effects on the plant (e.g., hormoligosis), (3) rapid resurgence of the primary target pest because of broad-spectrum decimation of beneficial arthropods and release from biological control, (4) toxic residues in food and forage crops, (5) hazards to insecticide handlers, and (6) litigation arising from all of the above. Today, we could add environmental contamination and regulatory costs associated with the above issues.

Stern et al. (1959) made two other critical observations. Population regulatory factors already work to "keep thousands of potentially harmful arthropod species permanently below economic thresholds," and their central thesis, "Chemical control should act as a complement to the biological control." They saw an opportunity where, with adequate understanding of the system, both methods could be made "to augment one another." Perhaps most prescient was their statement that our failure to recognize the complex ecology inherent to pest management, "leads to the error of imposing insecticides on the ecosystem, rather than fitting them into it." Of course, at the time, their idea about selective insecticides was in the context of a pest control system dominated by the broad-spectrum chlorinated hydrocarbon, DDT, and in the development of less persistent and less broad-spectrum organophosphate and carbamate alternatives.

Plimmer (1985) tied the term biorational to the process by which the control agent is designed and synthesized. He explained that biorational is "the exploitation of knowledge about plant or animal biochemistry in order to synthesize a new molecule designed to act at a particular site or to block a key step in a biochemical process." However, this process-oriented definition fails to explicitly address selectivity towards a target or safety to non-target organisms. Bowers (2000) took this process approach one step further to incorporate the idea of selectivity by stating, "However, future needs clearly command the development of selective, pest-suppressive methodologies that rationally perturb those discrete aspects of pest biology and behavior that have arisen from the divergent evolutionary changes that separate invertebrate and microorganism from human biology."

Definitions vary greatly, but others suggest that biorational agents should have limited or no affect on non-target organisms, including humans and their domestic plants and animals (i.e., least toxic, Diver and Hinman 2008). They are therefore generally narrower in spectrum, if not specific to phylum, class or even species of organisms. That being the case, the bio- prefix should not refer to source (as from a living system, or of 'natural' origin or makeup) so much as function (as compatible with living systems). Some define biorationals as biologicals or botanicals only. In this case, the former should be referred to as biocontrol agents and the latter may or may not be biorational depending on their degree of compatibility with living systems (e.g., rotenone or nicotine).

This limited or lack of adverse effects, while still controlling or suppressing the target pest, can be accomplished in any number of ways as outlined by Stern et al. (1959; *sensu* 'selective insecticides'). One way in particular has placed otherwise potentially broad biocides into the rubric of biorationals. Those who view a lack of environmental persistence as a key attribute have labeled various salts, soaps and oils as biorational. It is true that many of these materials have direct contact action only and as such act on a population instantaneously with little to no residual effects. However, most agents of this kind are in fact broadly biocidal and thus can be used in a manner not compatible with living systems. Thus, the term biorational should be used descriptively as an adjective denoting compatibility with living systems within specific contexts. That is to say an insecticide may in fact be alternatively biorational in one system and decidedly not so in another [e.g., the insect growth regulator (IGR), pyriproxyfen, targeting a whitefly, *Bemisia tabaci*, in Arizona cotton (Ellsworth and Martinez-Carrillo 2001; Naranjo et al. 2003, 2004) as compared with the same compound used against scale insects in South African citrus (Hattingh 1996)]. Taken one step further, ecorational is another term used in many respects synonymously with biorational. Again, the compatibility of the control agent with the system in which it is used appears to be the governing principle (Ware and Whitacre 2004; Antwi et al. 2007). This context will be important in appreciating the scientific advances made in the pest control industry and described in this volume.

Finally, a discussion of the term biorational would not be complete without reviewing its first usage in the scientific literature by Carl Djerassi (Djerassi et al. 1974), a pioneer in the development of the first birth control pill. The term was introduced in this paper in order to avoid confusion between chemical and biological control of insects. Here again, biorational has seemed to occupy an important rhetorical space where the two approaches are compatible. Djerassi et al. (1974) gave examples of biorationals as pheromones, insect hormones, and hormone antagonists. While no definition was proposed, they noted that many biorationals are species-specific, often active at low concentrations, generally not persistent or toxic, and innocuous to vertebrates. It is true that the origins of this term suggest that the agents do not kill outright. However, Djerassi's influence on the regulatory system of the time (US-EPA, which had only recently formed in 1970) likely led to this idea being captured and held by the term "biopesticide" as used today by the EPA (reviewed above). As a result, biorational has now come to include agents that either impact pest populations more subtly and indirectly as well as those that have

direct toxic action on their targets, but with great specificity. Either way, both Stern et al. (1959) and Djerassi et al. (1974) saw the importance of and greater need for education of the user of selective technologies (e.g., see Ellsworth and Martinez-Carrillo 2001).

Unfortunately, US-EPA has not clarified the issue of biorational pesticides. So they do not neatly fit within their regulatory framework. In 1997, the Food Quality and Protection Act brought forward new pesticide designations. "Reduced-risk" status is given to those candidate pesticides that do one or more of the following: reduce pesticide risks to human health, non-target organisms, or environmental resources, or help make IPM more effective (Uri 1998). Operationally, a biorational agent should be reduced-risk, but many potential reduced-risk pesticides may not be viewed as biorational. Without the weight of the US-EPA, it will be more difficult for a stable definition of biorational to gain traction in the literature. However, for this volume, we propose that the term be used in a bipartite manner to (1) describe substances or processes that when applied in a specific system or ecological context have little or no adverse consequence for the environment and non-target organisms, but (2) cause lethal or other suppressive or behavior modifying action on a target organism and augment the control system. Regardless of origin, these agents might be developed from natural or synthetic models, and generally exploit the evolutionary divergence of physiological systems in the target organism from non-target species including humans. If properly designed and deployed, a biorational agent should be nearly fully compatible with biological controls as envisioned for 'selective insecticides' by Stern et al. (1959) (also see Stansly et al. 1996).

3 Crop Protection Targeting Specific Biochemical Sites in Insect Pests

Various biochemical specific-sites of insects have been suggested as targets for developing pest control agents. By disrupting the activity of these sites, important processes could be affected resulting in pest mortality. Some examples of such methods using insect's target sites have been summarized in other books (Ishaaya and Horowitz 1998; Ishaaya 2001; Horowitz and Ishaaya 2004). In this volume, we focus on specific targets such as insect neuropeptides, tyramine and octopamine receptors, GABA receptors, and on insect hormones such as ecdysone and juvenile hormones (analogs).

Insect neuropeptides have potential in the development of novel insecticides, because they are involved in many primary physiological and behavioral processes of insects (Gäde 1997; Altstein et al. 2000; Altstein and Hariton, Nachman – this volume). Insect neuropeptides are considered less toxic than small organic molecules and many of them are insect-specific. Two major limitations have delayed their use in pest management: (1) the linear nature of these peptides results in high susceptibility to proteolytic degradation and difficulties in penetrating biological tissues, and (2) it is difficult to design insect-neuropeptide antagonists because of their flexible 3D structures (Altstein et al. 2000). Altstein and Hariton (this volume)

summarize a strategy they have developed in their laboratory to overcome these limitations. The strategy, named INAI (Insect Neuropeptides Antagonist Insecticide), was applied to the insect PK/PBAN (Pyrokinin/Pheromone Biosynthesis Activating Neuropeptide) family. After they discovered a lead antagonist of PK/PBAN, they designed backbone cyclic (BBC) peptides that showed antagonistic activity; and thereafter, they improved the selectivity and stability of these antagonists. Following injection of these antagonists into *Helicoverpa peltigera* females, up to 70% inhibition of sex pheromone biosynthesis was achieved and treatment of *Spodoptera littoralis* larvae resulted in almost a full inhibition of cuticular melanization. These findings can be used for further development of other and more effective PK/PBAN antagonists-based insect control agents.

Another study (Hirashima, this volume) also suggests inhibiting pheromone production using agonists for the tyramine receptor. Arylaldehyde semicarbazones and 5-aryloxazoles highly inhibited *in vitro* pheromone production and calling behavior in the Indianmeal moth *Plodia interpunctella*. The author assumed that the targets of tyramine are the pheromone glands, resulting in a decline of PBAN concentration in the insect's haemolymph. Besides tyramine, other biogenic amines such as dopamine and octopamine, which widely exist in the central nervous system of insects (Evans 1980), might be involved in sex-pheromone production in connection with mating stimuli and mechanical stressors (Hirashima et al. 2007). However, the potential of these agonists in pest control are not yet achieved.

Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both vertebrates' and invertebrates' central nervous systems (Roberts et al. 1976). GABA is released from the presynaptic neuron and moves across the synaptic cleft. It creates an electrical signal in the postsynaptic neuron by binding to its specific receptors. In their chapter on GABA receptors, Ozoe et al. (this volume) focus on the various GABA receptor ligands, their toxicity to insects and nematodes, their binding potencies and the effect of their agonists/antagonists on the receptors. Although GABA receptors have been considered an excellent source for developing specific insecticides, so far only two relatively new phenylpyrazole insecticides, fipronil and ethiprole (Caboni et al. 2003), have been discovered following the ban on use of organochlorine insecticides in use hitherto (dieldrin, and related organochlorine insecticides that are blocked the GABA-gated chloride channels were banned because of their persistence in the environment). However, a number of noncompetitive antagonists binding to GABA receptors and exhibiting insecticidal activity have been discovered. Similar to fipronil, they exhibited selectivity for insect vs. mammalian GABA receptors. This would enable the design of selective noncompetitive antagonists of GABA receptors, and due to the fact that they have ligand-multiple sites, various other sites could also be used to develop the third generation of safe and insect-specific insecticides targeting GABA receptors (Ozoe et al., this volume).

Ecdysteroids (20E) and juvenile hormones (JHs) regulate most of the physiological and biochemical processes during the insect's life cycle. Ecdysteroids affect and coordinate molting and metamorphosis processes, and JHs are responsible for the character of juvenile stages and for adult reproduction. Novel insecticides, which mimic 20E and JH action have been developed during the past three decades

(reviewed by Dhadialla et al. 1998) such as ecdysone agonists, JH analogs (JHAs) and JH mimics, e.g., methoprene, fenoxycarb and pyriproxyfen. In insects, metamorphosis is induced by ecdysteroid hormones in the absence of JHs. Therefore, persistence of JH or JHA during that time results in abnormal larvae or nymphs that are unable to develop to normal adults. Although some of the JHAs and JH mimics have been considered effective insecticides against various agricultural and medical pests, resistance to these agents, for example to pyriproxyfen in the whitefly *B. tabaci*, has developed a short time after its introduction into crop protection (Horowitz and Ishaaya 1994). The biological activities of JH have been well studied along with its molecular modes of action, especially in recent years. Palli (this volume) describes the genes and proteins involved in JH activity, mode of action of JH and JHA and their interaction with ecdysteroids. Finally he shows a model describing the possible molecular modes of action of JHs and JHA.

As an example, the gene '*Cfjhe*' (*Choristoneura fumiferana* [Spruce Budworm] Juvenile Hormone Esterase) is among those whose expression is regulated by both JH and 20E. This gene is induced by JH and its expression is suppressed by 20E. Expression of *Cfjhe* was found in four other insect species: *Trichoplusia ni*, *Heliothis virescens*, *Tribolium castaneum* and *Drosophila melanogaster* (Palli, this volume). Many other genes induced by JH were identified in *D. melanogaster*, which is an insect-model used for comprehensive study on molecular modes of action in insects (e.g. Dubrovsky et al. 2002; Li et al. 2007). A methoprene-tolerant gene (*met*) was also identified in this insect and this is the only example of target site resistance for JHAs (Wilson and Fabian 1986). Further research studies have shown the importance of *met* proteins in JH regulation along with its homologues in other insects, such as in *T. castaneum* (Parthasarathy et al. 2008).

Other studies suggested that JH and JHA, which antagonize 20E, are involved in specific processes in insects such as enhancing expression of immune protein genes, affecting programmed cell death of larval cells, and also connecting with the phosphorylation process. The proteins involved in their action interact and mediate cross talk between these hormones. Palli (this volume) concludes that although several proteins are significantly involved in JH signal transduction, the mechanism is unknown. Some possible modes of action were suggested, following the completion of whole genome sequencing. Available novel techniques make the insect *T. castaneum* an excellent model for further studies on JH action.

4 Exploitation of Plant Natural Products as a Source of Environmentally-Friendly Pesticides

Isman and Akhtar (2007) have reviewed various plant natural products with insecticidal activities. These natural substances are part of defensive chemistry that helps to defend plants from herbivores and pathogens. As they are naturally-occurring chemicals, the exploitation of such products may be useful for developing ecologically sound pesticides. Although there are several natural products that have been

developed as insecticides, there are numerous compounds that affect insects by interfering in their physiological processes. Azadirachtin (the major bioactive chemical in Neem) is an important natural product, which acts on insects in a manner similar to IGRs. However, to gain acceptable control one needs multiple applications. Its high cost and fast degradation render this product economically unfeasible (Isman 2004). Other products that have been reviewed (Isman and Akhtar 2007) are derivatives such as acetogenins from Annonaceae, alkaloids from Stemonaceae, napthoquinones from the Scrophulariaceae, rocaglamides from Aglaia, and monoterpenoids from plant essential oils. Although many compounds of plant origin have been found fully or partly effective as potential insecticides, Isman and Akhtar (2007) concluded that these natural compounds should be used as the starting point for the development of synthetic insecticides, as was done in the successful development of the pyrethroids from natural pyrethrins.

In this volume, Vandenborre et al. review the potential for insecticides based on plant lectins. Plant lectins are carbohydrate-binding proteins that are involved in plant defense and found in many plant species (Peumans and Van Damme 1995). Lectins incorporated in the diet or cloned in transgenic plants have shown some insecticidal activity to various insect orders (Van Damme 2008), especially Lepidoptera, Coleoptera, Diptera and Hemiptera. The main site of action for these compounds is the insect digestive system. The GNA- (*Galanthus nivalis*, the common snowdrop) related family is one of the major families that have been tested. An unclear point with regard to lectins is their impact on beneficial arthropods such as parasitoids, predators and pollinators. Many research studies, which incorporated lectins either in diets or in transgenic plants, have shown that low (and even high) doses of GNA had no effect on beneficial insects. However, others did show adverse effects on bumblebees, solitary bees and lady beetles, and also on some parasitoids, especially at high doses (Birch et al. 1999; Romeis et al. 2003; Babendreier et al. 2007). Vandenborre et al. (this volume) concluded that although the lectins apparently have minor impact on beneficial insects there is still a great potential to exploit them as expressed in transgenic plants, especially targeting hemipteran pests, which are insensitive towards Bt-transgenic crops.

5 Utilization of Semiochemicals (Pheromones) and Other Insect Communication Signals for Controlling Insect Pests

El-Sayed (2008) defined pheromones and semiochemicals as "…signaling chemicals that organisms can detect in its environment, which may modify its behavior or its physiology". Semiochemicals are considered safe and environmentally friendly substances because they are natural, generally volatile chemicals and are specific to the target insect without leaving any residues. The term pheromone was suggested by Karlson and Lüscher in a letter to "Nature" (1959). They wrote: "…various active substances which, though they resemble hormones in some respects,

cannot be included among them…Unlike hormones, however, the substance is not secreted into the blood but outside the body… (and it serves as) communication between individuals". Since then, pheromone identification has triggered their use in pest control. However, difficulties in practical application resulted in some loss of interest in their use. Cokl and Millar (this volume), in their comprehensive review on utilization of insect signaling for monitoring and controlling of insect pests, believe that these signals should be exploited for insect-pest management. In their chapter, they focus on two types of insect signals, chemical and mechanical. The chemical signal includes mainly sex pheromones, which mediate behavioral communication between the sexes, and aggregation pheromones that are produced to attract conspecific individuals to a host resource (a host tree – as is found in various bark beetles) or attract both sexes for reproduction. Other types of pheromones are used as alarm, trail (especially in social insects), and marking (territory or host).

5.1 Ways for Exploiting Pheromones

Pheromones have been exploited in several ways:

1. Monitoring of insect populations using "pheromone traps" for species such as the gypsy moth, the boll weevil, the pink bollworm (PBW) and the med fly. In general, sex pheromones that attract insect males have been used as a practical monitoring tool for early detection of local or invasive pests (Cokl and Millar, this volume).
2. Mating disruption is a common and successful use of sex pheromone technology (reviewed by Cardé 2007). The idea is to saturate an area-wide crop with synthetic insect pheromone, which interferes with the sexual communication of a specific insect pest. Among the main target pests have been the gypsy moth, codling moth, grapevine moths and pink bollworm. The mating disruption method was an important IPM tactic for controlling the pink bollworm in the Southwest of the USA before the era of Bt-cotton, after which its use has been reduced dramatically. A new eradication effort to control this pest was the use of the mating disruption method in conjunction with other strategies: (a) extensive survey; (b) transgenic Bt cotton; (c) pheromone application for mating disruption; and, (d) sterile PBW moth releases. Adoption of Bt cotton in Arizona exceeds 98% of cotton acres, with mating disruption concentrated on the remaining 2% of the area. So far, this eradication action seems to be highly successful (Grefenstette et al. 2008).
3. Mass trapping of insect pests is technically feasible but its cost and the extensive labor required has limited its use. However, pheromone-based mass trapping is still a major component of IPM programs for tropical fruits in many tropical and subtropical regions (Cokl and Millar, this volume).
4. "Attract and kill", a method combining the use of pheromone as attractant and an insecticide as a toxicant, is an effective method to control the pink bollworm, the codling moth, fruit flies and the boll weevil (Butler and Las 1983; Lösel et al. 2000; Bostanian and Racette 2001; Spurgeon 2001).

Mealybugs are pests of worldwide importance that could potentially be managed through pheromone and other environmentally friendly technologies as suggested by Franco et al. (this volume). Mealybugs are severe sap-sucking pests attacking many agricultural crops such as citrus, apple, vineyards, persimmon and other subtropical fruit trees, coffee and fresh herbs. After extensively reviewing the biological characteristics of mealybugs, their host plants and natural enemies, these authors propose reducing mealybug males to prevent mating during periods when they are at low level. In practice, one can use mating disruption; however, there are still some difficulties in synthesizing mealybug sex pheromones. For example, the synthesis and therefore use of the *Planococcus citri* homolog is easier and cheaper than the pheromone (A. Zada et al. unpublished data, 2008). 'Attract and kill' using pheromone bates and contact poisons was mentioned as an additional means to annihilate mealybug males. This approach should be accompanied by other tactics such as monitoring, detecting and chemically treating mealybug hotspots and detection and augmentation of natural enemies (Franco et al., this volume).

5.2 Insect's Mechanical Signals

Cokl and Millar (this volume) describe a possible exploitation of mechanical signals in controlling insect pest. These signals are used by arthropods for communication and can be defined as contact (tactile) signals, airborne sound, waterborne sound or substrate-borne sound. Their model for sound communication is the stink bug, *Nezara viridula*. Polajnar and Cokl (2008) have shown that it is feasible to disrupt the vibrational sexual communication in stink bugs by using artificial signals. These experiments need to be verified under field conditions, with the major obstacle of this method being how to vibrate large numbers of plants outdoors. Notwithstanding, the use of such artificial signals to disrupt acoustic communication is intriguing.

6 Biotechnology Manipulations (Genetic Approach) as Novel Strategies Against Arthropod Pests

The application of biotechnology for crop protection commenced on a large scale in 1996, and had increased 67-fold by 2007, reaching more than 114 million hectares worldwide; making it the fastest adopted crop technology in recent history (James 2008).

In 1987, Vaeck et al. published their classic article regarding transgenic tobacco plants expressing genes of Bt, which protect them from insect damage. Since then, Bt crops have become a great success of applied biotechnology in agriculture, especially to control lepidopteron pests in cotton and maize. So far, most of the genetically modified crops, which exhibit insecticidal activity, consist mainly of

the toxin Cry1Ac in transgenic cotton and Cry1Ab in transgenic corn (Tabashnik and Carriere 2009). In 1996, Bt crops were first commercially planted reaching 42 million hectares during 2007, and this technology has reduced substantially pesticide applications (Brookes and Barfoot 2008; James 2008).

As not all pests are susceptible to Bt toxins (e.g. sucking pests and stored product pests) solutions other than Bt should be developed, such as plant defensive proteins (see Vandenborre et al., this volume), secondary metabolic compounds and RNA interference (RNAi) (Gatehouse 2008).

In this volume, Alphey et al. have reviewed recent advances in biotechnology for crop protection and insect suppression. These include the use of transgenic insects with conditional lethal genes, symbiotic control of insect pests and disease vectors, and other use of symbionts for controlling pest populations (see also Darby, this volume).

A potential pest suppression approach involves modification of insects by engineering conditional lethal genes. This method is intended to replace irradiation as it is currently used for SIT (sterile insect technique). The lethal gene accumulates in a population and, over several generations, causes a decrease in population size (Alphey 2007). In support of the PBW eradication program that is underway in the Southwestern USA, an engineered strain of PBW was developed with a dominant lethal mutation (RIDL; though not yet implemented for PBW control). 'Genetic sterilization' of progeny results from the mating of wild types with RIDL insects, eventually leading to widespread pest suppression. Release of this strain under laboratory conditions resulted in 60% to 92% larval mortality. This method can be used for replacing the radiation-based sterilization process or as a supplement to the classic SIT (Simmons et al. 2007). RIDL technology also has been successfully introduced into other major pest species (Alphey et al. this volume).

Other advances in biotechnology of insects provide a new strategy for controlling insect borne pathogen transmission of human diseases and also vectors of plant pathogens. One application of this strategy that was tested in mosquitoes under laboratory conditions is the modification of genes that suppress pathogens' development (e.g., *Plasmodium* sp.) (Alphey et al. 2002; Abraham et al. 2007). One problem is how to efficiently spread these genes (the 'effector' or 'refractory' genes) in the field by an additional gene-driven system. Although there are considerable technical and regulatory problems to using this strategy, it has the potential to be very promising with major positive health consequences for the human population (Alphey et al. this volume).

A similar strategy is "paratransgenesis" or symbiotic control, also studied in mosquito-borne pathogen systems. Paratransgenesis is a genetic manipulation of the insect's symbiotic bacteria. The idea is to alter the disease vector's ability to transmit a pathogen (Beard et al. 2002; Riehle et al. 2003; Miller et al. 2007; Ren et al. 2008). One critical step to success with this strategy is to select an appropriate candidate symbiont, a major stumbling block for this research (Ren et al. 2008). Symbiotic control is a biologically-based method and may be the least disruptive to the ecosystem. However, it is still subject to severe regulatory inspection (Miller et al. 2007). This strategy has been adopted for controlling the Pierce's disease – a serious disease of grapevines (Miller et al. 2007). In this study the researchers

manipulated the symbiont *Alcaligenes xylosoxidans* var. *denitrificans* that occupies the same niche at the pathogen, *Xylella fastidiosa*, causing Pierce's disease is transmitted by the glassy-winged sharpshooter (*Homalodisca vitripennis*).

The bacterium, *Wolbachia pipientis* ('Wolbachia') is a widespread and abundant secondary cytoplasmic-symbiont of arthropods and nematodes (Stouthamer et al. 1999). *Wolbachia* spp. are involved to a great extent in manipulation of its host's reproductive properties including the induction of parthenogenesis, feminization, male killing and cytoplasmic incompatibility (CI). CI most associated with Wolbachia infection is a biological phenomenon causing a decrease in population numbers of those not carrying the specific cytoplasmic factors. There are many suggested ways that Wolbachia can be used to control insect pests (e.g. Ioannidis and Bourtzis 2007; Alphey et al. this volume). For example, Wolbachia-induced CI has been used for population suppression of the medfly, *Ceratitis capitata* (Zabalou et al. 2004). This technique is similar to SIT and is termed 'IIT' ("Incompatible Insect Technique"). In IIT, the males released into the field are infected with a Wolbachia strain and the target population is either uninfected or infected with an incompatible strain, hence, it results in a target-population decrease. Another suggested application is to spread desired genotypes in pest populations by using the driving ability of the CI phenotype (Ioannidis and Bourtzis 2007); in the same way, the virulent strain of Wolbachia can modify age structure of disease-borne vector populations resulting in suppression of disease transmission (see also Alphey et al., and Darby this volume).

All the above illustrate the importance of symbiotic control and its potential in pest and disease management.

7 Pesticide Resistance and Management Strategies

Pesticide resistance in arthropod pests, insects and mites, is a seriously increasing phenomenon in crop protection. Based on the Arthropod Pesticide Resistance Database (APRD), more than 550 species of arthropods have developed resistance to pesticides (Whalon et al. 2008). Pesticide resistance causes the disuse of effective pesticides and diminishes the pesticide diversity that is one of the basic principles in pest resistance management.

Managing resistance requires a practical solution to problems resulting from the adaptability of the insect pests to the used insecticides. In general, pests most prone to developing resistance are those with primary targets of numerous insecticide treatments, because they often are most abundant and damaging (Denholm et al. 1998). The whitefly, *B. tabaci*, and the two-spotted spider mite, *Tetranychus urticae* exemplify this phenomenon of rapid evolution of pesticide resistance. Through their resistances, these pests cause a decline in the number of effective pesticides and place new insecticides under severe threat of overuse.

Insecticide resistance management (IRM) is an important part of any IPM program in crops where pesticides are used frequently (Wise and Whalon, this

volume). The main purposes of IRM are: rational use of pesticides, reducing the number of treatments, combating resistance to available and effective insecticides, prolonging the life span of new insecticides by optimizing their use, and preserving natural enemies (Horowitz et al. 1995); all of the above goals are in high accordance with most IPM programs, though compatibility should not always be assumed (e.g., see Crowder et al. 2008).

Resistance management is based in resistance prevention, a positive outcome for IPM; however, resistance management goals are not always entirely consistent with IPM goals. The two concepts can ostensibly be at odds. IPM depends on pest prevention and maintenance of pest levels below economic levels. Resistance management, on the other hand, depends on a certain abundance and mobility of susceptible insects in a system. Occasionally, the densities of pests needed to secure resistance management are above economic thresholds established under IPM. Or, the assumptions for insect movement by resistance management models (to facilitate mating of susceptible and resistant individuals) are counter to tactics of isolation or strategic crop placement implemented for IPM. In another example, resistance models that optimize resistance evolution towards one technology by limiting its use place greater resistance pressure on other classes of chemistry, which are used alternatively and potentially multiple times. When this occurs, growers may fail to reach the economic goals stipulated by their IPM program. Few resistance studies have attempted to reconcile these issues; however, feedback of risks for future resistances should be incorporated into the IPM framework, most likely through adjusted economic thresholds (e.g. Caprio 1998; Hurley et al. 2002; Onstad et al. 2003; Crowder et al. 2006, 2008).

Knowledge of the target pest's biological characteristics, and the genetics and mechanisms of pesticide resistance in the pest is essential in pest resistance management programs. However, despite our rapidly increasing knowledge of the biochemical and molecular nature of resistance, it seems that management strategies still rely largely on common sense and practical tactics even though different resistance mechanisms may be present (Horowitz and Denholm 2001). Common sense is grounded in practical resistance management truisms: (1) *limit insecticide use to the lowest practical level*; (2) *diversify insecticide use patterns; and* (3) *partition insecticides among crops and pests such that modes of action are segregated as much as is practically possible* (e.g., see Ellsworth and Diehl 1998).

Van Leeuwen et al. (this volume) thoroughly reviewed the extent and mechanisms of resistance in the two-spotted mite, *T. urticae*, one of the most serious pests of glasshouses worldwide. Although biological control of *T. urticae* using predatory phytoseid mites has been implemented with success, chemical control by acaricide applications is still essential in both glasshouses and outdoors. This pest is extremely prone to developing rapid resistance and cross-resistance to many acaricides that have been used against it; however, in depth studies on mechanisms of acaricide resistance are scanty.

Resistance to OPs, the first chemical group used to control *T. urticae,* has occurred a short time after their introduction in the USA and Europe, with the main mechanism being reduced sensitivity of the enzyme acetylcholinesterase (AChE).

Resistance in this pest to pyrethroids, another important group of insecticides, is also widespread and the suggested main resistance mechanisms are metabolic, either enzymatic hydrolysis by carboxylesterases or oxidation by microsomal monooxygenases (e.g. Van Leeuwen et al. 2007). The pyrethroid target site is the voltage-gated sodium channel in nerve cells. Target site insensitivity to bifenthrin (a pyrethroid that has been used often against *T. urticae*) has been investigated at the molecular level (A. Tsagkarakou et al., unpublished data, 2009) and this study proved that target site insensitivity in *T. urticae* is also involved in resistance to pyrethroids.

Since the 1990s, a group of acaricides with similar modes of action have been introduced and classified as mitochondrial electron transport inhibitors (METI), inhibiting complex I of the respiratory chain (e.g. Hollingworth et al. 1994). Resistance to the METI group in *T. urticae* has been detected since the mid 1990s, and cross-resistance among different acaricides of this group has been observed worldwide (Van Leeuwen et al., this volume). The mechanisms of resistance to METI pesticides include mainly oxidative detoxification (metabolic). Based on molecular studies, it is also suggested that single point mutations that affect the binding of METI acaricides to complex I cause target site resistance in *T. urticae* (Van Pottelberge et al. 2008).

An interesting approach to integrated pest and resistance management in tree fruit orchards is described by Wise and Whalon (this volume). Following the introduction of new insecticides that replaced the conventional broad spectrum insecticides, they posit that a total switch to the new insecticides and abandoning the conventional ones is risky and can result in field control failures. Hence, they recommend a return to a 'system approach' in IPM "…to meet the demands of global markets, economics, and human and environmental safety expectations".

The first task in a system approach is to define its components such as crop area, pests and beneficial-insect populations, and the production factors of the growers. The next step is to clarify the interactions between each component. The authors demonstrated their system approach through a simple model called the 'PIC (Plant-Insect-Chemical) Triad', and noted that we must consider all the inherent interactions to create a powerful data-based system designed to optimize insecticide performance and other components relevant to IPM. When new insecticides are introduced, significant ecosystem impacts may occur, because the whole pest complex is generally not controlled. Thus, the loss of broad spectrum insecticides engenders the introduction of several new insecticides. In their field experiments, Wise and Whalon (this volume) examined the levels of natural enemies in cherry orchards, in contrasted regimes of conventional and new insecticides. Surprisingly, beneficial levels are significantly reduced following applications of new insecticides ("reduced risk"). Notwithstanding, their finding is in contrast with other research studies that were conducted in various cotton fields (e.g. Naranjo et al. 2003, 2004; Mansfield et al. 2006). Wise and Whalon (this volume) conclude that the effect of both types of insecticides should be examined under field conditions with a consideration of the whole ecosystem. Here again, determination of a biorational pest control approach is system-specific and context-sensitive. A broad-based IPM

program (including IRM) should use suitable monitoring tools to identify the contribution of natural enemies as they play an important role in pest and resistance management, in addition to the study on the impact of the insecticides used on mortality and resistance in key pests (*sensu* 'biorational' and life table analyses; Ellsworth and Martinez-Carrillo 2001; Naranjo 2001; Naranjo and Ellsworth 2005).

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Agonists/Antagonists of the Insect Kinin and Pyrokinin/PBAN Neuropeptide Classes as Tools for Rational Pest Control

Ronald J. Nachman

1 Introduction

While insect neuropeptides of the insect kinin (IK) and pyrokinin/PBAN (PK/PBAN) family are both potent and specific, these molecular messengers are not suitably designed to be effective either as pest insect control agents and/or tools for insect neuroendocrinologists. Neuropeptides are rapidly degraded by peptidases in the hemolymph and tissues within insects and generally exhibit poor bioavailability (Nachman et al. 2001, 2002a, b). The development of potent agonists and antagonists with enhanced biostability and bioavailability can overcome these limitations and can represent a key step in the development of pest management techniques based on neuropeptide analogs capable of disrupting critical life processes regulated by the IK and PK/PBAN families. In two separate sub-sections, a review is presented on what is known about chemical, conformational, and stereochemical aspects of the interaction of the IK and PK/PBAN families with their putative receptors, and how this knowledge can be harnessed to design and develop biostable mimetic analogs that retain an ability to bind, and potentially activate, those receptors. Strategies for the modification of the PK/PBAN neuropeptides to enhance bioavailability characteristics are also discussed and should be applicable to other insect neuropeptide classes.

2 Insect Kinin Neuropeptide Family

Insect neuropeptides of the insect kinin (IK) class share a common C-terminal pentapeptide sequence Phe_1-Xaa_2-Xaa_{23}-Trp_4-Gly_5-NH_2 (Xaa_2 = His, Asn, Phe, Ser or Tyr; Xaa_3 = Pro, Ser or Ala). They have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera, Diptera and Orthoptera. The first members of this insect neuropeptide family were isolated on the basis of their

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ability to stimulate contractions of the isolated cockroach hindgut (Holman et al. 1987, 2002a; Coast 1998), but they are also potent diuretic peptides that stimulate the secretion of primary urine by Malpighian tubules, organs involved in the regulation of salt and water balance (Nachman et al. 1990, 2002a; Coast et al. 1990, 1998). In addition, the IK have been implicated in the regulation of digestive enzyme release (Sajjaya et al. 2001; 2002 Harshini et al., 2003). IK, and/or analogs, have also been reported to inhibit weight gain by larvae of the tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) (Seinsche et al., 2000; Nachman et al., 2002a,2003), both important agricultural pests.

2.1 Chemical, Conformational and Stereochemical Aspects of IK/Receptor Interaction

Myotropic and diuretic assays of tissues in vitro, as well as assays using stably expressed IK receptors, show that the full biological activity of the IK resides in the C-terminal pentapeptide, which is the active core (Nachman et al. 1991, 1993, 2003), with the exceptions of the housefly Malpighian tubule fluid secretion assay (Coast 2001) and an expressed IK receptor from the mosquito *Aedes aegypti*, where the C-terminal pentapeptide core is less potent than native peptides by several orders of magnitude. Diuretic, myotropic, and/or receptor-interaction activity in these assays is completely lost when the C-terminal amide of the insect kinins is replaced with a negatively charged acid moiety (Nachman et al. 1995). Within the core pentapeptide, the aromatic residues Phe_1 and Trp_4 are the most important for activity whereas a wide range of variability is generally tolerated at position 2, from acidic to basic residues and from hydrophilic to hydrophobic (Nachman et al. 1991, 1993b). The expressed IK receptor from the mosquito *A. aegypti* represents a particular exception, as it clearly prefers an aromatic residue in position 2, which is consistent with the presence of aromatic residues in position 2 of all three of the native aedeskinins (Taneja-Bageshwar et al. 2006).

NMR spectroscopic data and molecular dynamics calculations on an active head-to-tail, cyclic analog (*cyclo*[AFFPWG]) reveal the presence of two major turn types within the active core region of the IK (Roberts et al. 1997). The more rigid of the two conformations featured a *cis*Pro in the third position of a type-VI β-turn over core residues 1–4, or Phe-Phe-**Pro-**Trp. ROESY spectra supported a well-defined Cβ-*exo*/Cγ-*endo* pucker for the *cis*Pro ring that was observed in unrestrained molecular dynamics for this cyclic analog. The other less rigid turn system involved a *trans*Pro and encompassed residues 2–5, or Phe-**Pro**-Trp-Gly (Roberts et al. 1997).

In an effort to provide definitive evidence that the most populous *cis*Pro type VI β-turn over residues Phe1 through Trp4 represented the 'active conformation' for receptor interaction, IK analogs incorporating restricted conformation components that preferentially mimic a *cis* peptide bond and a type VI β-turn were synthesized and evaluated. NMR studies with IK analogs incorporating either the tetrazole or 4-aminopyroglutamate (APy), moieties that mimic one turn over the other, indicate

a predominant population of a β-turn involving the Phe_1 to Trp_4 region (Nachman et al. 2002b, 2004). These restricted conformation analogs in the natural L,L (or S,S) configuration demonstrate significant retention of both diuretic activity in the cricket Malpighian tubule fluid secretion assay and interact with the expressed IK receptor from the tick *B. microplus* (Taneja-Bageshwar et al. 2008a).

In a more detailed investigation, all four stereochemical variants of tetrazole and APy moieties were incorporated into the C-terminal region of the insect kinin sequence and evaluated in a cricket Malpighian tubule fluid secretion assay and an expressed IK receptor from the tick *Boophilus microplus*. The optimal stereochemistry for the two turn mimic moieties in both the cricket Malpighian tubule fluid secretion assay and expressed tick receptor for agonist activity was identified as (D,L) and (2R,4S)(Taneja-Bageshwar et al. 2008a), respectively; with APy analog Ac-RF[APy]WGa (*R,S*) demonstrating an EC_{50} of 7 nM in the cricket diuretic assay. In contrast, the (L,D)-tetrazole analog demonstrated an ability to antagonize the diuretic response of natural achetakinins (Kaczmarek et al. 2007) in the in vitro cricket diuretic assay, but was inactive on the expressed tick receptor. It was suggested that the change in stereochemistry of the α-carbon at the N-terminal end of the tetrazole moiety from L to D appears to inhibit the activation response by interfering with the electrostatic interaction that occurs between the side chains of the Phe_1 and Trp_4 that allows these two critical side chains to present an aromatic surface to the receptor (Nachman et al. 2004).

The critical nature of the sidechains of Phe_1 and Trp_4 and the *cis*Pro, type VI turn conformation to the activity of the IK was also confirmed by evaluation of a small series of pseudotetrapeptide analogs that featured only these minimal constructs. These 'minimalist' analogs, based on an amino piperidinone carboxylate scaffold, retained very weak, but statistically significant diuretic activity in the in vitro cricket Malpighian tubule secretion bioassay (Kamoune et al. 2005).

In summary, structure/conformation-activity data are consistent with a receptor interaction model for the IK in which the C-terminal pentapeptide region adopts a type VI turn over residues Phe_1 to Trp_4, and that the aromatic side chains of Phe_1 and Trp_4 are oriented towards the same region and interact with the receptor. Conversely, the side chain of residue 2 lies on the opposite face pointing away from the receptor surface, explaining why this position shows greater tolerance to modifications (Nachman et al. 2002b).

2.2 *Biostable, IK Analogs That Interact with Receptors and Bioassays*

Members of the IK family are inactivated by tissue-bound peptidases of insects. Two susceptible hydrolysis sites in insect kinins (Nachman et al. 2002a) have been reported. The primary site is between the Pro_3 and the Trp_4 residues, with a secondary site N-terminal to the Phe_1 residue in natural extended IK sequences. Experiments demonstrate that angiotensin converting enzyme (ACE) from the housefly can

cleave at the primary hydrolysis site, whereas neprilysin (NEP) can cleave insect kinins at both the primary and secondary hydrolysis sites (Nachman et al. 1990, 2002a ; Cornell et al. 1995; Lamango et al. 1996;).

Incorporation of β-amino acids can enhance both resistance to peptidase attack and biological activity (Cheng et al. 2001; Juaristi and Soloshonok 2005). Recent work has described the synthesis of a number of analogs of the IK C-terminal pentapeptide core in which the critical residues Phe_1, Pro_3 and Trp_4, and/or adjacent residues, are replaced with β_3-amino acid and/or their β_2-amino acid counterparts.

It was anticipated that incorporation of β-amino acids in key positions of the IK would afford some measure of resistance to hydrolysis by the peptidases that degrade and inactivate the natural peptides. Indeed, several analogs including Ac-R**[β_3Phe]**FF**[β_3Pro]**WGa (1577), and Ac-RF**[β_3Phe]**-**[β_3Pro]**WGa (1578) have been shown to exhibit significantly enhanced resistance to peptidases that attack at insect kinin susceptible sites (Zubrzak et al. 2007). Under conditions in which the native IK from *Leucophaea*, leucokinin-I, is degraded 100% by NEP and 82% by ACE, analog 1578 shows no degradation by NEP and only 4% by ACE. These analogs are also blocked at the N-terminus with an Ac group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases (Gregory et al. 1964).

A single-replacement β-amino acid analog Ac-RFF[β_3**Pro**]WGa (1460), that involves modification of the Pro_3, was the most potent of the β-amino acid analogs in both mosquito (*A. aegypti*) and tick (*B. microplus*) receptors (Taneja-Bageshwar et al. 2008b) (Table 1). Analog 1460 proved to be more active than the positive control agonist FFFSWGa in the mosquito, and considerably more active than the minimum active core sequence FFSWGa. In the tick, 1460 was equipotent with the positive control agonist FFFSWGa and more active than the C-terminal pentapeptide active core. Analog 1460 therefore is a non-selective, biostable agonist for these two receptor systems. However, it is clear that the mosquito receptor is considerably more sensitive to modifications at Trp_4 than the tick receptor. In particular, the mosquito receptor is intolerant to the replacement of Trp_4 with β_2Trp, as 1656 (Ac-RFFP[β_2**Trp**]Ga) is inactive (Taneja-Bageshwar et al. 2008b) (Table 1). This complete loss of activity by the β_2Trp analog 1656 in the mosquito receptor cell line may result from an increase in the distance between the α-carbons of the critical aromatic residues at positions 2 and 4 over what is found in either the natural peptide. On the contrary, the tick receptor remains relatively tolerant to the introduction of a methylene group between the α-carbon and the amino group of Trp_4, as the potency of 1656 was not statistically different from the C-terminal pentapeptide core analog FFSWGa, and the analog retained 57% of the maximal response (Taneja-Bageshwar et al. 2008b). By virtue of this difference in receptor-interaction requirements, analog 1656 is a selective agonist for the tick receptor.

The double replacement analog 1577 features modification of non-critical regions; the non-critical Pro_3 residue and the residue just outside of the critical C-terminal pentapeptide core region. Accordingly, this analog retains significant activity in both the mosquito and tick receptor assays, demonstrating potency that

exceeds that of the minimum active core and which is not significantly different from that of the positive control agonist FFFSWGa (Taneja-Bageshwar et al. 2008b). It is a nonselective, biostable agonist of both arthropod receptors. Double replacement analog 1578 features modification of non-critical residues Phe_2 and Pro_3 that effectively changes the distance between the sidechains of the critical residues Phe_1 and Trp_4. Like analog 1656, this modification is tolerated to a much greater extent by the tick receptor than the mosquito receptor. Consequently, the analog 1578 demonstrates retention of activity in the tick receptor assay, with a potency that is not significantly different from that of the active core analog FFSWGa; but remains essentially inactive in the mosquito receptor system (Taneja-Bageshwar et al. 2008b) (Table 1). Thus, biostable agonist analog 1578 demonstrates selectivity between the two expressed arthropod receptor cell lines.

The β-amino acid IK analogs have not as yet been evaluated in in vitro mosquito or tick diuretic assays. However, data for these analogs has been reported in an in vitro insect kinin Malpighian tubule fluid secretion assay from the cricket *Acheta domesticus* (Zubrzak et al. 2007). As with the two expressed arthropod insect kinin receptors, analog 1460 was the most potent β-amino acid analog in the cricket fluid secretion bioassay, demonstrating a potent EC_{50} of 0.03 nM and a 100% maximal response. This analog exceeds by an order of magnitude the activity of at least one of the native achetakinins, AK-III (EC_{50} = 0.3 nM), and essentially matches the activity of the most potent of achetakinins (Coast et al. 1990). As in both expressed arthropod receptor assays, the double-replacement analog 1577 retained potent activity in the cricket diuretic assay, with an EC_{50} value of 0.1 nM and gave a 100% maximal response (Zubrzak et al. 2007). Although inactive, or virtually so, in the mosquito IK receptor, analogs 1656 (EC_{50} = 4 nM; 97% maximal response) containing β_2Trp and double replacement 1578 (EC_{50} = 1 nM; 100% maximal response) retain activity in the in vitro cricket diuretic assay (Zubrzak et al. 2007). The assay results obtained with the β-amino acid IK analogs suggest that the receptor associated with the Malpighian tubules of the cricket is more similar to the expressed IK receptor from the tick than that of the mosquito.

Another set of IK analogs that demonstrate enhanced resistance to peptidases feature a replacement of the third position of the C-terminal pentapeptide core (Pro or Ser) with α-aminoisobutyric acid (Aib), a sterically hindered α,α-disubstituted amino acid which effectively protects the primary tissue-bound peptidase hydrolysis site (Nachman et al. 1997a, 2002a). Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site, as with analog **[Aib]**FS**[Aib]**WGa (781), further enhances biostability (Nachman et al. 2002a). In the cricket Malpighian tubule assay, analog 781 exceeds the potency of the native achetakinins by an order of magnitude. The di-substituted Aib-containing analog **[Aib]**FF**[Aib]**WGa (**K-Aib-1**) also demonstrates a potency that either matches or exceeds that of the positive control FFFSWGa and/or the native *Aedes*kinin-2 in recombinant tick and mosquito receptors, respectively (Fig. 1) (Taneja-Bageshwar et al. 2009). **K-Aib-1** also demonstrates potent activity in an in vitro Malpighian tubule secretion assay in mosquito *A. aegypti*, demonstrating a potency that exceeds Aedeskinin-2 and matching that of Aedeskinin-3 (Fig. 2) (Taneja-Bageshwar et al. 2009).

Table 1 Estimated potencies (EC_{50}) and percentage of maximal bioluminescence response of different analogs in reference to FFFSWGa, and the minimum active core sequence FFSWGa, tested on mosquito (E10) and tick (BmLK3) receptor expressing cell lines (Taneja-Bageshwar et al. 2008b, 2009). EC_{50} values are an estimate of the concentration required to induce a half-maximal response

	Mosquito receptor (E10 cell line)		Tick receptor (BmLK3 cell line)	Selectivity	
Analogs	EC_{50} (10–8M)	[a]Maximal Response (%)	EC_{50} (10^{-8}M)	[a]Maximal Response (%)	
Double-Aib Analog					
[Aib]FF**[Aib]**WGa (K-Aib-1)	4.9	100	8	100	Non-selective
Beta Analogs					
1460 Ac-RFF[β_3**Pro**]WGa	36.7	50	29	72	Non-selective
1656 Ac-RFFP[β_2**Trp**]Ga	N.D.	0	68	57	Selective (tick)
1577 Ac-R[β_3**Phe**]FF [β_3**Pro**]WGa	65.3	65	50	64	Non-selective
1578 Ac-RF[β_3**Phe**-β_3**Pro**]WGa	N.D.	7	69	62	Selective (tick)
Controls					
FFFSWGa	68.5	100	27	100	
FFSWGa IK active core	N.D.	29	59	100	
*Aedes*kinin-2 (mosquito)	16.4	100	–	–	

[a]Maximal response is the maximal bioluminescence response of each analog expressed as a percentage of the maximal response of the known agonist FFFSWGa (positive control) at 10 μM. for tick and mosquito receptors. N.D.: The analog was tested but was not sufficiently active to determine an EC50 value. It should be noted that the NOVOstar bioluminescence method used to evaluate the response of the insect kinin analogs on expressed receptors reported in these studies is less sensitive as compared with a less practical confocal fluorescence cytometry method previously employed (Holmes et al. 2003). The bioluminescence plate assay is between 50- to 70-fold less sensitive. This difference should be taken into account when estimating the potency that these analogs would likely demonstrate in in vitro or in vivo physiological bioassays.

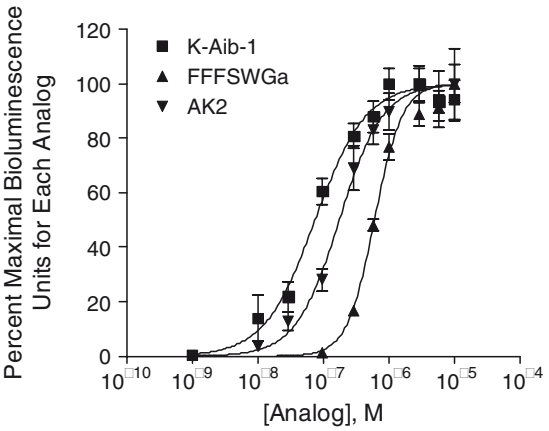

Fig. 1 Activity comparison of biostable analog **[Aib]**FF**[Aib]**WGa (K-Aib-1), native aedeskinin 2, and positive control hexapeptide FFFSWGa on a mosquito kinin receptor expressing cell line by a calcium bioluminescence plate assay (Taneja-Bagehswar et al. 2009c). The y-axis represents percent maximal bioluminescence units for each analog expressed as a percentage of bioluminescence observed at a concentration versus the maximal response observed among all concentrations tested for each analog. Statistical analysis and graphs were created with GraphPad Prism 4.0 software. Vertical lines on graph represent standard errors of independent experiments from maximum of six to minimum of three repetitions each consisting of two wells each. Analog FFFSWGa is the positive control for the receptor activity

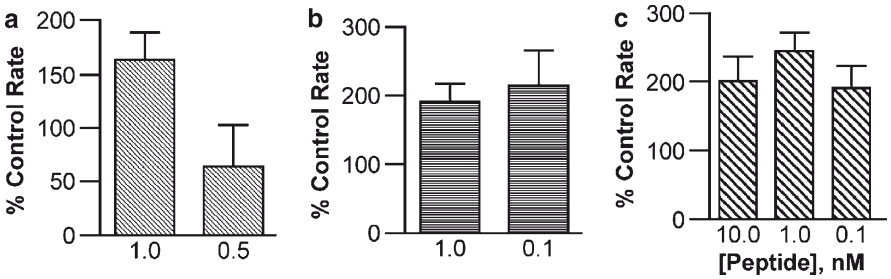

Fig. 2 In vitro *A. aegypti* Malpighian tubule secretion assay of control, FFFSWGa (panel A), native *Aedes* kinin 1 (panel B) and biostable analog **[Aib]**FF**[Aib]**WGa (K-Aib-1) (panel C). Results are expressed as a percentage of the control rate of secretion measured prior to peptide addition. Bars indicate the mean and vertical lines + 1 s.e.m. of 4–5 replicates. (Reprinted from Taneja-Bagehswar et al. (2009) with the permission of Elsevier)

These potent, biostable analogs represent ideal new tools for arthropod endocrinologists studying IK regulated processes, particularly in ticks for which a role for the insect kinins has yet to be established.

While in vivo activity studies of biostable IK analogs have not as yet been completed for the cricket, tick or mosquito, some in vivo results have been obtained in the housefly (*Musca domestica*) and in larvae of the corn earworm moth,

H. zea (Nachman et al. 2002a). Due to the different structure-activity requirements of the IK receptor associated with the Malpighian tubule fluid secretion assay in the housefly (*M. domestica*) (Coast et al. 2002), the in vitro activity of analog 781 is over four orders of magnitude less than that of the native muscakinin. Nonetheless, the in vivo diuretic activity of 781 is *equipotent* with that of the native muscakinin. Evidence indicates that analog 781 demonstrates a longer hemolymph residence time in the housefly than the peptidase-susceptible muscakinin, and it is this extended presence that likely explains the remarkable in vivo activity observed for this analog (Nachman et al. 2002a). Injection of helicokinins into developing larvae of the related moth *H. virescens* has been observed to inhibit weight gain (Seinsche et al. 2000). A helicokinin-II analog (VRFSSWGa) and a biostable Aib helicokinin analog **pQ**RFS**[Aib]**WGa (Hek-Aib) were injected daily (0.5 nmol) into 5-day old *H. zea* larvae for 5 or 6 days until pupation occurred. The helicokinin analog demonstrated a developmental trend that reached a peak on day 5 post-treatment, with a statistically insignificant 20% weight reduction as compared with controls. In contrast, the biostable, helicokinin Aib analog elicited a stronger, statistically significant effect spanning days 4–7, reaching a peak at day 5 of about a 50% reduction in mean larval weight as compared with controls. The time of pupation was delayed by a factor of 25% (Nachman et al. 2002a). Therefore, biostable characteristics can enhance the in vivo activity of IK analogs.

2.3 Nonpeptide Mimetic Agonists/Antagonists of Expressed IK Receptors

Perhaps the ultimate goal in the search for biostable, bioavailable analogs would be the design and/or discovery of nonpeptide mimetic agonists or antagonists of the IK. The availability of expressed IK receptors can accelerate the discovery process through the evaluation of nonpeptide libraries. A recent biorational approach has based the selection of a nonpeptide library on the presence, within its structure, of the side chain moiety of the most critical residue of the peptide (R.J. Nachman, uupublished). As discussed in an earlier section, the most critical residues for the interaction of the insect kinins with expressed receptors from the tick *B. microplus* and mosquito *A. aegypti* have been determined to be the Phe_1 and Trp_4 within the C-terminal pentapeptide core region (Nachman et al. 1990; Roberts et al. 1997; Taneja-Bageshwar et al. 2006). Data obtained from an in vitro Malpighian tubule fluid secretion assay indicate that a C-terminal pentapeptide IK analog in which the Phe is replaced with an Ala demonstrates an antagonist response against native achetakinins, whereas the analog in which Trp is replaced with Ala is devoid of activity (Nachman et al. 1993c; Nachman and Holman 1991). Furthermore, a C-terminal aldehyde analog in which Ala replaces Phe retains weak activity in an in vitro cricket diuretic assay (Nachman et al. 2007). This would suggest that Trp, which contains an indole side chain moiety, represents the most critical amino acid for the binding of IK with the receptor.

Consequently, a 400 member nonpeptide library based on the imidopyrido**indole** ('Ipi') scaffold (Reixach et al. 2000), which contains an indole ring embedded in its structure, was constructed and evaluated in expressed insect kinin receptors from the tick *B. microplus* and mosquito *Aedes aegypti*. One of the Ipi recombinant library analogs demonstrated significant activity in both of the expressed arthropod insect kinin receptor assays (R.J. Nachman et al., unpublished), as well as statistically significant activity in in vitro Malpighian tubule secretion assays of the mosquito *A. aegypti* and cricket *A. domesticus* at concentrations between 0.05 and 1 μM (R.J. Nachman et al., unpublished). Future evaluations of this and other rationally designed, indole-containing libraries may identify other nonpeptide mimetic agonists as well as antagonists. The data further underscores the preeminent importance of the indole moiety of Trp_4 to the interaction of the IK with their receptors.

2.4 C-Terminal Aldehyde IK Analogs

Aldehydes can form reversible imine bonds with amino groups. Peptide analogs containing reversible binding moieties, such as an aldehyde, at the C-terminus have been reported to inhibit various classes of proteolytic enzymes (Fehrentz et al. 1984; Chapman 1992; Sarubbi et al. 1993). It has been further postulated that a C-terminal aldehyde moiety could form a covalent, reversible Schiff base (imide linkage) with the amino group of a Lys residue (Lehninger 1970; Nachman et al. 2007) in an IK receptor pocket, thereby modifying the ligand–receptor interaction characteristics of the resulting IK analog. Evaluations of two C–terminal aldehyde IK analogs, R-LK-CHO (Fmoc-RFFPWG-H) and V-LK-CHO (Boc-VFFPWG-H), in developmental and diuretic assays have been reported (Nachman et al. 2003, 2007). Both aldehyde analogs demonstrated in vitro stimulation of fluid secretion in isolated cricket Malpighian tubules in the physiological concentration range and full efficacy, thereby providing evidence that they could interact with an IK receptor site.

2.5 H. Zea Larval Weight-Gain Inhibition Bioassay

Injection of R-LK-CHO into 5-day old *H. zea* larvae induced statistically significant reductions in weight gain in comparison with control animals on days 2 and 4–6 at the 5 nmol dose, but not at 500 pmol. Day 6 larvae experienced a significant reduction in weight gain at the 5 nM dose, with treated animals observed to be about 65% of the weight of controls. No significant difference in mortality was observed between treated and control groups (Nachman et al. 2003). The other aldehyde analog, V-LK-CHO, demonstrated a more pronounced effect than R-LK-CHO in the *H. zea* larval weight gain inhibition assay. V-LK-CHO induced significant reductions in weight gain on days 2, and 4 through 6, after initiation of the treatment at *both* the 500 pmol and

5 nmol dose. At day 6, treated larvae were observed to be 65% and 40% that of the weight of the control animals at doses of 500 pmol and 5 nmol, respectively. Notably, in those animals treated with V-LK-CHO a significant increase in mortality was observed at both doses (45%{500 pmol}and 67%{5 nmol})(Nachman et al. 2003).

The significant increase in mortality observed in larvae treated with V-LK-CHO is not likely a result of some general toxic effect of the aldehyde moiety itself, as increased mortality was not observed in R-LK-CHO, which also features a C-terminal aldehyde (Nachman et al. 2003).

2.6 In vitro and in vivo Housefly Diuretic Bioassays

As mentioned previously, IK analogs in which the C-terminal amide was replaced by an aldehyde moiety retained an ability to stimulate fluid secretion by cricket Malpighian tubules. Although a reliable in vivo assay for diuresis in crickets did not exist, such an assay was available for houseflies (Coast 2001, 2004). Neither of the two aldehyde analogs V-LK-CHO and R-LK-CHO stimulated fluid secretion of housefly Malpighian tubules, although notably tubules exposed to R-LK-CHO did not respond when subsequently challenged with a supramaximal concentration (10 nM) of native muscakinin (Musdo-K). In contrast, the same concentration of Musdo-K elicited a marked diuretic response in tubules that had first been exposed to V-LK-CHO. The inhibitory effect of R-LK-CHO on the diuretic activity of 10 nM Musdo-K was dose-dependent with an IC_{50} of 12 μM, which compares favorably with the EC_{50} of an N-terminal truncated Musdo-K analog of similar length (Coast et al. 2002) (Musdo-$K_{[9–15]}$ EC_{50} ~1 μM).

Other aldehyde analogs of the insect kinins were also tested on housefly tubules for diuretic activity and they demonstrated no ability to inhibit stimulation of fluid secretion by 10 nM Musdo-K (Nachman et al. 2007). The inhibitory activity of R-LK-CHO is thus highly specific and not a generalized effect of the aldehyde moiety.

By using a high sensitivity flow through humidity analyzer the excretion of urine from intact houseflies treated with the aldehyde analog and a control was recorded (Coast 2004). R-LK-CHO inhibits the in vivo activity of Musdo-K as evidenced by a marked reduction in the amount of urine voided in flies injected with 50 pmol each of the analog and the kinin compared with the kinin alone (Nachman et al. 2007). The diuretic response to hypervolemia is partly attributable to the release of Musdo-K from neurohaemal sites into the circulation, and the inhibitory effect is consistent with a selective effect of R-LK-CHO at the kinin receptor.

R-LK-CHO would not necessarily be expected to have any effect on the autonomous response of Malpighian tubules to haemolymph dilution, and yet it reduced the total amount of urine voided from flies injected with 1 μL of distilled water by almost 50% (Fig. 3). The markedly reduced urine output from flies injected with 1 μL distilled water containing 50 pmol R-LK-CHO suggests this analog may have a toxic effect on Malpighian tubules. In support of this it has been shown that it not only blocks the activity of Musdo-K, but also blocks

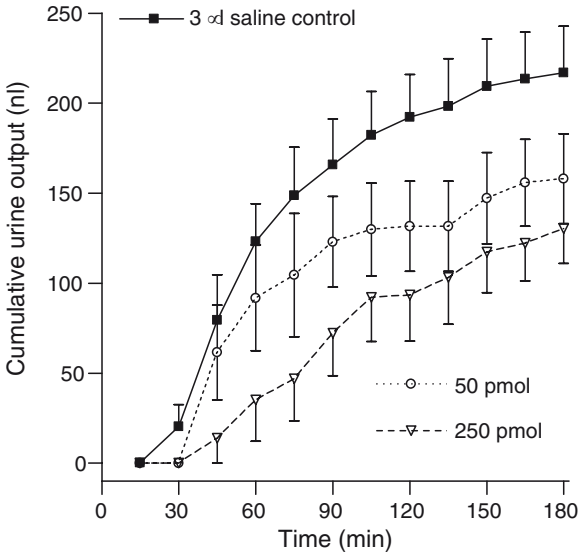

Fig. 3 R-LK-CHO attenuates the in vivo diuretic response to hypervolemia induced by the injection of 3 μL of saline. The volume of urine excreted by individual flies was measured over 15 min periods for 3h in insects injected with saline alone (solid squares, solid line) or saline containing either 50 pmol (open circles, dotted line) or 250 pmol (open triangle, dashed line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in 10 (saline alone) and 6 (+ R-LK-CHO) flies. (Reprinted from Nachman et al. (2007) with the permission of Elsevier)

stimulation of fluid secretion by both thapsigargin, a SERCA inhibitor, and by ionomycin, a calcium ionophore (Nachman et al. 2007). Kinin neuropeptides use Ca^{2+} as a second messenger to open a paracellular or transcellular chloride conductance pathway. This is mimicked by thapsigargin and ionomycin, which increase the level of intracellular calcium by promoting Ca^{2+} release for intracellular stores and the influx of Ca^{2+} from the bathing fluid, respectively. The ability of R-LK-CHO to block the activity of these pharmacological probes shows it is not acting as an antagonist of the kinin receptor, but must act downstream of the second messenger pathway.

At present, the cellular action(s) of R-LK-CHO on housefly Malpighian tubules is unknown. It is clearly not a generalized toxic effect, because the same analog has diuretic activity in the cricket Malpighian tubule assay. Moreover, closely related aldehyde analogs tested on housefly tubules, among them those that do not contain the complete IK core sequence, either have no activity or stimulate fluid secretion. R-LK-CHO may act as a 'magic bullet' and bind with the IK receptor on housefly tubules, become internalized, and thereby gain access to intracellular processes that couple a rise in intracellular calcium levels to the opening of the chloride conductance pathway (Nachman et al. 2007).

Compounds in the hemolymph, including pesticide toxins, are actively transported into the lumen of the Malpighian tubules, and their rate of elimination is dependent on

the rate of fluid secretion (Maddrell 1981; O'Donnell and Maddrell 1983). At high rates of secretion, the toxins do not reach the high concentrations in the tubule lumen, which minimizes diffusion back into the hemolymph down a concentration gradient. An agent capable of selective depression of fluid secretion would therefore be expected to reduce the rate of clearance of pesticides from the hemolymph allowing them to achieve higher concentrations and, in turn, likely reduce the amount of toxin required to kill an insect.

3 Pyrokinin/PBAN Neuropeptide Family

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides represent a multifunctional family that plays a significant role in the physiology of insects. Leucopyrokinin (LPK), isolated from the cockroach *Leucophaea maderae* in 1986 (Holman et al. 1986), was the first member of the family to be discovered. Since that time, over 30 peptides have been identified. They include PKs, myotropins (MTs), PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone (DH), pheromonotropin (PT), all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) (Altstein 2004; Predel and Nachman 2006; Rafaeli and Jurenka 2003). Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths (Altstein 2004; Matsumoto et al. 1992; Predel and Nachman 2006; Raina and Klun 1984; Rafaeli and Jurenka 2003; Raina et al. 1989), and mediation of key aspects of feeding (gut muscle contractions) (Nachman et al. 1986; Schoofs et al. 1991), development (embryonic diapause, pupal diapause and pupariation) (Imai et al. 1991; Nachman et al. 1993a, 1996, 1997b, 2006; Xu and Denlinger 2003; Zdarek et al. 2002) and defense (melanin biosynthesis) (Altstein et al. 1996; Matsumoto 1990) in a variety of insects (cockroaches, flies, locusts and moths). All of the above functions can be stimulated by more than one peptide, and they demonstrate considerable cross-activity between various PK/PBAN assays, thereby lacking any species-specific behavior (Abernathy et al. 1996; Altstein 2003; Choi et al. 2003; Nachman et al. 1993a; Rafaeli and Jurenka 2003). The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects. Agonists and/or antagonists, particularly selective ones, of the PK/PBAN family can shed light on this issue, and provide leads for insect management agents capable of disrupting PK/PBAN regulated systems.

3.1 Chemical and Conformational Aspects of PK/PBAN Activity

The C-terminal pentapeptide FXPRLa is highly conserved and thus, shared by PBAN and other pyrokinins. It has further been identified as the ligand core for activity in pheromonotropic bioassays (X = S) (Abernathy et al. 1995; Altstein et al. 1995) and the expressed PBAN receptor from the moth *H. virescens*

(HevPBANR-C) (Kim et al. 2008), although the C-terminal hexapeptide YFXPRLa (X = S) exhibits much greater potency. The C-terminal pentapeptide also has been shown to represent the core region for activity retention in such other PK/PBAN mediated physiological systems as the cockroach *L. maderae* hindgut contractile (Nachman et al. 1986) and melanization assays in *Spodoptera littoralis* (Altstein et al. 1996). However, the core region for activity is the C-terminal heptapeptide LWFGPRLa in the pupal diapause termination bioassay in *H. zea* (Zhang et al. 2008) and the C-terminal tetrapeptide TPRLa in the pupariation bioassay in the fly *Neobellieria bullata* (Nachman et al. 1997).

Several turn conformations have been proposed for the pentapeptide region of the PK/PBAN core based on NMR experiments of PBAN and/or core analogs in solution. Using the C-terminal hexapeptide PBAN analog [D-Phe$_{29}$, 28–33]PBAN in an NMR solution conformation study, Wang et al. reported that it adopts a type II β-turn; although the authors concluded that this may have resulted from the conformational averaging of a type I β-turn and an extended structure (1994). Clark and Prestwich investigated the solution conformation of the natural HezPBAN and reported a type I' β-turn with a *cis*-Pro in the C-terminal pentapeptide region (1996). They found no interaction between the C-terminal turn and the rest of the PBAN peptide chain, providing evidence that the turn is the critical conformation recognized by the PBAN receptor. Drawbacks to the studies conducted by Wang et al. and Clark and Prestwich are that they were investigating highly flexible structures and NMR experiments were conducted in solutions containing organic solvents (Wang: DMSO; Clark/Prestwich: 2,2,2-trifluoroethanol), which can promote formation of secondary structure that is not necessarily relevant to the conformation adopted during receptor docking. Nachman et al. conducted a conformational study of the rigid, cyclic PK/PBAN analog *cyclo*[NTSFTPRL] (*cyclo*[Asn1]LPK) in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics (Nachman et al. 1991, 2003b). The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro in the second position of a type-I β-turn over residues Thr-Pro-Arg-Leu within the core region. Indeed, a *trans*Pro is a defining characteristic of a type I β-turn (Chou and Fasman 1977). The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo*[Asn1]LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values (Nachman et al. 1991). This analog (*cyclo*[Asn1]LPK) demonstrated significant activity in several PK/PBAN bioassays, including hindgut contractile (cockroach *L. maderae*) (Nachman et al. 1991), oviduct contractile (cockroach *L. maderae*) (Nachman et al. 1995), pheromonotropic (silk worm *Bombyx mori*) (Nachman et al. 2003b), egg diapause induction (silk worm *B. mori*) (Nachman et al. 1995), pupal diapause termination (*H. zea*) (Zhang et al. 2009), and pupariation (flesh fly *N. bullata*) (Nachman et al. 1997) assay systems. Recently, a structure for the HezPBAN receptor has been predicted using the X-ray diffraction structure of the GPCR rhodopsin as a template; and this calculated structure has been used to build a binding model for the HezPBAN C-terminal hexapeptide fragment adopting each of the three

proposed β-turn types. The model clearly supports the presence of a β-turn in the receptor bound conformation of the PBAN core, but is not precise enough to provide evidence for the specific type of β-turn (Stern et al. 2007).

In order to provide more definitive evidence that a *trans*Pro, and a type I β-turn, represented the active conformation for pheromonotropic activity, a PK/PBAN analog incorporating a *trans*Pro, (*E*)-alkene mimetic component was evaluated in five PK/PBAN bioassay systems (pheromonotropic, pupariation, hindgut myotropic, and melanization) and found to retain high activity (Nachman et al. 1995, 2009f). The (*E*)-alkene mimetic analog demonstrated activity that was not statistically different from that of a PK/PBAN C-terminal pentapeptide parent analog in an expressed HevPBANR receptor (Nachman et al. 2009c) and proved to be an order of magnitude more potent than the native 24-residue diapause hormone (DH) in the pupal diapause termination assay in *H. zea* (Zhang et al. 2009). The greater potency observed in the latter in vivo assay is believed to be due to greater biostability of the mimetic analog to degradative peptidases. In the (*E*)-alkene moiety of the aforementioned PK/PBAN analog, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond (Wang et al. 2003).

The establishment of a *trans*Pro orientation has important implications concerning the preferred β-turn conformation. Previous NMR studies have led to the three proposed β-turn types (type I, type II and type I′) for the PBAN core region discussed above. Of the three studies, only Nachman et al. (1991, 2003b) used both a conformationally rigid PK/PBAN analog along with aqueous solutions free of added organic solvents that artificially promote the formation of secondary structure. Of further interest is the admission by Wang et al. (1994) that their finding of a type II β-turn could have resulted from the conformational averaging of a type I β-turn (identified in the study by Nachman et al.) and an extended conformation in the flexible analog used. The type I β-turn proposed by Nachman et al. features a *trans*Pro that was clearly evident in the rigid, cyclic analog *cyclo*[Asn1]LPK and has now been confirmed by the potent activity of the (*E*)-alkene analog, which locks in a *trans* orientation. This finding is not consistent with the type I' β-turn proposed in the study by Clark and Prestwich (1996) that used the highly flexible HezPBAN as it features a *cis*Pro as opposed to a *trans*Pro. The activity of the (*E*)-alkene analog not only provides evidence for the orientation of Pro and a core conformation for the interaction of PK/PBAN peptides with their receptors, but also identifies a scaffold with which to design mimetic PK/PBAN analogs.

3.2 Development of a Selective PK/PBAN Agonist Analog

While it had been established that Pro in the ligand core of PK/PBAN analogs preferentially adopts a *trans* orientation during receptor interaction, it was unknown whether differences existed between the PK/PBAN receptors associated with the

various bioassay systems in terms of tolerance to any variance from the natural *trans*Pro conformation. Any differences could be exploited to develop mimetic analogs with selectivity for one of the related PK/PBAN receptors over others. In this regard, the dihydroimidazoline moiety (Fig. 5) was proposed as a novel mimic of a *trans* peptide bond, particularly one associated with Pro, and then incorporated into a PK/PBAN core sequence. Molecular modeling indicated that the dihydroimidazoline moiety (labeled 'Jones' moiety; abbreviated 'Jo') could serve as a surrogate of a *trans* peptide bond and/or a *trans*Pro, although the fit is not as close as with the (*E*)-alkene moiety above (Nachman et al. 2009a). An analog incorporating the 'Jones' moiety **PPK-Jo**, Ac-YF[**Jo**]RLa, was evaluated in four PK/PBAN assays (pheromonotropic, melanotropic, pupariation and hindgut myotropic). In the in vivo *S. littoralis* melanotropic assay **PPK-Jo** demonstrated strong activity, reaching a 100% maximal response at doses of 100 pmol and 1 nmol; matching the efficacy of the natural peptide HezPBAN1–33 if not its potency. The parent PK/PBAN hexapeptide YFTPRLa demonstrated a strong response in this assay as well,

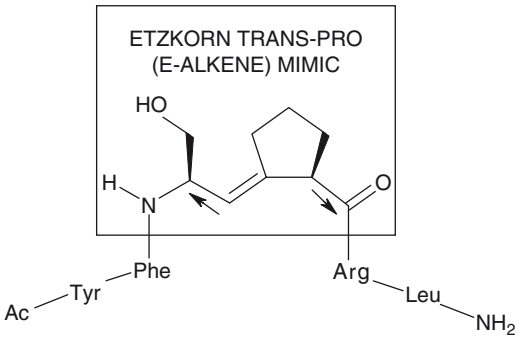

Fig. 4 Structure of the analog PK-Etz (Ac-Tyr-Phe-SerΨ[***trans-CH = C***]Pro-Arg-Leu-NH2), containing an (E)-alkene, *trans*Pro motif ('Etzkorn'). In this motif, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond. (Wang et al. 2003; Nachman et al. 2009a)

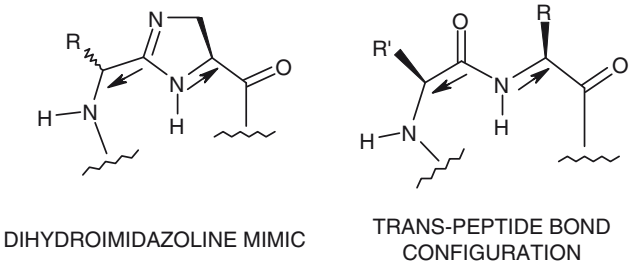

Fig. 5 Comparison of the *trans*-peptide bond (right) and the dihydroimidazoline ('Jones') mimetic motif. A '*trans*' orientation is frozen within the five-membered ring structure (Nachman et al. 2009a)

but, unlike **PPK-Jo** it also inhibited the melanotropic activity of control peptide HezPBAN1–33. Therefore, **PPK-Jo** proved to be a pure agonist in the melanotropic assay. **PPK-Jo** did not elicit any significant agonist or antagonist activity in the pheromonotropic, pupariation or hindgut contractile bioassays, indicating that it is a pure, selective agonist for the melanotropic response in *S. littoralis* (Nachman et al. 2009a). In contrast, control peptides elicited strong agonist responses in all three of these PK/PBAN bioassays. It is apparent that the receptor associated with the melanotropic assay in *S. littoralis* is more promiscuous than those of the other PK/PBAN assays, demonstrating more tolerance to deviations from the natural *trans*Pro structure. The development of a selective PK/PBAN agonist can lead to a better understanding of the endogenous mechanisms of this important peptide class and can serve as a probe to study PK/PBAN regulated systems in insects and as a lead for insect management agents capable of disrupting these systems in a selective manner.

Another analog incorporating a structural modification at the Pro residue of the PK/PBAN core region that features a selective agonist profile is covered under the next section on biostable analogs of the PK/PBAN family. In other work, Altstein et al. investigated cyclized versions of the linear lead PK/PBAN antagonist RYF[dF]PRLa that feature selective inhibition of either the pheromonotropic or melanotropic activity of control PK/PBAN peptides (Altstein 2003; Altstein and Hariton 2008). This work is covered extensively in another chapter of this book (Altstein et al. this book), and therefore will not be further addressed here.

3.3 Biostable PK/PBAN Analogs

The PKs are hydrolyzed by tissue-bound peptidases at a primary susceptibility site between the Pro and Arg residues within the C-terminal pentapeptide sequence that defines members of this family of neuropeptides (Nachman et al. 2002b). Incorporation of β-amino acids can enhance resistance to peptidase attack and modify biological activity (Cheng et al. 2001; Juaristi and Soloshonok 2005), as demonstrated in the first section of this chapter with the insect kinin neuropeptide family (Taneja-Bageshwar et al. 2008b; Nachman et al. 2009d). This strategy can lead to the identification of potent selective and nonselective agonists with enhanced biostability characteristics. Analogs of the PK/PBAN C-terminal hexapeptide core (YFTPRLa) that incorporated β_3-Pro as a replacement for Pro were synthesized with the expectation that this modification could protect the adjacent peptidase-susceptible peptide bond linking the Pro and Arg residues. The two analogs, **PK-βA-1** (**Ac**-YFT[**β_3P**]RLa) and **PK-βA-4** (**Ac**-[**β_3F**]FT[**β_3P**]RLa), were tested against two peptidases (neprilysin and angiotensin-converting enzyme [ACE]) that degrade and inactivate natural PK/PBAN peptides and found to demonstrate greatly enhanced resistance to hydrolytic cleavage. For neprilysin, the observed rates of hydrolysis for the parent peptide YFTPRLa, **PK-βA-1**, and **PK-βA-4** were found to be about 700, 0 and 30 pmol/h, respectively. For ACE, the rates of hydrolysis were observed to be

about 2,360; 0 and 210 pmol/h, respectively (Nachman et al. 2009b). **PK-βA-1** is a particularly biostable analog to these two degradative peptidases. Despite the insertion of an additional methylene group into each of two residues of the PK/PBAN core region, analog **PK-βA-4** demonstrates an agonist response in all four of the PK/PBAN bioassays in which it was evaluated (pheromonotropic, melanotropic, pupariation, and hindgut myotropic), but is especially potent in the *N. bullata* pupariation assay, where it essentially matches the potency of a natural control PK. Analog **PK-βA-1** demonstrates a more selective profile than control PK/PBAN peptides as it is essentially inactive in the pupariation bioassay and represents a biostable antagonist of the pheromonotropic and melanotropic assays, without eliciting the significant agonist activity of the parent PK/PBAN hexapeptide.

Other β-analogs of the PK/PBAN family with either a β_3-Phe or β_2-homo-Phe replacement for the Phe, a residue far removed from the primary peptidase-susceptible site, were not expected to feature the extent of biostability observed for the aforementioned β_3-Pro analogs but did modify the biological activity profile of the parent PK/PBAN hexapeptide. Analog **PK-βA-2** (**Ac**-Y**[$\beta_2$homoF]**TPRLa) proved to be an especially potent agonist in the in vivo *S. littoralis* melanotropic bioassay, demonstrating full efficacy at 1 pmol. Analog **PK-βA-3** (**Ac**-Y**[β_3F]**TPRLa) was found to be a non-selective agonist in all four PK/PBAN bioassays, but elicits a particularly unusual response in the *N. bullata* pupariation bioassay (Nachman et al. 2009b). **PK-βA-3** accelerates pupariation in this fly at a potency (0.2 pmol/larva) that matches that of the native pyrokinin factor. At higher concentrations, this β-amino acid pyrokinin analog induces irregular pupariation behavior patterns that are suggestive of neurotoxic properties. Specifically, the immobilization (*I*) phase began almost immediately after recovery from chilling (there was no wandering) and violent convulsive contractions of overall musculature were observed during the *I* phase (Nachman et al. 2009f). Additional modifications intended to protect the primary peptidase-susceptible peptide bond that links the Pro and Arg residues of **PK-βA-3** may lead to biostable analog leads with the potential to further disrupt the important pupariation process in flies.

Other PK/PBAN analogs have been synthesized that feature sterically-hindered replacements for Pro in the core region to enhance resistance to peptidase hydrolysis at the adjacent primary peptidase-susceptible site. The Pro analogs hydroxyproline (Hyp) and octahydroindole-2-carboxylate (Oic) were incorporated into the core region of the PK/PBAN pentapeptide core region. The resulting analogs, **Hex**-FT**[Hyp]**RLa (901) and **Hex**-FT**[Oic]**RLa (904), proved to be completely resistant to degradation by peptidases bound to Heliothine Malpighian tubule tissue over a 120 min period, whereas a natural pyrokinin was completely degraded in 30 min. Despite the structural changes incorporated into the biostable Hyp (901) and Oic (904) analogs, they retained significant activity in a *H. virescens* pheromonotropic assay, with ED_{50} values of 9 and 114 pmol, respectively (Nachman et al. 2002b). The ED_{50} of the parent PK/PBAN pentapeptide FTPRLa was 2.3 pmol. The two analogs matched the efficacy of natural HezPBAN and/or its fragment-analogs. Although Oic analog 904 was inactive in a *H. zea* pupal diapause termination assay, Hyp analog 901 proved to be 5-fold more potent than the native DH hormone (Zhang et al. 2009). The enhanced

potency of the biostable analog is likely a result of the longer in vivo half life as compared with the native hormone. Therefore, the Hyp analog 901 is a biostable, *nonselective* agonist for both the pheromonotropic and pupal diapuase termination response in Heliothine insects, whereas Oic-containing 904 is a biostable analog that is *selective* for the pheromonotropic bioassay.

3.4 PK/PBAN Analogs with Enhanced Topical and/or Oral Bioavailability

Insect neuropeptides in general are not suitably designed to efficiently penetrate either the outer cuticle or the digestive tract of insects. Nonetheless, studies have shown that the PK/PBAN class of insect neuropeptides can be modified to enhance bioavailability characteristics.

3.5 Topical Activity

Topical experiments have been conducted with a couple of insect neuropeptide families. Topical application of members of the adipokinetic hormone (AKH) family in mixed aqueous/organic solvent to the cuticle of the cricket *Gryllus bimaculatus* did lead to a significant AKH-like increase in hemolymph lipids (Lorenz et al. 2004). The presence of organic solvent likely served as a carrier for the AKH peptides in penetrating the cuticular waxes. In addition, the AKHs are particularly hydrophobic, which may also aid penetration through the cuticle.

On the other hand, experiments involving topical application of aqueous solutions of members of the PK/PBAN family did not produce significant pheromone production in the tobacco budworm moth *H. virescens* (Nachman et al. 1996; Abernathy et al. 1996). Structural modification to produce PK/PBAN analogs that feature amphiphilic properties greatly enhances their ability to both penetrate the hydrophobic cuticle, and also to maintain the aqueous solubility required to reach their target receptor once they encounter the hemolymph (Nachman et al. 1996; Abernathy et al. 1996). The development of a series of pseudopeptide analogs of this neuropeptide family was accomplished with the addition of various hydrophobic groups to the N-terminus of the C-terminal pentapeptide active core, which in conjunction with the polar/charged Arg side chain, confer an amphiphilic property. Hydrophobic groups appended to the N-terminus included fatty acids of various chain lengths, cholic acid, carboranylpropionic acid, and aromatic acids (Nachman et al. 1996, 2001, 2002b; Nachman and Teal 1998; Teal and Nachman 1997,2002). Many of these amphiphilic analogs showed greater in vivo potency in a pheromonotropic assay than the native 33-membered PBAN when delivered via injection in female *H. virescens* moths. In studies involving topical application in aqueous solution, neither PBAN nor its C-terminal pentapeptide active core

elicited pheromone production when applied at 1–2 nmol/female. By contrast, various amphiphilic analogs induced significant pheromone production 15 min after topical application of aqueous solutions to the lateral abdominal surface of the moths with ED_{50} values ranging from 60 to 500 pmol/female and ED_{max} values of 60–2,000 pmol/female (Nachman and Teal 1998), dependant on the individual analog. No assistance by organic solvents was needed. Following application to dissected pieces of *H. virescens* cuticle, 24-h recoveries of a series of amphiphilic pyrokinin analogs ranged from 5–70%, depending on the individual analog. In addition, prolonged pheromone production exceeding 20 h was observed following a single topical application of one amphiphilic PK/PBAN analog to the moth *H. virescens*. Furthermore, the nature of the hydrophobic moiety was observed to influence the duration of the slow release of a particular amphiphilic analog. It is clear that the insect cuticle can serve as a reservoir for the time-release of a physiologically active, amphiphilic analog of an insect neuropeptide (Nachman and Teal 1998).

One amphiphilic PK/PBAN analog, **2Abf-Suc**-FTPRLa (PK-2Abf), featured an appended brominated fluorine aromatic ring as the hydrophobic moiety and demonstrated highly unusual in vivo activity following delivery via injection (Teal and Nachman 2002). Unlike other amphiphilic analogs, a single injection of 500 pmol of this brominated fluorine (2Abf) pyrokinin analog into female *H. virescens* moths induced a highly unnatural response; continuous production of high levels of pheromone for as long as 20 h (Teal and Nachman 2002). While such a result might be expected from the time-release of an amphiphilic analog following topical application, the observed prolonged pheromone production following injection suggested that the 2Abf analog might have a strong affinity for the pheromone receptor. Studies on an expressed PK/PBAN receptor from *H. virescens*, the Abf analog proved to be more active than the native 33-membered PBAN neuropeptide, and much more active than the parent C-terminal pentapeptide fragment (R.J. Nachman and M.E. Adams, unpublished). When evaluated in an in vivo pupal diapause termination assay in *H. zea*, this Abf analog proved to be hyperpotent, with an EC^{50} nearly 50-fold more potent than the natural DH hormone (Zhang et al. 2009). However, in the pheromonotropic assay the analog had an interesting side effect in that it led to mortality in 100% of the treated *H. virescens* adults. The LC^{50} value for this potent toxic side effect was found to be 0.7 pmol, whereas 100% mortality was achieved with a 5 pmol dose. Related analogs such as **2Abf-Suc**-AARAAa, retaining similar amphiphilic and solubility properties, failed to demonstrate toxicity (Teal and Nachman 2002). Therefore, the effect was not a result of any inherent toxicity of the 2Abf moiety itself. Furthermore, the toxic effect was highly specific to the presence of the PK/PBAN sequence. Although the mechanism of the insecticidal activity of the PK-2Abf analog in *H. virescens* is unknown, it is hypothesized that the toxicity results from an interaction with receptor sites for the PK/PBAN class of insect neuropeptides (Teal and Nachman 2002).

The same strategy used to develop PK/PBAN agonists with enhanced bioavailability characteristics was recently employed with the linear lead PK/PBAN *antagonist* RYF[dF]PRLa (Ben-Aziz et al. 2005; Zeltser et al. 2000). Addition of the hydrophobic hexanoic acid to the N-terminus led to the amphiphilic analog

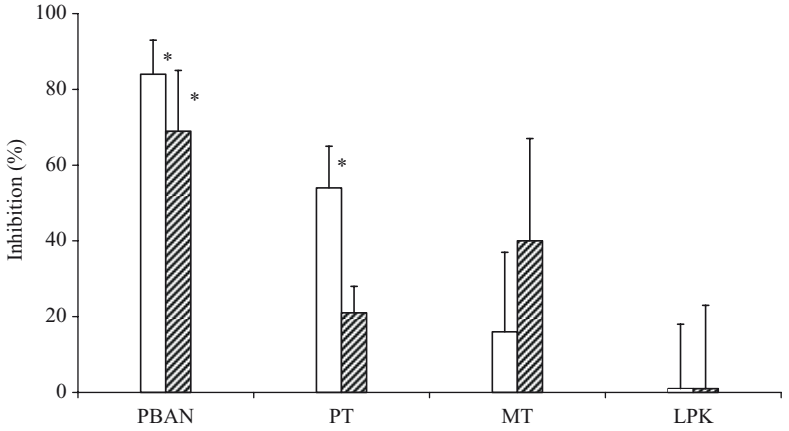

Fig. 6 In vivo inhibition of sex pheromone biosynthesis elicited by PBAN, PT, MT and LPK by 100 pmol (*open bars*) and 1 nmol (*dark bars*) of the amphiphilic analog PPK-AA in adult female *H. peltigera*. The data represent means ± SEM (n = 8–10). An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. (Reprinted from Nachman et al. (2009e) with the permission of Elsevier)

Hex-Suc-A[dF]PRLa (PPK-AA), which was able to inhibit the pheromonotropic activity of PBAN by a factor of 84% at a dose of 100 pmol in *H. peltigera* (Fig. 6). PPK-AA is a pure, selective antagonist as it demonstrates no significant agonist response in either the heliothine pheromonotropic assay or in the *S. littoralis* melanotropic assay and no inhibition of control PK/PBAN peptides in the melanotropic assay. Testing on isolated cuticle dissected from the abdominal region of adult heliothine females demonstrates that from an initial topical application of 500 pmol, a high percentage (25–30%, or 130–150 pmol) penetrates through the outer cuticular surface to the hemolymph side (Fig. 7) (Nachman et al. 2009e). The cuticle-penetration data indicates that the quantity of the amphiphilic antagonist PPK-AA capable of transmigration through the abdominal heliothine cuticle is sufficient to reach a physiologically significant dose. The amphiphilic analog PPK-AA is a selective pheromonotropic antagonist featuring enhanced bioavailability, and represents a significant addition to the arsenal of tools available to arthropod endocrinologists studying the endogenous mechanisms of PK/PBAN regulated processes, as well as a prototype for the development of environmentally friendly pest management agents capable of disrupting the critical process of reproduction.

3.6 Oral Activity

Generally, oral activity for unmodified insect neuropeptides is poor to nonexistent. Small quantities of members of the PK/PBAN (Raina et al. 1995) and the proctolin classes of neuropeptides (Bavoso et al. 1995) have been previously reported to

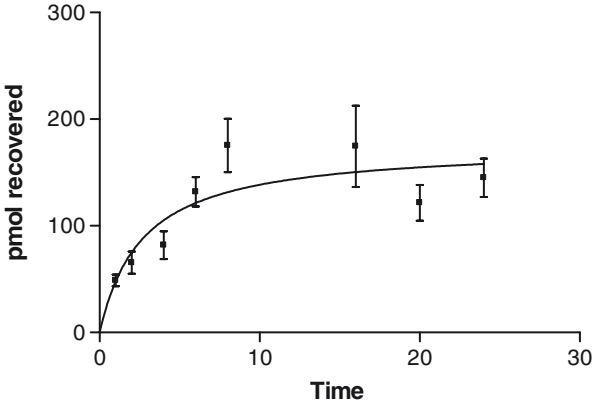

Fig. 7 Penetration of the amphiphilic antagonist analog PPK-AA through the isolated cuticle dissected from the abdominal region of adult female *H. virescens* at 1, 4, 6, 8, 16, 20, and 24 h intervals, as monitored via HPLC. The data represent means ± SEM (n = 6). (Reprinted from Nachman et al. (2009e) with the permission of Elsevier)

survive exposure to the digestive enzymes of the digestive tract and penetrate through to the hemolymph to reach their target receptors. In addition, small quantities (<3%) of A-type allatostatins have been shown to be transported across dissected foregut tissue of the moth *Manduca sexta* (Audsley and Weaver 2007). An attempt to feed PBAN to adult females of the moth *H. zea* led to very low and inconsistent levels of pheromone production that were not progressively dose-dependent (Raina et al. 1995). In other experiments, no statistically significant pheromone production was observed in starved adult females of the related moth species *H. virescens* 1–2 h after ingestion of a sugar solution of 50 pmol/μL of PBAN or the C-terminal pentapeptide core FTPRLa. However, the aforementioned biostable amphiphilic, pyrokinin analogs **Hex**-FT**[Hyp]**RLa (901) and **Hex**-FT**[Oic]**RLa (904) demonstrated an ability to penetrate the dissected portions of the insect digestive tract as well as elicit significant pheromonotropic activity following oral delivery (Nachman et al. 2002c).

Direct penetration of the two analogs 901 and 904 through dissected cockroach foregut and midgut were investigated. The digestive system of the cockroach was chosen because the guts of adult moths are not of sufficient size or stability to allow for practical delivery of peptide analog solutions. Out of a total of 2.5 nmol placed within the lumen of a sealed foregut, 800 nmol (over 30%) of Oic analog 904 penetrated the tissue preparation. It is interesting to note that Oic analog 904 demonstrates time-release properties, as equal amounts were recovered over the 0–4 h period as over the 4–24 h period. The majority of Hyp analog 901 penetrated in the first 0–4 h period (Nachman et al. 2002c). The lumen of the insect foregut features a cuticular component, which could explain why the time-release effect is similar to that observed for the outer cuticle for these amphiphilic analogs. It also suggests that the foregut can serve as a reservoir for the time-release delivery of neuropeptide analogs in insects, thereby bypassing the hostile, peptidase-rich environment of the midgut.

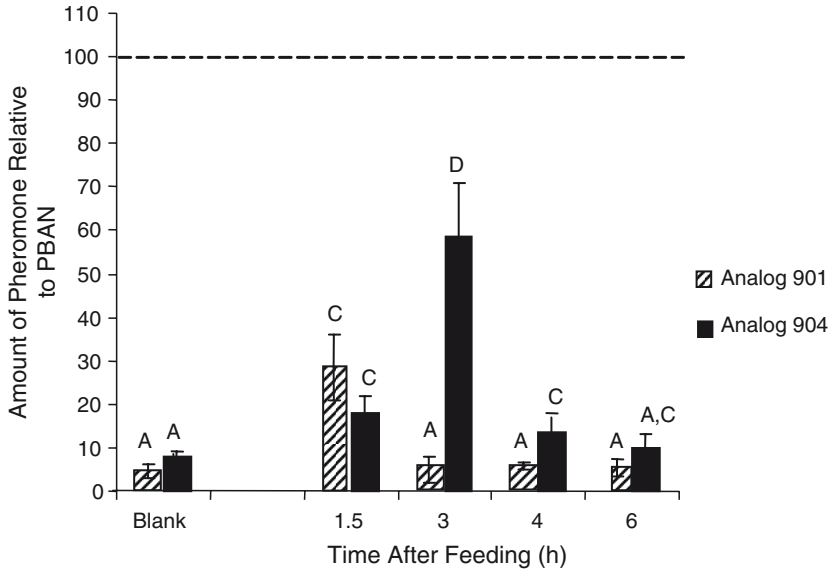

Fig. 8 Amount of pheromone, relative to maximal levels produced by injected PBAN, produced by Hyp-pyrokinin analog 901 and Oic-pyrokinin analog 904, at 1.5, 3, 4, and 6 h following oral administration. The dotted line at 100% denotes maximal production of pheromone by injected PBAN (positive control). (Reprinted from Nachman et al. (2002c) with the permission of Elsevier Press)

These in vitro penetration studies of analogs 901 and 904 were followed by in vivo oral pheromonotropic activity trials in adult female *H. virescens* (Nachman et al. 2002c). Pheromone production was monitored following ingestion of 30 μL of a sugar solution containing 50 pmol/ μL of either 901 or 904 at 1.5, 3, 4 and 6 h post feeding (Nachman et al. 2002b). A statistically significant increase in pheromone titer was observed at 1.5 h post-feed with 901 with a 17% maximal response. Oral administration of the analog 904 induced statistically significant levels of pheromone at 1.5, 3 and 4 h post-feed, but not at 6 h. Optimal pheromone production was achieved at 3 h, with a highly significant 60% maximal response (Nachman et al. 2002c). The shift in the pheromone spike from 1.5 h for 901 to 3 h post-feed for 904 is consistent with the greater time-release effect observed for the direct penetration of the more hydrophobic 904 in both ligated fore-and midgut preparations.

4 Summary

Structure-activity studies employing restricted-conformation analogs have led to a greater understanding of the chemical and conformational aspects of the interaction of the IK and PK/PBAN neuropeptide classes with expressed arthropod receptors.

In the process, several turn-mimic motifs have been identified as scaffolds for the development of mimetic agonist and antagonist neuropeptide analogs with enhanced biostability. These include the tetrazole and Apy *cis*Pro-mimetic motifs for the IK family and (*E*)-alkene and dihydroimidazoline *trans*Pro-mimetic motifs for the PK/PBAN class. Biostable mimetic analogs of both neuropeptide families have been shown to match or exceed the in vitro and in vivo activity, disrupt normal IK or PK/PBAN neuropeptide-regulated physiological processes, feature enhanced selectivity for particular physiological processes and/or species specificity, and in some cases result in increased mortality in insects. A 'magic bullet' C-terminal aldehyde IK analog selectively targets housefly Malpighian tubules, the major organ of diuresis in insects, and leads to marked inhibition of urine release. An agent capable of selective depression of fluid secretion would be expected to allow pesticides to achieve higher concentrations in the hemolymph; and, in turn, likely reduce the amount of toxin required to kill an insect.

While neuropeptides are not generally designed for penetration of the outside cuticle or the gut wall in large quantities, enhancement of bioavailability has been demonstrated in the PK/PBAN neuropeptide class. Amphiphilic agonist PK/PBAN analogs have shown an ability to efficiently penetrate in vitro preparations of insect cuticle and foregut, as well as demonstrate potent activity in in vivo pheromonotopic bioassays when administered via topical or oral routes. An amphiphilic PK/PBAN analog that features a pure, selective antagonist profile in a heliothine pheromonotropic bioassay demonstrates an ability to penetrate in vitro preparations of heliothine cuticle in quantities that have been shown to inhibit the pheromonotropic activity of PBAN by 84%.

In conclusion, the studies presented here have led to the identification of interesting tools for arthropod endocrinologists and promising mimetic analog leads that when either delivered in isolation, or possibly in concert with mimetic analogs of other neuropeptide classes that regulate different critical physiological processes, may be effective in managing arthropod pests.

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Rational Design of Insect Control Agents: The PK/PBAN Family as a Study Case*

Miriam Altstein and Aliza Hariton

1 Introduction

The success of modern agriculture in developing and maintaining high-yield crops depends strongly on controlling insect pests by means of heavy use of insecticides, and at present organo-synthetic chemical insecticides remain the main weapon in this armory. However, in recent decades, uncontrolled application of chemical insecticides has led to acquisition of resistance by insects, has contaminated the environment with toxic residues that endanger humans and other life forms, and has disrupted the ecological balance in and around cultivated fields. The growing concern regarding the toxic effects of insecticides has led to the implementation of strict regulations in the Western World, and these are being adopted by other countries too. These regulations limit the application of some organo-chemical insecticides and ban continued use of the more toxic ones.

The strategic approach that characterizes worldwide R&D efforts is based on identification and development of novel families of non-toxic, insect-specific compounds that eventually could replace the organo-synthetic chemicals. In seeking a new class of non-toxic insecticides that eventually could replace the existing toxic organo-chemical compounds and overcome the limitations associated with the existing bio-insecticides, entomological studies are focused on the search for targets and compounds that could form a basis for the development of highly effective, selective and environmentally friendly insect control agents and/or insecticides, and emerge as a new mainstream product of the insecticide industry.

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1.1 Insect Neuropeptides as Control Agents/Insecticides

Insect neuropeptides (Nps) are prime targets in the search for novel insecticides, since they regulate many physiological and behavioral processes during development, reproduction and senescence of insects. Their blockers (antagonists) may disrupt and interfere with the normal growth, development and behavior of insects and can yield, therefore, receptor-selective, insect-specific insecticides. Such antagonists would be derived from and resemble the natural peptides, but would have to be of a peptidomimetic nature. The chemical nature of Nps enables them to be used as the basis for the design of a generic group of insect-specific and non-toxic insecticides – an approach that has been applied to human Nps as a novel direction in the drug industry. The major roles Nps play in the physiology of organisms, and their high potential for practical applications, have stimulated active interest in Np studies in general, and in those of insects in particular.

1.2 Insect Nps: Historic Perspective

Since the early 1980s, many insect Nps have been identified, the basic principles of their action (e.g., biosynthesis, processing, release, transport, activation of the target cell, and degradation) have been discovered, and their roles in the physiology of organisms have been determined by means of genome sequencing, peptidomics, gene micro-arrays, receptor characterization, and targeted gene interference, all applied in association with physiological, electrophysiological and behavioral analyses. For review see (Altstein and Nässel 2007; Gäde and Marco 2006; Hauser et al. 2006; Hewes and Taghert 2001; Nassel and Homberg 2006).

The first insect Nps were identified (prior to the genomic/proteomic era) by the classical techniques of isolation and Edman degradation sequencing, and their putative functions were tested in a variety of in vivo and in vitro bioassays. This approach was applied mainly to functionally identified Nps. Later, Np primary sequences were predicted on the basis of their cloned genes. In parallel, localization studies were carried out using immunohistochemical and in situ hybridization techniques and Np receptors, obtained from fractionated cell membranes were subjected to specific binding assays. These studies led to the discovery of a few tens of Nps and less than five receptors before the late 1990.

In the past few years, several major advances in research on insect Nps have enhanced our familiarization with their structures and functions. One of the most important of these advances was the sequencing of several insect genomes (*Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori*, *Anopheles gambiae* and *Aedes aegypti*), which yielded information about Np precursor genes, on the basis of which many new Nps were identified. About 30–40 genes encoding Np precursors have been predicted in each species, and a total of over 150 insect Nps have been characterized, most of which were isolated from the fruit fly *D. melanogaster*, cockroaches (e.g., *Leucophaea maderae, Periplaneta americana*), locusts (e.g., *Locusta migratoria* and *Schistocerca gregaria*), various kinds of

moths (e.g., *Manduca sexta*, *B. mori* and various Heliothinae species), and from mosquitoes (*An. gambiae* and *Ae. aegypti*). The great majority of these Nps were insect-specific.

Genome sequencing also accelerated identification of Np G-protein coupled receptors (GPCRs). Within a few years 48 Np receptor genes, which constitute the vast majority, perhaps all, of the peptide GPCRs encoded by the *D. melanogaster* genome have been predicted and, to date, nearly 30 GPCRs in this fly have been characterized with respect to their preferred peptide ligands. Peptides from other insects have also been identified, and knowledge related to *D. melanogaster* GPCRs was used to annotate 35 Np GPCRs in the recently sequenced genome of the honey bee *A. mellifera*. The genomic information also extended our knowledge regarding Np prohormone-processing enzymes and several other genes that encode proteins involved in the regulation of cell-specific Np expression. This knowledge greatly extended our potential for gaining better and deeper insight into Np functions in insects. More recently, mass spectrometry (MS) followed by sequence determination was employed for Np identification and localization. This method enabled analysis of Nps (in fentomole quantities or less) in the hemolymph, in tissue extracts and single cells by matrix-associated laser desorption/ionization (MALDI) MS, and examination of neuronal homogenates by electrospray ionization (ESI) MS. A variety of MS-based approaches (direct tissue MS or MS in combination with laser capture micro-dissection) have accelerated the development of rapid and accurate identification of predicted Nps in small amounts of tissues, and even in single neurons, and have led to both identification and localization of hundreds of new Nps in a single step.

This novel information, together with that obtained by means of more traditional approaches, opened many opportunities for the use of "rational design" approaches to the discovery of receptor-selective Np agonists and antagonists. The resulting discoveries can further advance our understanding of the mode of action of insect Nps and may serve as potential insecticide/insect management agents. Many recently published, detailed, well documented reviews of insect Nps address all the above issues (Altstein and Nässel 2007; De Loof 2008; Gäde and Marco 2006; Hauser et al. 2006; Hewes and Taghert 2001; Hummon et al. 2006; Li et al. 2008; Nassel and Homberg 2006; Predel et al. 2007; Southey et al. 2008; Veenstra 2000).

Although a vast amount of knowledge on insect Nps and their receptors has been accumulated in recent years, and in spite of the great potential of Np antagonists as insecticides, not a single Np-based antagonist insect control agent has been made commercially available up to now, and the application of Np antagonists as pest-control compounds does not exist. There are many reasons for this. First and foremost, no methodology has yet emerged for the conversion of an agonist to an antagonist and then, into an insecticide or insect-control agent. Second, even when such antagonists have been made, the poor metabolic stability and the low bioavailability of these peptides – which stems partly from their difficulty in crossing membrane barriers and, especially, the insect cuticle – would hinder their practical application as insect-control agents.

The very same problems have severely limited the development of therapeutic drugs based on human Nps, therefore, recent studies in medicinal chemistry have

aimed to develop strategies to overcome these problems. These studies have yielded impressive progress in rational design, modification, and synthesis of chemicals, and have led to the development of several strategies for producing modified peptides and mimetic agonists and antagonists with reduced susceptibility to proteolysis and enhanced bioavailability. These new approaches, have improved the chances of obtaining useful drugs that are structurally related to the parent peptides, and have led to the development of a few peptide-based drugs for several human diseases (Hökfelt et al. 2003). Notwithstanding this progress the technology has not yet been optimized and most of the non-peptide ligands for Np receptors were found by random screening of large chemical libraries and were then chemically adapted to impart the desired selective and pharmacokinetic properties. There is still very limited information available on the conversion of an agonist to an antagonist, and new approaches are still being sought, to the generation of such antagonists and to the modification of native peptides to create stable and bioavailable peptidomimetic compounds with the desired features. For detailed reviews on conversion of Nps to drugs see (Adessi and Soto 2002; Hökfelt et al. 2003).

In the past few years, we have devised a novel integrated approach, designated Insect Np Antagonist Insecticide, INAI – based on rational design – which overcomes some of the limitations associated with the application of Nps as insect control agents and paves the way toward the development of Np antagonists. The method was applied to the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family. In this chapter we summarize the INAI approach, review the current knowledge of the PK/PBAN family of peptides, and describe the implementation of this approach to the generation of highly potent, selective and highly bioavailable PK/PBAN antagonists.

2 INAI Approach to the Development of Novel Insect Np-Based Antagonist Insecticides

2.1 The INAI Approach

The INAI approach comprises two main steps:

1. Conversion of an agonist into an antagonist;
2. Conversion of the antagonist into an insect-control agent prototype.

Conversion of an agonist into an antagonist requires identification of a lead antagonist. The first steps towards this comprise:

1. Identification of the minimal sequence that constitutes the active site of the agonistic Np.
2. Modification of the active sequence identified in step 1.

3. Determination of the bioactivity and structure-activity relationship (SAR) of the peptides that are generated in step 2.
4. Identification of a linear lead antagonist (among the modified peptides examined in step 3).

The rationale behind these steps derives partly from practices developed in the field of medicinal chemistry, in attempting to convert vertebrate Np agonists to antagonists. Many vertebrate Np antagonists were discovered by simple modifications of their primary sequences (Cody et al. 1995; Collins et al. 1996; Coy et al. 1989; Folkers et al. 1984; Heinzerian et al. 1987; Hruby et al. 1990, 1992; Llinares et al. 1999; Maretto et al. 1998; Piercey et al. 1981; Rees et al. 1974; Rhaleb et al. 1991; Rodriguez et al. 1987; Rosell et al. 1983; Sawyer et al. 1981; Vale et al. 1972; Vavrek and Stewart 1985). In order to minimize the number of possible combinations to be examined and, therefore, the number of peptides that require subsequent bioactivity evaluation, it is necessary to identify the shortest possible active sequence in the native Np. Once that is known, sequential modifications (mostly based on substitution by D-Phe or D-Trp) are made to it, and the resulting small linear libraries are tested for bioactivity.

Once a linear lead antagonist has been made available it is necessary to improve its characteristics and to optimize it in accordance with its intended applications. In the present case, this involves generating a more efficient, highly potent antagonist that is metabolically stable, and that exhibits receptor-selectivity and bioavailability. Line1ar peptides cannot serve such a purpose because they are highly susceptible to proteolytic degradation, they lack sufficient bioavailability, and their high conformational flexibility leads to lack of selectivity. An effective approach to overcoming these limitations is through the introduction of conformational constraint into the linear lead peptides, which leads to higher resistance to proteolytic degradation, slower equilibrium rate and reduces the flexibility of the molecule.

Conformational constraint can be imposed on peptides by various means (for reviews see (Hruby et al. 1990; Giannis 1993; Goodman 1995), of which cyclization is one of the commonest and most attractive (Kessler et al. 1986). Conformational constraint: (i) imparts high selectivity by restricting the conformational space of the peptide to a conformation that mediates one function and excluding those that mediate other functions; (ii) enhances metabolic stability by excluding conformations that are recognized by degrading enzymes and thereby preventing enzymatic degradation; (iii) increases biological activity, because of the much slower equilibrium between the conformations; and (iv) improves bioavailability by reducing polarity. However, these advantages are gained only when the conformational space of the cyclic peptide overlaps the bioactive conformation.

Following the above steps, generation of an optimized antagonist involves:

5. Conversion of the linear peptide(s) to conformationally constrained molecule(s).
6. Determination of the bioactivity, SAR, selectivity, stability and bioavailability of the molecule(s) obtained in step 5.

7. Selection of a conformationally constrained antagonist with the required properties (on the basis of step 6.)

Once an improved antagonist has been identified, it must be developed into a prototype control agent. This requires the introduction of features that are normally found in commercial insecticides, i.e.: the antagonist must be converted into a low-molecular-weight compound, and able to readily penetrate through the insect cuticle and/or gut; the resulting compounds must have high environmental stability; and, especially, must be cost effective in production. The generation of small, cost-effective molecules with the above characteristics necessitates conversion of the peptide antagonist into a non-peptide small molecule (SM), and this, in turn, requires identification of the biophores that are essential for the antagonistic activity, and their incorporation into novel, scaffold SM libraries.

Biophores can be identified through detailed conformational and computational analysis of the active (agonistic or antagonistic) and inactive compounds (by means of nuclear magnetic resonance, NMR and X-Ray crystallography) (Gilon et al. 1998b; Grdadolnik et al. 1994; Kasher et al. 1999; Saulitis et al. 1992). Further SAR information can be obtained by examination of the pharmacokinetic relationships of the native and the conformationally constrained peptides with native and mutated Np receptors. This requires availability of both a cloned receptor that can be mutated, and of a functional binding assay that can be developed into a high-throughput assay (HTA) for the fast screening of the non-peptide small-molecule (SM) libraries and selection of the biologically active compounds. Once biophores have been identified, SM libraries can be generated and screened for insect control agent prototypes. This final stage involves the following steps:

8. Determination of the antagonistic conformational requirements of the peptides identified in step 7.
9. Determination of the SAR of the native and of the mutated Np receptor, and the mode of its interaction with the native and the conformationally constrained molecules.
10. Design of non-peptide SM libraries on the basis of the information gathered in steps 8 and 9.
11. Synthesis of non-peptidic SM combinatorial libraries on the basis of step 10.
12. High throughput screening (HTS) of the resulting SM libraries for bioactivity.
13. Identification of bioactive non-peptidic SMs.

Once such non-peptidic SMs have been obtained, their in vivo bioactivity, bioavailability and stability must be re-evaluated. The most potent, stable and bioavailable of them can then be subjected to appropriate formulation and preliminary toxicology evaluation and to testing in preliminary field trials. Although this strategic approach has been used in the development of some vertebrate Np agonists and antagonist-based drugs (for reviews see (Adessi and Soto 2002; Hökfelt et al. 2003), it has not, so far, been used in relation to insect pest control, and still needs to be developed in this context.

An overall multidisciplinary strategy is necessary in designing an insect control agent prototype based on the INAI approach, and its success depends on the fulfillment of several preliminary requirements:

- Knowledge of the primary sequence of the target Np, on which step 1 depends.
- Availability of in vivo or in vitro bioassays for screening the libraries and for selection of the most potent compounds, on which steps 3, 6 and 13 depend.
- Knowledge and know-how in molecular design and combinatorial chemistry, on which steps 5 and 10 depend.
- Availability of or access to an advanced chemistry facility for synthesis of linear peptides and of conformationally constrained combinatorial and SM combinatorial libraries, on which steps 2, 5 and 11 depend.
- Availability of or access to specialized facilities for determination of the molecular structural conformations on which step 8 depends.
- Availability of cloned native and mutated receptors that are required for steps 9 and 12.
- Availability of a receptor-based HTS assays (HTSA) for fast screening of SM libraries and selection of the biologically active compounds, on which step 13 depends.

We applied the above strategy to the PK/PBAN family of insect Nps, and thereby identified several highly potent conformationally constrained, bioavailable antagonists, both selective and non-selective. There follows a short summary of the PK/PBAN family and the steps that led to the identification of these antagonists.

2.2 PBAN and PK/PBAN Family

2.2.1 Isolation and Identification of PBAN and Other Pheromonotropic Peptides

PBAN was first reported by Raina and Klun (Raina and Klun 1984) as the Np that regulates sex pheromone production in female moths (*Helicoverpa zea)*. In 1989 Raina et al. (Raina et al. 1989) isolated and characterized PBAN from *H. zea*, as a 33-amino-acid, C-terminally amidated Np, which was designated Hez-PBAN (for nomenclature see (Raina and Gade 1988)). Since 1989 the primary sequence of 19 PBAN molecules has been determined in many other moth species, by several methods: sequencing of the purified Np; from cloned cDNA; and from gene sequence or genomic data (for review see (Altstein 2004) and Table 1). PBAN molecules were also found in insects belonging to orders other than Lepidoptera (e.g., in *A. gambiae* and in *A. aegypti*). Those peptides were not cloned but were predicted from the genome sequences of these insects (Holt et al. 2002; Nene et al. 2007). Comparison among the primary sequences of the above molecules revealed that the peptides share differing degrees of homology ((Jing et al. 2007; Wei et al.

2008), and Table 1). Regardless of their length and degree of homology all of them share a common C-terminal pentapeptide sequence FSPRLa.

Further studies based on molecular cloning have revealed that the sequence of the cDNA encoding the PBAN pre-prohormone also encodes four other peptides, in addition to PBAN, and that they are separated by prohormone processing sequences, (single or pairs of the basic amino acids Lys or Arg, and Gly, for amidation) and that they are cleaved into four peptides which have the PBAN consensus sequence FXPRLa (X = S, T, G or V): diapause hormone, (DH), two subesophageal ganglion Nps (SGNP β and γ), and one FXPKLa peptide (α-SGNP) (see Table 1). As the number of available sequences has increased, the X in the consensus sequence has been extended to include X = I (as in *B. mori* and *B. mandarina*-β-SGNP) and K (as in *D. melanogaster* PK-2, see Table 1). Recent studies revealed additional variations in this family of peptides, such as the occurrence of a bioactive PBAN homolog, (in *Ascotis selenaria cretacea*) that was found to be connected to β-SGNP by a GR sequence (Kawai et al. 2007). The gene that encodes these five peptides was termed the DH-PBAN gene (Sato et al. 1993; Xu et al. 1995), and it was found to be expressed only in seven pairs of neurosecretory cells, termed the DH-PBAN-producing neurosecretory cells (DHPGs) in the subesophageal ganglion (SOG) (Sato et al. 1993, 1994). Examples of the DH-PBAN peptides from a few moth species are listed in Table 1.

Advances in insect Np studies revealed that other peptides, which do not originate from the DH-PBAN prohormone, also have the same signature sequence. They include the PKs (Lem-PK, Lom-PK-I and Lom-PK-II); the myotropins (Lom-MT-I to IV), which are myotropic peptides isolated from the cockroach *L. maderae* and the migratory locust, *L. migratoria* (Nachman and Holman 1991; Nachman et al. 1986; Schoofs et al. 1991, 1993b); and a myotropic peptide from *S. gregaria* (Scg-MT-1) (Veelaert et al. 1997). Additional peptides that were found to share the same consensus sequence are: pheromonotropin (Pss-PT) an 18-amino-acid peptide isolated from *Pseudaletia (Mythimna) separata* (Matsumoto et al. 1992) that shares high homology with β-SGNP; peptides from *D. melanogaster* – Drm-PK-1 (Kean et al. 2002) and Drm-PK-2 (Meng et al. 2002) – each of which is encoded by a different gene (Olsen et al. 2007): and two PK peptides from *P. americana* – Pea-PK-5 and Pea-PK-6 – (Predel and Eckert 2000; Predel et al. 1999). For details of the amino acid sequences of some of these peptides see Table 1. All peptides sharing the above sequences were grouped into one family, which was designated the FXPR(K)La family or the PK/PBAN family, named after PBAN and Lem-PK which was the first member of this family to be identified, (Holman et al. 1986), and had myostimulatory ("kinin"-like) action and a pGlu residue at its N-terminus, (and thus termed pyro-kinin). Recently, a few additional peptides sharing just the PRLa C-terminal sequence were added to the family; they are not listed in Table 1 and for further information on their origins and sequences the reader is referred to Rafaeli and Jurenka (Rafaeli and Jurenka 2003).

The discovery of PBAN and the other PK/PBAN peptides stimulated many studies on isolation, identification and quantification of new molecules, their gene expression and localization in various insects under various conditions, their expression during

Table 1 Amino acid sequence of PBAN and other peptides of the PK/PBAN family

Code name	Insect species	Amino acid sequence	Reference
Hez-PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRLa**	(Raina et al. 1989)
Bom-PBAN-I	*Bombyx mori*	LSEDMPATPADQEMYQPDPEEMESRTRY**FSPRLa**	(Kitamura et al. 1989)
Bom-PBAN-II	*Bombyx mori*	RLSEDMPATPADQEMYQPDPEEMESRTRY**FSPRLa**	(Kitamura et al. 1989)
Bma-PBAN	*Bombyx mandarina*	LSEDMPATPADQEIYQPDPEVMESRTRY**FSPRLa**	(Xu et al. 1999)
Lyd-PBAN	*Lymantria dispar*	LADDMPATMADQEVYRPEPEQIDSRNKY**FSPRLa**	(Masler et al. 1994)
Has-PBAN	*Helicoverpa assulta*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRLa**	(Choi et al. 1998)
Agi-PBAN	*Agrotis ipsilon*	LADDTPATPADQEMYRPDPEQIDSRTKY**FSPRLa**	(Duportets et al. 1999)
Mab-PBAN	*Mamestra brassicae*	LADDMPATPADQEMYRPDPEQIDSRTKY**FSPRLa**	(Jacquin-Joly and Burnet 1998)
Spl-PBAN	*Spodoptera littoralis*	LADDMPATPADQELYRPDPDQIDSRTKY**FSPRLa**	(Iglesias and Marco 2002)
Plx-PBAN	*Plutella xylostella*	RLKDSGLAPPDEYRTPELLDARAQY**FSPRLa**	(Lee and Boo 2005)
Har-PBAN	*Helicoverpa armigera*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRLa**	(Zhang et al. 2004c)
Hev-PBAN	*Heliothis virescens*	ADDMPATPADQEMYRQDPEQIDSRRTKY**FSPRLa**	(Xu and Denlinger 2003)
Spe-PBAN	*Spodoptera exigua*	LSDDMPATPADQELYRPDPDQIDSRTKY**FSPRLa**	(Xu et al. 2007)
Anp-PBAN	*Antheraea pernyi*	LSDDMPATPKDQEMYHQDPEQVDTRTRY**FSPRLa**	(Wei et al. 2008)
Scr-PBAN	*Samia cynthia ricini*	LTEDMPATPTDQEMFDQDPEQIDTRTRY**FSPRLa**	(Wei et al. 2004)
Ado-PBAN	*Adoxophyes* sp	QSEAVTSSDEQVYRQDMSPVDGRLKY**FSPRLa**	(Choi et al. 2004)
Mas-PBAN	*Manduca sexta*	ISEDMPATPSDQEYPMYHPDPEQIDTRTRY**FSPRLa**	(Xu and Denlinger 2004)
Assc-PBAN	*Ascotis selenaria cretacea*	QLVDDVPQRQQIEEDRLGSRTRF**FSPRLa**	(Kawai et al. 2007)
Cla-PBAN	*Clostera anastomosis*	LADDMPATPSDQEYYRQDPEQIDSRSNY**FSPRLa**	(Jing et al. 2007)
Ort-PBAN	*Orgyia thyellina*	LSDDMPATPPDQEYYRPDPEQIDSRTKY**FSPRLa**	Unpublished[a]
Bom-DH	*Bombyx mori*	TDMKDESDRGAHSERGALC**FGPRLa**	(Imai et al. 1991)
Hez-DH	*Helicoverpa zea*	NDVKDGAASGAHSDRLGLW**FGPRLa**	(Ma et al. 1994)
Has-DH	*Helicoverpa assulta*	NDVKDGAASGAHSDRLGLW**FGPRLa**	(Choi et al. 1998)
Har-DH	*Helicoverpa armigera*	NDVKDGAASGAHSDRLGLW**FGPRLa**	(Zhang et al. 2005)
Agi-DH	*Agrotis ipsilon*	NDVKDGGADRAHSDRGGMW**FGPRLa**	(Duportets et al. 1999)
Spl-DH	*Spodoptera littoralis*	NEIKDGGSDRGAHSDRAGLW**FGPRLa**	(Iglesias and Marco 2002)
Lom-PK-I	*Locusta migratoria*	pEDSGDGWPQQP**FVPRLa**	(Schoofs et al. 1991)
Lom-PK-II	*Locusta migratoria*	pESVPT**FTPRLa**	(Schoofs et al. 1993a)

(continued)

Table 1 (continued)

Code name	Insect species	Amino acid sequence	Reference
Lem-PK	*Leucophaea maderae*	pETS**FTPRLa**	(Holman et al. 1986)
Pea-PK-5	*Periplaneta americana*	GGGGSGETSGMW**FGPRLa**	(Predel et al. 1999)
Pea-PK-6	*Periplaneta americana*	SESEVPGMW**FGPRLa**	(Predel and Eckert 2000)
Drm-PK-1	*Drosophila melanogaster*	TGPSASSGLW**FGPRLa**	(Choi et al. 2001)
Drm-PK-2[b]	*Drosophila melanogaster*	SVP**FKPRLa**	(Choi et al. 2001)
Lom-MT-I	*Locusta migratoria*	GAVPAAQ**FSPRLa**	(Schoofs et al. 1990a)
Lom-MT-II	*Locusta migratoria*	EGD**FTPRLa**	(Schoofs et al. 1990b)
Lom-MT-III	*Locusta migratoria*	RQQP**FVPRLa**	(Schoofs et al. 1990b)
Lom-MT-VI	*Locusta migratoria*	RLHQNGMP**FSPRLa**	(Schoofs et al. 1990b)
Scg-MT-1	*Schistocerca gregaria*	GAAPAAQ**FSPRLa**	(Veelaert et al. 1997)
Pss-PT	*Pseudaletia separata*	KLSYDDKVFENVE**FTPRLa**	(Matsumoto et al. 1992)
Hez-β-SGNP	*Helicoverpa zea*	SLAYDDKSFENVE**FTPRLa**	(Ma et al. 1994)
Has-β-SGNP	*Helicoverpa assulta*	SLAYDDKSFENVE**FTPRLa**	(Choi et al. 1998)
Har- β-SGNP	*Helicoverpa armigera*	SLAYDDKSFENVE**FTPRLa**	(Zhang et al. 2005)
Agi-β-SGNP	*Agrotis ipsilon*	SLSYEDKMFDNVE**FTPRLa**	(Duportets et al. 1999)
Mab-β-SGNP	*Mamestra brassicae*	SLAYDDKVFENVE**FTPRLa**	(Jacquin-Joly and Burnet 1998)
Spl-β-SGNP	*Spodoptera littoralis*	SLAYDDKVFENVE**FTPRLa**	(Iglesias and Marco 2002)
Bom-β-SGNP[b]	*Bombyx mori*	SVAKPQTHESLE**FIPRLa**	(Kawano et al. 1992)
Hez-γ-SGNP	*Helicoverpa zea*	TMN**FSPRLa**	(Ma et al. 1994)
Has-γ-SGNP	*Helicoverpa assulta*	TMN**FSPRLa**	(Choi et al. 1998)
Har- γ-SGNP	*Helicoverpa armigera*	TMN**FSPRLa**	(Zhang et al. 2005)
Agi-γ-SGNP	*Agrotis ipsilon*	TMN**FSPRLa**	(Duportets et al. 1999)
Mab-γ-SGNP	*Mamestra brassicae*	TMN**FSPRLa**	(Jacquin-Joly and Burnet 1998)
Spl-γ-SGNP	*Spodoptera littoralis*	TMN**FSPRLa**	(Iglesias and Marco 2002)
Bom-γ-SGNP	*Bombyx mori*	TMS**FSPRLa**	(Kawano et al. 1992)

Bold letters indicate conserved amino acid sequences. DH – diapause hormone; MRCH – melanization and reddish coloration hormone; PBAN – pheromone biosynthesis activating neuropeptide; PT – pheromonotropin; PK – pyrokinin; MT – myotropin; SGNP– sub–esophageal ganglion neuropeptide.
The sequence of α-SGNP is the same in all insects (VIFTPKLa). Additional sequences of DH, α-, β- and γ-SGNP can be found in (Jing et al. 2007).
[a] GenBank accession No. AB259122.
[b] A member of the family but with the difference that X = Lys or Ilu.

embryonic and post-embryonic development stages, bioactivity, modes of action (e.g., cellular activity, second-messenger mediation, and their effects on the enzymatic pathway involved in sex pheromone production), and characterization of the receptor(s) that mediate their functions. These studies are reviewed in (Rafaeli and Jurenka 2003). Phylogenetic trees, based on the open reading frame (ORF) sequences of known DH-PBAN genes were also constructed and were matched with taxonomic characteristics of the insects (Jing et al. 2007; Kawai et al. 2007). More advanced studies focus on gene organization, identification of transcription factors that activate the DH-PBAN gene in different moth species (e.g., *B. mori* and *Helicoverpa armigera*) and the role of this activation in the differentiation of neuroendocrine cells (Hong et al. 2006; Shiomi et al. 2007; Zhang et al. 2004a, 2005). Earlier studies, some of which are still being evaluated, addressed transport routes, release sites, target organs, and degradation of the peptides; these are reviewed in (Altstein 2004; Rafaeli and Jurenka 2003). In the present review we briefly summarize our studies and those of other workers on a few topics in this field: (i) biological activity of the PK/PBAN family; (ii) structure-activity relationship; and (iii) characterization of the PK/PBAN receptor. Additional topics were covered in three comprehensive recent reviews (Altstein 2004; Rafaeli 2002, 2005; Rafaeli and Jurenka 2003), to which the reader is referred for further information.

2.2.2 Biological Activity of the PK/PBAN Family

The PK/PBAN family of peptides is a ubiquitous multifunctional family that plays a major physiological role in regulating a wide range of developmental processes in insects: pupariation (Nachman et al. 1997); diapause (Imai et al. 1991; Nachman et al. 1993; Sun et al. 2005; Xu and Denlinger 2003; Zhang et al. 2004b; Zhao et al. 2004); cuticular melanization (Altstein et al. 1996; Matsumoto et al. 1990); feeding, i.e., gut muscle contraction (Nachman et al. 1986; Schoofs et al. 1991); and mating behavior, i.e., sex pheromone production (Raina and Klun 1984; Altstein 2004).

The first function that was discovered to be mediated by a member of the PK/PBAN family – a function that also provided the name of one of its peptides, PBAN – was stimulation of sex pheromone biosynthesis in female moths (Raina and Klun 1984). Now, after nearly 25 years of research, it is well established that PBAN and other peptides of the FXPR/KLa family regulate sex pheromone production in many moth species. PBAN stimulates sex pheromone production in insects that synthesize type I pheromones (which are composed of unsaturated primary alcohols with a straight C_{10}–C_{18} chain and their functional derivatives are produced from acetyl-CoA by de novo synthesis in the pheromone gland), and type II pheromones (which are composed of polyalkenes with a C_{17}–C_{23} chain and their epoxy derivatives use linolenic and linoleic acids that originate in plants transported to the pheromone gland *via* the hemolymph after association with lipophorin, and only the epoxidation proceeds in the pheromone gland) (Kawai et al. 2007).

As studies on the bioactivity of the PK/PBAN family of peptides progressed, it became apparent that the main roles of this group of Nps are in development

processes, such as metamorphosis and diapause. Studies since the late 1990s have found that members of the family induce embryonic diapause, terminate pupal diapause, accelerate pupariation and regulate the final stage in molting, i.e., cuticular melanization. Studies on the role of the FXPR/KLa peptides have indicated that they induce embryonic diapause in *B. mori*, through a mechanism that is yet not fully understood; it could be that the peptides act on the developing oocytes during pupal-adult development, and thereby induce diapause in the next generation (Yamashita 1996); or it could be that the hormone targets the developing ovaries, either directly, or indirectly through the DH receptors of the prothoracic gland (PG) during the later stages of the 5th instar larva and the early pupal stages, thereby regulating production of ecdysteroid hormones that might influence post-embryonic development (Watanabe et al. 2007).

These peptides are also involved in termination of pupal diapause, through their activation of ecdysteroidogenesis in the PG, in the course of larval-pupal development (Zhang et al. 2004b). Whether this activation is direct or indirect, e.g., via another factor, is also unknown so far. Recent studies suggest that DH might stimulate the PG by up-regulating expression of a DBI/ACBP gene and thereby causing an increase of ecdysteroids, which would result in diapause termination (Liu et al. 2005). Termination of pupal diapause has been demonstrated in a few *Helicoverpa/Heliothis* species (*H. virescens* (Xu and Denlinger 2003), *H. armigera* (Zhang et al. 2004c) and *H. assulta* (Zhao et al. 2004). PK/PBAN Nps also accelerated pupariation in wandering larvae of the fleshfly (*Sarcophaga bullata*). Studies of *S. bullata* revealed that Lem-PK accelerated the switch from wandering behavior to immobilization/retraction behavior, and subsequently affected puparial tanning. By accelerating both aspects of puparium formation, Lem-PK mimicked the effects of the pupariation factors, in exerting an effect on the central motor neurons (Nachman et al. 1997).

PK/PBAN peptides have been reported to be involved in cuticular melanization – the last step in the molting processes – in many noctuid moths. The possible involvement of this family of Nps in the control of larval cuticular melanization was first observed in the common army worm, *Leucania separata*, by Ogura and co-workers (Ogura 1975; Ogura and Saito 1972; Suzuki et al. 1976). The hormone, which was termed melanization and reddish coloration hormone (MRCH), was later found to be identical with Bom-PBAN, which initiates the melanization of the integument of many moth larvae, (Ben-Aziz et al. 2005, 2006; Hiruma et al. 1984; Matsumoto et al. 1981; Morita et al. 1988).

PBAN neurosecretory cells, which express all five DH-PBAN gene peptides, were recently associated with another developmental function: development and maturation of the flight motor system of holometabolous insects. Studies of *B. mori* revealed correlation between the rhythmic electrical activity of immature dorsal longitudinal flight muscles, on the one hand, and the bursting activity of PK/PBAN neurosecretory cells, on the other hand, which suggested that the development and maturation of flight muscles during pupal-adult development is regulated by PK/PBAN peptides (Kamimoto et al. 2006).

Most studies of the various functions of this peptide family were carried out moths, although a few were performed with other insects, e.g., gut contraction stud-

ies in cockroaches and locusts; and pupariation in flies. Studies performed in several laboratories, including ours, have shown that most functions can be stimulated by more than one member of this peptide family, and that the peptides are not species-specific; this subject is reviewed in (Gade 1997; Rafaeli 2002; Rafaeli and Jurenka 2003; Kamimoto et al. 2006; Ma et al. 1996). As the number of DH-PBAN-sequenced genes has increased, particular attention recently has been focused on the multifunctionality of peptides derived from the DH-PBAN-gene, i.e., DH, PBAN, α, β and γ-SGNP, as demonstrated in their pheromonotropic and diapause inducing (in embryos) and terminating (in pupae) activities. It has been found that all peptides derived from the DH-PBAN gene, except for α-SGNP, can activate pheromone biosynthesis and embryonic diapause; α-SGNP activates only on the former and not the latter (Ma et al. 1996; Sato et al. 1993).

The involvement of PK/PBAN Nps in the above functions was demonstrated in a variety of in vivo and in vitro bioassays, e.g., pheromonotropic, melanotropic, diapause, pupariation and myotropic assays, that were developed and optimized in several laboratories (Altstein et al. 1996; Gazit et al. 1990; Holman et al. 1991; Matsumoto et al. 1990; Nachman et al. 1993, 1997; Raina and Klun 1984; Schoofs et al. 1991, 1993b; Zdarek et al. 1998). All these assays, apart from the myotropic one, were carried out in vivo.

2.2.3 Structure Activity Relationship of the PK/PBAN Family

Identification of the amino acid sequences of PBAN and of other members of the PK/PBAN family made possible detailed SAR studies that used synthetic peptides derived from their sequences. Early studies on a variety of moth species have shown that the C-terminal region of the Np is essential for the pheromonotropic activity, and that within this region, the signature pentapeptide (FXPRLa; X = S) represents the minimal sequence required for induction of pheromonotropic activity, although in most cases its activity was lower than that of full-length PBAN. The amide group and the X position were shown to be of major importance (Altstein et al. 1993, 1995, 1996, 1997; Kochansky et al. 1997; Nachman and Holman 1991; Nagasawa et al. 1994; Raina and Kempe 1990, 1992). The N-terminal part of the molecule was much less important for the onset of pheromonotropic activity (Altstein et al. 1993; Raina and Kempe 1990; Kitamura et al. 1989; Kuniyoshi et al. 1992b). A variety of Hez-PBAN-derived fragments in a range of doses applied to *Heliothis peltigera* at various times post-injection were used to demonstrate, that peptides lacking 12 or even 16 amino acids from their N-terminus were as active as the full-length PBAN, and that a C-terminal-derived hexapeptide that contained the signature sequence (YFXPRLa) was capable of stimulating a similar level of sex pheromone production to PBAN1–33NH_2 when its activity was analyzed after shorter post-injection intervals (Altstein et al. 1995); this indicated that the hexapeptide might be the biologically active site of the Np.

Structure-function studies were also performed on other insect Nps that contain the PBAN pentapeptide C-terminal region, i.e., PKs, Bom-DH and Pss-PT. All of these

peptides showed pheromonotropic activity, and confirmed the importance of the C-terminal region in its onset (Abernathy et al. 1995; Fonagy et al. 1992; Kuniyoshi et al. 1992a; Nachman et al. 1993; Schoofs et al. 1993b).

Further evaluation of PK/PBAN SAR and conformational studies of the PK/PBAN active core (based on NMR and activity analysis of conformationally constraint analogs) hinted that a β-turn constitutes the active conformation of these peptides. In order to provide a more definitive evidence that the β-turn represents the active conformation for the PK/PBAN Np family and to try and identify the exact type of the β-turn (I, I′ or II), a PK/PBAN analog PK-Etz, incorporating an (*E*)-alkene *trans*Pro isosteric component has recently been evaluated by us in four diverse PK/PBAN bioassay systems. The conformationally constrained PK-Etz analog demonstrated activity equivalent to parent PK/PBAN peptides of equal length in all four PK/PBAN bioassays, and matched and/or approached the activity of peptides of natural length in three of them. The relatively potent agonist activity of PK-Etz provides strong evidence that a *trans*Pro type I β-turn represents an important conformational aspect of the PK/PBAN Nps during their interaction with receptors associated with a range of disparate PK/PBAN bioassays (Nachman et al. 2009d). A detailed description of these results is presented by R.J. Nachman in another chapter in this book.

The ability of a variety of peptides to stimulate sex pheromone production raised the question whether the pheromonotropic activity of the different peptides might be mediated by multiple PBAN receptors or receptor sub-types, or if all peptides mediate this activity by one receptor to which the C-terminal part of the PK/PBAN Nps binds. The multi-functionality of the PK/PBAN family in several different insect species raised similar questions as to whether the different functions are mediated by different receptors, located on different target organs, each activated by one or more of these Nps or whether all functions are mediated by the same receptor. Those and other questions led to an extensive study on the PK/PBAN receptors as indicated below.

2.2.4 PK/PBAN Target Organ and Receptors

A variety of techniques – biochemical, molecular, physiological, pharmacological and histochemical – were used to localize, isolate, clone and characterize the receptors of this family of Nps in various insects. The great majority of the studies focused on the receptors that mediate the pheromonotropic activity. Early studies had suggested that PBAN might act on a target other than the pheromone gland (see (Altstein 2004) and references therein), but since the late 1990s it became clear, beyond any doubt, that the pheromonotropic activity of PBAN is mediated via the PK/PBAN receptor in the pheromone gland.

A histochemical study (Altstein et al. 2003) of the pheromone gland cells of *H. peltigera* was the first direct indication of the presence of the PK/PBAN receptors in the pheromone gland; The study was carried out using two biotinylated photoaffinity (benzophenon substituted) PBAN ligands (a full-length PBAN1–33NH_2 molecule, BpaPBAN1–33NH_2, and a shorter fragment derived from its C-terminus, Arg^{27}-PBAN28–33NH_2, BpaPBAN28–33NH_2), and revealed the presence of the

receptor in columnar epithelial cells throughout the intersegmental membrane between, the eighth and the ninth abdominal segments, and also in the ventral and dorsal epithelial cells in the ninth abdominal segment. Staining revealed a polar pattern, with intense staining at the basal part of the epithelial cells. This polarity of the PBAN receptor most likely facilitates efficient contact with the hemolymph and the blood-borne hormones, e.g., PBAN, that stimulate sex pheromone production in these cells. A detailed summary of the histochemical study was presented by Altstein et al. (Altstein et al. 2003).

As in the case of other vertebrate and invertebrate Nps, the PK/PBAN Nps activate cellular processes via G-protein-coupled receptors (GPCRs), which are seven transmembrane domain proteins (7TM) that sense molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses. Many studies on insect Np GPCR cloning, including those of the PK/PBAN family were stimulated by the *Drosophila* Genome Project (Hewes and Taghert 2001), and by the completion of the *Drosophila* genome sequence in 2000 and of the *Anopheles* genome sequence in 2002 (Adams et al. 2000; Holt et al. 2002). In less than 5 years the cDNA of 12 PK/PBAN receptors from six insects (mainly moths) were cloned and another five were only annotated (Table 2). Most of the receptors were cloned in light of the assumption of Hewes and Taghert (Hewes and Taghert 2001) that the PK/PBAN receptor (PK/PBAN-R) may be homologous to the mammalian neuromedin U receptor (NmU-R), since their ligands – NmU and the PK/PBAN Nps – contain similar motifs at their C-terminus, i.e., FRPRNa and FSPRLa, respectively. Primers based on the consensus sequences of NmU and on sequences of genes CG8784 and CG8795, CG9918 and CG14575, which were annotated as coding for GPCRs in the *Drosophila* Genome Project, were designed and were successfully used to clone two *D. melanogaster* receptors (PK-2-1 and PK-2-2), two *Anopheles* receptors (PK-1 and PK-2), *D. melanogaster* PK-1 receptor (PK-1-R), and also a *B. mori* and an *H. zea* PBAN receptor (PBAN-R) (Table 2). A few receptors were cloned later on based on the already published PK/PBAN-R sequences (from *Spodoptera littoralis* and *H. armigera*, Table 2). Receptors were cloned from pheromone glands of adult female moths, from several different larval instars and from pupal ovaries. Most receptors were expressed in Chinese hamster ovary (CHO) or Sf-9 cell lines; one was expressed in *Xenopus oocytes* (*B. mori* pupal ovary), and one (*S. littoralis* larvae) in NIH3T3 and human embryonic kidney cell lines (HEK293). In all of the studies, other than the *B. mori* pupal ovary, expression was monitored by Ca influx; in the case of the *B. mori* pupal ovary receptor it was monitored by voltage clamping.

The receptors were characterized with respect to their gene structure, homology with other cloned PK/PBAN receptors, tissue distribution, and ability to bind FXPRLa or PRLa peptides. Phylogenetic trees were constructed in searching for orthologous receptors. All the cloned receptors were found to be GPCRs, and the pheromone gland receptors from *H. zea* (Choi et al. 2003), *H. virescens* (Kim et al. 2008) and *B. mori* (Hull et al. 2004) were found to share a significant homology. However, the homology between the various PBAN-Rs deviated most at the N-terminus and, in some cases (Bom-PBAN-R), also in the C-terminus. *B. mori*-R

Table 2 Summary of FXPR/KL-HN_2 cloned receptors

Insect	Code	Tissue	GenBank Acc. #	Expression sys.	Reference
Pheromone gland					
Helicoverpa zea	Hez-PBAN-R	Pheromone gland	AY319852	Sf-9	(Choi et al. 2003)
Bombyx mori	Bom-PBAN-R	Pheromone gland	AB181298	Sf-9	(Hull et al. 2004)
Heliothis virescens	Hev-PBAN-R (C)	Pheromone gland	EU000527	CHO	(Kim et al. 2008)
Larvae					
Drosophila melanogaster	Drm-PK-1-R	Whole larvae (3rd instar)	AF368273	CHO	(Cazzamali et al. 2005)
Spodoptera littoralis	Spl-PBAN-R	Whole larvae (5th instar)	DQ407742	NIH3T3, HEK293 Sf-9	(Zheng et al. 2007) Hariton, 2008 (unpublished)
Heliothis virescens	Hev-PBAN-R (A)	Larvae CNS (5th instar)	EU000525	CHO	(Kim et al. 2008)
Heliothis virescens	Hev-PBAN-R (B)	Larvae CNS (5th instar)	EU000526	CHO	(Kim et al. 2008)
Pupae					
Bombyx mori	Bom-DH-R	Developing ovary (pupa)	AB164386	Xenopus oocytes	(Homma et al. 2006)
Whole insect					
Drosophila melanogaster	Drm-PK-2-R-1	Whole insect	AY277898	CHO	(Rosenkilde et al. 2003)
Drosophila melanogaster	Drm-PK-2-R-2	Whole insect	AY277899	CHO	(Rosenkilde et al. 2003)
Anopheles gambiae	Ang-PK-1-R	Adult whole insect	AY900218	CHO	(Olsen et al. 2007)
Anopheles gambiae	Ang-PK-2-R	Adult whole insect	AY900219	CHO	(Olsen et al. 2007)
Annotated only					
Anopheles gambiae	APR-1 (PK-2)	–	BK001383	–	(Rosenkilde et al. 2003)
Anopheles gambiae	APR-2 (PK-2)	–	BK001384	–	(Rosenkilde et al. 2003)
Plutella xylostella	Plx-PBAN-R	–	AY974334	–	2005
Helicoverpa armigera	Har-PBAN-R	–	AY792036	–	2005
Orgyia thyellina	Ort-DH-R	–	AB283041	–	2007

CHO: Chinese hamster ovary cells; HEK293: Human embryonic kidney cells.

contained an extra amino acid sequence on its C-terminus, which was found to be responsible for the regulation of clathrin-mediated internalization of this receptor after it was challenged by PBAN during pheromone biosynthesis (Hull et al. 2004, 2005). All pheromone gland receptors responded to PBAN doses in the nanomolar range, in a dose-dependent manner, and also to peptides derived from the DH-PBAN gene products and to other FXPRLa and PRLa peptides – but at lower affinities. The pheromone gland receptor of *B. mori* was found to be tissue-specific, and it underwent significant up-regulation on the day preceding eclosion. Another *B. mori* receptor of the family was cloned from pupal ovaries during pupal-adult development (Homma et al. 2006). The receptor exhibited high affinity toward DH doses in the nanomolar range. The affinity of the receptor to other DH-PBAN-derived peptides was much lower.

We have cloned another PBAN-R from fifth-instar larval tissue of *S. littoralis* (Zheng et al. 2007). The receptor also showed high homology with the pheromone gland receptors, with differences in the N-terminal region, the second outer loop and the third inner loop. PBAN induced activation of an MAP kinase via a signaling mechanism that was protein kinase C (PKC)-dependent but Gαi-independent. As in the case of the pheromone gland receptors, the larval receptor also responded to other PK/PBAN peptides by MAP kinase activation.

Three PK receptors were cloned from *D. melanogaster*: *Drm*-PK-1-R (Cazzamali et al. 2005) and two *Drm*-PK-2 receptors – *Drm*-PK-2-1 and *Drm*-PK-2-2 (Rosenkilde et al. 2003). These receptors are the first PK receptors predicted on the basis of the findings of the *Drosophila* Genome Project, based on the homology with neuromedin U, CG9918, CG8784 and CG8795, respectively. The *Drm*-PK-1-R was highly specific to *Drm*-PK-1; it reacted with all other FXPRLa and PRLa peptides but at much lower affinities. The other two receptors, *Drm*-PK-2-1 and *Drm*-PK-2-2, exhibited high affinity, in dosages in the nanomolar range, toward *Drm*-PK-2 only. Gene silencing by means of RNA-mediated interference (RNAi) techniques showed that silencing of the PK-2-1 gene killed embryos, whereas silencing of the PK-2-2 gene resulted in reduced viability of both embryos and first instar larvae (Rosenkilde et al. 2003). Two PK receptors, *Ang*-PK-1-R and *Ang*-PK-2-R, were also cloned from the malaria mosquito *A. gambiae* (Olsen et al. 2007). Similarly to the *Drosophila* receptors, the *Ang*-PK-1-R was selectively activated by *Ang*-PK-1 peptides but not by *Ang*-PK-2. The *Ang*-PK-2-R was less selective and could be activated by both *Ang*-PK-2 and *Ang*-PK-1, but with a somewhat lower affinity for the latter.

A few other receptors that belong to the PRL/I/Va Np family (Capa-1, Capa-2 and Hug-γ) and which share some homology with the FXPR/KLa family and are encoded by a capa prohormone and PK prohormone genes (which also encode for PK-1 and PK-2 in *Drosophila* and *Anopheles*), have also been cloned and characterized. Further information on their structure and characterization can be found in (Iversen et al. 2002; Olsen et al. 2007). Two other PBAN-Rs (from the moth *Plutella xylostella* and *H. armigera*) and one DH-R from *Orgyia thyellina* have been annotated (see Table 2).

2.3 *Implementation of the INAI Strategy for the PK/PBAN Family*

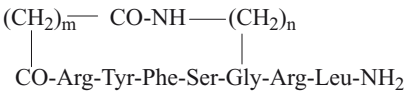
2.3.1 Discovery of PK/PBAN Antagonists

Initially we optimized two in vivo bioassays for evaluation of agonistic and/or antagonistic activities of linear and conformationally constrained peptides: a pheromonotropic bioassay with female *H. peltigera* adults, and a melanotropic bioassay with *S. littoralis* larvae (Altstein et al. 1995, 1996). Once the assays were available we synthesized a variety of linear peptides derived from the sequence of Hez-PBAN1-33NH_2, and used SAR techniques to identify the minimal active sequence of PBAN that constitutes the active core of the PK/PBAN molecule. The sequence that exhibited the same activity as that of the full length PBAN was YFSPRLa (Altstein et al. 1993, 1995, 1997). A "biased library" of linear peptides, based on this hexapeptide active sequence, was synthesized, with each amino acid being sequentially substituted with the amino acid D-Phe. We tested the peptides in the D-Phe substituted linear library for their agonistic and antagonistic pheromonotropic activities by using the full length PBAN, *Hez*-PBAN1-33NH_2, as a stimulator, and discovered a highly potent antagonist – RYFdFPRLa – that was capable of inhibiting sex pheromone biosynthesis by 80%, at a dosage of 100 pmol (Altstein et al. 2000; Zeltser et al. 2000).

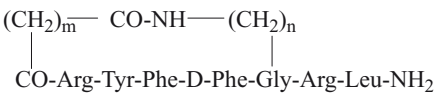
The discovery of a lead antagonist enabled us to take the next step: the search for improved selective and metabolically stable antagonists. For this we designed backbone cyclic (BBC) peptides, which exhibit conformational constraint and offer many advantages compatible with the requirements of improved antagonists (Gilon et al. 1991, 1993; Kessler et al. 1986). The minimal active sequence (that includes the consensus sequence of the PK/PBAN family YFSPRLa), and that of the lead antagonist (RYFdFPRLa) were used as a basis for the design of two conformationally constrained chemical BBC libraries (Altstein et al. 1999a). The first, the Ser sub-library, was based on a slight modification of the active sequence (RYFSPRLa). The second, the D-Phe sub-library, was based on the sequence of the lead antagonist. All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring; they differed in their bridge sizes and in the position of the amide bond along the bridge (Fig. 1). This part of the study was carried out in collaboration with the laboratory of C. Gilon, of the Hebrew University of Jerusalem, who had developed the BBC methodology and the cycloscan concept (Gilon et al. 1991, 1993, 1998a). Screening of the two sub-libraries for pheromonotropic antagonists revealed that all the antagonistic peptides originated from the D-Phe sub-library, and led to the discovery of four compounds that, at 1 nmol dosage, fully inhibited sex pheromone biosynthesis elicited by 1 pmol of PBAN1-33NH_2 and exhibited no agonistic activity (BBC 20, 22, 25 and 28; n + m = 2 + 3; 3 + 2; 4 + 2; 6 + 2, respectively, see Fig. 1). Substitution of the D-Phe amino acid with a Ser resulted in a loss of antagonistic activity (Altstein et al. 1999a; Zeltser et al. 2001). Four small BBC peptides, modified at their C-terminus (Fig. 2), and four precyclic peptides (Fig. 3), based on two of the

Fig. 1 General structure of backbone cyclic (BBC) peptides

A. Ser BBC sub-library



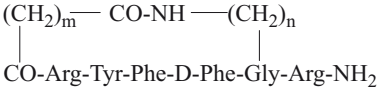
B. D-Phe BBC sub-library



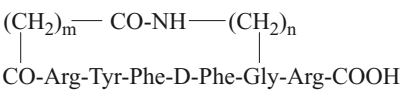
n=2,3,4,6; m=2,3,4

Fig. 2 General structure of the "small" BBC peptides

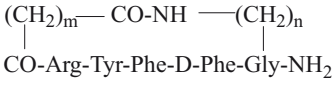
28.1



28.2

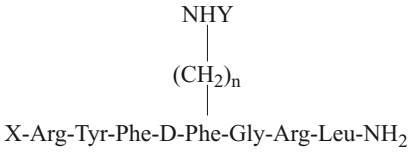


28.3



n=6; m=2

Fig. 3 General structure of the precyclic peptides



Peptide	X	Y	n	m
20-L-1	Ac	Ac	2	
28-L-1	Ac	Ac	6	
20-L-2	OC-$(CH_2)_m$-COOH	H	2	3
28-L-2	OC-$(CH_2)_m$-COOH	H	6	2

BBC antagonists (BBC20 and BBC28; n + m = 2 + 3; 6 + 2. respectively), were also synthesized; their activities revealed that a negative charge at the N-terminus of the peptide eliminated the antagonistic activity (Altstein 2004; Ben-Aziz et al. 2006; Zeltser et al. 2001). Assessment of the metabolic stability of the BBC peptides indicated that they were much more stable than their linear parent molecules (Altstein

et al. 1999a, 2001). These compounds were the first antagonists of the PK/PBAN Nps and served as a basis for the design of other antagonistic peptides (R.J. Nachman et al., 2009c) of this family.

2.3.2 Determination of the Bioactivity, SAR, Selectivity, and Bioavailability of the BBC Antagonists

The availability of a library of conformationally constrained BBC peptides (which confers high receptor selectivity) in which some peptides were potent antagonists of PBAN-evoked pheromonotropic activity and some were devoid of any biological activity made the BBC peptides also very powerful tools for examining the modes of action of the PK/PBAN family that exhibits a multifunctional pattern of activity, enabling identification of potential selective and non-selective antagonists for the various activities, which can further provide important information on whether the various functions regulated by the different PK/PBAN peptides are mediated by the same receptor or by different receptor subtypes, and/or more then one Np. Such information is indispensable for further design of insect control agents that are meant to interfere with the activity of this family of Nps. The high metabolic stability of the BBC peptides as well as their potentially increased biological activity and bioavailability also enabled to evaluate their SAR, bioavailability, and ability to interfere with the endogenous, i.e., native mechanism of action. The last of these examinations was necessary because the bioassays that were used to select the antagonists were based on injection of a synthetic stimulator and elicitation of an "artificial" response which may not fully reflect the natural (*in vivo*) mechanism of action.

Evaluation of the ability of the BBC peptides to inhibit sex pheromone biosynthesis elicited by endogenous factors, i.e., by the natural peptides, confirmed that the BBC peptides did inhibit the endogenous mechanism. Four highly potent antagonistic BBC peptides: BBC-20, 23, 25 and 28 (n + m = 2 + 3; 3 + 3; 4 + 2; 6 + 2, respectively, see Fig. 1), were able to inhibit sex pheromone biosynthesis by 68%, 57%, 54% and 70%, respectively, for 5 h, following injection of a 1 nmol dosage (Altstein 2003). Further examination of the time response of the most potent antagonist (BBC-28; n + m = 6 + 2, see Fig. 1) showed that significant inhibition of sex pheromone production in *H. peltigera* females could last up to 11 h (Altstein 2003), indicating the high potency and metabolic stability of this antagonist. The BBC peptides were also tested for their bioavailability by applying them topically on female moths at scotophase and examining the resulting inhibition of endogenously elicited sex pheromone production. The BBC peptides (BBC-25 and BBC-28; n + m = 4 + 2; 6 + 2, respectively, see Fig. 1) exhibited high bioavailability (i.e. cuticular penetrability) and were able to inhibit pheromone production by 40–60% and 50–67%, respectively, similarly to their performance when injected to the insect (A. Hariton and M. Altstein, 2008 unpublished).

Further evaluation of the bioactivity of the BBC peptides and of some of their analogs, i.e., small BBC peptides and precyclic peptides, involved a series of

experiments that examined their ability to inhibit other PK/PBAN-mediated functions – cuticular melanization, pupariation and hindgut contraction – elicited by PBAN and other member of the PK/PBAN family such as PT, Lom-MT-II, Lem-PK. Particular emphasis was placed on the question of whether elicitation of a given function by several different peptides and elicitation of several different functions by a given peptide were affected by the same or by different BBC peptides.

Interestingly, the experiments revealed differing patterns of inhibition by the BBC peptides when a given function was elicited by different peptides (Altstein et al. 2007). In the pheromonotropic assay, BBC peptides that inhibited pheromone biosynthesis elicited by PBAN1-33NH_2, did not exhibit antagonistic activity against any other tested elicitor. In the melanotropic assay most BBC peptides inhibited more than one elicitor but only a few of the compounds inhibited all elicitors (Altstein et al. 2007). The inhibition patterns differed among the other PK/PBAN-mediated functions also, and in general there were marked differences between the BBC inhibition patterns of the pheromonotropic and the pupariation and myotropic assays (Altstein et al. 2007; Ben-Aziz et al. 2006). No BBC peptide inhibited the myotropic activity, although this activity was stimulated by Lem-PK, which was effectively inhibited by two BBC peptides – BBC-22 and BBC-23 (n + m = 3 + 2, and 3 + 3, respectively, see Fig. 1) – in the melanotropic assay. Lem-PK-elicited pheromone biosynthesis was also not inhibited by any of the BBC peptides. Major differences were also found between the BBC peptides that inhibited the pheromonotropic and melanotropic activities. Four selective antagonists (e.g., exhibiting an inhibitory activity toward only one function) were found, three of which were melanotropic selective antagonists (BBC-23, n + m = 3 + 3, BBC-28-1 and 20-L-1, see Figs. 1 through 3) and one pheromonotropic selective inhibitor (BBC-22, n + m = 3 + 2) (Altstein et al. 2007). Five non-selective melanotropic and pheromonotropic antagonists were found (BBC-20, 25, 28, n + m = 2 + 3, 4 + 2, and 6 + 2, respectively, 20-L-1 and 28-L-1, see Figs. 1 through 3), only one of which (BBC-25, n + m = 4 + 2) also inhibited pupariation. However, it is interesting to note that this peptide inhibited pupariation when it was elicited by the endogenous mechanism, whereas it did not affect it when it was elicited by exogenously injected LPK. This indicates that the endogenous mechanism may not be mediated by LPK. Selective agonists were also found amongst the BBC peptides. Six selective pure melanotropic agonists and one non-selective (melanotropic and pheromonotropic) pure agonistic compound were discovered (Altstein et al. 2007; Ben-Aziz et al. 2006).

The differing inhibitory and stimulatory patterns that were found in different assays indicated that the various functions may be mediated by structurally different receptors which do not equally recognize the BBC peptides. Although the selectivity may result from differences between the assays themselves, e.g., different insects, developmental stages, assay conditions, etc., it is also possible that it may indicate diversity in the binding pockets or in the ligand docking regions of the elicitor on the receptors that mediate the different functions resulting in an inability of the BBC peptides to block all of them. The differences obtained

within a given assay also suggested, that the PK/PBAN peptides might mediate their activity via different receptor subtypes, or that the various ligands might exhibit differing binding properties, with respect to docking or affinity, toward a given receptor and would, therefore, differ in their degrees of inhibition by the BBC peptides. Given that all the elicitors used in this study contained the same signature sequence – FXPRLa – which constitutes the active site, the differing inhibitory capabilities of the BBC peptides toward different elicitors in a given assay also suggest that their mode of inhibition is non-competitive (see also item 2.3.3, below).

The completion of this stage of the study was marked by the accumulation of a large amount of information on the SAR of the BBC peptides, on their endogenous bioactivity and bioavailability, and on their selective properties. The data also identified the most potent antagonists for each function, highlighted the residues that give the compounds their inhibitory properties and ability to penetrate the cuticle, and shed important light on their non-competitive nature. All of the above information together with the large amount of data gained recently on: (i) the high bioavailability of the native linear elicitors (PBAN, PT, LPK and MT) (Hariton et al. 2009a); (ii) the SAR and high bioavailability of β^2 and β^3 amino acid substituted peptides (Hariton et al. 2009b; Nachman et al. 2009a); (iii) the bioactivity and selectivity of the *trans*Pro mimic analogs Ac-YF[Etz]RLa and Ac-YF[Jo]RLa (Nachman et al. 2009b; Nachman et al. 2009d) (which contain an (*E*)-alkene termed "Etzkorn" abbreviated "Etz" moiety or a dihydroimidazoline moiety termed "Jones" abbreviated "Jo", respectively); and on the bioactivity of a variety of amphiphilic and hydrophobic linear and cyclic molecules, peptidase resistant peptides and cytotoxic magic bullet analogs, (M. Altstein and R.J. Nachman, unpublished), set the basis for further structural analysis in support of the design of improved antagonists. One example of a successful implementation of our approach is the design of the novel amphiphilic linear D-Phe analogs Hex-Suc-AdFPRLa (in which a hexanoyl-(Hex) moiety linked by a succinic acid was incorporated to the N-terminus). The design of the peptide was based on our finding that substitution of the second amino acid in the consensus sequence of the PK/PBAN family (FXPRLa) by D-Phe results in conversion of an agonist (RYFSPRLa) to an antagonist (RYFdFPRLa) (Zeltser et al. 2000). The analogs Hex-Suc-AdFPRLa was proven to be a potent and efficacious inhibitor of sex pheromone biosynthesis elicited by PBAN (84% at 100 pmol) and PT (54% at 100 pmol), but not by MT and LPK. The analog was also found to be a selective pure antagonist as it failed to inhibit melanization elicited by any of the natural PK/PBAN peptides and most important was shown to transmigrate isolated cuticle dissected from adult female *Heliothis virescens* moths to a high extent of 25–30% (130–150 pmol), representing physiologically significant quantities (Nachman et al. 2009c). Results on the bioactivity of all of the above peptides (amphiphilic D-Phe peptides, β-substituted analogs, PK-Etz, and PPK-Jo) are not detailed in this review and are summarized by R.J. Nachman in another chapter in this book.

2.3.3 PK/PBAN Receptor Cloning and Characterization

In order to gain additional SAR information studies focused also on the PK/PBAN receptor. The studies in this part pursued two parallel avenues: (i) cloning of the receptor in order to be able to mutate it and to study its SAR and pharmacokinetic properties more deeply; and (ii) development of a binding assay, which could later be converted into an high throughput assay, for screening libraries of chemical, molecular, and natural compounds in the search for small molecules that interact with the receptor, and which later might serve as insecticide/insect-control prototypes.

In pursuing this objective we recently cloned a PK/PBAN receptor from *S. littoralis* larvae, which mediates cuticular melanization (Zheng et al. 2007), and evaluated the structure of its gene. The receptor exhibited high homology with the pheromone gland receptors, with differences in the N-terminal region, the second outer loop and the third inner loop. Currently, the structural mechanisms of the interactions between ligands and GPCRs are still not clear, but the data on GPCRs that have been accumulated so far suggest that the extracellular N-terminus/loops are responsible for the ligand docking/interaction (Leff 1995). It is thus possible that the major difference observed in the N-terminal region of the PBAN-R may, therefore, account for optimal conspecific ligand/receptor docking and interaction, which results in differing binding patterns of different ligands, (e.g., elicitors), to the pheromone gland and larval receptors. The TM helices that are highly conserved between different PBAN-Rs might form a precise ligand interaction pocket which is required for the FXPRLa motif-induced receptor conformational change and receptor activation.

The proposed model may explain the differences, (both within and between assays), between the inhibitory patterns of the BBC and those of the other antagonistic peptides, and may provide an insight into the nature (competitive or non-competitive) of the inhibitors. It is most likely that the common sequence of the PK/PBAN elicitors (FXPRLa) docks in the highly conserved region of the receptors, whereas the other sequences of the elicitor molecule dock at different sites, which may differ among the various assays. In light of the above suggestions, we may further hypothesize that the various BBC and other antagonists bind outside the FXPRLa docking pocket, and may, therefore be considered as non-competitive inhibitors, whose inhibitory activity results, most likely, from interference with the docking of the other parts of the elicitor ligand with its receptor. Since different ligand molecules may dock in different regions (because of structural differences between the receptors), the degree of inhibition of a given elicitor imposed by a given BBC compound results in differing inhibitory potencies. We have previously suggested that the BBC inhibitors are non-competitive, in light of the results of an in vitro radioisotope-receptor binding study carried out with the native *H. peltigera* receptor (Altstein and Ben-Aziz 2001) (see below) and based on the in vivo assays described above. A definite answer to all of the above questions will depend on further evaluation based on SAR analysis by means of 3-D modeling of cloned receptor(s) and evaluation of their interactions with the various ligands and inhibitors. These issues are currently under investigation in our laboratory.

In parallel to cloning of the receptor, two binding assays were developed with the native pheromone gland PK/PBAN receptor of *H. peltigera*. The first was a radio-receptor assay (RRA) in which ^{3}H-tyrosyl-PBAN28-33NH$_2$ was used as a radio-ligand (Altstein and Ben-Aziz 2001; Altstein et al. 1999b); the second was a microplate binding assay in which a biotinylated PBAN (BpaPBAN1-33NH$_2$) was used as a tagged ligand (Ben Yosef et al. 2009). The microplate assay was designed to facilitate its further development into a HTS binding assay. A method for obtaining an active receptor preparation from the pheromone gland of the moth *H. peltigera* was developed, and the optimal membrane preparation and incubation conditions, (e.g., buffer pH values, divalent ions, and protease inhibitors) for receptor-ligand binding were determined. The experimental set up will be applied to the cloned receptor for the development of HTS binding assays.

In summary, the availability of the conformationally constrained selective and non-selective agonists and antagonists, and also of bioassays that have been developed for each of the above-mentioned functions has opened the way to a better understanding of the endogenous mechanisms of the PK/PBAN peptide family in moths and other insects. The knowledge gained in the course of the present study, regarding the effects of the compounds on the endogenous (native) mechanism, regarding their bioavailability (cuticular penetration) and their selectivity toward the various activities, as well as the data on the differing activity patterns of the PK/PBAN elicitors and on the structural changes in the receptors that mediate their activities, are all of major importance for the design of additional, improved (i.e., more potent, highly selective, metabolically stable and cost effective) agonistic and antagonistic compounds, which may, once they become available, be candidates for agrochemical applications. After formulation and preliminary field experiments, they could serve as prototypes in the development of a novel group of highly effective, insect-specific and environmentally friendly insecticides. Indeed, some of the above antagonists have already been used as a basis for the design and synthesis of novel and improved compounds with enhanced inhibitory potency and bioavailability. These compounds are currently being screened for their ability to stimulate or inhibit cuticular melanization in *S. littoralis* larvae and sex pheromone biosynthesis in *H. peltigera*. Results of these studies are not detailed in this review and are summarized by R.J. Nachman in another chapter in this book.

3 Concluding Remarks and Future Prospects

Intensive studies of the FXPRLa peptides have yielded very interesting information on the chemical and molecular nature of the PK/PBAN family of peptides, their origin, localization, target organ, route of transport, etc. In spite of this, however, many questions about these peptides are still unresolved, especially regarding their mode of action, and much remains to be learned about the structural, chemical and cellular basis of their activity, downstream cellular events, species specificity, receptor heterogeneity and the mechanisms that underlay their functional diversity.

The information that has already been accumulated and the tools, e.g., bioassays, in vitro binding assays, receptor-selective agonists and antagonists, receptor genes, etc., that are currently available to us and to other laboratories offer great potential for further exploration of the above issues. Receptors are most significant in understanding the biological function of any Np, especially in families where several peptides exhibit similar bio-activities, and they play a central role in providing information on the direct correlation between the activity of a given Np and its target. Antagonists, especially those that are receptor-selective, such as the BBC peptides described above, form excellent research tools for studying multi-peptide families that exhibit functional diversity. We anticipate that the availability of conformationally constrained antagonists, the high-affinity ligands that were developed in our laboratory, the cloned receptor and the binding assays that were developed, together with the in vivo bioassays will provide a solid basis for further studies that aim to gain a better insight into the mode of action of PBAN and the other PK/PBAN peptides in moths and other insects. Beyond the high scientific value of the above findings, the strategies and approaches that were developed in the course of the PBAN research also offer high potential for practical application, by providing a basis for generation of insect Np antagonist-based insect control agents, as described in this chapter.

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Tyramine and Octopamine Receptors as a Source of Biorational Insecticides

Akinori Hirashima

1 Introduction

Biogenic amines such as dopamine (DA), octopamine (OA) and tyramine (TA) are widely distributed in the central nervous system of insects (Evans 1980). The administration of biogenic amines and agonists or antagonists for their receptors and direct measurement of concentrations of biogenic amines under various conditions indicate that these agents function as neurotransmitters, neuromodulators and neurohormones. They are involved in regulating many physiological phenomena such as learning (Dudai 1986), memory (Yovell and Dudai 1987), circadian rhythms (Muszynska-Pytel and Cymborowski 1978), contraction rhythm of muscles, flight (Goosey and Candy 1980), walking, feeding behaviour (Long et al. 1986), juvenile hormone (JH) (Lafon-Cazal and Baehr 1988; Granger et al. 1996; Grutenko et al. 2007), mating behaviour, pheromone production (Rafaeli and Gileadi 1995) and the reaction to various stressor stimuli (Davenport and Evans 1984).

OA [2-amino-1-(4-hydroxyphenyl)ethanol] is the monohydroxyllic analogue of the vertebrate hormone noradrenalin (NA). OA was first discovered in the salivary glands of octopus by Erspamer and Boretti (1951). It has been found that OA is present in a high concentration in various invertebrate tissues (Axelrod and Saavedra 1977). This multifunctional and naturally occurring biogenic amine has been well studied and established as (1) a neurotransmitter, controlling the firefly light organ and endocrine gland activity in other insects; (2) a neurohormone, inducing mobilization of lipids and carbohydrates; (3) a neuromodulator, acting peripherally on different muscles, fat body, and sensory organs such as corpora cardiaca (CC) and the corpora allata (CA); (4) a centrally acting neuromodulator, influencing motor patterns, habituation and even memory in various invertebrate species (Orchard 1982; Evans 1985; Orchard et al. 1993). Three different OA receptor (OAR) classes OAR1, OAR2A and OAR2B had been distinguished from

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non-neuronal tissues (Evans 1981). The action of OAR2 is mediated through various messengers, which is coupled to G-proteins and is specifically linked to an adenylate cyclase (Nathanson 1985). Thus, the physiological actions of OAR2 have been shown to be associated with elevated levels of cAMP. In the nervous system of locust *Locusta migratoria* L., a particular receptor class was characterized and established as a new class OAR3 by pharmacological investigations of the OA binding site using various agonists and antagonists (Roeder and Gewecke 1990; Roeder 1990, 1992, 1995). According to the new classification scheme recently proposed based on the identities and properties of cloned receptors, OAR3 class does not exist as a separate class (Evans and Maqueira 2005; Maqueira et al. 2005).

The TA receptors (TARs) have generated considerable pharmacological interest as targets for the identification of selective drugs/chemicals that may interact with specific receptor subtypes (Smith et al. 2007). To understand the basis of the increased susceptibility of Lepidoptera to agonists or antagonists for TAR, there is a need to characterize and compare TAR subtypes found in moths with those of other insect species. Identification of ligands with a high degree of selectivity enables the characterization and differentiation of TAR populations in various tissues and species and will lead to potential insecticides. Potent and selective agonists or antagonists for TAR with low toxicity in vertebrates could be useful toxins in invertebrates and particularly insects. It is therefore of critical importance to provide information on the pharmacological properties of TAR types and subtypes. Thus, we have been prompted to construct in vitro bioassay system using radioisotope (Hirashima et al. 2003b). Future study is therefore designed to evaluate pharmacological activities of the ligands against the hypothetical TAR and OAR.

2 Effect of TA and OA on Sex-Pheromone Production

Female moths emit a species-specific male attractant (sex-pheromone blend) from a glandular area at the abdominal tip. Sex-pheromone production in many species of moths is controlled by pheromone biosynthesis activating neuropeptide (PBAN) or PBAN-like factors (Raina 1993). To date, PBANs have been isolated and their primary structures were determined from following species of moths: *Helicoverpa zea* (Raina et al. 1989), *Bombyx mori* (Kitamura et al. 1989, 1990), *Pseudaletia separata* (Matsumoto et al. 1992), *Lymantria dispar* (Masler et al. 1994) and *Agrotis ipsilon* (Duportets et al. 1998). PBAN, which acts on pheromone glands (PGs) to stimulate pheromone biosynthesis (Rafaeli 2002), was first identified as a 33-amino acid C-terminal amidated peptide (Raina et al. 1989). Subsequently, it was determined that the five C-terminal amino acids, FXPRLamide, represented the minimal sequence required for activity (Raina and Kempe 1990). More structures have been determined through cDNA (see recent review: Rafaeli 2008). This motif has been identified from a variety of peptides and most will stimulate pheromone biosynthesis when tested on pheromone glands isolated from moths (Ma et al. 1996).

A temporary or permanent suppression of pheromone production after mating has been reported for many species of moths. Reports on this process show that different mechanisms are used by different species of Lepidoptera. In a few species of *Helicoverpa* moths, factors originating from the testis or the accessory gland of the male reproductive system and acting through the mated female's haemolymph, are implicated in suppressing pheromone production (Raina 1989; Ramaswamy et al. 1994). A polypeptide with such pheromonostatic activity has been identified in the male-accessory glands of *H. zea* (Kingan et al. 1993, 1995). *Drosophila* sex-peptide-like substance in moths was discovered by Fan et al. (1999, 2000) and Nagalakshmi et al. (2004). It is not clear what role is played by the pheromonostatic peptide. In the gypsy moth and two tortricid moths, suppression of pheromone production after mating seems to be induced by a neural signal that originates from the abdomen and runs through ventral nerve cord (VNC) to inhibit the release of PBAN, because transection of the VNC fails to induce the post-mating inactivation of pheromone production (Giebultowicz et al. 1991; Foster 1993; Jurenka et al. 1993; Foster and Roelofs 1994). In the silkworm moth, *B. mori*, a decrease in pheromone titers after mating seems to be caused by the same mechanism (Ichikawa et al. 1996a). Mating inhibits the release of PBAN into the hemolymph and inhibition of PBAN release was demonstrated by Nagalakshmi et al. (2007). The neural signal travelling through the VNC to the brain-suboesophageal ganglion (SOG) complexes triggers an inactivaion of PBAN release. The sex peptide does not affect the VNC, indeed some of the mating effect is through the VNC but not sex peptide, since sex peptide inhibits pheromone production by injecting it to the hemolymph of virgin females (Fan et al. 1999, 2000; Nagalakshmi et al. 2004, 2007). However, the neural mechanism remains to be clarified.

Post-mating inactivation of pheromone production is inhibited or recovered by application of stressors, such as restrictions and anesthesia, in *B. mori* (Ichikawa et al. 1996a). The elucidation of such phenomenon could lead to clarification of the neural mechanism. A relationship between sex-pheromone production and biogenic amines in brain-SOG complexes in *B. mori* was investigated using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The changes in OA, DA and TA levels in brain-SOG complexes relative to mating stimuli and mechanical stressor were observed, in which TA levels increase in mated females (Hirashima et al. 2007; Hirashima 2008). Futhermore, the effects of external DA, OA and TA on sex pheromone (bombykol) production of *B. mori* in vivo and in vitro at its candidate target organ, PG, were examined.

There was no difference in OA levels between virgin and mated females in brains of *A. domesticus*. It was not determined whether levels of other biogenic amines, such as DA, changed after mating (Woodring et al. 1988). Suppression of pheromone production after mating seems to be induced by a neural signal that originates from the abdomen and runs through the VNC to inhibit the release of PBAN, because transection of the VNC fails to induce the post-mating inactivation of pheromone production (Hirashima et al. 2007). The bombykol titers of females mated with penis-less male, which was obtained by surgical operation removing the

accessory glands, were significantly higher than those of mated control females. PBAN is released by the CC and many studies showed that PBAN production in the neurosecretory cells of the SOG (Ichikawa 1995; Ando et al. 1996; Ichikawa et al. 1996b) is present at similar levels at various ages, photoperiods and after mating (see reviews: Rafaeli 2002; Rafaeli and Jurenka 2003). Thus, to induce complete suppression of PBAN release, it is necessary that the CC receives another signal that may be induced by the sperm and/or testicular factors transferred from a male along with mechanical stimuli. The duration of mating necessary for permanent inactivation of PBAN release is between 30 min and 1 h (Ichikawa et al. 1996a). In this period, the neural signal induced by two kinds of stimuli seems to be sent to the brain through VNC and once the signal is received, it is no longer necessary to maintain PBAN release inactive. A similar two-step inactivation mechanism of sex-pheromone release has been inferred from observation of mated gypsy moth (Giebultowicz et al. 1991).

Production of the sex-pheromone in many species of moths is controlled by PBAN or PBAN-like factors (Raina 1993). These hormones that are produced in the SOG and secreted into the haemolymph through CC (Soroker and Rafaeli 1989). The target tissue was later on refined as the PG tissue by Rafaeli and Gileadi (1995). A G protein-coupled receptor (GPCR) or PBAN was identified from PGs of the moth *H. zea* (Choi et al. 2003). In addition, the computer-based molecular modeling on the binding of PBAN to its receptor was studied (Stern et al. 2007). Recently, the effects of the biogenic monoamine TAR and TAR ligands have been reported on pheromone biosynthesis of some species of moths (Hirashima et al. 2004a,b). In *H. armigera* females, TA seems to act on the receptor, which exists in the intersegmental membrane and suppresses PBAN-stimulated pheromonotropic activity (Rafaeli and Gileadi 1995, 1996; Rafaeli et al. 1997). In *H. virescens* and *H. zea* females, OA is nearly as effective as PBAN in stimulating pheromone biosynthesis in vivo (Christensen et al. 1991). However, it is also reported that OA has no effect on stimulating pheromone production of *H. zea* and *Mamestra brassicae* females (Jurenka et al. 1991). Mechanisms of these pheromonostatic and pheromonotropic effects of TA and OA have not yet been clarified.

In vitro assay was conducted using the method described by Fonagy et al. (1999). There was a significant difference in the in vitro pheromone-production of the PG: TA inhibited pheromone production by the PG in a dose dependent manner (Hirashima et al. 2007; Hirashima 2008), suggesting that the target of TA is the PG, whereas OA had no effect. TA showed stronger inhibitory activity on pheromone production than that of OA in *H. armigera*, whose action was nullified (Rafaeli and Gileadi 1995) by yohimbine (YHN), an antagonist of the TAR (Hiripi et al. 1994). Some of the so-called TARs respond better to OA than TA when one considers different second messenger pathways (e.g. see Robb et al. 1994). Previous work has demonstrated inhibition by several aminergic compounds on sex-pheromone production and cAMP synthesis in *H. armigera* (Rafaeli et al. 1999). Inhibitors of calling behaviour and pheromone production in *Plodia interpunctella* have also been demonstrated (Hirashima et al. 2003a,b,c), which inhibited PBAN-induced sex-pheromone production competitively with YHN

(Hirashima et al. 2004b). TA suppressed pheromone production in *B. mori* and its target is likely the PG.

In a cell-free system the receptor–ligand interaction is still present in the case of the OAR (Hirashima et al. 2004b). Thus, a homogenate was used in this experiment to determine whether it contains receptors for PBAN and biogenic amines, and whether the receptor–ligand interaction is still present. VNC transection in females, penis removal in males and various amine injections can interfere and reduce this inhibition by a maximum of 46%. Stressed females also caused a reduction in the usual mating effect only by ~10%. The significant inhibitory influences by TA are possible only at very high pharmacological levels of 2–20 mM, considering ID_{50} values in the nanomolar range reported for inhibition of in vitro pheromone production by other insects. In addition, these mated females stressed or treated with TA are still not producing the high levels of pheromone that can be observed in virgins. It is not clarified yet whether this interference in the mating effect actually affects whether the females are again attractive for a second mating. Thus, other factors could be responsible for the pheromonostatic function, although the importance of neural/amine channel cannot be denied.

In conclusion, TA in brain–SOG complexes may play a part in regulating pheromone production. Clarification of the distribution of TA and its metabolites in brain-SOG complexes may serve as a major breakthrough to elucidate the mechanism of action. In *B. mori*, the level of pheromone fluctuates diurnally, with little pheromone present during the scotophase and the peak of pheromone titers occurring during the mid-photophase, and mating causes a permanent suppression of pheromone production (Ichikawa 1995). Both of these phenomena are due to a decline in the concentration of PBAN in the haemolymph, but a mechanism controlling PBAN release remains unknown. In the CC PBAN has been shown to be lower in the scotophase, thereby implicating release during that time, when it can also be found in the hemolymph. The possibility is suggested that TA is a candidate in regulating the pheromone production in the PG. In *B. mori* females, TA is also a possible candidate for regulating the shift in PBAN release from an active to inactive state after the start of mating. TA could act on the neurosecretory cells of the SOG, in which twelve neurosecretory cells of PBAN exist (Ichikawa et al. 1996b), or the CC and the release of PBAN could be blocked.

3 Stress Reaction

In studies with cloned receptors, evidence is emerging that the OAR and TAR are positively and negatively coupled to adenylate cyclase, respectively (Arakawa et al. 1990; Saudou et al. 1990; Vanden Broeck et al. 1995; Gerhardt et al. 1997; Han et al. 1998; Blenau et al. 2000; Chang et al. 2000; Bischof and Enan 2004). In *B. mori*, the cAMP is not involved in the PBAN signal transduction cascade (Hull et al. 2007), whereas in heliothine species, the second messenger is a crucial component in PBAN signaling (Rafaeli and Jurenka 2003; Rafaeli 2002). While it has been clearly

shown that OA acts as a neuromediator in various insects, there have only been suggestions that TA might be a neuromediator in invertebrates (Robertson and Juorio 1976). Consequently, TA could not only be a biosynthetic precursor to OA, but may also play an independent role as a neuromediator as shown in Fig. 1, although it still remains to be clarified. Thus, the pheromonostatic receptor, acting in a neuromodulatory role, could represent a TAR. It is therefore of critical importance to provide information on the pharmacological properties of the TAR types and subtypes.

When *Schistocerca gregaria*, *Periplaneta americana* and crickets *Acheta domesticus* were subjected to various stressors, such as mechanical, temperature and chemical (insecticide) stressors, the concentration of OA in the haemolymph increased within a few minutes after the onset of stressor application (Davenport and Evans 1984; Woodring et al. 1988). In *S. gregaria* and *A. domesticus*, recovery to resting levels of OA after stressor application is rapid, requiring only 15 min. In contrast, OA levels in *P. americana* after stressor application remain elevated for at least 1 h after the end of stressor application. Orchard et al. (1981) reported the elevation of lipids in haemolymph corresponding to the increase in haemolymph OA in response to stressor in *L. migratoria*, and Gole and Downer (1979) found that OA induced hypertrehalosemia in *P. americana*. Thus, stressor-induced haemolymph OA influences carbohydrate and lipid metabolism and may stimulate the motor responses aimed at avoidance of danger in insect (Chernysh 1991). In *Apis mellifera*, mechanical stressor results in peak elevation of OA in brain after 10 min (Harris and Woodring 1992). In individuals of line 101 of *Drosophila virilis* responding to stressor by a hormonal stress reaction, the contents of OA were lower than those of line 147 that did not respond to the stressor (Hirashima et al. 2000). However,

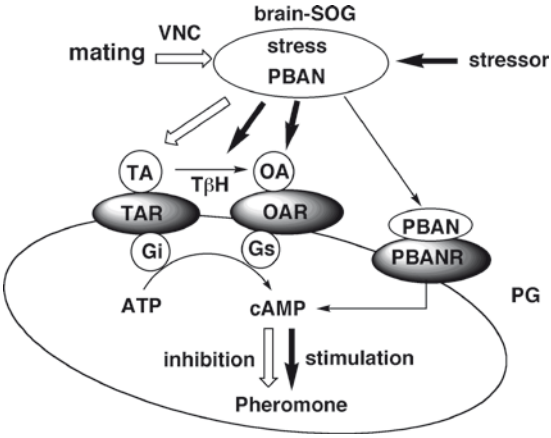

Fig. 1 Involvement of TA and OA on sex-pheromone production in *Helicoverpa* spp. VNC, ventral nervous cord; SOG, subesogeal ganglion; PBAN, pheromone biosynthesis activating neuropeptide; PG, pheromone gland; TA, tyramine; TβH, tyramine-β-hydroxylase; OA, octopamine; TAR, tyramine receptor; OAR, octopamine receptor; PBANR, pheromone biosynthesis activating neuropeptide; PG, pheromone gland

heat stressor caused an increase in the contents of OA in line 101, whereas the equivalent titers in line 147 remain unchanged.

Stressor-induced increases in the levels of OA and DA were observed both in virgin and mated females of *B. mori* 24 h after the start of stress application (Hirashima et al. 2007). In *B. mori*, anesthesia caused by ether or carbon dioxide (chemical stressor) as well as mechanical stressor is able to induce the resumption of bombykol production in mated female. Any changes to activate PBAN release which are suppressed after mating could occur in brain–SOG complexes within 3 h after the start of stressor application (Hirashima et al. 2007). OA and DA may be related to stressor-induced resumption in mated female. Whether effects of stressors such as restriction and anesthesia on activation of PBAN release are caused through the same mechanisms is unknown. Studies in locusts have shown that OA is implicated in the modulation of oviductal visceral muscle by two lines of evidence: innervation of the oviductal muscle by octopaminergic median unpaired neurons (Kalogianni and Pflüger 1992) and physiological evidence that OA modulates activity of the oviductal muscle (Kalogianni and Theophilidis 1993). Considering the involvement of OA in oviposition of *Drosophila melanogaster* (Monastirioti et al. 1996) and locusts, the stressor-induced resumption of bombykol production in mated stressed female of *B. mori* could be caused by an increase of OA levels in the haemolymph.

4 Effect of TA and OA on Metamorphosis

The rate of pupation of the "yellow" black carpet beetle *Attagenus elongatulus* larvae is reduced by increased larval crowding (Barak and Burkholder 1977). Pupation of mature larvae of several species of Tenebrionid beetles is inhibited by crowding (Tschinkel and Willson 1971; Tschinkel 1978, 1981). Stressors induce changes in the OA levels of insect haemolymph (Davenport and Evans 1984). The authors have focused on effect of stressor including crowding on OA contents and larval growth of the red flour beetle *Tribolium castaneum* (Herbst) (Hirashima et al. 1992b, 1993a,b). *T. castaneum* is a well known cosmopolitan and experimental stored-product insect, whereas *Tribolium freemani* (Hinton) is a potential pest in stored products (Nakakita 1982). Although these insects belong to the same genus and both are stored-product pests, they respond to larval population density differently. Under crowded conditions the last instar *T. freemani* larvae fail to pupate and instead enter a series of stationary moults that may continue for more than 6 months (Nakakita 1982). However, optical stressor (light), i.e. continuous illumination, accelerated pupation of *T. freemani* (Hirashima et al. 1995a). In *T. castaneum*, cannibalism is the chief mechanism to control the population size. In *T. freemani*, however, prevention of pupation by crowding may be the main regulatory factor in controlling population. Suppression of pupation under crowded conditions in *T. freemani* may be due to hyperactivity of the CA resulting in high levels of JH (Nakakita 1990; Kotaki et al. 1993). Pupation in *Zophobas atratus* (Tenebrionidae)

is dependent on isolation and larvae under grouped conditions may delay the onset of metamorphosis through modulation of JH titers (Quennedey et al. 1994). Kotaki and Fujii (1995) suggested that a substance(s) on the body surface of larvae may be involved in the crowding response. In our previous reports (Hirashima et al. 1995a,b), however, the involvement of OA in metamorphosis of *T. freemani* has been suggested. At the prepupal stage of the cabbage looper, *Trichoplusia ni*, juvenile-hormone esterase (JHE) activity is regulated through its induction by JH (Jones and Hammock 1983). A decrease in JHE activity occurred in *Drosophila virilis* larvae when metamorphosis was delayed by crowding (Rauschenbach et al. 1987). This report describes effects of larval density and stresses on metamorphosis, whole-body biogenic amine contents, especially OA, JHE activity and ecdysteroid levels in order to identify neuromediators responsible for triggering pupation in *T. freemani* larvae.

Isolation, optical stressor, vibration and OA agonists appear to affect JH system of *T. freemani*, since exogeneous JH I antagonized their effects on pupation. JH I inhibited even pupation of isolated larvae and induced stationary moults. Since allatectomized chilled *Galleria* larvae fail to undergo supernumerary moults (Pipa 1976), it seems obvious that a high JH titer in chilled larvae has been caused by activation of the CA. In crowded *T. freemani* larvae the CA may be more active than in the isolated larvae or the isolated larvae may stop production of JH. The results in this report agree with the observations in the migratory locust *L. migratoria* by Fuzeau-Braesch et al. (1979), in which OA titers were shown to increase in the larval life and the moult was characterized by a sharp decrease in OA titer. The nervous systems of insects contain high levels of OA (Evans 1980, 1986). It plays important roles in regulating the nervous system, acting as a neurotransmitter, neurohormone and neuromodulator (Bodnaryk 1980). Thus, taken together with the results in this report, OA may produce some biological effects on programming of larval-pupal development. OA inhibits JH release in the cockroach, *Diploptera punctata* (Thompson et al. 1990) and the cricket, *Gryllus bimaculatus* (Woodring and Hoffmann 1994), whereas in *L. migratoria* (Lafon-Cazal and Baehr 1988) and the honey bee, *A. mellifera* (Kaatz et al. 1994; Rachinsky 1994) it enhances the release of JH. At the prepupal stage JHE activity is regulated through direct induction by JH (Jones and Hammock 1983). According to Rountree and Bollenbacher (1986), Gruetzmacher et al. (1984), and Gilbert and Schneiderman (1959), JH controls the release of the prothoracicotropic hormone (PTTH), which activates PG to synthesize ecdysone in the tobacco hornworm, *Manduca sexta*.

When the last instar larvae of *T. freemani* are isolated, they pupate within 6 days, whereas the number of days required for pupation of the crowded larvae (two larvae in a vial) is 12 days. In both groups and optically-stressed larvae, there was a peak of whole-body OA content 3 days before pupation. This peak could trigger the developmental program that elicits wandering behaviour, cessation of feeding and initiation of pupation that occurs 3 days later (Hirashima et al. 1998). An ecdysteroid peak elicits wandering behaviour (Sakurai et al. 1998). OA seems to function as a pacemaker in the metamorphosis of *T. freemani*. Mellanby (1954) has reported that high temperatures delayed the larval metamorphosis of the mosquito, *Aedes aegypti*, and the mealworm, *Tenebrio molitor*, almost by a month.

Cold exposure (0°C) as short as a few minutes of the last instar larvae of the wax moth, *Galleria mellonella*, caused supernumerary moultings (Cymborowski and Bogus' 1976). McCaleb and Kumaran (1980) reported that JHE activity is low in cooled wax moth larvae. The isolated larva of the blowfly, *Calliphora erythrocephala*, stimulated by placing in a rolling round-bottomed flask or larvae maintained together in a small volume delayed the release of ecdysone and pupation (Berreur et al. 1979). *G. mellonella* larvae reared in a constant light regime terminated feeding and pupated 1 day earlier than insects kept in constant darkness (Bogus' et al. 1987). Our results agree with theirs, in which the peak value of JHE activity was 22% higher in *G. mellonella* reared under constant light conditions as compared with animals kept in constant darkness. Hence, light seems to decrease the JH titer by activating JHE which leads to a stimulation of ecdysteroid production, thus accelerating pupation. Thus, optical stressor also resulted in an increase of OA content (Hirashima et al. 1995a), and some agonists for OA receptor actually had the same effect as isolation and optical stressor did: an increase in JHE activity and ecdysteroid level, followed by pupation, suggesting that OA is responsible for pupational programming. Thus, these results, together with our previous reports (Hirashima et al. 1995a,b), suggest that increase in OA content followed by an increase of JHE activity and ecdysteroid level is correlated with a change in the developmental program in *T. freemani* larvae. Furthermore, the results in experiments of agonists for OA receptor suggest that OA is a causative agent in larval programming (Hirashima 2004; Hirashima et al. 1998).

At days 2–4 under the crowded conditions a broad peak of OA was observed, probably a reprogramming peak which may influence JHE activity. This peak also appeared in optically-stressed larvae. It delays or represses the intervening of ecdysteroid sources during the feeding/wandering phase, leading to a longer larval phase, then it allows the larval moult to operate instead of pupal moult. The second OA peak appears to be related to pupal ecdysis, since under all culture conditions (uncrowded, crowded and optically stressed), the peak appeared 3 days before pupation. On the other hand, isolation activates the events preparatory to a pupal moult, i.e. the secretion of OA and increase of JHE activity followed by stimulation of ecdysteroid release, without a programing OA peak. Alternatively the first OA peak may play as both reprogramming and ecdysis peaks. Thus, commitment to metamorphosis has been initiated at day 2–4. The *T. freemani* larvae treated with optical stressor or agonists for OA receptor completed adult development like the control insects, about 6 days after pupation (Hirashima et al. 1995a,b). Hence, adult development could not be altered for the change from larva to pupa by optical stress or agonists for OA receptor, once metamorphogenic events have been initiated.

Coordination of the initiation of pupation in *T. freemani* larvae with external factors that control OA titer allows the larvae to escape unfavorable environmental situations. Under conditions of decreased locomotory activity OA titer may increase in response to the decreased contact with other larvae, thus initiating the program associated with metamorphosis. The mechanism by which OA titer is regulated by external conditions might have an adaptive value for animal survival, but this is still to be clarified. An increase in OA titers seems to be a common response to a variety

of stressful stimuli in insects (Bailey et al. 1983; Davenport and Evans 1984; Hirashima et al. 1992a, 1993a,b). Alteration of OA titer, influenced by stressor, however, reflects only one of the actions of numerous pharmacologically active compounds which are released under stressfull conditions. In our previous reports (Hirashima and Eto 1993a,b,c), increases of TA titers, in addition to OA titer, were observed in mechanically-stressed *P. americana*. The function of TA in vertebrates and invertebrates remains to be elucidated. Furthermore, various other biogenic amines and related substances, other than OA, may be candidates as mediators controlling metamorphosis of *T. freemani* larvae.

Release of biogenic amines and related substances seems to be stressor-specific, indicating difference in the mechanisms of action of these stressors. Differences in the membrane permeability between subtypes of the protocerebral neurosecretory cells (NSC) have resulted in selective responses of NSC to the effect of different chemical stressors (Jankovic'-Hlandni et al. 1983; Ivanovic' et al. 1985). Hence, NSC may respond selectively to various stressors, leading to selective release of various biogenic amines and related substances. Thus, some of the actions of these stressors in metamorphosis may result from an imbalance of these neuroactive amines and related substances, thereby disturbing metamorphosis in *T. freemani*. In order to study the relationship between the responses of biogenic amines and metamorphosis in *T. freemani*, more detailed experiments are in progress.

5 Prevention of Progeny Formation in *D. melanogaster*

DA and NA seem to be the principal catecholamines and there are much lower levels of NA compared to DA in insects (Anderson 1979; Evans 1980). Black et al. were unable to identify NA in adult of *D. melanogaster* among the alumina-extracted and electrochemically active compounds separated by HPLC reviewed by Wright (1987). Hodgetts and Konopka (1973) also failed to detect any NA in flies. However, the reports of Tunnicliff et al. (1969) and O'Dell et al. (1987) do support the presence of NA in *Drosophila*. Watson et al. (1993) have measured the content of NA in the heads of flies of *D. melanogaster* and have established that the content of the amine is very low in fruit fly. They showed that DA content is 11 times higher than the titer of NA in the flies of wild type line Canton-S of *D. melanogaster* (Watson et al. 1993). The concentrations of DA and OA are similar in the cockroach *P. americana*, while NA is present in very low amount (Evans 1978; Dymond and Evans 1979).

In *B. mori*, mated females start ovipositional behaviour in scotophase on the day when they have mated, but stressor-applied and mated females started the behaviour immediately after the onset of stressor application and in photophase they deposited about 20% of the total eggs laid within 24 h after the start of mating (unpublished observation). In *D. melanogaster*, OA biosynthesis requires TA β-hydroxylase (TβH) to convert TA to OA and the creation of null mutations at the TβH locus results in complete absence of the OA biosynthesis (Monastirioti et al. 1996). TβH-null female flies are sterile, although they mate normally. This defect in egg laying is due to

the null mutation of TβH and associated with the OA deficit, because females that have retained eggs initiate egg laying when transferred onto OA-supplemented food (Monastirioti et al. 1996).

Newly eclosed *D. melanogaster* flies were systemically depleted of DA by feeding on an inhibitor of the DA β-hydroxylase (DβH), and analyzed for abnormalities in courtship behaviour (Pendleton et al. 1996). DA-depleted females were significantly less receptive to males than were control females, although males were strongly attracted to treated females. Female receptivity may be regulated via interactions with hormonal pathways, since depletion of DA levels via inhibition of DβH activity in *D. melanogaster* adult females has established that DA is required for normal ovarian maturation and fecundity. Actually, the inhibition of DβH had significant effects on prevention of progeny formation (Pendleton et al. 1996). The effects of an inhibitor of DβH were examined on progeny formation and the contents of NA, OA and its precursor TA in *D. melanogaster*, using a simple method based on HPLC with ECD (Hirashima et al. 2006). Furthermore, it determined whether the prevention of progeny formation is due to inhibition of DβH or TβH (Hirashima et al. 2006).

The control over contraction of the oviducts of the African migratory locust *L. migratoria* has been physiologically well characterized. Two of the main neuromodulators that affect oviduct contractions are the biogenic amine OA and the pentapeptide proctolin. Both of these are released onto the oviducts from nerve terminals (Orchard and Lange 1987), but may also have a hormonal effect, after being released into the haemolymph. OA works primarily to inhibit contractions of the oviduct muscle by decreasing the tonus and has been shown to inhibit neurally-evoked contractions of the oviducts (Orchard and Lange 1985). Proctolin on the other hand, is a strong stimulant of contraction of the oviducts, and increases the frequency and amplitude of spontaneous phasic contractions, and results in a sustained basal contraction (Lange et al. 1986, 1987; Lange 1992). The mechanism of contraction of this insect visceral muscle, as well as the intracellular signal transduction pathways involving OA and proctolin, has been more clearly elucidated as shown in the review by Lange and Nykamp (1996).

According to Restifo and White (1990), it is possible that a minor catecholamine, present only in a few neurons, could have been easily lost in some of the analyses including the prepurification of the sample by alumina column. There is an agreement between the results of this study in *D. melanogaster* and published results (Rauschenbach et al. 1993; Watson et al. 1993) for the catecholamines titers in *D. melanogaster* and in different parts of the nervous system of insect species (Brown and Nestler 1985; Evans 1980,1985; Tanaka and Takeda 1997). NA is known to be present in brain and nervous tissues of some insect species; *Periplaneta* (Hirashima and Eto 1993a,b) and *Locusta* (Brown and Nestler 1985) in relatively low quantities ranging between 0.1 and 1 ng per tissue. Martínez-Ramírez et al. (1992) used HPLC-ECD technique to detect catecholamines in whole body extracts of *D. melanogaster*. The lability of catecholamines and their low concentrations in *Drosophila* demands the detection methods as simple as possible. The HPLC-ECD method described in the section "Materials and methods" by Hirashima et al. (2006) provides a simple way of measurement of various biogenic amines in *Drosophila* samples without preliminary

purification of the extracts. The identity of chromatographic peak of the biogenic amines has been demonstrated by comparison with the standard. TβH and DβH, which catalyze the final hydroxylation step in the production of OA and NA, are functionally homologous and are probably related evolutionarily.

6 Agonists and Antagonists for TA and OA Receptors

The antagonists for TAR inhibited calling behaviour (Hirashima et al. 2003a,c, 2004a,b) and in vitro pheromone synthesis of *P. interpunctella* (Hirashima et al. 2003b, 2004b), thereby providing evidence of the involvement of a TA receptor. Antagonists for TA receptor were tested for inhibitory specificity using a modified radiochemical bioassay to monitor de novo pheromone production in *P. interpunctella*. Inhibitory activity of a set of molecules in sex-pheromone production in *P. interpunctella* was tested. Arylaldehyde semicarbazones (AASs) and 5-aryloxazoles (AONs), whose structure are shown in Fig. 2, had the highest potency in inhibition of pheromone production in vitro and calling behaviour, respectively (Hirashima et al. 2004b).

We have reported a successful short-term in vitro pheromonal bioassay technique, in which PBAN-stimulated incorporation of radioactivity was inhibited by various compounds (Hirashima et al. 2003b). We used here calling behaviour and the short-term in vitro pheromonal-bioassay technique for screening inhibitors in sex-pheromone production. In this study, highly active compounds have been identified as specific pheromonostatic compounds which inhibited calling behaviour of female *P. interpunctella*. They also inhibited in vitro pheromone production of *P. interpunctella* at the ID_{50} ranges expected from the calling-behaviour experiment, taking into account that the compounds were administered in vitro. On the basis of dose-response studies a putative hypothetical model for active analogs could be determined.

Compounds 1-(2,6-diethylphenyl)imidazolidine-2-thione (DEIT) and 2-(2,6-diethylphenyl)iminoimidazolidine (NC-5) only were full agonists (Hirashima et al. 1992b, 2002, 2003d) for OAR in this study, since it generated the same amount of cAMP as OA and all other compounds were partial agonists, since they did not increase cAMP levels significantly, compared to OA. However, they were poor inhibitors both in calling behaviour and pheromone production. There are several receptors that can be activated by OA in the central nervous system that can either activate or inhibit adenylate-cyclase activity (Evans and Maqueira, 2005; Maqueira et al. 2005). In addition, at least one of these receptors may be expressed in up to six alternatively spliced forms which may have different functional properties. Maximal stimulation of nerve cord adenylate-cyclase activity by 2-(4-methyl-2-chlorophenyl)-iminooxazolidine (AC-6), DEIT and NC-5 was inhibited by several antagonists, including mianserin, cyproheptadine, chlorpromazine and gramine (Hirashima et al. 1992b). The rank-order ability of these antagonists to block the maximal adenylate-cyclase activation by AC-6, DEIT and NC-5 was identical to the rank-order

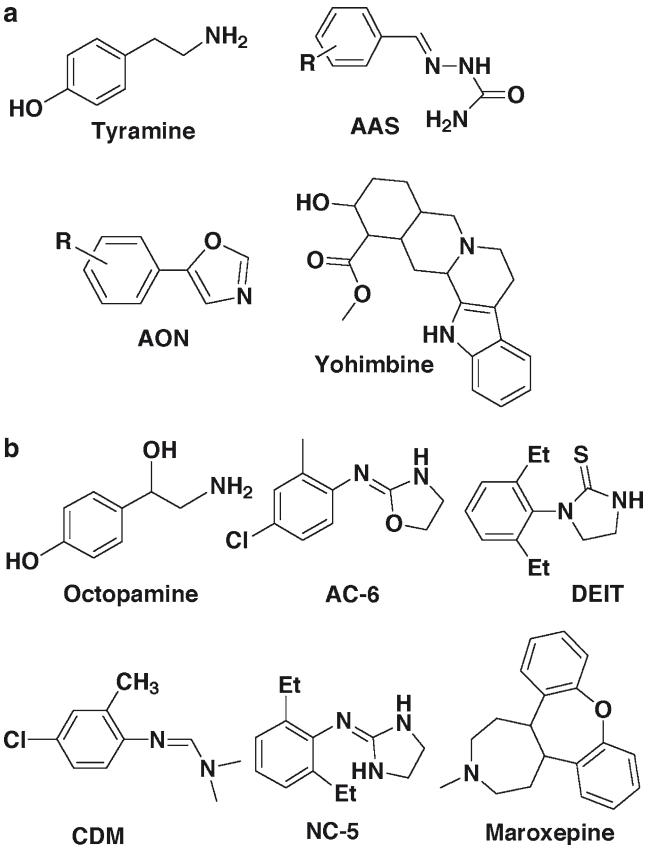

Fig. 2 Structures of agonists and antagonists for (**a**) TAR and (**b**) OAR: AAS, arylaldehyde semicarbazone; AON, 5-aryloxazole; AC-6, 2-(4-methyl-2-chlorophenyl)iminooxazolidine; DEIT, 1-(2,6-diethylphenyl)imidazolidine-2-thione; CDM, chlordimeform; NC-5, 2-(2,6-diethylphenyl) iminoimidazolidine. Maximal stimulation of nerve cord adenylate-cyclase activity by AC-6, DEIT and NC-5 was inhibited by several antagonists, including mianserin, cyproheptadine, chlorpromazine and gramine (Hirashima et al. 1992b). The antagonists for TAR including AASs, AONs and yohimbine inhibited calling behaviour and in vitro pheromone synthesis of *P. interpunctella*, thereby providing evidence of the involvement of a TAR (Hirashima et al. 2003b, 2004b)

ability of the same antagonists to block the enzyme activation by an optimally effective concentration of OA. The β-adrenergic antagonist propranolol was less potent in this respect. Thus, the action of AC-6, DEIT and NC-5 is same as that of OA, due to elevated levels of cAMP via OAR2.

Isolation, chlordimeform (CDM), some agonists for OAR and precocene II accelerated pupation of larvae of *T. freemani* reared under the crowded conditions (Hirashima et al. 1995a). These effects were antagonized by JH I. Optical stress caused an increase in OA content, JHE activity, ecdysteroid level and resulted in early pupation of crowded larvae. Hence, both isolation and optical stressor resulted

in an increase of OA content, followed by increase of JHE activity, thus decreasing JH titer, stimulation of the ecdysteroid level and advancing metamorphosis. Additionally, CDM, AC-6 and DEIT stimulated JHE followed by increase of ecdysteroids leading to stimulation of pupation in *T. freemani* (Hirashima et al. 1995a,b, 1998) whose action is supposed to be via TAR (Fig. 3). CDM, AC-6 and DEIT have been originaly found to work on OAR (Hirashima et al. 1995b). This could be due to structural similarity of TA and OA, suggesting possible cross reactivity of TAR and OAR. Decreased levels of cAMP caused via TAR may stimulate pupation. TA seems to be responsible for pupational programming: TA may activate the events preparatory to a pupal moult, i.e. the secretion of TA could stimulate ecdysteroid release. On the other hand, stressor such as heat and cold shock deactivated JHE followed by decrease of ecdysteroids leading to delay of pupation in *T. freemani* whose action is supposed to be via OAR (Fig. 3). Stressor (crowding)-induced increase in the levels of OA was observed in *T. freemani* (Hirashima et al. 1993c). Suppression of pupation under crowded conditions in *T. freemani* may be due to hyperactivity of the CA resulting in high levels of JH. Increased levels of cAMP caused via OAR may be responsible for high levels of JH, leading to delay of pupation. In the presence of both JH and ecdysteroids, *B. mori* repeats larval ecdysis, whereas in the presence of ecdysteroids and absence of JH, it engages in pupal ecdysis. Thus, taken together, in addition to corazonin, ecdysis triggering hormone, eclosion hormone, crustacean cardioacceleratory peptide, bursicon etc., TA and OA may produce some biological effects on the events preparatory to a pupal moult (Hirashima et al. 1995a,b, 1998).

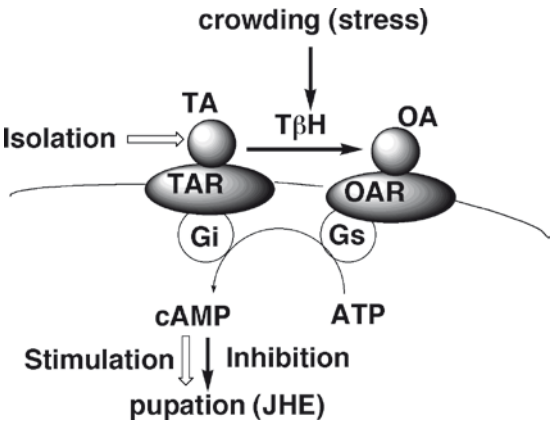

Fig. 3 Involvement of TA and OA on pupation in *T. freemani*: TA, tyramine; TβH, tyramine-β-hydroxylase; OA, octopamine; TAR, tyramine receptor; OAR, octopamine receptor; JHE, juvenile hormone esterase. CDM, AC-6 and DEIT stimulated JHE followed by increase of ecdysteroids leading to stimulation of pupation in *T. freemani* (Hirashima et al. 1995a,b, 1998) whose action is supposed to be via TAR. Stressor (crowding)-induced increase in the levels of OA was observed in *T. freemani* (Hirashima et al. 1993c). Suppression of pupation under crowded conditions in *T. freemani* may be due to hyperactivity of the CA resulting in high levels of JH. Increased levels of cAMP caused via OAR may be responsible for high levels of JH, leading to delay of pupation

7 Computer-Assisted Drug Design

Quantitative structure–activity relationship (QSAR) modeling is an area of research pioneered by Hansch and Fujita. The QSAR study assumes that the difference of the molecules in the structural properties experimentally measured accounts for the difference in their observed biological or chemical properties (Hansch and Fujita 1964; Hansch and Leo 1995). The result of QSAR usually reflects as a predictive formula and attempts to model the activity of a series of compounds using measured or computed properties of the compounds. More recently, QSAR has been extended by including the three-dimensional information. In drug discovery, it is common to have measured activity data for a set of compounds acting upon a particular protein but not to have knowledge of the three-dimensional (3D) structure of the active site. In the absence of such 3D information, one may attempt to build a hypothetical model of the active site that can provide insight on the nature of the active site. This approach using comparative receptor surface analysis (CoRSA) and molecular field analysis (MFA) is effective for the analysis of data sets where activity information is available but the structure of the receptor site is unknown. MFA is quantitative and a pharmacophore is qualitative. MFAs differ from pharmacophore models in that the former tries to capture essential information about the receptor, while the latter only captures information about the commonality of compounds that bind.

7.1 Homology Modeling, Agonist Binding Site Identification, and Docking

OAR2 has been defined as one of class A GPCR. This past year has seen a steady and exciting growth of novel inhibitors identified through computational analysis of target structure. A combination of more structural comparison, advances in homology modeling, better docking and scoring tools, fragment-based methods, and advances in virtual screening has been fundamental in this progress. Protein structure based small molecule design is clearly becoming a valuable and integral part of the inhibitor discovery, which has been proven to be more efficient and productive. In order to understand OAR2 protein-ligand interaction, the 3D model of OAR2 was predicted, and then its agonist binding site was identified, followed by docking study (Hirashima and Huang 2008) using Discovery Studio (DS Modeling1.1/1.2, Accelrys Inc.).

GPCRs include receptors for sensory signal mediators. While in other types of receptors ligands bind externally to the membrane, the ligands of family 1 GPCRs typically bind within the transmembrane domain (Bockaert and Pin 1999). The transduction of the signal through the membrane by the receptor is not completely understood. It is known that the inactive G protein is bound to the receptor in its inactive state. Once the ligand is recognized, the receptor shifts conformation and thus mechanically activates the G protein, which detaches from the receptor. The receptor can now either activate another G protein, or switch back to its inactive state. It is believed that a receptor molecule exists in a conformational equilibrium between active

and inactive biophysical states (Rubenstein and Lanzara 1998). The binding of ligands to the receptor may shift the equilibrium toward the active receptor states. Three types of ligands exist: agonists are ligands which shift the equilibrium in favour of active states; inverse agonists are ligands which shift the equilibrium in favour of inactive states; and neutral antagonists are ligands which do not affect the equilibrium. It is not yet known how exactly the active and inactive states differ from each other.

If a receptor in an active state encounters a G protein, it may activate it. Some evidence suggests that receptors and G proteins are actually pre-coupled. For example, binding of G proteins to receptors affects the receptor's affinity for ligands. Activated G proteins are bound to GTP. The enzyme adenylate cyclase is an example of a cellular protein that can be regulated by a G protein. Adenylate-cyclase activity is activated when it binds to a subunit of the activated G protein. Activation of adenylate cyclase ends when the G protein returns to the GDP-bound state.

Based upon this study, several models for the agonist-OAR2 interactions have been proposed. Those models are considered to be useful in designing new leads for hopefully more active compounds, although the numbers of compounds tested are still limited. Hence, a further comparison study of 3D models responsible for the agonist-OAR2 interactions is in progress and expected to clarify the mode of action of these compounds acting on the OAR2. Such work will surely help to elucidate the mechanisms of OAR2-ligand interactions. The molecular modeling studies (Hirashima and Huang 2008) show that agonists with certain substituents can be potential ligands to OAR2. In order to optimize the activities of these compounds as agonists for OAR2, more detailed experiments are in progress.

7.2 Hypothesis Generation

A pharmacophore model postulates that there is an essential three-dimensional arrangement of functional groups that a molecule must possess to be recognized by the receptor. It collects chemical features distributed in 3D space that is intended to represent groups in a molecule that participates in important binding interactions between ligands and their receptors. Hence, a pharmacophore model provides crucial information about how well the chemical features of a subject molecule overlap with the hypothesis model. It also informs the ability of molecules to adjust their conformations in order to fit a receptor with energetically reasonable conformations. Pharmacophore models tend to be geometrically underconstrained (while topologically overconstrained); this steric underconstraint leads to false positives, that is, compounds that are deemed active by the model but which are inactive when tested. They postulate a 3D arrangement of atoms recognizable by the active site in terms of the similarity of functional groups common to the set of binding molecules.

A 3D pharmacophore hypothesis that is consistent with known data should be useful and predictive in evaluating new compounds and directing further synthesis. A pharmacophore model postulates that there is an essential 3D arrangement of functional groups that a molecule must possess to be recognized by the active site.

It collects common features distributed in 3D space that is intended to represent groups in a molecule that participates in important interactions between drugs and their active sites. Hence, a pharmacophore model provides crucial information about how well the common features of a subject molecule overlap with the hypothesis model. It also informs the ability of molecules to adjust their conformations in order to fit an active site with energetically reasonable conformations. Such characterized 3D models convey important information in an intuitive manner.

Catalyst modeling environment from Accelyrys (San Diego, CA) automatically generated conformational models for each compound using the Poling Algorithm. The number of conformations needed to produce a good representation of a compound's conformational space depends on the molecule. Conformation-generating algorithms were adjusted to produce a diverse set of conformations, avoiding repetitious groups of conformations all representing local minima. The conformations generated were used to align common molecular features and generate pharmacophoric hypotheses. HipHop used conformations generated to align chemically important functional groups common to the molecules in the study set. Maroxepine showed the highest activity as an antagonist of the locust OAR (Roeder 1990). A pharmacophoric hypothesis then was generated from these aligned structures of agonists (Hirashima et al. 1999b, 2002) and antagonists (Pan et al. 1997) for OAR, and TA ligands (Hirashima et al. 2003e, 2004a,b).

The work of antagonists and agonists for OAR and TAR shows how a set of activities of various compounds may be treated statistically to uncover the molecular characteristics which are essential for high activity. These characteristics are expressed as common features disposed in 3D space and are collectively termed a hypothesis. Hypotheses were obtained and applied to map the active or inactive compounds. Important features of the surface-assessable models were found which are the minimum components of a hypothesis for effective compounds. It was found that more active compounds map well onto all the features of the hypotheses. For some inactive compounds, their lack of affinity is primarily due to their inability to achieve an energetically favorable conformation shared by the active compounds. Taken together, the antagonist YHN, which has a selectivity for TAR (Hiripi et al. 1994), showed an inhibitory activity of pheromone production. Thus, inhibition of calling behaviour and PBAN-stimulated incorporation of radioactivity is via antagonistic effect to TA receptor.

7.3 Comparative Receptor Surface Analysis

Receptor surface models (RSMs) are predictive and sufficiently reliable to guide the chemist in the design of novel compounds. These descriptors were used for predictive QSAR models of agonists (Hirashima et al. 2003d) and antagonists (Hirashima et al. 2003c) for OAR, and TA ligands (Hirashima et al. 2004a). Currently, work on GPCRs suggests that agonists and antagonists may bind to additional sites on GPCRs which affect the true ligand binding site by allosteric modulation. In the present case it is

clearly not known how many different sites their different substances are binding to. The assay read out is many steps away from the actual binding of the compounds to their receptor site(s) which makes things even more difficult to interpret. In such case we have presented an application of CoRSA that can be applied to study ligand–receptor interactions whenever the structure of the biological target is not known. The in vitro interaction between chemical compounds and their biological targets (transporters, receptors, ion channels, enzymes) can be efficiently predicted with the 3D QSAR models. A subset of the most active molecules was selected to generate the virtual receptor model; these molecules represent the receptor generating set of compounds. The central assumption is the complementarity between the shape and properties (atomic charges, hydrophobicity, hydrogen-bonding properties) of these molecules and the virtual receptor. Unlike real receptors (formed by atoms), the CoRSA virtual receptor is represented by points situated on a surface.

TA and OA are not likely to penetrate either the cuticle or the central nervous system of insects effectively, since it is fully ionized at physiological pH. Derivatization of the polar groups would be one possible solution to this problem in trying to develop potential pest-control agents. The above CoRSA studies show that phenyl ring substitution requirements for agonists for TAR and OAR differ substantially from each other and other various types of ligands could be potent, although the type of compounds tested here is still limited to draw any conclusions. These derivatives could provide useful information in the characterization and differentiation of TAR and OAR. The agonists for TAR and OAR showed reasonable predicted activities according to CoRSA. The result may imply that the process of calculating an RSM treats these structures reasonably. The CoRSA could provide useful information in the characterization and differentiation of TAR and OAR. It may help to point the way towards developing extremely potent and relatively specific TA and OA ligands, leading to potential insecticides, although further research on the comparison of the 3D QSAR is necessary. In order to optimize the activities of these compounds as TA ligands, more detailed experiments are in progress.

7.4 Molecular Field Analysis

MFAs tend to be geometrically overconstrained (and topologically neutral), since in the absence of steric variation in a region, they assume the tightest steric surface which fits all training compounds. MFAs do not contain atoms, but try to directly represent the essential features of an active site by assuming complementarity between the shape and properties of the receptor site and the set of binding compounds. The MFA application uses 3D surfaces that define the shape of the receptor site by enclosing the most active members (after appropriate alignment) of a series of compounds. The global minimum of the most active compound in the study table (based on the value in the activity column) was made as the active conformer. When there is no information on the actual “active conformation” of the ligands, MFA does

not really describe the receptor; it describes a self-consistent field around the molecules that can explain activity. It really is just one of possibly many self-consistent models that fit the biological activity data.

MFA models are predictive and sufficiently reliable to guide the chemist in the design of novel compounds. These descriptors were used for predictive QSAR models of agonists and antagonists for OAR (Hirashima et al. 1999a), and TA ligands (Hirashima et al. 2003a). When agonists and antagonists were analyzed together, correlation was drastically decreased. Whereas, correlation was unexpectedly good, when agonists and antagonists were analyzed separately, suggesting that agonists and antagonists have different structural features. However, although antagonists may not interact with the same part of the membrane with which the agonists interact, the presence of some common structural elements, such as the phenyl ring, suggests the binding sites may have some features in common. The ionophore, one component of the cell membrane, may be activated between a ligand-receptor interaction. Antagonists may act via the receptor or via the ionophore or a combination of both. Taken the part of the membrane with which the agonist interacts as the true receptor, the antagonist may well interact with an area surrounding the receptor including the ionophore.

8 Conclusions

Effect of TA and OA on insect behaviour such as sex-pheromone production, egg laying and metamorphosis has been discussed in detail. TA inhibited pheromone production in vitro in a dose dependent manner and DA had a lower inhibitory activity than TA, whereas OA had no effect, suggesting that TA is a candidate for regulating pheromone production in the PG of *B. mori*, although other factors could be responsible for the pheromonostatic function. The inhibition of progeny formation in *D. melanogaster* could be due to inhibition of TβH and DβH. TA is a causative agent in pupational programming of *T. freemani*, by inducing an increase of JHE activity, thus decreasing JH titer followed by an increase of ecdysteroid level and delayed pupation. AASs and AONs had the highest potency in inhibition of pheromone production in vitro and calling behaviour of *P. interpunctella*, respectively.

Agonist docking into OAR model was done and the superimposition of two top poses of representative agonists was performed with a soft surface generated. Those models are considered to be used in designing new leads for hopefully more active compounds. Future design of new compounds will be directed at maximizing activity and provide ideas and details of how to advance this knowledge so that indeed future design will reveal some interesting compounds. It may point the way towards developing extremely potent and relatively specific agonists for OAR and TAR, leading to potential pest-control agents. Additionally, considering new findings of the 3D structure of the adrenergic GPCR (Rasmussen et al. 2007), one would prevent these newly designed compounds from affecting the adrenergic receptors of vertebrates, i.e. designing truly specific agents.

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Recent Advances in the Mode of Action of Juvenile Hormones and Their Analogs

Subba Reddy Palli

1 Introduction

Ecdysteroids (steroid hormones, 20 hydroxyecdysone, 20E, is the most active form) and juvenile hormones (JH, sesquiterpenoids) regulate almost every aspect of the insect's life including growth, development, immune response and reproduction. While 20E initiates and coordinates molting and metamorphosis, JH maintains the juvenile status and when the JH level goes down, it signals the insect to undergo metamorphosis (Riddiford 1994, 1996). Both 20E and JH along with other hormones regulate vitellogenesis, oogenesis, fertilization and choriogenesis during reproductive maturation of insects (Wyatt et al. 1996). In some insects such as the mosquito *Aedes aegypti*, 20E plays a critical role in the regulation of vitellogenin genes (Raikhel et al. 2002), whereas in others such as *Locusta migratoria*, JH plays a key role in the regulation of vitellogenin genes (Dhadialla and Wyatt 1983). In *Drosophila melanogaster*, 20E enhances the expression of some of the antibacterial peptides such as diptericin that are involved in the suppression of bacterial infection and JH suppresses this enhancement (Flatt et al. 2008). In *Spodoptera exigua* 20E enhances the sensitivity of plasmatocytes to an insect cytokine, plasmatocyte-spreading peptide whereas JH and JHA antagonize this enhancement (Kim et al. 2008). In addition, JH regulates other transformative processes such as diapause, migratory behavior, wing length, color polymorphism and caste determination (Futahashi and Fujiwara 2008; Giray et al. 2005; Hartfelder 2000; Hrdy et al. 2006; Min et al. 2004; Singtripop et al. 2000).

Numerous analogs of JH have been synthesized and tested for their pest control potential (Fig.1). JH analogs (JHA) have also been found to be naturally occurring in many plants supposedly as a defense mechanism. The JHAs, methoprene and hydroprene produce the characteristic effects of JH inducing precocious metamorphosis during early larval stages (Parthasarathy et al. 2008) and larval-pupal deformities

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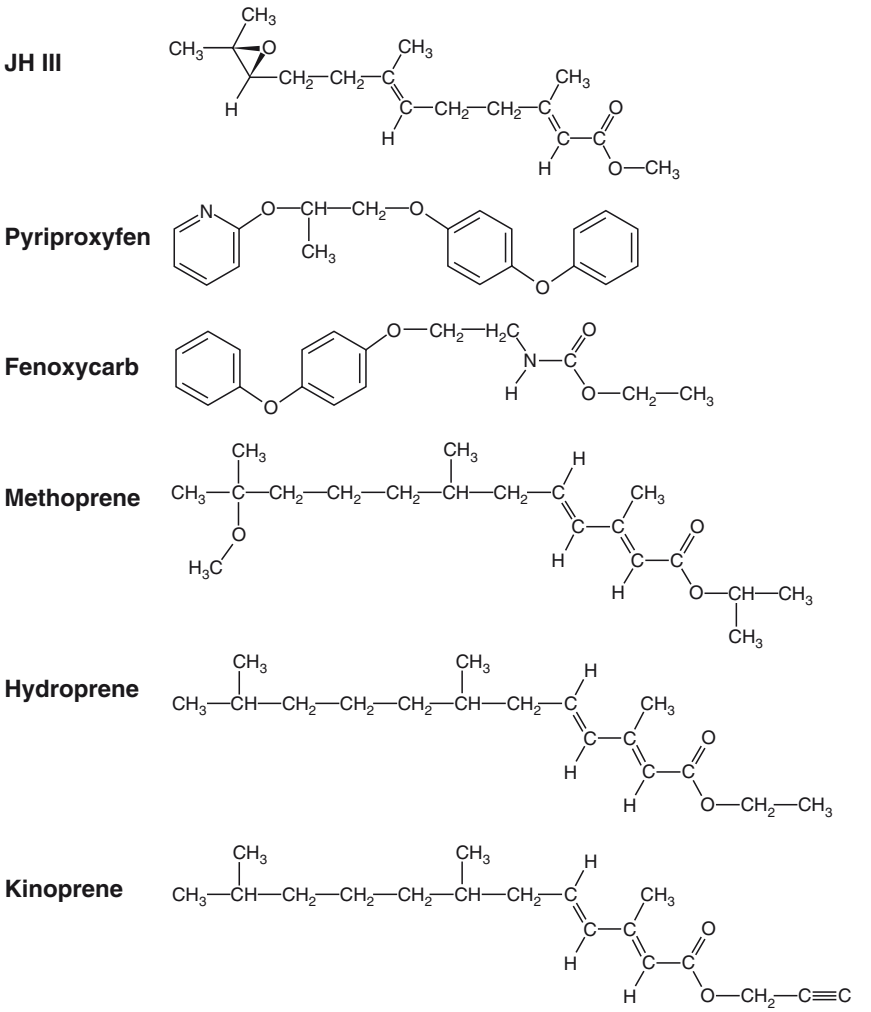

Fig. 1 Chemical structures of JH III and JHAs, pyriproxyfen, fenoxycarb, methoprene, hydroprene and kinoprene

during the final instar (Parthasarathy and Palli 2007). Fenoxycarb is a JHA that has a carbamate moiety in its structure and mimics JH action (Davey and Gordon 1996). Juvenile hormone analogs such as methoprene, hydroprene, kinoprene and pyriproxyfen have been used to control several pests and disease vectors such as white flies and mosquitoes (Dhadialla et al. 2005; Henrick 2007; Lee 2001; McCarry 1996; Sjogren et al. 1986; Staal et al. 1976). However, there are reports of development of resistance to methoprene in some insects, such as *Aedes nigromaculis*, (Cornel et al. 2000), *Oclerotatus nigromaculis* (Cornel et al. 2002), *Aedes taeniorhynchus* (Dame et al.

1998), *Anopheles gambiae* (Kadri 1975) *Culex fatigans* (Brown and Brown 1974) and *Musca domestica* (Cerf and Georghiou 1972; Georghiou et al. 1978; Pospischil et al. 1996). Pyriproxyfen resistance in *M. domestica* (Bull and Meola 1994; Zhang et al. 1997; Zhang and Shono 1997) and *Bemisia tabaci* (Devine et al. 1999; Crowder et al. 2006, 2007, 2008; Horowitz et al. 2002, 2003, 2005; Li et al. 2003) has also been reported. The mechanisms of resistance to JHAs are not well understood. Methoprene resistance in *M. domestica* thought to be caused by increased metabolism of methoprene by oxidation (Bull and Meola 1994; Zhang et al. 1998). *O. nigromaculis* resistance to methoprene is likely not due to increased metabolism of methoprene because the synergists have no effect on resistance (Cornel et al. 2002). The only known example of target site resistance for JHAs is the methoprene tolerant (Met) mutation identified in *D. melanogaster* (see below for details on Met (Wilson and Fabian 1986)). Although the biological actions of JH and JHA have been well studied, the molecular mode of action of JH and JHA is not well understood. More recently, during the past few years, there has been significant progress in our understanding of the mode of action of JH and JHA. In this chapter, I will summarize the recent progress made in our understanding of the mode of action of JH and JHA. First a brief description of the candidate proteins involved in JH action indentified to date will be presented. This will be followed by the recent progress in our understanding of the mode of action of JH and JH modulation of ecdysteroid action. Finally, a model based on these findings will be proposed. This model could serve as the basis for future studies on the molecular mode of action of JH and JHA.

2 JH-Response Genes

Several JH-responsive genes including calmodulin, vetellogenin and diapause protein have been identified (Glinka et al. 1995; Hirai et al. 1998; Iyengar and Kunkel 1995). Another JH-responsive gene, jhp21, was identified in *L. migratoria*, and a response element has been identified in the promoter region of this gene (Zhang et al. 1996). In *Manduca sexta* fat body cells, a JH-binding protein (JHBP) is induced by JH I within 2 h after treatment and appears to be a primary response gene (Orth et al. 1999). Six putative regulatory elements, Hunchback, Heat shock factor binding element, Ultrabithorax, Broad-Complex Z3 and Chorion factor 1 were identified in the 2,000 bp promoter region of the JHBP gene from *Galleria mellonella* (Sok et al. 2008). Footprinting of this promoter identified a response element (TCAACA-AAC-TGTTCA) that binds with high-affinity to EcR:USP heterodimer. Deletions of region of JHBP gene promoter containing this response element enhanced the transcriptional activity of luciferase reporter gene in the *Trichoplusia ni* High Five cell line suggesting that this response element mediates inhibitory effect on the expression of JHBP gene.

Differential display of mRNAs after JH treatment was used to identify *Choristoneura fumiferana* juvenile hormone esterase (Cfjhe) as a primary JH-response gene in CF-203 cell line developed from *C. fumiferana* (Feng et al. 1999). This

Cfjhe gene is directly induced by JH I within one hour after adding the hormone. The expression of Cfjhe is suppressed by 20E even in the presence of cyclohexamide (Feng et al. 1999). Cfjhe is one of the few examples in which the direct regulation by JH and 20E has been demonstrated experimentally. Expression of jhe gene in *T. ni* (Jones et al. 1994), *Heliothis virescens* (Wroblewski et al. 1990) and *D. melanogaster* and *Tribolium castaneum* is induced by JH. Thus, at least in these five species of insects the jhe gene is a direct target for JH.

The promoter region of *T. ni* JHE gene was characterized, and a putative DR-1 element and fat body consensus elements were identified (Jones et al. 1998, 2000; Schelling and Jones 1995; Venkataraman et al. 1994). A 1,270-bp promoter region of the Cfjhe gene was cloned and a 30-bp region that is located between −604 and −574 and is sufficient to support both JH I induction and 20E suppression was identified (Kethidi et al. 2004). This 30-bp region contains two conserved hormone response element half-sites separated by a 4-nucleotide spacer similar to the direct repeat 4 element, DR4. The luciferase reporter placed under the control of this DR4 element and a minimal promoter was induced by JH I in a dose- and time-dependent manner. In addition, nuclear proteins isolated from JH I-treated CF-203 cells bound to DR4 and the binding was competed by a 100-fold excess of the cold probe but not by 100-fold excess of double-stranded oligonucleotides of an unrelated sequence.

Dubrovsky et al. (Dubrovsky et al. 2000, 2002) showed that *D. melanogaster* S2 cells responded to JH; they used differential display of mRNAs technique and identified four JH response genes, JhI-1, Jhl-26, JhI-21 and minidisks (*mnd*). Three of these genes JhI-1, JhI-26 and *mnd* are induced rapidly, indicating that they may be regulated directly by a JH-receptor complex. The biological functions and the nature of the promoter regions of these genes are not yet known. Dubrovsky et al., (2004) showed that E75A is regulated by JH in *D. melanogaster* S2 cells. Beckstead et al., (2007) used microarray analysis and identified E74B, pepck, and CG14949 as JH-response genes. Among these three genes, CG14949 can be induced by JH independently of protein synthesis. Li et al., (2007) compared JH-response genes identified by microarray analysis in honey bee and *D. melanogaster* L57 cells and identified 16 genes that are induced by JH in both the honey bee and the L57 cells. In *D. melanogaster*, kruppel homologue, Kr-h1 mRNA is induced in the abdominal integument by JH application at pupariation and acts upstream.

3 Proteins Identified to be Involved in JH and JHA Action

Several hemolymph, cytosolic and nuclear JH binding proteins have been identified and characterized (de Kort and Granger 1996; Palli et al. 1991). Human retinoic acid receptor cDNA was used as a probe to identify a steroid/thyroid superfamily member from *M. sexta*. Further characterization of this cDNA revealed that this is not a JH receptor but rather a 20E-induced transcription factor which plays a critical role in ecdysone signal transduction and is related to the *Drosophila* hormone

receptor 3 (Koelle et al. 1992), and was subsequently named as *Manduca* hormone receptor 3 (Palli et al. 1992).

3.1 64 kDa Cockroach Protein

A 64 kDa protein was purified from the fat body of the cockroach, *Leucophaea maderae* and this protein was shown to bind with high affinity to JH III (Engelmann 1995). This protein was detected in the last instar nymphs and its titer increased throughout this stage, but none of this protein was detected in the penultimate nymphal instar. Lack of expression in the penultimate instar correlated with a lack of competence to make vitellogenin upon exposure to the JH analog, methoprene. This protein is an attractive candidate for JH receptor, because of its high affinity binding to JH and correlation of its presence with JH action.

3.2 29 kDa Manduca Protein

Photoaffinity analogs of JH I and methoprene were used to identify a 29 kDa protein in the epidermis of *M. sexta*. This protein was purified and partially sequenced and the sequence was used to isolate cDNA (Palli et al. 1994). The protein expressed from this cDNA showed high affinity binding to JH I in the initial analysis (Palli et al. 1994), but subsequent analysis showed that the high affinity JH I binding observed for the recombinant 29 kDa protein was an artifact caused by endogenous cellular esterases that co-purified with a recombinant 29 kDa protein (Charles et al. 1996). The abundance of the 29 kDa protein that was identified through photoaffinity labeling was very low, but the abundance of mRNA for the gene identified based on the sequence from the purified 29 kDa protein was high. Therefore, there is a discrepancy between the protein and the mRNA abundance. This could be due to several reasons, including a regulation of translation of mRNA for 29 kDa protein. It is also possible that the protein purified is not the low abundant photoaffinity labeled 29 kDa protein but another abundant protein with a molecular weight of 29 kDa. It is not known whether the 29 kDa protein that bound to the photoaffinity analogs of JH I and the 29 kDa protein that was purified are one and the same.

3.3 35 kDa Membrane Protein

The action of JH on the uptake of yolk protein, vitellogenin (Vg), was studied in the locust and a 35 kDa JH binding protein was identified and it was proposed that it is a critical protein involved in JH action (Sevela et al. 1995). Polyclonal antibodies produced against the partially purified protein were used to screen a locust ovarian cDNA library and a cDNA coding for a 35 kDa protein was isolated. The

cDNA was expressed in a baculovirus system, and a 35 kDa protein was produced. This protein produced in a baculovirus system did not show high affinity JH binding. Since this is a membrane protein, there could have been problems in modification and translocation of this protein to the proper site within the cell during its expression in the baculovirus system.

3.4 TFP1

The JH-response gene, jhp21, was identified in *L. migratoria*, and a response element has been identified in the promoter region of this gene (Zhang et al. 1996). Characterization of the protein that binds to this response element lead to the hypothesis that this protein is a transcription factor activated by JH and participates in the regulation of expression of the JH-response genes (Zhou et al. 2002). Walker and her colleagues (Zhou et al. 2006) used the JHRE identified in the promoter of JHP21 in a yeast one-hybrid assay and identified a transcription factor (tfp1) and a putative lipid-binding protein. This protein is constitutively present in the fat body and is most likely recruited to a larger transcription complex in the presence of JH.

3.5 USP

Binding of JH III, but not JH acid or methoprene to FXR and RXR heterodimer has been reported (Forman et al. 1995). Activation of RXR by methoprene and methoprene acid has also been reported (Harmon et al. 1995). These two studies suggested that RXR or its insect homologue USP could play an important role in signal transduction of JH or JH-related compounds. Jones and Sharp (Jones and Sharp 1997) showed that both JH III and JHB_3 bind to a *D. melanogaster* USP homodimer. Subsequent studies showed that *D. melanogaster* USP can bind DR12 response element, and JH III can induce a reporter gene placed under the control of the DR12 response element and *jhe* core promoter (Jones et al. 2001; Xu et al. 2002). Interestingly, methyl farnesoate, the precursor of JH III, bound to USP with nearly a 100-fold higher binding affinity as compared to the binding affinity of JH III to USP raising the possibility of USP being the methylfarnesoate receptor.

3.6 Met

Most of the studies on Met come from the model insect, *D. melanogaster*. Mutagenic screening of *D. melanogaster* resistant to methoprene, a JH analog

used as an insecticide, identified Met (Methoprene tolerant) allele and examination of Met allele phenotypes and rescue by Met + transgenes clearly showed Met gene product is directly responsible for methoprene resistance (Wilson and Fabian 1986). Further characterization of mutant flies showed no changes in penetration, degradation or sequestration of methoprene between the wild-type and mutant flies. In addition, protein extracts isolated from the fat body and accessory glands of Met mutant flies showed reduced JH binding activity as compared to the proteins isolated from the wild-type flies suggesting that methoprene resistance is most likely due to target site insensitivity (Shemshedini et al. 1990) The Met mutation in *D. melanogaster* resulted in 100-fold enhancement in resistance to methoprene and surprisingly, studies of fitness components of Met allele in comparison to the wild-type showed that loss of Met was not as severe as might be expected should Met play a key role in JH signal transduction (Minkoff and Wilson 1992). Cloning and characterization of the Met gene identified it as a member of the bHLH-PAS transcription factor family (Ashok et al. 1998). Gamma-ray induced allele, Met 27 flies that completely lacked the Met transcript were viable and showed resistance to both methoprene and JH (Wilson and Ashok 1998). Localization of Met proteins showed developmental and tissue specific expression in early embryos, and various JH target tissues of larvae, pupae and adults of *D. melanogaster* (Pursley et al. 2000). Although, Met mutants could not unravel the mechanisms of JH action during metamorphosis, the involvement of JH in stress response (Gruntenko et al. 2000a,b) and male reproduction (Wilson et al. 2003) were determined using Met mutants. Also, pleiotrophic effects of Met allele in JH-regulated life history traits of *D. melanogaster* were reported (Flatt and Kawecki 2004). Met protein produced *in vitro* bound to JH III with high affinity, consistent with the physiological concentration of JH and Met exerted JH and methoprene dependent activation of a reporter gene (Miura et al. 2005). This study raised the possibility of Met being a JH receptor. Later, identification of a paralogous gene in *D. melanogaster*, germ-cell expressed (gce) (Moore et al. 2000), whose protein product dimerized with Met in a JH sensitive manner (Godlewski et al. 2006) suggesting that GCE could complement functional redundancy in Met mutants rendering them viable. Recent studies have proposed a genetic interaction of Met with Broad in *D. melanogaster* (Wilson et al. 2006b). Also, wide spectrum mutation analysis of Met in *D. melanogaster* identified its role as a transcriptional regulator of ecdysone secondary response genes during metamorphosis (Wilson et al. 2006a). Search for Met homolog in other insects identified single ortholog of *Drosophila* Met and gce in lower dipteran mosquitoes (Wang et al. 2007) and coleopterans (Konopova and Jindra 2007a). RNAi knock-down in the expression of Met/gce homolog in the red flour beetle, *T. castaneum* rendered the beetles resistant to the effects of JH treatment and caused the early-stage beetle larvae to undergo precocious metamorphosis (Konopova and Jindra 2007a). RNAi knock-down in the expression of TcMet in the red flour beetle, *T. castaneum* rendered the beetles resistant to the effects of ectopic JH and caused the early-stage beetle larvae to undergo precocious metamorphosis (Konopova and Jindra 2007b). The phenotypes of TcMet RNAi insects shared similarity with the phenotypes of some allatectomized lepidopteran larvae that were attempting to undergo

precocious larval-pupal metamorphosis (Parthasarathy et al. 2008). *T. castaneum* larvae injected with TcMet dsRNA demonstrated resistance to a JH analog (JHA), hydroprene and also caused down regulation of JH-response genes, JHE and Kr-h1 suggesting that TcMet might be involved in the expression of these genes. These RNAi studies in *T. castaneum* confirmed a key role for Met in JH action but the mechanism of Met action in JH signal transduction remains unclear.

3.7 FKBP39 and Chd64

Sixteen genes induced by JH or JHA in both the fruit fly, *D. melanogaster* and the honey bee, *Apis mellifera* were identified after analyzing microarray data on JH-induced genes obtained after treating *Drosophila* L57 cells and the honey bee, *A. mellifera* with JH or JHA. Analysis of the promoter regions of these 16 *D. melanogaster* JH inducible genes identified a cis regulatory element present in the promoters of these genes and named as DmJHRE1 (*D. melanogaster* JH response element 1). The reporter gene regulated by DmJHRE1 transfected into L57 cells was induced by JH III. A DNA affinity column prepared with multimers of DmJHRE1 was used to purify the nuclear proteins isolated from the L57 cells treated with JH III and two proteins (FKBP39 and Chd64) were identified that bound to DmJHRE1 (Li et al. 2007). In L57 cells, FKBP39 and Chd64 double-stranded RNA inhibited JH III induction of the luciferase gene regulated by DmJHRE1 and a minimal promoter. Gel shift assays were used to demonstrate binding of FKBP39 and Chd64 proteins to DmJHRE1. Two-hybrid and pull-down assays showed that these two proteins interact with each other as well as with the ecdysone receptor, ultraspiracle and methoprene-tolerant protein. The developmental expression profiles of the genes coding for these two proteins, JH induction of FKBP39 and Chd64 mRNA and interaction of these proteins with proteins known to be involved in both JH (methoprene-tolerant protein) and ecdysteroid action (ecdysone receptor and ultraspiracle) suggests that these proteins probably play important roles in cross talk between JH and ecdysteroids.

4 JHA Methoprene Blocks Midgut Remodeling by Modulating 20E Action

In holometabolous insects, the midgut undergoes remodeling during metamorphosis. In the mosquito, *A. aegypti* larvae, the programmed cell death (PCD) of larval midgut cells and the proliferation and differentiation of imaginal cells are initiated around 36 h after ecdysis to the 4th instar and are completed by 12 h after ecdysis to the pupal stage (Wu et al. 2006). When the *A. aegypti* larvae are treated with methoprene, the proliferation and differentiation of imaginal cells are initiated at the same time as in untreated control larvae, but the PCD is initiated only after

ecdysis to the pupal stage. In this insect methoprene blocks the terminal events that occur for completion of PCD during pupal stage. As a result, the pupae developed from methoprene-treated larvae contain two midgut epithelial layers and these methoprene-treated larvae die during the pupal stage. Further studies using quantitative PCR showed that methoprene affected midgut remodeling by modulating the expression of ecdysone receptor B, ultraspiracle A, broad complex, E93, ftz-f1, dronc and drice, the genes that are shown to play key roles in 20E action and PCD suggesting that methoprene blocks midgut remodeling in *A aegypti* by interfering with the expression of genes involved in 20E action resulting in the death of treated insects during the pupal stage.

In lepidopteran insect, *H. virescens*, both programmed cell death (PCD) of larval midgut cells as well as proliferation and differentiation of imaginal cells begin at 108 h after ecdysis to the final instar and proceeded through the pupal stages (Parthasarathy and Palli 2007). In this insect methoprene blocks PCD by maintaining high levels of inhibitor of apoptosis, down-regulating the expression of caspase-1, ICE and inhibiting an increase in caspase-3 protein levels. The differentiation of imaginal cells is also impaired by methoprene treatment therefore, unlike in *A aegypti*, the formation of pupal/adult midgut was affected in *H. virescens*. However, in this insect as well, methoprene blocks midgut remodeling by modulating the action of 20E (Parthasarathy and Palli 2007).

5 JHA Hydroprene Blocks Metamorphosis and Midgut Remodeling by Modulating 20E Action

Application of hydroprene to *T. castaneum* penultimate or final instar larval stages blocked larval-pupal metamorphosis and induced supernumerary larval molts (Parthasarathy and Palli 2008a). Down-regulation of expression of transcription factor, Broad and up-regulation of other genes involved in ecdysteroid action (FTZ-F1, E74) were observed in JHA treated larvae undergoing supernumerary larval molts.

In the red flour beetle, *T. castaneum* the midgut remodeling is initiated at 96 hr after ecdysis into the final instar larval stage. Intestinal stem cells undergo proliferation and differentiation to form new midgut epithelium (Parthasarathy and Palli 2008b). *In vitro* midgut culture experiments showed that 20E induces proliferation of intestinal stem cells as well as programmed cell death of larval midgut epithelial cells. Both JH and hydroprene block 20E induced proliferation and differentiation of intestinal stem cells as well as programmed cell death of larval cells. Application of hydroprene to the final instar blocked midgut remodeling by affecting programmed cell death of larval cells and proliferation and differentiation of imaginal cells to pupal midgut epithelium. In this insect hydroprene suppresses the expression of EcRA, EcRB, Broad, E74, E75A, and E75B genes involved in ecdysteriod action resulting in a block in programmed cell death as well as proliferation and differentiation of imaginal cells. In this insect also, the JH analog hydroprene functions by modulating the action of ecdysteroids.

6 JH and JHA Potentiation of EcR Transactivation

In mammalian cells *D. melanogaster* EcR isoforms were responsive to 20E when they were paired with USP, and the transcriptional activity of EcRB2:USP heterodimer was potentiated by JHIII in the presence of 20E (Henrich et al. 2003). These studies showed that the ligand-binding domain of EcR is the prerequisite component for JH III potentiation of 20E action. The authors concluded that both the N-terminal domain of EcR and the particular ecdysteroid affect JHIII potentiation. In *D. melanogaster* S2 cell line JH III was shown to act as USP ligand and cause suppressive effects on 20E-induced EcR transactivation (Maki et al. 2004). The authors of these studies suggested that JH and JHAs serve as USP ligands and antagonize EcR-mediated ecdysone actions through the recruitment of histone deacetylase complexes. In a recent study, Sion and his colleagues showed that JHAs does not affect EcR activity in two insect cell lines, *D. melanogaster* S2 and *Bombyx mori* Bm5 (Soina et al. 2004). Interestingly, both these groups used the same cell line but employed different approaches and reached different conclusions. These studies demonstrate the complexity of JH and JHA action in modulation of ecdystriod action.

7 JH and JHA Suppress 20E Enhancement of Antimicrobial Peptide Gene Expression

JH has a profound effect on the immune response in insects. Although not universal, it appears in general that 20E enhances and JH suppresses the immune response. In the tobacco hornworm, *M. sexta*, JH suppresses the granular phenoloxidase synthesis and cuticular melanization (Hiruma and Riddiford 1988). JH also suppresses phenoloxidase synthesis and encapsulation in the mealworm, *Tenebrio molitor* (Markus et al. 2003; Rolff and Siva-Jothy 2002). In the larvae of the flesh fly, *Neobellieria bullata* JH suppresses the 20E-induced nodulation reaction (Franssens et al. 2006). In the fruit fly, *D. melanogaster* JHA and 20E showed antagonistic effects on the induction of antimicrobial peptide genes (Flatt et al. 2008). In *D. melanogaster* Schneider S2 cells 20E enhanced immune stimulation of antimicrobial peptide genes. In contrast, JH III and JHA, methoprene and pyriproxyfen, strongly interfered with this 20E-dependent immune potentiation. In vivo analyses in adult flies confirmed that JH is an immuno-suppressor. RNAi mediated silencing of ecdysone receptor complex partners EcR or USP prevented the 20E-induced immune potentiation. In contrast, silencing methoprene-tolerant (Met), a candidate JH receptor, did not impair suppression of 20E potentiation of immune response by JH III and methoprene suggesting that suppression of 20E potentiation of immune response by JH may not function through MET (Flatt et al. 2008). These studies

suggest that JH and JHA antagonize 20E enhanced expression of immune protein genes.

8 JH Modulation of 20E Action Could be Mediated by Protein: Protein Interactions Among Proteins Involved in JH and Ecdysteroid Action

As discussed above, JH modulates ecdysteriod action to regulate many developmental and physiological processes including midgut remodeling during metamorphosis. Bitra and Palli (2007) employed two-hybrid assays in yeast and showed that EcR:USP heterodimers, USP:USP homodimers, Met:Met homodimers as well as Met:EcR and Met:USP heterodimers induced reporter activity in the absence of ligand and addition of ecdysteroid or JH analogs did not increase the reporter activity regulated by either homodimers or heterodimers of Met protein. Among the receptors tested, only EcR:USP heterodimers showed ligand dependent induction of reporter gene activity. Two-hybrid assays in insect cells and in vitro pull-down assays confirmed the interaction of Met with EcR and USP. These studies showed that the proteins involved in ecdysteroids (EcR and USP) and juvenile hormones (Met) action interact and mediate cross talk between these two important hormones.

In a recent study, Li et al. (2007) showed that two proteins (FKBP39 and Chd64) that were identified using DNA affinity column prepared using conserved juvenile hormone response element present in 16 genes induced by JH both in fruit fly cells and honey bee interact with EcR, USP and Met suggesting that these two key hormones that regulate insect development and reproduction may function through multiple protein complexes that include proteins involved in signal transduction of both these hormones (Fig. 2).

9 Phosphorylation Plays a Key Role in JH Action

In *D. melanogaster* L57 cells, the reporter gene regulated by the JH response element (JHRE) identified in Cfjhe gene was induced by JH I, JH III, methoprene, and hydroprene (Kethidi et al. 2006). Interestingly, protein kinase C inhibitors in the absence of JH induced the luciferase gene placed under the control of JHRE. Nuclear proteins isolated from L57 cells bound to the JHRE and exposure of these proteins to ATP resulted in a reduction in their binding to JHRE. Interestingly, JH III or calf intestinal alkaline phosphatase (CIAP) was able to restore the binding of nuclear proteins to the JHRE. Protein kinase C inhibitors increased the binding and protein kinase C activators reduced the binding of nuclear proteins to the JHRE.

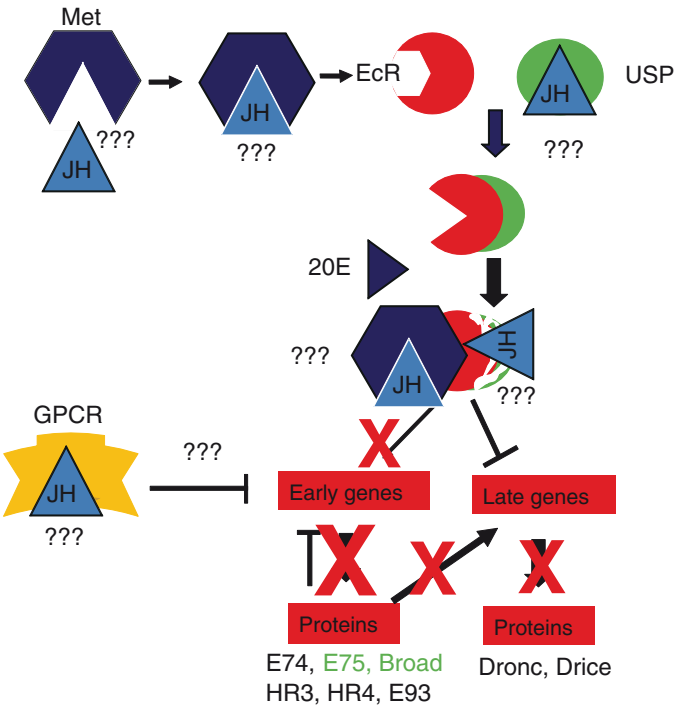

Fig. 2 Possible mode of action for JH and JHA modulation of ecdysteriod action. Based on previous studies that showed binding of JH to Met and USP; binding of ecdysteriods to EcR:USP heterodimer and observed interactions between EcR:USP, EcR:Met and Met:USP, the possible mode of action of JH in modulation of ecdysteriod action include: (1) Met disrupts ecdysteriod binding to EcR:USP heterodimer in the presence of JH. (2) In the presence of JH, USP is not available for EcR:USP heterodimerization and binding to ecdysteriods. A third possibility which does not have experimental evidence yet is JH to function through an existing lipid signal transduction mechanism through a membrane receptor such as G-protein-coupled receptor to modify either receptors (EcR and USP) or transcription factors (early genes) involved in ecdysteriod action

These studies point to a critical role for phosphorylation in JH action. Some previous studies also showed the involvement of protein kinases and phosphorylation in JH action (Yamamoto et al. 1988; Zhou et al. 2002).

10 Prospectives on the Mode of Action of JH and JHA

Recent studies suggest that JH signal transduction occurs through multiple proteins. Although, several possible proteins such as Met, USP, Kr-h1, FKBP39 and Chd64 that play key roles in JH signal transduction have been identified, the mechanism of action of these proteins in JH action is not understood. Except for two studies that showed *D. melanogaster* Met binding to JH with nanomolar affinity (Miura et al. 2005) and *D. melanogaster* USP binding to JH III with micormolar affinity

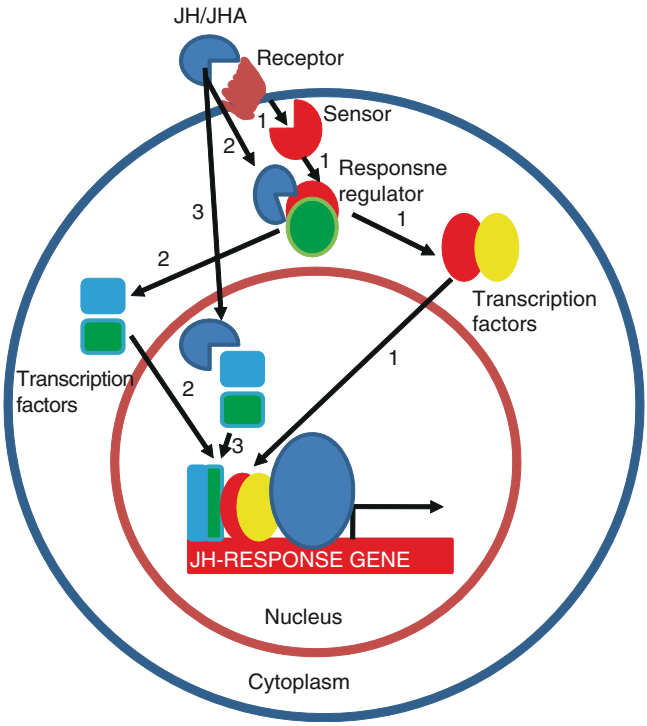

Fig. 3 Three possible modes of action of JH are shown. 1. JH functioning through an existing lipid signal transduction mechanism through a membrane receptor such as G-protein-coupled receptor, 2. JH could enter the cells and activate proteins such as protein kinases and phosphatases. The protein modifiers in turn could modify other proteins resulting in their translocation into the nucleus or binding to other proteins to form multi-protein complexes that bind to response elements present in the promoter regions of JH-response genes and 3. JH could enter nucleus and bind to nuclear proteins and induce conformational changes. The nuclear protein in turn could bind to the promoters of JH-response genes or induce chromatin remodeling

(Jones and Sharp 1997), there is very little information on JH binding affinity to proteins that have been identified to be involved in JH action. In a recent review Wheeler and Nijhout (2003) noted that a number of lipid-soluble signaling molecules in vertebrates, invertebrates and plants show similarities with the juvenile hormones and argued that JH could used in existing signal activation and transduction systems used by lipid-soluble signaling molecules. In light of the recent work on JH action that showed involvement of multi-protein complexes in JH signal transduction (Li et al. 2007) and proteins that bind to juvenile hormone response elements are modified through phosphorylation/dephosphorylation mechanisms (Zhou et al. 2002; Kethidi et al. 2006) it is likely that JH transduces its signals through existing signal activation and transduction mechanisms (Fig. 3). In one possible mode of action, JH signals from outside the cells could be transmitted into the cells via membrane receptors such as G-protein-coupled receptors, receptor

protein kinase, transmembrane scaffolds, guanylyl cyclase or other membrane receptors. The signals from these receptors are received by sensor molecules that interact with these membrane receptors. The sensors then interact with response regulators to modify transcription factors. Transcription factors in turn could bind to JH response elements to regulate the expression of JH response genes. In a second possible mode of action, JH could enter the cells and modify signaling proteins by binding to protein modifiers such as protein kinases and phosphatases. The protein modifiers in turn could modify other proteins resulting in their translocation into the nucleus or binding to other proteins to form multi-protein complexes that bind to response elements present in the promoter regions of JH-response genes. In a third possible mode of action, JH could enter the nucleus and bind to the nuclear proteins present in the nucleus inducing conformational changes in these nuclear proteins. The nuclear protein in turn could bind to the promoters of JH-response genes or induce chromatin remodeling. Completion of whole genome sequencing, availability of microarrays, functioning of systemic RNA interference (RNAi) and excellent response to JH and its analog make *T. castaneum* an excellent model system for studies on JH action. The work on JH action in this insect is currently underway in our laboratory as well as others and the results of these investigations should help us elucidate the mechanism of action of juvenile hormone.

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γ-Aminobutyric Acid Receptors: A Rationale for Developing Selective Insect Pest Control Chemicals

Yoshihisa Ozoe, Makio Takeda, and Kazuhiko Matsuda

1 Introduction

It has long been known that γ-aminobutyric acid (GABA) participates in a bypass of the tricarboxylic acid (TCA) cycle in plants and bacteria. In a single1950 issue of the Journal of Biological Chemistry, Awapara et al., Roberts and Frankel, and Udenfriend independently reported the presence of a large quantity – approximately 1 mg/g tissue – of GABA in vertebrate brains; later similarly high contents were found in the inhibitory neurons of the Atlantic lobster (Kravitz et al. 1963; Otsuka et al. 1967).

Though found in the brain, GABA was not immediately recognized as a neurotransmitter until Bazemore et al. (1957) demonstrated that GABA from bovine brain inhibited spontaneous discharges of the stretch receptor neuron of the crayfish. These findings sparked many subsequent studies to determine whether or not GABA fulfills standard criteria as a neurotransmitter: its presence and biosynthesis in the presynaptic neuron, its release from the inhibitory nerve terminal upon stimulation, its uptake in the nerve terminal, and the presence of specific receptors on the postsynaptic neuron. Together, these efforts eventually established GABA as an inhibitory neurotransmitter in vertebrates and invertebrates (Roberts et al. 1976).

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GABA is released from the presynaptic neuron upon stimulation. The released GABA diffuses across the synaptic cleft and provokes an electrical change in the postsynaptic neuron by binding to specific receptors localized in the postsynaptic membrane. Additionally, GABA also activates an intracellular signaling pathway(s) by binding to another type of receptors. The GABA receptors (GABARs) are targets for drugs and pesticides. Functional differentiation and differences in receptor type or subtype provide a rationale for designing target-specific and therefore selective control chemicals to combat insect and other arthropod pests.

Vertebrate GABARs are widely distributed in the central nervous system, and are divided into types A and B (Hevers and Lüddens 1998; Bettler et al. 2004). Peripheral endocrine tissues also express a variety of their subunits, but the physiological relevance of GABARs in peripheral tissues is unclear (Akinci and Schofield 1999). The type A receptors ($GABA_A$Rs) are ligand-gated ion channels (LGICs) containing a built-in chloride channel, and are thus described as ionotropic GABARs or GABA-gated chloride channels (Fig. 1). In general, LGICs are membrane-bound proteins mediating fast synaptic neurotransmission. Opening of the cation-permeable channels results in depolarization of the postsynaptic membrane, triggering the generation of action potentials. Inversely, opening of the anion-permeable channels leads to membrane hyperpolarization, inhibiting nerve excitation elicited by membrane depolarization. The latter scenario is the case with $GABA_A$Rs. $GABA_A$Rs are formed as combinations of five homologous subunits (Fig. 1), which in mammals are chosen from a pool of 19 distinct subunits (α_{1-6}, β_{1-3}, γ_{1-3}, ε, θ, δ, π, and ρ_{1-3}) (Enna 2007). The major receptor subtype in the brain is assembled from α_1, β_2, and γ_2 subunits with a stoichiometry of 2:2:1. GABARs containing ρ

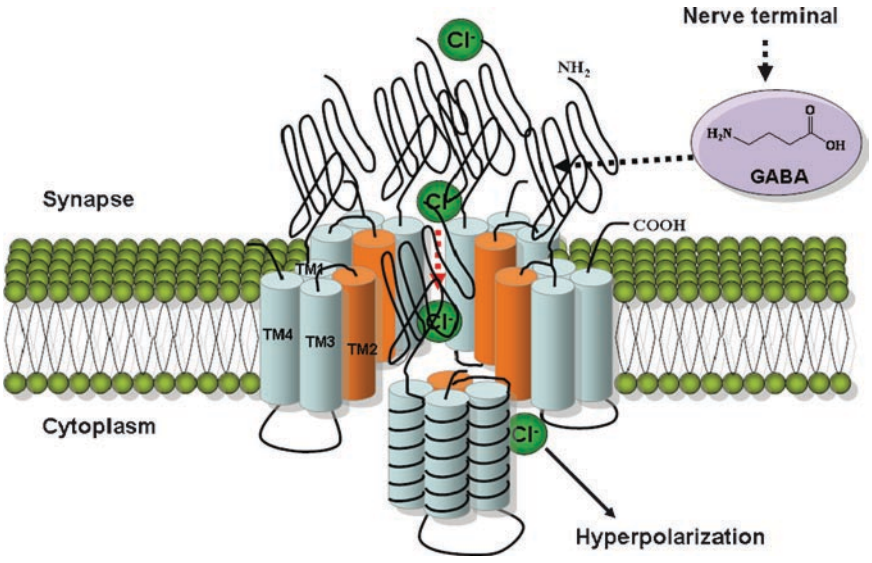

Fig. 1 Schematic representation of ionotropic GABAR

subunits were designated type C, but the classification of these receptors as a specialized set of $GABA_A$Rs has been recommended (Barnard et al. 1998). Every subunit of $GABA_A$Rs is comprised of a large N-terminal extracellular domain, four transmembrane segments (TM1-TM4), a long intracellular loop connecting the TM3 and TM4 segments, and a short extracellular C-terminus (Fig. 1). Five subunits are assembled to form a central channel with the TM2 segment lining the channel pore. The GABA binding site resides in the extracellular interface between the N-terminal domains of two subunits. The $GABA_A$R is an allosteric protein that is affected by binding of compounds such as barbiturates, benzodiazepines, anesthetics, avermectins, and picrotoxinin as well as neurosteroids (Sieghart 1995).

The type B receptors ($GABA_B$Rs) are heterodimeric G-protein-coupled receptors (GPCRs), which modulate effector proteins (adenylate cyclase, K^+ channels, and Ca^{2+} channels) via activation of specifically coupled G-proteins, and are thus referred to as metabotropic GABARs. $GABA_B$Rs are distributed widely throughout the nervous system, and co-localize with $GABA_A$Rs in certain cell types (Waldvogel et al. 2004). They occur at both presynaptic and postsynaptic membranes. Presynaptically, they inhibit synaptic release of GABA or glutamate by activating K^+ conductance and diminishing Ca^{2+} conductance via an autoreceptor- or heteroreceptor-like mechanism (Thompson and Gähwiler 1992; Bonanno et al. 1998). Postsynaptic action is mediated by pertussis-toxin-sensitive G-proteins that cause slow hyperpolarization by enhancing K^+ conductance. Cloning of this type of GABARs revealed a high sequence similarity to the family of metabotropic glutamate receptors but not to other GPCRs (Kaupmann et al. 1997). Detailed information on the molecular structures and physiological functions of $GABA_B$Rs can be found in two excellent recent reviews (Bowery et al. 2002; Bettler et al. 2004).

Insects also have both ionotropic and metabotropic GABARs (Buckingham and Sattelle 2004). Insect GABARs are found not only in the central nervous system (CNS) but also in the neuromuscular junction (Lummis 1990). Insect ionotropic GABARs functionally resemble vertebrate $GABA_A$Rs, although they have a unique pharmacology (Buckingham et al. 2005). A gene encoding a GABAR subunit was first isolated from *Drosophila melanogaster* and named *Rdl* (*R*esistant to *d*iel*d*rin). The so-called *Rdl* mutation (see Section 5.3) confers resistance to the cyclodiene insecticide dieldrin (ffrench-Constant et al. 1991). The *Rdl*-coded protein (RDL) is probably a common subunit of insect ionotropic GABARs as the orthologs have been identified from other insect species such as Diptera (*Aedes aegypti*, *Lucilia cuprina*, and *Musca domestica*), Lepidoptera (*Heliothis virescens*, *Spodoptera litura*, *Spodoptera exigua*, *Pultella xylostella*, and *Bombyx mori*), Coleoptera (*Tribolium castaneum*), and Hemiptera (*Laodelphax striatella*) (http://www.ncbi.nlm.nih.gov/). RDL-containing GABARs were shown to be involved in mediating synaptic inhibition in *D. melanogaster* neuronal circuits (Lee et al. 2003). Two other GABAR-like subunits, LCCH3 (Henderson et al. 1993, 1994) and GRD (Harvey et al. 1994), were cloned from *D. melanogaster* (see Section 4.1 for co-assembly of both subunits). RDL not only forms a homopentamer that imitates the pharmacology of native GABARs, but also forms a heteromer with LCCH3 (see Section 4.1) (Zhang et al.

1995). In addition to this variation, alternative splicing and RNA editing of *Rdl* further increase the complexity of insect GABAR functions and pharmacology (ffrench-Constant and Rocheleau 1993; Hosie et al. 2001; Buckingham et al. 2005; Es-Salah et al. 2008). Metabotropic $GABA_B$Rs were also identified in *D. melanogaster* and partially characterized (Bai and Sattelle 1995; Mezler et al. 2001).

In this chapter, we focus on the variety of GABAR ligands investigated to date for their insect/nematode toxicities, their binding potencies on insect/nematode membrane preparations, and their electrophysiological agonist/antagonist actions on native and recombinant receptors. We also focus on the structure-activity relationships (SARs) of noncompetitive antagonists (NCAs) and on the identification of the NCA binding site based on molecular functional analysis of mutant GABARs and homology modeling/docking studies. Mechanisms underlying NCA insecticide resistance are dealt with in relation to target site insensitivity. Furthermore, the immunohistochemical localization of insect GABARs in the nervous system and their pharmacological features are described. We discuss the achievement of each study discussed and the problems remaining to be solved.

2 Immunohistochemical Distribution of GABA, Glutamate Decarboxylase, GABA Transporter, and GABARs in the Central Nervous Systems of Insects and Other Arthropods

GABA is mainly metabolized via a short pathway (the GABA shunt) bypassing the TCA cycle. Three enzymes, glutamate decarboxylase (GAD), GABA transaminase, and succinic semialdehyde dehydrogenase are involved in this pathway. By and large, GABA-, GAD-, RDL-, and GABA transporter (GAT)-like immunohistochemical reactivities (ir's) occur widely in the CNS of insects and other arthropods (Homberg et al. 1987, 1993; Callaway et al. 1989; Takeda and Ohnishi 1994; Harrison et al. 1996; Brotz et al. 1997; Strambi et al. 1998; Judge and Leitch 1999; Wegerhoff 1999; Sattelle et al. 2000; Ganeshina and Menzel 2001; Umesh and Gill 2002). An antibody against *Drosophila* RDL failed to detect GABAR-ir in the midgut of the American cockroach (*Periplaneta americana*) (M. Takeda unpublished data). The same antibody revealed numerous cell bodies and dendrites in the cockroach brain-suboesophageal ganglia (SOG) and the ventral nerve cord ganglia (Sattelle et al. 2000). Blechschmidt et al. (1990) counted 1,000 neurons reactive to their antibody against GABA in the thoracic ganglia. On the other hand, the extracerebral tissues that showed RDL-ir include the corpora cardiaca (CC) (Sattelle et al. 2000). The CC also showed GABA-ir in *P. americana* (Blechschmidt et al. 1990). In two representative hemimetabolla, *P. americana* and *Acheta domesticus,* the same authors compared RDL-ir and GAD-ir (Takeda and Ohnishi 1994; Sattelle et al. 2000) as well as RDL-ir (with the same antibody) and GABA-ir (Strambi et al. 1998). Although distinct neurons and dendrites were shown to be immunoreactive at least in *P. americana*, shared structures, indicative of autocrine secretion,

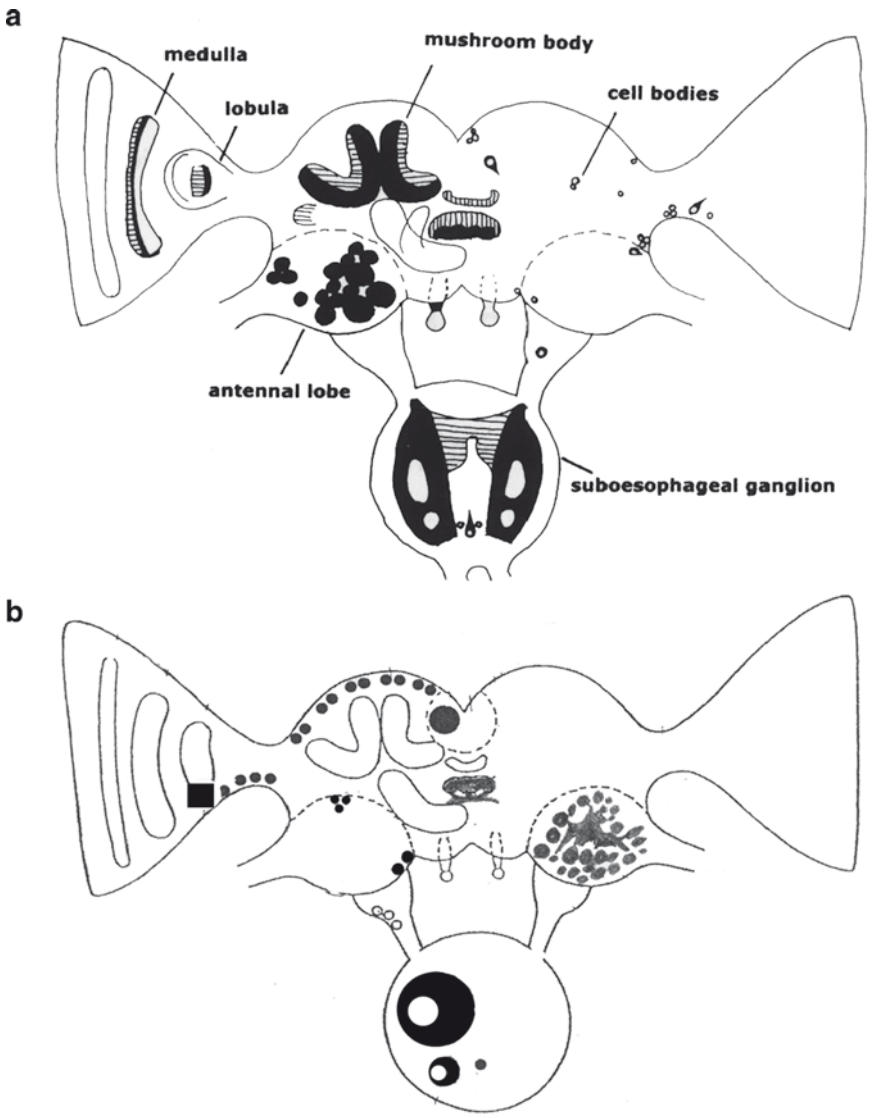

Fig. 2 Schematic illustrations of RDL-ir and GAD-ir in the brain-SOG of *P. americana.* (**a**) General anatomy and RDL-ir (modified from Sattelle et al. 2000). Left hemisphere shows neuropil reactivity, and right shows cell body locations. (**b**) GAD-ir (Takeda and Ohnishi 1994). The *dots* indicate the locations of GAD-ir cell bodies, but they do not correspond to the actual number. The *square* shows the putative locus of the circadian pacemaker. Calyces of MB show GAD-ir forming a loose mesh-work

seemed to be present in certain neuronal cell bodies, such as cell bodies at the pars intercerebralis and proximal frontoventral parts of the optic lobe as well as the unpaired neuron of the SOG (Fig. 2). The deutocerebral glomeruli and lower body

(ellipsoidal body) of the central body (CB) showed strong reactivity to both antibodies, although the identity or close association remains undetermined (Fig. 3). The calyx of the mushroom body (MB), the site of association between the input neurons from the photoreceptor and intrinsic neurons, was strongly positive to RDL-ir, but the GAD-ir was much weaker than in the antennal lobe glomeruli and the CB. The SOG had a wide distribution of GAD-ir of modest intensity, particularly in the frontal field, while very intense RDL-ir appeared in a much broader field. Both tracts and commissures were positive, while the commissures were free from staining with anti-GAD serum (Takeda and Onishi 1994; Sattelle et al. 2000). In the pedunculus of MB the intrinsic neurons form synapses with output neurons.

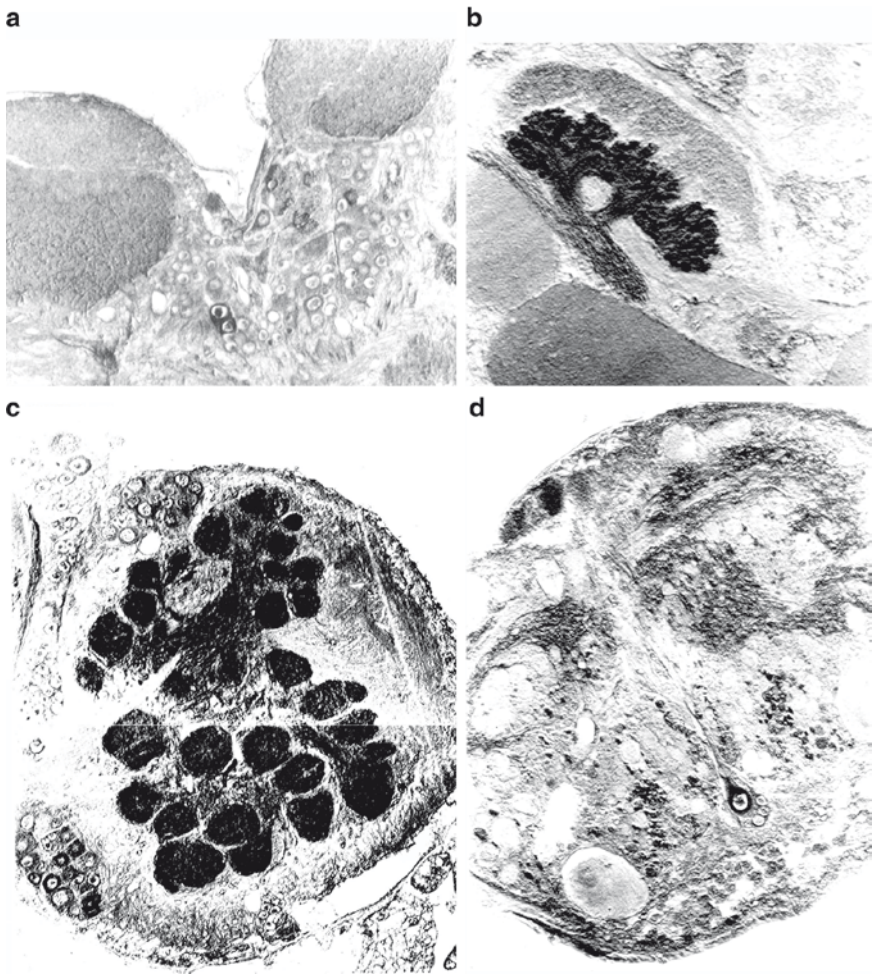

Fig. 3 GAD-ir in the brain-SOG of *P. americana.* (**a**) The pars intercerebralis. Note the meshwork of GAD-ir in the calyces of MB. (**b**) The central complex. (**c**). Antennal lobe showing intense GAD-ir in the glomeruli. (**d**) The SOG. Note a GAD-ir median unpaired neuron

Yamazaki et al. (1998) observed three classes of GABA-ir neurons in the MB: (1) four large neurons (30–50 μm) that arborize in the diffused neuropil surrounding the α lobe, projecting to a wide area of the calyces; (2) seven to nine neurons ascending from the circamesophageal connectives, projecting to the calyces, which represent inhibitory input neurons; and (3) ca. 40 neurons with dendritic arborizations in the junctions between the pedunculus and the lobes, probably inhibitory output neurons. In *A. domesticus*, the number of neurons showing GABA-ir exceeded that of RDL-ir. The PI and distal optic neurons do not seem to have shared reactivity. The upper pedunculus of MB, which plays a critical role in learning and memory in the cricket, was strongly positive for RDL-ir, suggesting that GABA is likely to control these processes (Strambi et al. 1998).

The most thoroughly studied holometabolla is probably *Manduca sexta*, by virtue of its size superiority to *Drosophila.* Homberg et al. (1987) counted 20,000 GABA-ir neurons per hemisphere in the brain and SOG alone. Most are optic lobe centrifugal neurons from the lamina and tangential neurons innervating the medulla and lobula. About 150 of the most prominent neurons are negative feed-back neurons connecting MB calyces and lobes to the lower CB. The calyces are strongly stained. *M. sexta* GAT (MasGAT)-ir has been investigated in this species (Umesh and Gill 2002). The optic neuropil staining pattern was almost identical to that of GABA-ir except that the lamina staining was much weaker with MasGAT-ir. The antennal glomeruli and central complex showed intense immunoreactivity. MasGAT-ir was not observed in the cell bodies. A slight difference may be that the stronger staining was observed with MasGAT-ir at the upper bodies of CB complex.

RDL-ir has been investigated in *Drosophila* and *Calliphora* (Harrison et al. 1996; Brotz et al. 1997). In *Drosophila*, positive RDL-ir was observed at the MB, optic neuropils, and ellipsoidal body, but not in the upper lobe. The location of RDL-ir cell bodies was not detected, due to either insufficient resolution or an actual absence (Harrison et al. 1996). Brotz et al. (1997) detected the reactivity in the MB, and they attribute the reactivity to Kenyon cells, which are intrinsic to calyces. Aronstein et al. (1996) investigated the expression patterns of RDL-ir and LCCH3-ir, and found that LCCH3 is expressed in the neuroblast and at a later embryonic age than is RDL, whose reactivity was found mainly in the neuropil. A more detailed examination was conducted by employing GABAR and GAT probes (Enell et al. 2007). In general, GABAR (i.e., RDL)-ir, $GABA_BR$-ir, GAD-ir, and GAT-ir are in agreement. The MB lobes displayed only RDL-ir and not $GABA_BR$-ir. GAT-ir occurred in the same area as $GABA_BR$-ir at presynaptic sites. RDL-ir is distributed in the calyx, protocerebral bridge, ellipsoid body, antennal glomeruli, and optic lobe neuropils. $GABA_BR$-ir was observed only in the periphery. RDL-ir was absent in the peduncles in β and γ lobes, whereas RDL-ir was present in these lobes. RDL-ir was weak in the lamina, while $GABA_BR$-ir was strong. In larval CNS, RDL-ir was observed in the cell body and the Kenyon cells, while $GABA_BR$-ir occurred in neuropils (Enell et al. 2007).

The Kenyon cells of calyces are considered the integrating center of both photic and olfactory inputs as well as the center of learning and memory in insects as

described above. Ganeshina and Menzel (2001) investigated GABAergic structures under electron microscopic observations using *Apis mellifera*, which is capable of sophisticated communication and outstanding memory. In the lip region of the calyx neuropil, GABA-ir profiles formed synapses with 76% of the small postsynaptic profiles (intrinsic neurons) and 4% of the large immunoreactive boutons (extrinsic neurons). Kenyon cells transmit information through the peduncle to α and β lobes. The MB is organized into microglomeruli, each of which is made up of a bouton, a dilator nerve profile, and numerous neural profiles surrounding it. The boutons are the neural terminals of extrinsic neurons, and small postsynaptic profiles belong to Kenyon cells numbering 340,000 in the calyx of the bee. Three types of GABA-ir profiles were found: (1) output synapses onto small profiles surrounding the principal boutons (76%), (2) output synapses onto GABA immunonegative principal neurons (4%), and (3) input synapses onto GABA-ir profiles (20%). These are inhibitory neurons. Recurrent inhibitory circuits are formed among Kenyon cells, antennal lobe projection neurons, and inhibitory GABAergic neurons within the calyx.

Enell et al. (2007) pointed out that $GABA_B$R-ir occurred in the circadian clock locus, lateral neurons ventral in *D. melanogaster*. However, RDL-ir and GAD-ir were observed in the cockroach and cricket (Sattelle et al. 2000; Strambi et al. 1998; Takeda and Ohnishi 1994). Also, these insects showed RDL-ir in the cell body. GABARs may differ between these insects and *D. melanogaster.*

Together with recent genome information, these histochemical investigations should enrich our resources and understanding for the basic structures and the diversity of GABAergic mechanisms. The rich natures of GABARs, and their physiological roles and versatile range of interactions with other receptors, promise to provide opportunities for the target-oriented development of novel insecticides and acaricides.

3 Overview of Ligands

3.1 NCAs: Structural Diversity

3.1.1 Terpenoids and Other Natural Products

A number of terpenoids isolated from plants have been reported to act as NCAs of GABARs. NCAs block GABA-induced chloride currents by binding to a site different from the GABA binding site. Picrotoxinin isolated from *Cocculus* species is the longest known terpenoid NCA of GABARs (Florey 1954; Usherwood and Grundfest 1965; Takeuchi and Takeuchi 1969). (Note that picrotoxin isolated is a 1:1 mixture of picrotoxinin and picrotin [Jarboe and Porter 1965]. Picrotoxinin is more potent than picrotin. The names used in the cited reports are employed in this chapter.) Picrotoxinin also blocks other ligand-gated chloride channels (Raymond and Sattelle 2002). Picrodendrin-Q (Fig. 4), a related terpene isolated

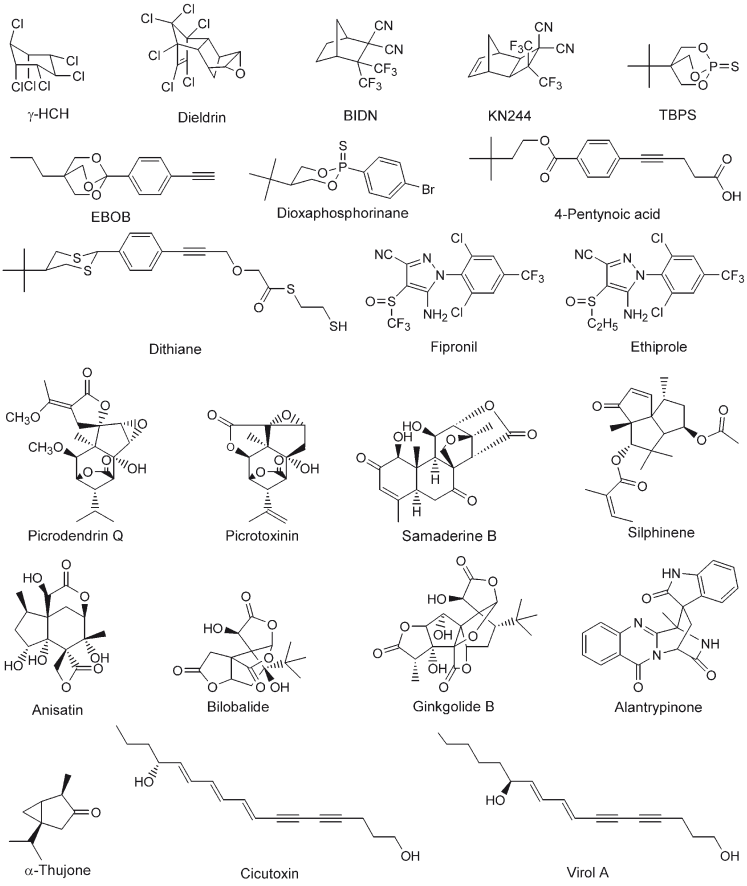

Fig. 4 Structures of NCAs of GABARs

from *Picrodendron baccatum*, is a more potent NCA than picrotoxinin at *Drosophila* RDL homo-oligomers expressed in *Xenopus* oocytes and native housefly (*Musca domestica*) GABARs (Hosie et al. 1996; Ozoe et al. 1998a). This terpene has IC_{50} values in the nanomolar range at these GABARs. Picrodendrin-Q exhibited insecticidal activity against German cockroaches (*Blattella germanica*) with an LD_{50} of 71 ng/roach, and was active against both housefly and rat GABARs (Ozoe et al. 1998a). More terpenoids of plant origin (Fig. 4) have been added to the list of NCAs of GABARs: e.g., anisatin and *seco*-prezizaanes (Ikeda et al. 1999; Kuriyama et al. 2002), α-thujone (Höld et al. 2000), bilobalide and ginkgolides (Ahn et al. 1997; Ivic et al. 2003; Huang et al. 2003, 2004), and silphinenes (Bloomquist et al. 2008). Several reports describe the positive allosteric modulation of GABARs by monoterpenes (essential oils) such as thymol, borneol, and menthol. It is particularly interesting that menthol (Watt et al. 2008) and valerenic acid (Khom et al. 2007) likely act via the propofol and loreclezole sites on GABARs, respectively. Cicutoxin and virol A (Fig. 4)

isolated from *Cicuta virosa* are straight-chain alcohols that contain uniquely conjugated double and triple bonds. Virol A inhibited GABA-induced chloride currents in rat neurons (Uwai et al. 2001). The potency of cicutoxin and that of virol A at insect GABARs remain to be investigated. While the alkaloid bicuculline is a competitive antagonist, two alkaloids, songorine and alantrypinone (Fig. 4), were reported to be among NCAs of GABARs (Zhao et al. 2003b; Kuriyama et al. 2004). Alantrypinone is selective toward insect GABARs and exhibits insecticidal activity against cockroaches and aphids (Kuriyama et al. 2004).

3.1.2 Trioxabicyclo[2.2.2]octanes and Their Derivatives

Caged phosphorus compounds that possess a trioxabicyclo[2.2.2]octane skeleton were discovered as the second class of NCAs of GABARs after picrotoxinin (Bowery et al. 1976; Korenaga et al. 1977). *tert*-Butylbicyclophosphorothionate (TBPS) (Fig. 4) and its analogs have high mammalian toxicity but low insecticidal activity (Ozoe and Eto 1986). As this compound has high affinity to mammalian GABARs, the radiolabeled compound is a good probe to specifically label the NCA site of mammalian GABARs (Squires et al. 1983). Some chemical modifications of TBPS were made to reduce its mammalian toxicity and increase its insecticidal activity. Consequently, an alkyl group added at the 3-position of the caged structure increased the NCAs' affinity and selectivity to housefly GABARs (Ju and Ozoe 1999, 2000). It is interesting to note that quassinoids (e.g., samaderine B) (Fig. 4), plant-derived nematocidal terpenoids, proved to have a possible structural overlap with bicyclophosphorothionates (Kuriyama et al. 2005).

Bicycloorthocarboxylates were derived from bicyclophosphorothionates. Compounds of this class are high-affinity NCAs for both mammalian and insect GABARs, and are toxic to both mammals and insects (Palmer et al. 1991). 4′-Ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB) (Fig. 4) provides a useful radioligand to label the NCA sites of both vertebrate and insect GABARs (Cole and Casida 1992). Further modifications of trioxabicyclo[2.2.2]octanes led to a variety of insecticidal NCAs such as dithianes, phenylthiophosphonic acid derivatives, and acyclic esters and ethers (Fig. 4) (Palmer and Casida 1992; Ozoe et al. 1998b; Hamano et al. 2000). These NCAs should include useful tools for investigating the molecular basis of ligand selectivity for insect GABARs. EBOB blocked both GABA- and glutamate-induced currents in *P. americana* neurons, with ca. 22-fold selectivity to GABARs (Ihara et al. 2005).

3.1.3 Organochlorine Insecticides and Related Cycloalkanes

γ-Hexachlorocyclohexane (γ-HCH or lindane), dieldrin, and related organochlorine (OC) insecticides (Fig. 4) exert their toxicity by blocking the GABA-gated chloride channels (they, with the exception of α-endosulfan, were banned because of their

persistence in the environment). This blocking action takes place in a noncompetitive fashion by binding to a site different from the GABA binding site. This discovery came from the observation that the cyclodiene insecticide heptachlor epoxide and γ-HCH structurally resemble the first NCA, picrotoxinin (Matsumura and Ghiasuddin 1983). Later, simple dechlorinated cyclic hydrocarbons or lactones were found to retain certain levels of insecticidal and NCA activities on GABARs, indicating that the chlorine atoms are not essential for the action, although they enhance it (Ozoe and Matsumura 1986; Ozoe et al. 1993b). Antagonist actions of OC insecticides were clearly demonstrated by electrophysiological studies in addition to initial binding studies. Co-application of dieldrin with GABA to rat native and recombinant GABARs produced a suppression of GABA-induced currents, which was preceded by an initial enhancement (Nagata et al. 1994; Nagata and Narahashi 1994). Dieldrin suppressed the amplitude of responses to GABA in S2 cells and *Xenopus* oocytes expressing *D. melanogaster* RDL channels (ffrench-Constant et al. 1993; Buckingham et al. 1996). OC insecticides were found to be more effective NCAs in *P. americana* coxal muscle than in motor neurons, suggesting the presence of pharmacologically distinct subtypes of GABARs in the central and peripheral nervous systems (Schnee et al. 1997). γ-HCH and its isomer δ-HCH (Fig. 4) showed similar enhancement of GABA-induced currents in rat native and recombinant GABARs, followed by more critical suppression, while α- and β-HCH had little or no effects (Nagata and Narahashi 1995; Nagata et al. 1996). The potentiation by δ-HCH was reproducible in *Drosophila* RDL GABARs expressed in *Xenopus* oocytes (Hosie and Sattelle 1996a). The potentiating effect of δ-HCH was proposed to be mediated through the barbiturate binding site on GABARs (Aspinwall et al. 1997). The toxicological implication of the potentiation remains to be investigated. It should be noted that γ-HCH also blocked glutamate-gated chloride currents in neurons of *P. americana*, although its action was weaker than that for GABA-induced currents (Ihara et al. 2005).

BIDN and its analog KN244 (Fig. 4) are not OC insecticides but are related bicyclo- and tricycloalkane NCAs with geminal cyano and trifluoromethyl groups. [^{3}H]BIDN binding to whole-body membranes prepared from the southern corn rootworm (*Diabrotica undecimpunctata howardi*) was inhibited by OC insecticides but not by trioxabicyclooctanes (Rauh et al. 1997). Co-application of BIDN with various concentrations of fipronil resulted in an additional dose-dependent reduction of the maximal probability of channel opening in a single channel analysis of *D. melanogaster* RDL homo-oligomers (Grolleau and Sattelle 2000). KN244 also blocked GABA-induced currents in *D. melanogaster* RDL homo-oligomers, and KN244 was less active at the dieldrin-insensitive A302S mutant (see Section 5.3 for this mutant) (Matsuda et al. 1999). The results suggest that these compounds may bind to a site that is close to the binding site of OC insecticides, but not to the same site as those of picrotoxinin, trioxabicyclooctanes, and fipronil. In the light of recent information, however, it seems more likely that their binding sites overlap.

3.1.4 Phenylpyrazoles and Other Phenylheterocycles

Fipronil (Fig. 4) is a phenylpyrazole insecticide with high activity against various insect pests and relatively low toxicity to mammals (Colliot et al. 1992). This class of insecticides can be considered the second generation of GABAR NCA insecticides after the classical OC insecticides. Fipronil inhibited GABA-induced currents in homo-oligomeric *D. melanogaster* RDL GABARs expressed in S2 cells (Grolleau and Sattelle 2000) and *P. americana* neurons (Zhao et al. 2003a). Fipronil inhibited [^{3}H]EBOB binding to housefly head membranes in a noncompetitive fashion with respect to [^{3}H]EBOB, indicating the action on a different or overlapping site (Cole et al. 1993). Recent information supports the action on an overlapping site rather than on a different site (see Section 5.2). Although fipronil and ethiprole are the only commercial phenylpyrazole insecticides, a number of other phenylheterocycles were reported to have insecticidal activity that possibly stemmed from NCA actions on GABARs (Pulman et al. 1996; Ozoe et al. 2000; Alam et al. 2007; Lyga et al. 2007). Most of these reported heterocyclic NCAs exhibited selectivity for insect vs. mammalian GABARs. The use of information from these studies should enable the design of selective NCA insecticides in the future. It is worth noting that fipronil potently acts as an open channel blocker of glutamate-gated chloride channels (GluCls) in neurons of *P. americana* while acting on both resting and activated GABARs (Zhao et al. 2004b). GluCls are not expressed in vertebrates, so the action at GluCls might provide one explanation for the selective toxicity of fipronil. However, the significance of GluCls as targets for insecticides remains to be validated in comparison with that of GABARs, because insects have two types of major ligand-gated chloride channels.

3.1.5 Avermectins and Related Macrolides

Avermectins and milbemycins are endectocidal, insecticidal 16-membered macrocyclic lactones from *Streptomyces* sp. (Shoop et al. 1995). While it is well established that these lactones exert their anthelmintic effects by activating GluCls (Cully et al. 1996; Wolstenholme and Rogers 2005), their effects on GABARs are still elusive. Information about their modes of action on cloned invertebrate GABARs is scarce, but some studies were performed using vertebrate GABARs. Avermectin B_{1a} (a component of avermectins) enhanced GABA-induced chloride currents in *Xenopus* oocytes injected with chick brain mRNA (Sigel and Baur 1987), and ivermectin (IVM; 22,23-dihydroavermectin B_1) activated rat $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$Rs in the absence of GABA (Adelsberger et al. 2000). IVM not only induced direct currents in the absence of GABA, but also potentiated GABA responses in rat cortical neurons and Ltk cells stably expressing human $\alpha_1\beta_3\gamma_{2S}$ GABARs (Dawson et al. 2000). In contrast, 22,23-dihydroxyavermectin B_{1a} (DHAVM) and IVM depressed GABA-induced currents in extrasynaptic GABARs in the muscle of the parasitic worm *Ascaris suum* (Martin and Pennington 1989) and GABARs in *Locusta migratoria* neurons (Bermudez et al. 1991), respectively.

IVM had different effects on GABA-induced currents in *Xenopus* oocytes expressing two types of the α/γ subunit of the parasitic nematode *Haemonchus contortus* with the β subunit of the free-living nematode *Caenorhabditis elegans*; IVM enhanced the amplitude of GABA-induced currents in one hetero-oligomer and attenuated the GABA response of another (Feng et al. 2002). It is interesting that changes of only four amino acids in the *H. contortus* subunits drastically changed the hetero-oligomers' responses to IVM. DHAVM at low concentrations (75 pg–7.5 ng/ml) induced irreversible increases in chloride permeability and partially blocked GABA-induced chloride conductances in *Schistocerca gregaria* muscle, whereas it induced an irreversible increase in chloride permeability and potentiated GABA-induced chloride conductance at high concentrations (10 ng–1 μg/ml) (Duce and Scott 1985). More experiments using cloned invertebrate GABARs would be needed to clarify GABARs' involvement in the insecticidal action of macrocyclic lactones.

3.1.6 Nodulisporic Acid

Nodulisporic acid (NA) is an insecticidal alkaloid isolated from the fungus *Nodulispolium* sp. *N*-(2-Hydroxyethyl-2,2-^{3}H)nodulisporamide, a radiolabled NA derivative, bound with high affinity to a specific site coupled to a [^{3}H]IVM binding site in *Drosophila* GluCls (Smith et al. 2000). The NA binding protein solubilized from *D. melanogaster* heads was immunoprecipitated with both antibodies raised against *Drosophila* RDL and GluClα subunits, suggesting that NA acts at a receptor in which GluClα and RDL subunits co-assemble (Ludmerer et al. 2002). The same group also suggested that the NA receptor is one of the two populations of IVM receptors. The possibility of the co-assembly of RDL and GluClα is discussed in Section 4.1

3.2 Agonists and Competitive Antagonists

The type or subtype specificity of GABARs has been conventionally examined on the basis of pharmacological responses to agonists and antagonists. Mammalian GABARs were classified into three types – $GABA_AR$, $GABA_BR$, and $GABA_CR$ – mainly on the basis of their pharmacological characteristics (see Introduction for the classification). For example, muscimol and isoguvacine are specific $GABA_AR$ agonists, and baclofen and 3-aminopropyl(methyl)phosphinic acid (3-APMPA) are selective $GABA_BR$ agonists. *cis*-4-Aminocrotonic acid (CACA) is a selective $GABA_CR$ agonist. Competitive antagonists, which inhibit GABA action by binding to the same site as GABA, are also useful for characterizing GABARs. Specific competitive $GABA_AR$ antagonists include bicuculline (and its methiodide) and SR95531 (2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide), and representative competitive $GABA_BR$ antagonists are phaclofen and saclofen.

The GABARs of insects and other arthropods cannot usually be classified in the same way as in mammals (Benson 1989; Sattelle 1990; Jackel et al. 1994). Pharmacological profiles are different even within the Arthropoda. For example, CACA was inactive in the heart of *Lymulus* and the visual interneurons of *Calliphora erythrocephala* (Benson 1989; Brotz and Borst 1996), while cultured crustacean thoracic neurons responded to CACA, although the potency was tenfold lower than that of GABA (Jackel et al. 1994). Cultured insect neurons from *P. americana* were activated by $GABA_AR$ and $GABA_CR$ agonists, but competitive $GABA_AR$ antagonists did not work (Aydar and Beadle 1999). The cloned *L. striatella* and *D. melanogaster* GABARs were also sensitive to both $GABA_AR$ and $GABA_CR$ agonists (Millar et al. 1994; Hosie and Sattelle 1996b; Narusuye et al. 2007). It is worth noting that SR95531 was an effective competitive antagonist in cloned GABARs of *L. striatella* and *D. melanogaster*, as well as native GABARs of *A. suum* and *S. litura* (Duittoz and Martin 1991; Hosie and Sattelle 1996b; Satoh et al. 2005; Narusuye et al. 2007), while insensitivity to bicuculline is a consistent feature of most invertebrate GABARs. Interestingly, 3-APMPA acted as an agonist in cloned *D. melanogaster* $GABA_BRs$, whereas baclofen, a specific mammalian $GABA_BR$ agonist, was without effect (Mezler et al. 2001). 3-APMPA has insecticidal activity (Fukunaga et al. 1999).

4 Molecular Assembly of Insect GABARs

4.1 Assembly: Homomer or Heteromer

The RDL subunit forms a homo-oligomeric ionotropic GABAR when expressed in *Xenopus* oocytes, Sf21 cells, or S2 cells (ffrench-Constant et al. 1993; Lee et al. 1993; Shotkoski et al. 1994, Millar et al. 1994; Wolff and Wingate 1998; Narusuye et al. 2007). The pharmacology of the recombinant RDL homo-oligomers resembles that of native receptors in terms of the agonist pharmacology and the insensitivity to bicuculline (Sattelle et al. 1988; Sattelle et al. 1991; Sattelle et al. 2003). However, the RDL homomer can be distinguished from native receptors in its sensitivity to benzodiazepines. For example, co-applying flunitrazepam with GABA potentiated the GABA-induced response of identified cockroach (*P. americana*) neurons at 1μM (Sattelle et al. 1988), whereas flunitrazepam and Ro 5–4865 (4′-chlorodiazepam) had negligible effects on RDL homo-oligomers (Buckingham et al. 1996; Hosie and Sattelle 1996a), suggesting the possibility that RDL could co-assemble with at least one of LCCH3 and GRD rather than standing alone in the nerve membranes. Co-expressing RDL with LCCH3 in Sf21 cells formed robust ion channels with a pharmacology distinct from that of the RDL homo-oligomer (Zhang et al. 1995). The RDL/LCCH3 receptor was insensitive to picrotoxin, which can block the GABA responses of the RDL receptor as well as native GABA receptors at low concentrations. In addition, RDL and LCCH3 are expressed with different

spatial patterns in insects (Hosie et al. 1997). These findings rule out the possibility of co-assembly of these two subunits *in situ*.

The GRD subunit forms a heteromeric GABA-gated cation channel when co-expressed with LCCH3 in *Xenopus* oocytes (Gisselmann et al. 2004). This sounds unusual, but several lines of evidence suggest that GABA also acts as an excitatory neurotransmitter in particular cases of invertebrates (Beg and Jorgensen 2003; Schuske et al. 2004). However, such cationic GABAR channels seem to be much less abundant than GABA-gated chloride channels, as no cationic response has been observed from isolated native neurons. It is of pharmacological interest to note that the GRD/LCCH3 receptor was sensitive to picrotoxinin but not to γ-HCH, dieldrin, or bicuculline.

4.2 GABA-vs. Glutamate-Gated Chloride Channels

The presence of GluCls was first demonstrated in locust muscle fibers (Cull-Candy 1976) and then in identified motor neurons of *P. americana* (Sattelle 1992). The molecular entity of GluCls has been disclosed by cloning a coding gene from *C. elegans* and shown to be the target of avermectins (Cully et al. 1994). Since then, its orthologs and paralogs have been identified when several genome sequencing projects were completed (Dent et al. 1997; Vassilatis et al. 1997; Delany et al. 1998; Horoszok et al. 2001; Raymond and Sattelle 2002; Tandon et al. 2006). Although multiple GluCl subunits were cloned from nematodes, only a single type of subunit, such as DrosGluCl-α and MdGluCl, was isolated from *D. melanogaster* and *M. domestica*, respectively (Cully et al. 1996; Eguchi et al. 2006).

GluCls are as abundant as RDL GABARs in the nervous systems of insects, and recent data show that NCAs of GABARs cross-react with GluCls (Zhao et al. 2003a, 2004b; Ihara et al. 2005; Eguchi et al. 2006). It is important to examine the cross-reactivity of NCAs and the toxicological/physiological significance of two similar chloride channels. Meanwhile, Ludmerer et al. (2002) reported that RDL may form a heteromer with GluCl, as *Drosophila* RDL and GluClα subunits were co-precipitated with antibodies specific to each subunit. However, patch-clamp electrophysiology failed to detect GABAR/GluCl heteromers in the thoracic ganglion neuron of *P. americana* (Zhao et al. 2004a). We have cloned the genes encoding RDL (MdRDL) and GluCl α (MdGluCl) subunit orthologs from *M. domestica* to investigate the pharmacology of chloride channels reconstituted by expressing each receptor subunit alone or co-expressing them in *Xenopus* oocytes (Eguchi et al. 2006). The pharmacology of the recombinant receptors resulting from the co-expression resembled that of MdRDL in terms of sensitivity to GABA and picrotoxinin, yet mixing a small amount of MdGluCl cRNA significantly promoted MdRDL expression (K. Matsuda et al. unpublished data). Presumably, MdGluCl works as a molecular chaperone helping either MdRDL folding, transport to the oocyte membranes, or both, but further studies are needed to elucidate the mechanism underlying this observation.

5 Exploring Pharmacophores for Pesticide Design and Resistance Management

5.1 NCA Structure-Activity Relationships

Since the first discovery of picrotoxinin as an NCA of GABARs, SAR studies have been performed separately for each class of NCAs. Jarboe et al. (1968) reported that the unmodified bridgehead hydroxyl group, the lactone group connecting the bridgehead carbons, and the axial isopropenyl group of picrotoxinin are critical for toxicity (Fig. 5). [^{35}S]TBPS binding assays of simplified picrotoxinin analogs showed that the axial (*trans*) isopropenyl group of this class of NCAs is preferable to the equatorial (*cis*) isopropenyl group in interaction with rat GABARs (Ozoe et al. 1993a). The importance of a small hydrophobic substituent combined with the electronegative oxygen atoms was also noticed in the SARs of bicyclic phosphates (Eto et al. 1976) (Fig. 5). A similar structural feature can be seen in other recently reported classes of NCAs, such as picrodendrane and *seco*-prezizaane terpenoids (Kuriyama et al. 2002; Schmidt et al. 2004) and ginkgolides and bilobalide (Ivic et al. 2003; Huang et al. 2003). Soloway (1965) suggested that biologically active cyclodienes possess two electronegative centers (i.e., pharmacophores), one composed of chlorines of the hexachloronorbornene nucleus (depicted as P2 and P3 in Fig. 5) and the other composed of a double bond or oxygen atom (depicted as P1 in Fig. 5) of the second ring system. The structural overlay of cyclodienes, picrotoxinin, and TBPS prompted Ozoe and Matsumura (1986) to point out the presence of an additional pharmacophore in NCAs: the above-mentioned small substituent (e.g., the *tert*-butyl group of TBPS) plays a role as a hydrophobic center, which might overlap with the geminal chlorines of cyclodienes (Fig. 5). To reconcile this pharmacophore model and the SARs of EBOB analogs, many hybrids of cyclodienes and EBOB analogs were synthesized (Ozoe et al. 1990, 1993b). This SAR study led to the suggestion that the NCA binding–site should have an additional pocket (P1) that accommodates the phenyl group of EBOB and related analogs as well as that of 2-phenyl-1,3-dithianes (Fig. 5). Consequently, the NCA binding-site model consisting of four subsites (P1-P4) was constructed (Ozoe et al. 1993b) (Fig. 5).

One of the important questions to be examined at this point is whether or not structurally diverse NCAs indeed compete for a common site of action. Analysis using computer chemistry software gave some support to the above binding-site model. Comparative molecular field analysis (CoMFA) is a landmark SAR tool that makes it possible to quantitatively and three-dimensionally investigate whether or not bioactive compounds with different skeletons share the same site of action (Cramer et al. 1988). The CoMFA procedure consists of the following steps, outlined briefly. (1) A set of bioactive molecules are superimposed according to a pharmacophore model, (2) steric and electrostatic interaction energies between each molecule and probe atoms (sp^3 carbon with a +1 charge) regularly placed surrounding the molecules are calculated, and (3) the relationship between the obtained

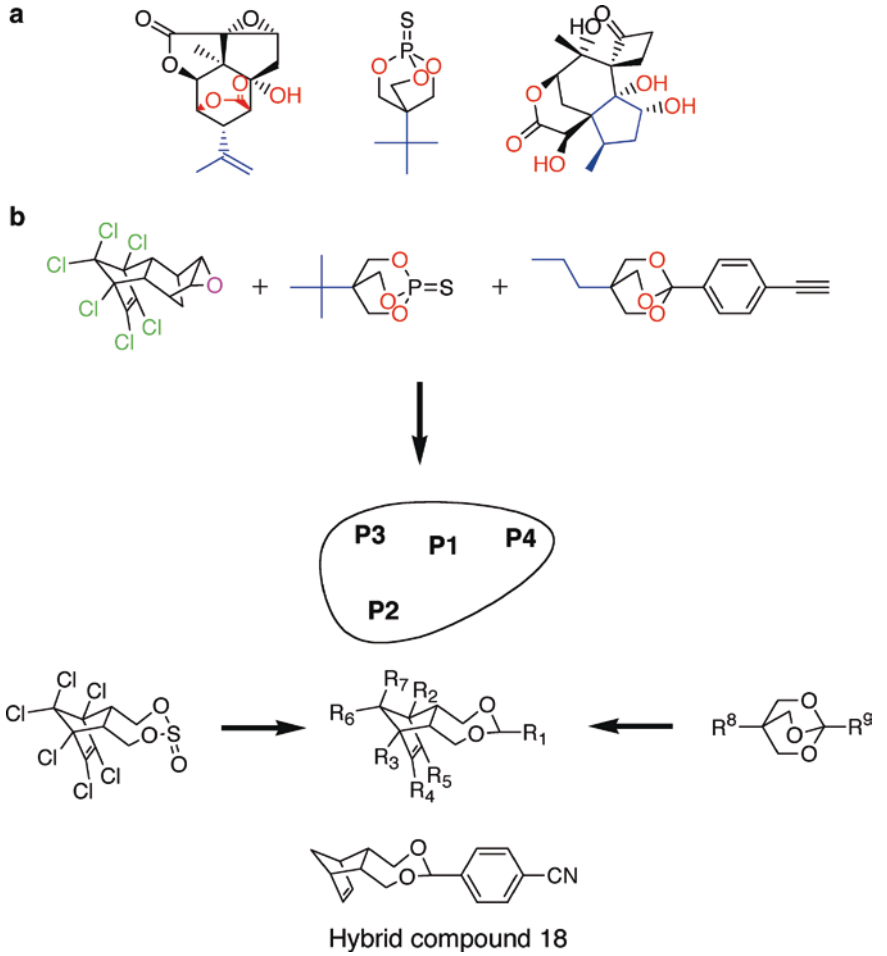

Fig. 5 Schematic representation of structure–activity relationships of NCAs. (**a**) Blue, hydrophobic moiety found in NCAs. Red, important hydrophilic moiety proposed by Jarboe et al. (1968) (picrotoxinin [*left*]), Ozoe et al. (1990, 1993), Kuriyama et al. (2002), and Schmidt et al. (2004) (TBPS [*center*] and anisatin [*right*]). (**b**) Generation of a pharmacophore model. Green and magenta: pharmacophores proposed by Soloway (1965). Convergence of several types of NCAs led to a pharmacophore model composed of four pharmacophores (P1-P4). P1: electronegative moiety. P2-P4: mainly sterically important moiety

potential energy fields and biological activities of the molecules is analyzed by the partial least squares method. When the CoMFA method was applied to a set of structurally diverse NCAs, statistically significant equations were obtained for the majority of the NCAs used, indicating the validity of the proposed model (Akamatsu et al. 1997). Furthermore the CoMFA analysis revealed a variety of differences between the NCA binding sites of rat and housefly GABARs. One of the conspicuous differences is that the rat binding site is sterically constrained compared with the

housefly binding site (Ozoe and Akamatsu 2001) (Fig. 6). This characteristic is represented as shorter distances between pharmacophores for the housefly receptor than those for the rat receptor in the DISCOtech analysis (DISCO is an acronym for DIStance COmparison and is used as a tool for identifying pharmacophores) of structurally diverse NCAs, where one hydrophobic ring and two hydrogen bond acceptor atoms are predicted as pharmacophores (Ju et al. 2007).

5.2 Location of the NCA Binding Site

Several reports containing important information about the location of the NCA binding site were published in the late 1900s. Specific [^{35}S]TBPS binding to rat brain membranes was entirely dependent on halide ions and potently inhibited by GABA (Squires et al. 1983). Inhibition of GABA-induced currents by picrotoxin was use-dependent (dependent on the presence of GABA) and noncompetitive in native vertebrate neurons, and the application of additional GABA reversed this inhibition (Inoue and Akaike 1988; Yoon et al. 1993). These findings prompted us to speculate that picrotoxin accesses the binding site through the open channel to stabilize the closed conformation and is trapped within the closed channel.

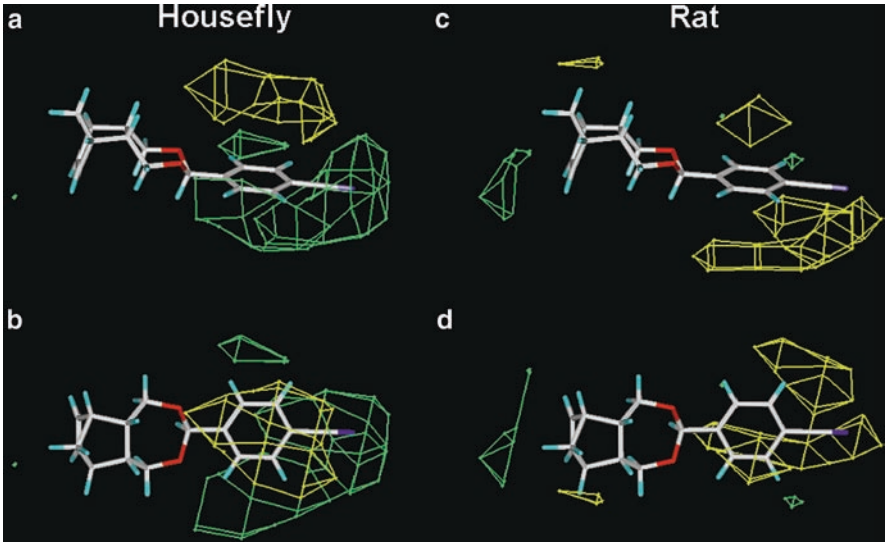

Fig. 6 CoMFA steric field maps with hybrid compound 18 in Fig. 5. The contours surround regions where a higher steric bulk of ligands increases (green) and decreases (yellow) insecticidal activity (housefly) or affinity to receptors (rat). (**a**) Housefly receptor side view. (**b**) Housefly receptor top view. (**c**) Rat receptor side view. (**d**) Rat receptor top view. Modified from Akamatsu et al. (1997)

Functional assays using cloned GABARs proved that the NCA site was within the GABAR channel. The channel lumen is formed by five TM2 segments of five subunits (Fig. 7c). As the TM2 segment is an α-helix, amino acids in this region face the channel pore every three or four residues, and thus these amino acid residues from five subunits form a kind of rings at several levels (Fig. 7d). These residues in the TM2 segment are occasionally referred to by an index numbering system, where the conserved positively charged residue at the cytoplasmic end is numbered 0′ and the number increases toward the extracellular end (the C-terminus) (Fig. 7a). Thus, amino acids at the -1′-, 2′-, 6′-, 9′-, 13′-, and 16′-positions are thought to be pore-facing amino acids.

Gurley et al. (1995) utilized site-directed mutagenesis and a two-electrode voltage clamp to identify the site of action of picrotoxin in the rat $\alpha_1\beta_2\gamma_2$ GABAR, and reported that substitution of a single 6′ Thr of the β_2 subunit with Phe conferred resistance to picrotoxin. Xu et al. (1995) found that picrotoxin protected α_1V2′C mutant of the rat $\alpha_1\beta_2\gamma_2$ GABAR from modification by an extracellularly applied sulfhydryl reagent that irreversibly inhibited GABA-induced currents in the mutant. Fipronil analogs with a chemically reactive functional group irreversibly reacted with α_1V2′C and α_1S17′mutants of the rat $\alpha_1\beta_1\gamma_2$ GABAR, and these reactions were prevented in the presence of picrotoxinin or TBPS (Perret et al. 1999). Using

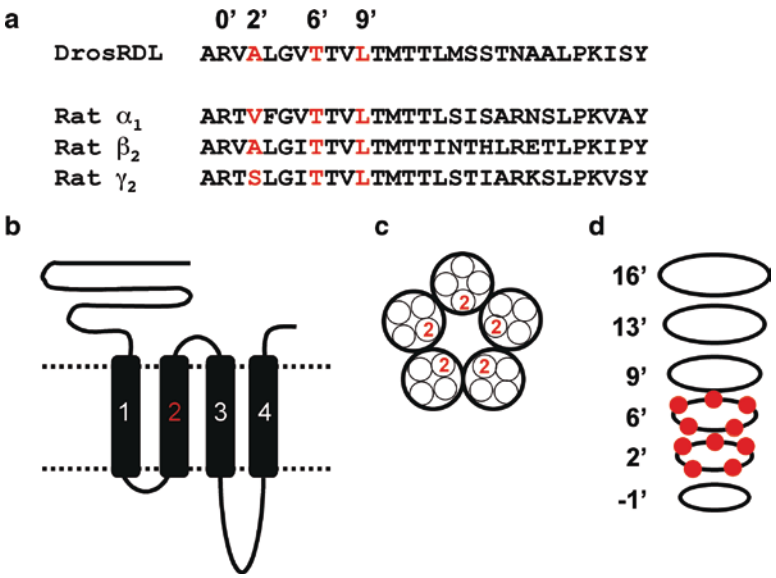

Fig. 7 Formation of the channel pore and the structure of the TM2 segment. (**a**) Alignment of amino acid sequences of the TM2 regions of *Drosophila* RDL, rat α_1, β_2, and γ_2 subunits. Index numbers are shown above the sequences. (**b**) Side view of a subunit. (**c**) Top view of a pentameric GABAR channel. (**d**) Formation of a ring by five amino acids (*red dots*) from five subunits at each level

hetero-oligomeric GABARs formed by chimeric β_3 (N-terminus)/α_1 (C-terminus) and α_1 subunits, it was shown that the introduction of 2′A and 3′L of β_3 subunit into the chimeric subunit was necessary to form the [^{35}S]TBPS binding site (Jursky et al. 2000). Furthermore, when the effects of A-1′S, A2′S, and T6′V mutations of the human β_3 homo-oligomeric GABAR on [^{3}H]EBOB binding were examined, 2′A and 6′T substitutions were found to affect the binding, with T6′ having the most profound effect (Hisano et al. 2007). This disagrees with the results of Chen et al. (2006) where mutations of almost all residues in the cytoplasmic half of the TM2 segment of the same receptor abolished [^{3}H]EBOB binding. All these research results consistently indicate that the mutations at the 2′- and 6′-positions in the intracellular half of the TM2 segment significantly affect the sensitivity of GABARs to structurally diverse NCAs. That is, NCAs bind to the channel-lining region formed by 2′ and 6′ amino acids. A number of similar results showing the importance of 2′ and 6′ amino acids in NCA actions have been reported to date in other ligand-gated chloride channels, such as glycine and serotonin type 3 receptors as well. In addition to the actions on 2′ and 6′ amino acids, the NCA actions on other amino acids have been reported. Chemically reactive fipronil analogs reacted with a 17′ amino acid (Perret et al. 1999), and N19′R mutation of the RDL homo-oligomer enhanced the potency of NCA penicillin G (Hosie et al. 2006).

A combination of homology modeling and ligand docking is another helpful approach for gaining insights into GABAR–NCA interactions. Zhorov and Bregestovski (2000) first built a ρ_1 GABAR homology model using the kinked-helices model of the nicotinic acetylcholine receptor (nAChR) in the open state as a template, and calculated Monte Carlo-minimized energy profiles for picrotoxinin pulled through the channel pore. They found that the ether and carbonyl oxygen atoms of picrotoxinin accept hydrogen atoms from 6′T and that the isopropenyl group enters the rings formed by 2′A. The X-ray crystallographic structure of acetylcholine binding protein at a 2.7 Å resolution was released in 2001 (Brejc et al. 2001), and the structure of the TM domain of the closed-state *Torpedo* nAChR at a 4 Å resolution (PDB entry 1OED) was published by Miyazawa et al. (2003). Since then, several groups generated GABAR homology models. NCA docking studies with a β_3 GABAR homology model based on 1OED showed that a wide range of NCAs fit the same site (2′–9′) within the channel (Chen et al. 2006). Consistent with the three-dimensional quantitative SAR model, hydrophobic substituents such the isopropenyl group of picrotoxinin interacted with the methyl group of 2′A, and the electronegative part of NCAs interacted with the hydroxyl group of 6′T. The 4-ethynylphenyl group of EBOB interacted with the methyl group of 6′T. As the β_3 subunit has high sequence identity to the RDL subunit in the TM2 region and the advantage of being easily expressed in cell lines, the homomeric β_3 GABAR proved a useful insect GABAR model to study the mechanisms underlying NCAs' interactions with insect GABARs; the potencies of insecticidal NCAs at the β3 GABAR were well correlated with the potencies at *M. domestica* native GABARs (Ratra and Casida 2001; Alam et al. 2006). Note that the β_3 subunit is not expressed as a homomeric channel in the human brain. In the docking of phenylpyrazole and phenyltriazole

NCAs into the β_3 GABAR homology model, a good correlation between docking scores (energies) and IC_{50} (or K_i) values obtained from [^{3}H]EBOB binding to housefly head membranes was observed, indicating that the binding mode of certain NCAs is predictable using this model (Alam et al. 2007; Ci et al. 2007).

5.3 NCA Site Sensitivity and Insects' Resistance to NCA Insecticides Due to Target Site Insensitivity

As described above, the 2′ and 6′ amino acids of the TM2 segment of GABARs are critical for the potency of NCAs, and therefore the sensitivity to NCAs depends on the composition of LGIC subunits (Ratra et al. 2001). Thr constitutes the 6′ amino acids of GABAR subunits of both mammals and insects, but amino acids at the 2′-position differ from one subunit to another. The α_1, β_2, and γ_2 subunits have a Val, an Ala, and a Ser, respectively, at the 2′-position, while the RDL subunits of most insects have an Ala at the equivalent position. It seems natural to speculate that the pore size at the 2′ ring formed by two Val's (isopropyl side chain), two Ala's (methyl side chain), and one Ser (hydroxymethyl side chain) of the $\alpha_1\beta_2\gamma_2$ GABAR is smaller than that formed by five Ala's of the homo-oligomeric RDL GABAR (Fig. 8). This difference might prove an important clue to designing NCA insecticides selective to insect pests. Actually, enlargement of the molecular size of TBPS-type NCAs by introducing a small alkyl group into their ring endocyclic methylene led to NCAs that fit housefly GABARs better than rat GABARs (Ju and Ozoe 1999).

A single amino acid change at the 2′-position is one of the causes of insects' resistance to NCA insecticides. A single A2′S mutation of insect RDL subunits (A2′G in the case of *D. simulans*) confers resistance to dieldrin (ffrench-Constant et al. 1991; Thompson et al. 1993). It was reported that the resistance of *D. melanogaster* to dieldrin was semidominant (ffrench-Constant et al. 1990), and that the cross-resistance of dieldrin-resistant *M. domestica* to fipronil was inherited as an incompletely recessive trait (Wen and Scott 1999). Subsequent patch-clamp analysis of NCA action using cultured neurons from wild-type and homozygous dieldrin-resistant *D. melanogaster* suggested that this mutation brings in two changes in GABA-gated channels: (1) structural changes to weaken the affinity of NCAs to the binding site and (2) destabilization of the NCA-favored (desensitized) conformation of GABARs (Zhang et al. 1994). According to this hypothesis, the mutant receptor should exhibit different levels of insensitivity to NCAs with different structures, based on the former mechanism (ffrench-Constant and Roush 1991; Zhang et al. 1994). The resistance of dieldrin-resistant insects to fipronil is lower than their resistance to dieldrin (Colliot et al. 1992; Cole et al. 1993; Wen and Scott 1999; Ozoe et al. 2007). Biochemical studies with dieldrin-resistant houseflies, which were homozygous for the 2′S *Rdl* allele, indicated that a similar level of affinity of fipronil to wild-type and A2′S mutant GABARs was responsible for the low resistance to fipronil

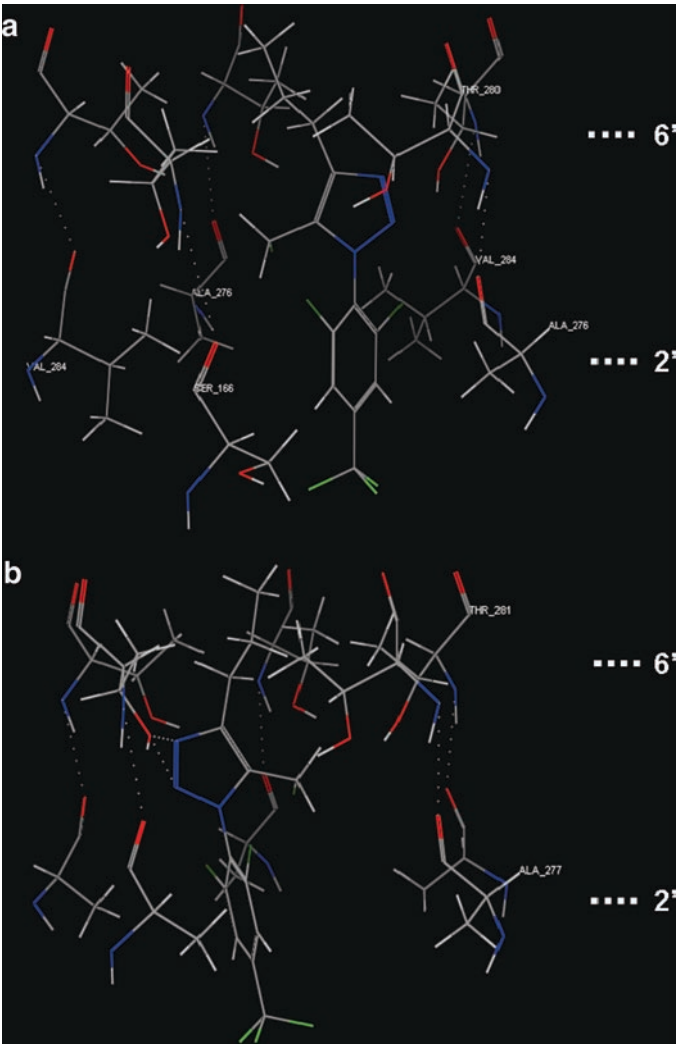

Fig. 8 Examples of docking of a phenyltriazole NCA into the 2′ and 6′ region of the TM2 segment of human β_3 (**a**) and $\alpha_1\beta_2\gamma_2$ (**b**) GABARs. Modified from Alam et al. (2007)

(Ozoe et al. 2007). Perhaps the resistance to fipronil is due to the second mechanism, although more experiments are needed to draw a conclusion. It should be noted that the mutant GABAR's high sensitivity was observed only in fipronil and phenylpyrazoles closely related to fipronil, but not in other phenylpyrazoles (Ozoe et al. 2007). Fipronil's high affinity probably arises from its peculiar substitution on the pyrazole ring.

Interestingly, insect GluCls have a Ser at the 2′-position of the TM2 segment, and this might explain why GABAR NCAs show selectivity toward GABARs over GluCls (Ihara et al. 2005; Eguchi et al. 2006). To test this view, the GluCl with an S2′A mutation at this position was generated and examined for its sensitivity to NCAs (Hirata et al. 2008). Consequently, the reverse *Rdl* (S2′A) mutant showed an enhanced sensitivity to γ-HCH, picrotoxinin, and fipronil, providing support for the result from GABARs that the amino acid at the 2′-position is a determinant of NCA action.

Recently, a strain of *D. simulans* with ca. 20,000-fold resistance to fipronil was obtained by laboratory selection (Le Goff et al. 2005). The RDL subunit of this strain carried two mutations, A301G at the 2′-position in the TM2 segment and T350M in the TM3 segment. Each of the two mutations reduced the sensitivity of the *Drosophila* RDL homo-oligomer to fipronil and picrotoxin, and the double mutant showed the highest insensitivity among mutants to fipronil. It is worthwhile to examine whether or not the amino acid residue in the TM3 segment is located in the fipronil binding site.

6 Summary and Future Perspective

GABARs have long been considered a gold mine for the development of safer insecticides (Eto 1983; Matsumura and Ghiasuddin 1983). However, GABAR-targeting insecticides that were put into practical use are only two phenylpyrazole insecticides, fipronil and ethiprole, after most of the classical OC NCA insecticides were banned. Fipronil was discovered in 1987, and pesticide scientists modified this structure to design and develop more NCA insecticides. Despite strenuous discovery efforts, no more novel NCAs have been developed as practical insecticides up to the point of writing this review.

The same year fipronil was discovered, the cDNAs encoding bovine $GABA_AR$ subunits were cloned for the first time (Schofield et al. 1987). This accelerated GABAR research, and led to an enhanced understanding of their structures and functions. We now know that NCAs interact with the region from the 2′ to 9′ amino acids within the channel and that there are structural differences between mammalian and insect GABARs. Furthermore, we have numerous tools for investigating the functions of GABARs and screening compounds, or even more elaborate techniques. The insect ionotropic GABAR is a validated target for insecticidal action, and the target sites are not restricted to the NCA site, since GABARs have multiple sites for ligands. Much progress can be expected toward the biologically rational design and development of a third generation of insecticides targeting the NCA site or of insect pest control agents acting at other sites.

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Natural Products: Plant Lectins as Important Tools in Controlling Pest Insects

Gianni Vandenborre, Els J.M. Van Damme, and Guy Smagghe

1 Introduction

During the long course of interaction and co-evolution with herbivorous insects, plants have evolved a broad range of defense mechanisms to counteract insect attack (Chen 2008). Next to the presence of structural defense barriers, plants have developed several chemical defense strategies towards herbivorous insects. These defense chemicals include plant secondary metabolites and proteins that can reduce the nutrient value of the plant material or have a direct effect by interfering with the normal insect metabolism. One particular class of these defense proteins is plant lectins, a heterogeneous group of proteins that specifically interact with sugars (Peumans and Van Damme 1995; Czapla 1997; Van Damme et al. 2007, 2008). Many plants including different food crops such as wheat, rice, potato, tomato, soybean and bean contain lectins (Van Damme et al. 1998). Nowadays, the term plant lectin is used for all plant proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide (Peumans and Van Damme 1995). Lectins from different plant species often differ with respect to their molecular structure and specificity. Based on sequence similarity, plant lectins can be divided in different subgroups of structurally and evolutionary related proteins (Table 1).

Each member of a lectin family shares one or more carbohydrate-binding domains with all other members. The major plant lectin families are: (1) amaranthins, (2) lectins with a Nictaba domain, (3) chitin-binding lectins composed of hevein domains, (4) the GNA-related lectins, (5) the jacalin-related lectins, (6) the legume lectins and (7) lectins with ricin-B domains (Van Damme et al. 2007). The

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Table 1 Overview of major plant lectin families, their carbohydrate binding properties and insecticidal activity

No.	Lectin family	Carbohydrate specificity	Example	Insecticidal activity*
1	Amaranthin family	GalNAc/T-antigen	Amarantin	+
2	Lectins with Nictaba domain	(GlcNAc)n N-glycans	Nictaba	?
3	GNA related lectins	Man High Man N-glycans	GNA, ASAL, ASAII, ACA, LOA	+++
4	Chitin-binding lectins composed of hevein domains	(GlcNAc)n N-glycans	WGA, UDA, OSA	++
5	Jacalin-related lectins	Gal/T-antigen Man N-glycans	Jacalin	+
6	Legume lectins	Man/Glc Gal	ConA, Gleheda, PHA, PSA, GS-II, BPA	++
7	Lectins with ricin-B domains	Gal/GalNAc Siaα2-6Gal/GalNAc	Ricin, SNA-I	+

*Insecticidal activity has been reported for many (+++), several (++), a few (+) or no (?) lectins from this plant lectin family. GNA, *Galanthus nivalis* agglutinin; ASAL, *Allium sativum* leaf agglutinin; ASAII, *Allium sativum* bulb agglutinin II; ACA, *Allium cepa* agglutinin; LOA, *Listera ovata* agglutinin; WGA, *Triticum aestivum* (wheat germ) agglutinin; UDA, *Urtica dioica* agglutinin; OSA, *Oryza sativa* agglutinin; BPA, *Bauhinia purpurea* agglutinin; ConA, *Canavalia ensiformis* agglutinin; PHA, *Phaseolus vulgaris* agglutinin; PSA, *Pisum sativum* agglutinin; GS-II, *Griffonia simplicifolia* agglutinin; SNA-I, *Sambucus nigra* agglutinin.

occurrence of these different carbohydrate-binding domains is not restricted to a certain plant family. Moreover, most carbohydrate-binding motifs are spread all over the plant kingdom. In addition, several carbohydrate-binding motifs can recognize similar sugar structures. The wide distribution of lectins in different plant tissues and the ubiquitous presence of some particular lectins suggest important roles for these proteins. As soon as it was found that plant lectins recognize specific carbohydrates, numerous hypotheses have been put forward suggesting different functions based on this particular biological activity. Most of these hypotheses were speculative and based on very little experimental evidence. However, a breakthrough in the search for the physiological role of plant lectins was achieved when it was realized that many of these proteins have a sugar specificity towards carbohydrates present in organisms outside the plant kingdom. Sugar moieties present on viruses, micro-organisms, fungi or the digestive tract of herbivorous insects, can be recognized by plant lectins. Accordingly, many known plant lectins are probably involved as part of the plant's defense. To explain the abundance of lectins in storage tissues, it has been proposed that lectins fulfill a dual role: they serve as storage

proteins in the absence of herbivores but act as defense proteins once the tissue is under attack. During the last two decades, important progress has been made in the study of the insecticidal activity of several plant lectins and the elucidation of their mode of action. This chapter will focus on the insecticidal activity of different plant lectins and their use as defense proteins in crop protection.

1.1 Classical Lectins

Many plant lectins are found in storage tissues where they are highly abundant. These lectins are constitutively expressed or start to accumulate in these storage tissues during a certain developmental period. Because of the high lectin concentrations, the isolation procedure results in relatively high amounts of purified lectin. These plant lectins are often referred to as the 'classical lectins', and this group comprises most lectins that have been characterized so far. Most classical plant lectins are present in seeds but a lot of them are also found in different vegetative tissues (Van Damme et al. 1998). Typically, lectins account for 0.1–5% of the total seed protein. However, some seed lectins are predominant proteins representing up to 50% of the total protein (e.g. in some *Phaseolus* seeds). The same holds true for non-seed lectins, which have been found in virtually all kinds of vegetative tissues such as leaves, stems, bark, bulbs, tubers, rhizomes, roots, fruits. Feeding experiments on insects with artificial diets supplemented with plant lectins have been performed almost exclusively with classical lectins for the simple reason that these proteins can be purified in reasonable amounts. Famous examples are members of the legume lectin family such as the lectins present in the seeds of jackbean (*Canavalia ensiformis*, ConA), pea (*Pisum sativum*, PSA) or bean (*Phaseolus vulgaris*, PHA). Several of the chitin-binding lectins composed of hevein domains have also been intensively studied for their effect on different insects. Examples are the lectins from the germs of wheat (*Triticum* sp., WGA) or rice (*Oryza sativa*, OSA), the rhizomes of the stinging nettle (*Urtica dioica*, UDA), or hevein, a lectin from the latex of the rubber tree (*Hevea brasiliensis*). A third family of lectins that has been examined intensively for its toxic effect on insects is related to a lectin from the bulbs of snowdrop (*Galanthus nivalis*, GNA). Similar lectins have been isolated from the bulbs or leaves of garlic (*Allium sativum*, ASA-II and ASAL, respectively), the bulbs of onion (*Allium cepa*, ACA) or the leaves of the twayblade (*Listera ovata*, LOA).

1.2 Inducible Lectins

Next to the pre-formed (constitutive) defense strategies, plants also possess different inducible defense mechanisms that become activated upon insect attack. Certain inducible mechanisms, like the accumulation of an array of defense proteins, play

an important role in resistance and result in different effects on phytophagous insects such as toxicity, a retardation of larval development or enhanced susceptibility to natural enemies. The synthesis of the inducible defense proteins in plants is regulated by the interplay between different signaling plant hormones like jasmonic acid, salicylic acid, abscisic acid and ethylene. Jasmonic acid is the key molecule that regulates the wound response and consequently also the induced defense against chewing insects like caterpillars or larvae of beetles. In contrast, salicylic acid is considered to be the regulating molecule for the induction of defense responses towards bacteria and some fungi.

Recently, different lectins have been described that can only be detected after applying certain stress conditions onto the plant tissue. These lectins distinguish themselves from the classical lectins by their cellular localization and their relatively low concentrations. Some of these lectins respond to plant hormones that regulate the inducible defense mechanisms and are thought to play a role in plant protection against insects. Moreover, a few lectins have been found to be expressed after insect feeding on plant material. A first example is the tobacco lectin called Nictaba, which has a sugar-binding specificity for oligomers of *N*-acetylglucosamine (GlcNAc), high-mannose and complex *N*-glycans (Lannoo et al. 2006). No agglutination activity can be detected in leaves of *Nicotiana tabacum*, but when they are treated with methyl jasmonate Nictaba starts to accumulate (Chen et al. 2002). Other plant hormones like salicylic acid, ethylene, gibberellic acid, auxins and cytokines were tested for their ability to induce the lectin, but they all failed to induce Nictaba expression (Lannoo et al. 2007). When tobacco leaves were infested with larvae of the cotton leafworm, *Spodoptera littoralis*, Nictaba started to accumulate. Figure 1 shows the accumulation over time of Nictaba in tobacco leaves after feeding by *S. littoralis* larvae. In addition to the local induction of Nictaba, this tobacco lectin is also induced systemically (Lannoo et al. 2007). In contrast, wounding alone was not able to induce the tobacco lectin expression, which strengthens the hypothesis that additional elicitors present in the oral secretions of the caterpillars are necessary for proper Nictaba expression. Purified Nictaba also exerted a toxic effect on insect cell lines, which supports a defensive role of Nictaba in tobacco leaves (Vandenborre et al. 2008).

Zhu-Salzman et al. (1998b) reported a lectin, GS-II, that is induced in the leaves of *Griffonia simplicifolia* in response to treatment with methyl jasmonate. After wounding the leaves, which typically increases jasmonate levels, no increase in GS-II levels was found locally at the site of wounding, whereas GS-II expression was clearly upregulated in the systemic leaves. The hypothesis was put forward that ethylene, which also accumulates after injury, suppresses GS-II expression in the local leaves, while ethylene levels in systemic leaves are insufficient to prevent GS-II accumulation. It was suggested that *G. simplicifolia* has evolved a rather specialized response to herbivore attack whereby local activation of defensive gene expression is attenuated to mount a more substantial defense distal to the site of invasion (Zhu-Salzman et al. 1998a). In contrast to the tobacco lectin, which can only be detected after induction, GS-II is also constitutively expressed at low but detectable levels in leaf tissues.

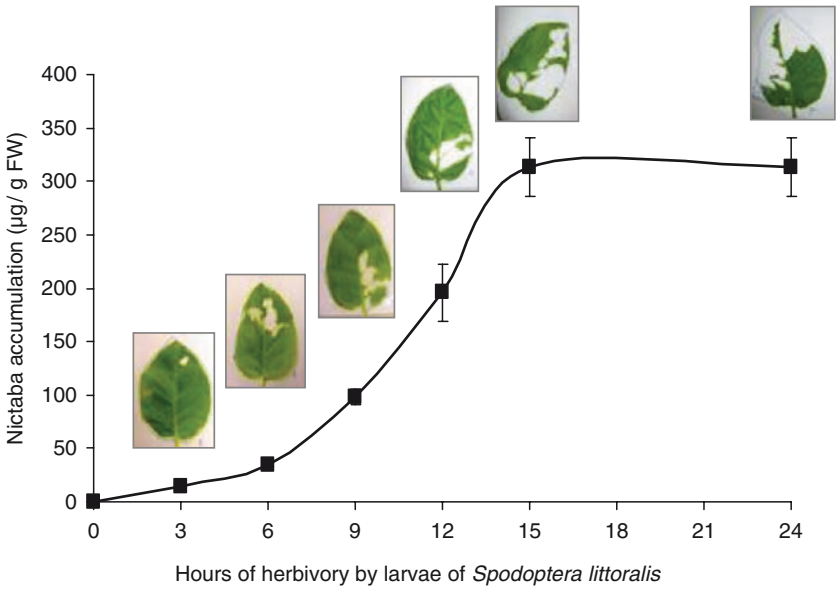

Fig. 1 Accumulation of Nictaba following herbivory. Quantitative analysis of Nictaba accumulation in a tobacco leaf after *Spodoptera* herbivory. One L4 *Spodoptera littoralis* larva per plant was allowed to feed for the time intervals indicated on the x-axis before being removed. At 48 h after the start of the experiment, lectin activity was quantified and expressed as microgram lectin per gram fresh weight (FW) of leaf tissue (mean values ± SD of three independent replicate leaves) (adapted from Lannoo et al. 2007). The amount of herbivory that corresponds to the different feeding periods is indicated in the pictures above the corresponding time periods

In wheat plants (*Triticum aestivum*) a gene called *Hfr-1* (Hessian fly responsive gene 1) that encodes a jacalin-related mannose-binding lectin was induced after infestation by larvae of the Hessian fly (*Mayetiola destructor*) (Williams et al. 2002). Detailed binding analyses revealed that the HFR-1 lectin has a high specificity towards oligomers of mannose and high-mannose *N*-glycans (Subramanyam et al. 2008). The accumulation of *Hfr-1* transcripts started within 22 h after egg hatching and returned to normal levels only after 5 days. The expression profile of *Hfr-1* corresponds to the critical period in which the first-instar larvae are attempting to establish feeding sites by injecting substances into the plant. Resistant wheat plants accumulated high levels of HFR-1 at the larval feeding sites which may deter avirulent larvae from feeding (Subramanyam et al. 2008). In addition, Puthoff et al. (2005) and Giovanini et al. (2007) identified two other Hessian fly-responsive wheat genes called *Hfr-2* and *Hfr-3*, containing lectin domains similar to amaranthin and hevein, respectively. Expression of *Hfr-2* is upregulated by methyl jasmonate, phloem feeding of bird cherry-oat aphids (*Rhopalosiphum padi*) and chewing of fall armyworm (*Spodoptera frugiperda*) caterpillars, whereas wounding, salicylic acid and abscisic acid treatment only had a slight effect (Puthoff et al. 2005). After hatching of Hessian fly larvae and induction by *R. padi*, wheat plants also responded by high expression levels of *Hfr-3*. In contrast feeding of the fall armyworm, wounding, treatment with methyl jasmonate,

salicylic acid or abscisic acid could not induce *Hfr-3* expression. All these data suggest the involvement of a set of inducible lectins in resistance of wheat against pest insects like Hessian fly and aphids.

2 Insecticidal Activity of Plant Lectins

Lectins incorporated in artificial diets or expressed in different transgenic plants have been shown to reduce the performance of several insect species belonging to the orders Lepidoptera, Coleoptera, Diptera and Hemiptera (Sharma et al. 2004; Van Damme 2008). During the last two decades, it became clear that lectins are potential tools in crop protection. However, it should be mentioned that the effect of lectins is usually restricted to one or a few insect orders. In addition, the insecticidal activity of a particular lectin can differ within the same insect order.

2.1 Lepidoptera

A lectin that has been used a lot in feeding experiments on pest herbivores is the mannose/glucose-specific lectin ConA from the legume *C. ensiformis*. When fed to larvae of the tomato moth (*Lacanobia oleracea*) in an artificial diet, larval development was retarded and 90% mortality was scored at a lectin concentration of 2% (w/w) of the total protein (Gatehouse et al. 1999). Bioassays with *L. oleracea* larvae on ConA-expressing potato plants also showed a retardation in the development and a decrease in larval weight of >45% compared to control larvae (Gatehouse et al. 1999). Another legume lectin from *P. sativum* (PSA) increased resistance of transgenic tobacco plants to the tobacco budworm *Heliothis virescens* (Boulter et al. 1990).

The mannose-specific *Galanthus nivalis* agglutinin (GNA) from snowdrop bulbs also exerts noxious effects on the larval growth and development of several Lepidoptera, including the tomato moth (*L. oleracea*), the Mexican rice borer (*Eoreuma loftini*) and the cotton bollworm (*Helicoverpa armigera*) (Gatehouse et al. 1997; Sétamou et al. 2003; Shukla et al. 2005) when feeding on GNA-containing diets or transgenic plants expressing GNA. However, a study by Wakefield et al. (2006) also investigated the effect of GNA and found contrasting results. After ingestion of transgenic tomato leaves expressing approximately 2% GNA, *L. oleracea* larvae consumed more leaf material resulting in an increased larval weight. As a consequence, the mean developmental time to the pupal stage was reduced by seven days. The garlic lectins ASAL and ASAII, both related to GNA, have also been expressed in transgenic tobacco plants (0.1–1% of total protein) and feeding of larvae of the cotton leafworm *S. littoralis* on the leaves significantly reduced the rate of weight gain (Sadeghi et al. 2008a). The lectin retarded the development of the larvae and their metamorphosis, and was also detrimental to

the pupal stage resulting in weight reduction and lethal abnormalities. Transgenic plants expressing ASAL and ASAII caused a reduction in larval weight gain of approximately 42% and 30%, respectively, after 3 days (Sadeghi et al. 2008a). The pupae that originated from the treated larvae showed reduction in size and pupal weight as compared to the control pupae, and resulted in a pupal mortality of 100% and 60% for the specimens fed on ASAL and ASAII, respectively. Similarly, Sadeghi et al. (2009) reported insecticidal effects of the leek lectin (APA) delivered via transgenic plants on the development of *S. littoralis*. Machuka et al. (1999) screened the effects of 25 lectins from 15 different plant families on the development of legume pod borer (*Maruca vitrata*) larvae. Although a total of 16 lectins had detrimental effects pertaining to larval survival, weight, feeding ability, pupation, adult emergence and/or fecundity, only the *Listera ovata* agglutinin (LOA) and GNA were effective against larvae of *M. vitrata* for all six parameters examined. Larval mortality and feeding inhibition caused by the most active lectin (LOA) was >60% compared to the control larvae for both parameters.

Czapla and Lang (1990) screened 26 plant lectins from six different plant families in artificial diets, for effects against the neonate larvae of the European corn borer *Ostrinia nubilalis*. They found two lectins, WGA and the *Bauhinia purpurea* lectin (BPA), out of the 26 to be effective against *O. nubilalis*. In a similar screening of 127 lectins against *O. nubilalis* two lectins could be added to the list, the rice lectin OSA (*Oryza sativa* agglutinin) and a lectin from couch grass (*Agropyrum repens*) (Cavalieri et al. 1995). Both lectins have a molecular structure that is very similar to that of WGA. Recently, Gupta et al. (2005) also reported an effect of WGA against neonates of the cotton bollworm (*H. armigera*).

A lectin family that has been studied less intensively for its insecticidal properties is the family of plant lectins with a ricin domain, which are type-2 ribosome inactivating proteins (RIPs). Type-2 RIPs are plant lectins consisting of a sugar-binding domain and a catalytic domain that can inactivate the large subunit of ribosomal RNA by preventing binding of the elongation factor EF2 to the ribosome. As a consequence, protein synthesis is arrested resulting in cell death (Van Damme et al. 1998). A study by Shahidi-Noghabi et al. (2009) analyzed the effect of the type-2 RIP SNA-I' from elderberry bark (*Sambucus nigra*) on growth and development of larvae of the beet armyworm (*Spodoptera exigua*). Transgenic tobacco plants expressing SNA-I' significantly reduced the rate of weight gain for *S. exigua* larvae and caused a retardation in development (Fig. 2). Two transgenic lines exhibiting an expression level of 21 and 40 μg SNA-I′ per gram fresh weight, caused a reduction of 39% and 55%, respectively, in the mean weight of *S. exigua* after 10 days of feeding (Fig. 2a). In addition, a delay in development of *S. exigua* and a significant increase in mortality were noted for the treated larvae (Fig. 2b). Recently, Shahidi-Noghabi et al. (2008) confirmed strong insecticidal activity of SNA-I against aphids and showed the importance of carbohydrate-binding activity for insecticide action. Another type-2 RIP with promising insecticidal activity is cinnamomin from *Cinnamomum camphora* (Zhou et al. 2000; Wei et al. 2004). Toxicity of cinnamonin was observed towards *H. armigera* and the domestic silkworm (*Bombyx mori*) larvae.

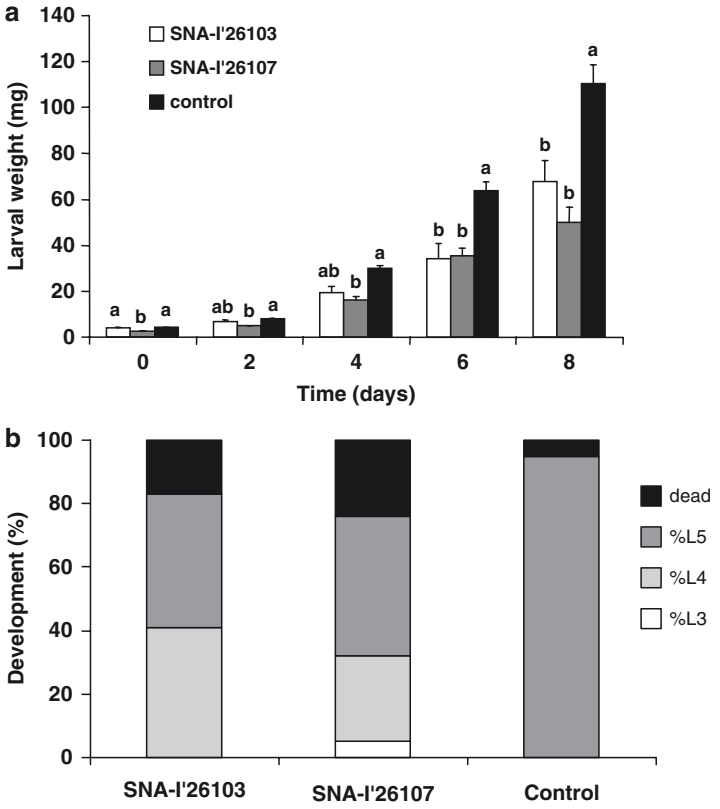

Fig. 2 In *planta* bioassay of beet armyworm *Spodoptera exigua* on transgenic lines (*Nicotiana tabacum*) expressing SNA-I′. (**a**) The inhibitory effect of transgenic lines SNA-I′26103 and SNA-I′26107 on larval weight gain is expressed as means ± SE after feeding for 2–10 days. The initial number of larvae was 30 for both treatments and control. Significant differences in weight gain are indicated using a letter code. (**b**) Effect of the different transgenic lines and wild type (control) plants on the development of *S. exigua* larvae in the third (L3), fourth (L4) and fifth larval stage (L5), after a period of 13 days feeding (adapted from Shahidi-Noghabi et al. 2009)

2.2 *Coleoptera*

Compared to Lepidoptera little research has been done to investigate the insecticidal effect of lectins on members of the order Coleoptera. The growth and development of the larvae of the cowpea weevil (*Callosobruchus maculatus*) were moderately inhibited by several plant lectins like WGA, OSA, UDA, the potato (*Solanum tuberosum*) lectin and thorn apple (*Datura stramonium*, DSA) lectin (Murdock et al. 1990; Huesing et al. 1991). It is striking that all these lectins have been reported to interact with chitin or GlcNAc oligomers. In addition, Czapla and Lang (1990) showed that WGA inhibits the larval growth of the Southern corn rootworm (*Diabrotica undecimpunctata*) after feeding on artificial diets containing the chitin binding lectin.

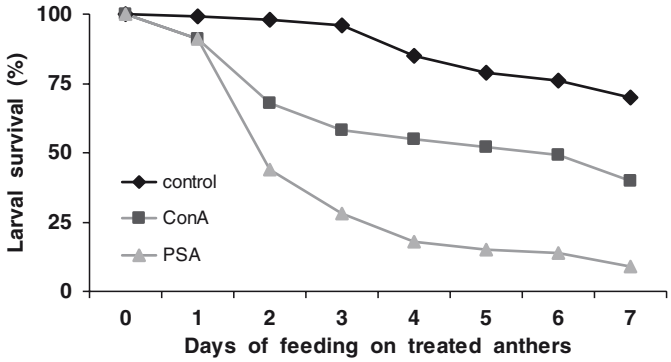

Fig. 3 Survival rates of pollen beetle larvae fed anthers soaked in solutions of plant lectins or controls. Each curve represents the percent of larvae surviving each day after ingesting anthers soaked in a 1% solution of ConA or pea lectin. The initial number of larvae was 47 for all three treatments. (Adapted from Melander et al. 2003)

Melander et al. (2003) evaluated the effect of two legume lectins ConA and PSA on the growth and survival of pollen beetle larvae (*Meligethes aeneus*) when fed with oilseed (*Brassica napus*) anthers soaked in a 1% solution containing either of the lectins (Fig. 3). After 7 days, a larval mortality of 60% and 91% was observed after feeding on anthers treated with ConA and PSA respectively, while the mortality in the control larvae was only 30%.

In addition, an *in planta* feeding experiment was performed to target the pollen beetle using transgenic oilseed rape with a tissue specific expression of PSA (1.2% of total soluble protein) in the anthers and pollen (Melander et al. 2003). Feeding on the anthers from this plant material resulted in lower weight gain of the pollen beetle larvae, but had no effect on the adult beetles (Lehrman et al. 2007). Another member of the legume lectin family that was recently discovered, is Gleheda found in ground ivy (*Glechoma hederacea*) (Wang et al. 2003). Insect feeding trials demonstrated that Gleheda is a potent insecticidal protein for larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*) (Wang et al. 2003). Potato leaves dipped in a 2% (w/v) solution of Gleheda resulted in a dramatic inhibition of feeding and weight gain of the larvae. Moreover, none of the Gleheda-fed larvae reached the pupal instar, indicating that the lectin caused complete mortality (Wang et al. 2003).

2.3 Hemiptera

Next to the insecticidal effects of certain plant lectins on chewing insects, a lot of evidence has emerged in the last decade that at least some lectins also have toxic effects on sap-sucking insects. This is of particular interest because sap-sucking insects are not targeted by the well known *Bacillus thuringiensis* (*Bt*) endotoxins (Sharma et al. 2000). Hemiptera, including the aphids, whiteflies and plant- and

leafhoppers represent an important order of sap sucking pest insects. These insects can damage the crops in several ways. First, Hemiptera feed on the phloem sap and directly deprive the plant of nutrients. Second, since they feed on the phloem, they also cause indirect damage to plants by transmitting plant viruses. Third, they secrete honeydew, a sugar-rich sticky substance, onto the plants which in turn is the favorite food source for sooty molds.

For different mannose-binding lectins, especially those from the Amaryllidaceae and ConA, an insecticidal effect was addressed towards sap-sucking insects. Among the Amaryllidaceae lectins, the snowdrop lectin GNA has been used in a lot of feeding experiments and is effective both in vitro and in planta towards Hemipteran pest insects like aphids or plant/leafhoppers. Using artificial diets supplemented with GNA or transgenic plants expressing GNA either constitutively or under the control of a phloem specific promoter, the pea aphid *Acyrthosiphon pisum* (Rahbé et al. 1995), the peach aphid *Myzus persicae* (Sauvion et al. 1996), the glasshouse potato aphid *Aulacorthum solani* (Down et al. 1996) and the grain aphid *Sitobion avenae* (Stoger et al. 1999) were shown to be sensitive. With respect to aphids, GNA acts primarily by reducing growth, development, and fecundity rather than increasing mortality. In contrast, transgenic rice expressing GNA does cause a significant increase in nymphal mortality of planthoppers and leafhoppers (Rao et al. 1998; Foissac et al. 2000; Powell, 2001; Sun et al. 2002; Nagadhara et al. 2004). When transgenic rice plants with selective GNA expression in the phloem sap were fed to the whitebacked planthopper (*Sogatella furcifera*), a highly significant reduction (>90%) in nymphal survival was shown compared to rearing on non-transformed rice plants (Nagadhara et al. 2004). Furthermore, GNA also decreased the development with a general delay of 5–7 days in the life cycle of *S. furcifera* and exerted >90% reduction of fecundity. In this study, the untransformed control rice plants with severe hopper burn failed to survive the 21-day bioassay period, while the transgenic plants expressing GNA survived the infestation and were grown to maturity with normal vigor and seed fertility. These results clearly illustrate the high level protection of GNA against planthoppers without interfering with the normal metabolism of rice plants. ASAII and ASAL, two other plant lectins derived from garlic and possessing similar carbohydrate-binding activities to GNA, were also shown to have insecticidal properties towards sap-sucking pest insects (Dutta et al. 2005; Saha et al. 2006; Sadeghi et al. 2007). Feeding experiments using transgenic tobacco expressing ASAL up to 2% of total protein, decreased survival of *M. persicae* to 20% or lower compared to 75% when fed on untransformed tobacco plants (Dutta et al. 2005). In contrast, Sadeghi et al. (2007) could not observe any effect on nymphal survival of *Myzus nicotianae* in feeding trials using transgenic tobacco, although the growth and development into adults was clearly retarded. Feeding of *M. nicotianae* on the transgenic plants also resulted in a significant decrease in aphid fecundity compared with that on control plants. The difference of the nymphal survival of *M. nicotianae* compared to the results from Dutta et al. (2005) was attributed to the low expression levels (0.03%) of ASAL in the transgenic tobacco plants (Sadeghi et al. 2007). In a study by Hossain et al. (2006), the

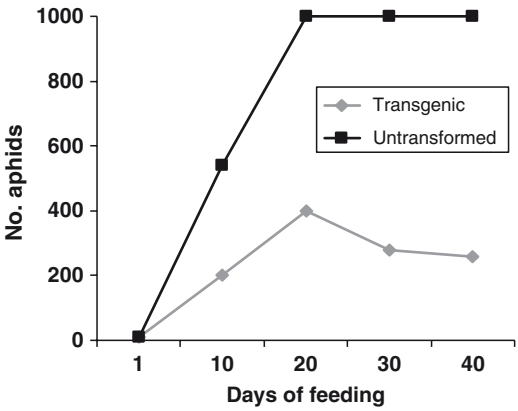

Fig. 4 Increase in aphid population for 40 days on a transgenic Indian mustard plant expressing the onion lectin ACA compared to control plants (Adapted from Hossain et al. 2006)

insecticidal effects of GNA, ASAL and the onion lectin ACA (*Allium cepa* agglutinin) were compared towards nymphs of the mustard aphid (*Lipaphis erysimi*). ACA was found to exert a higher toxicity than GNA or ASAL when added to artificial diets. Using transgenic Indian mustard plants (*Brassica juncea*) expressing ACA, Hossain et al. (2006) were able to control the growth of the *L. erysimi* population (Fig. 4). Interestingly, Saha et al. (2006) reported that transgenic rice plants expressing ASAL (1% of total soluble protein) exhibited less or no incidence of tungro disease after attack by the green leafhopper *Nephotettix virescens*. These results show that expression of a lectin not only helps to control the phloem feeding insects but also the viral diseases they transmit.

Feeding trials with transgenic tobacco plants expressing the *Helianthus tuberosus* lectin (HTA) or *Pinellia ternata* lectin (PTA) demonstrated that lectins also have a devastating effect on the peach-potato aphid (*M. persicae*) (Chang et al. 2003; Yao et al. 2003). When fed on the transgenic tobacco expressing HTA, the aphid population was reduced to 30% of the control level after 11 days, fecundity was inhibited by >50% and the development of the aphids was notably retarded (Chang et al. 2003). Interestingly, ConA and PSA, two legume lectins with similar sugar-binding specificities, exerted different anti-metabolic effects towards the tara planthopper *Tarophagous proserpina* (Powell 2001). When ConA was added to artificial diets (0.1% w/v) and fed to *T. proserpina*, a corrected mortality of 93% was noted, while PSA (0.1% w/v) did not exert any significant effect towards the survival of the aphids.

3 Lectins and Insect Behavior

The question whether plant lectins can have an influence on the behavior of insects has largely been unsolved. But recently, two studies have opened this research topic.

Subramanyam et al. (2008) analyzed the behavior of Hessian fly larvae during infestation of resistant wheat plants that induces the HFR-1 lectin and susceptible

wheat plants that can only express the lectin to very low amounts. One day after egg hatch, when the HFR-1 lectin is clearly detectable in resistant plants, the avirulent and virulent larvae did not differ in size or shape but behavioral differences became evident. Two days after egg hatch, all virulent larvae were sessile, while the avirulent larvae were displaying abnormal writhing and rearing behaviors. The avirulent larvae became sessile only after 4 days. The avirulent larvae did not show any visible gut contractions indicating that no food was digested. Eight days after hatch, the avirulent larvae were dead, whereas the virulent larvae were already in their second instar.

Another study focused on the repellent or deterrent activity of several plant lectins on the cowpea weevil *C. maculatus* (Sadeghi et al. 2006). Using binary choice experiments, it was clearly demonstrated that coating of chickpeas with a panel of lectins with different carbohydrate-specificities dramatically reduces the oviposition of *C. maculatus* and that the reduction in egg number is dose dependent. The results of these binary choice experiments leave no doubt that insects are able to distinguish lectin-coated from control seeds and that lectins influence their oviposition behavior. However, no clear correlation could be found between deterrent activity and sugar-binding specificity of the lectins.

4 Mode of Action

The insecticidal activity of plant lectins is assumed to be related to the sugar binding capacity of these proteins. During the last two decades many efforts have been undertaken to unravel the mechanism behind the defense properties of plant lectins. Detailed analyses of the carbohydrate-binding properties have shown that many lectins recognize sugar structures that are not present in plants but can be found in other organisms (Van Damme et al. 1998) which favors a role in plant defense. Furthermore, it has become obvious that the binding specificity of different plant lectins towards sugars is not directed against simple sugars but rather against more complex sugar structures like *O*- and *N*-glycans (Van Damme et al. 2007, 2008). Most of these complex glycans are present on glycoconjugates along the intestinal tract of insect or mammalian herbivores. Receptors for plant lectins can be defined as glycoconjugates that possess a carbohydrate moiety with a structure complementary to that of the binding site of the lectin (Peumans and Van Damme 1995). This implies that in the same insect glycoconjugates of different nature (e.g. glycoproteins, polysaccharides, glycolipids) but with identical (or structurally similar) carbohydrates can act as different receptors for the same plant lectin. One lectin molecule can also exert different insecticidal effects against different insect orders.

4.1 Stability of Plant Lectins

A prerequisite to be successful as a protein in exerting an insecticidal effect is the resistance to proteolytic degradation by the insect digestive enzymes and, in addi-

tion, to survive the environmental pH conditions present in the insect digestive tract. Indeed, it is a common property of many plant lectins to withstand proteolytic degradation and to remain stable and active in a broad pH range. For example, GNA was shown to be resistant to gut proteolysis in lepidopteran larvae (Gatehouse et al. 1995). Furthermore, structure/function analysis by site-directed mutagenesis indicated that the insecticidal activities of the GlcNAc-specific lectin GS-II and its mutant forms were correlated with the resistance to proteolytic degradation by the midgut extracts of the larvae of *C. maculatus* (Zhu-Salzman et al. 1998 b). Experiments with mutants lacking carbohydrate-binding and insecticidal activity revealed that these proteins are rapidly digested by two purified cathepsin L-like gut proteases (Zhu-Salzman and Salzman 2001).

4.2 Potential Targets for Plant Lectins

When plant material is ingested by insect herbivores, the primary site of action for lectins is the insect digestive tract. The foregut and the hindgut are lined with a chitinous layer that is replaced after each molting cycle (Billingsley and Lehane 1996). For the chitin-binding lectins, the foregut can be the first potential target site after food ingestion. However, most research has focused on the midgut to find target sites for plant lectins. The midgut is the principal site for digestion of food and uptake of nutrients and does not have a continuous cuticular layer, but in many insects the midgut cells secrete a detached peritrophic membrane (PM). The PM serves as a shield to protect the microvilli from direct contact with abrasive food particles and as a barrier against the entry of viruses, bacteria or other parasites that are too large to pass through the membrane (Billingsley and Lehane 1996). The PM is an acellular, semi-permeable matrix lining the midgut and is composed of chitin microfibrils with associated proteoglycans and glycoproteins (Lehane 1997). Proteins, glycoproteins and proteoglycans account for 22–55% of the total PM components (Lehane 1997), while chitin contributes only a small but important proportion of the PM, ranging from 3.7% to 13% of the total mass (Tellam and Eisemann 2000). The chitin microfibrils form an intersecting mesh-like network, providing a strong but elastic scaffold for PM assembly. The PM can be divided into two types referred to as type-I and type-II. A type-I PM is secreted as a continuous delamination all along the length of the midgut, while a type-II PM is secreted from a ring of cells at the anterior margin of the midgut (Lehane 1997). Since chitin is an important component of the PM, it is likely that several chitin-binding lectins will bind on this structure and interfere with the conformation or the functionality of the PM. Hopkins and Harper (2001) studied the effect of WGA on the formation of the PM in the European corn borer (*O. nubilalis*) and the tobacco hornworm (*Manduca sexta*) after feeding on a WGA-containing diet. In the European corn borer, it was shown that WGA can bind to the chitinous network, which caused a hypersecretion of unorganized PM in the midgut, resulting in cessation of feeding. In contrast, WGA had no effect on the organization of the PM of *M. sexta*. Next to binding on the chitin microfibrils, different proteins like peritrophins or intestinal mucins are

potential target sites for lectins. Shi et al. (2004) described a mucin-like protein present in the PM of *Mamestra configurata*, which is heavily glycosylated. It should be mentioned, however, that the formation of a PM is not universally present in all insects. Hemiptera and more particularly the Homoptera appear not to form a PM, which in part can help to explain the differences in insecticidal activity for certain lectins on insects from different orders.

Since digestive enzymes secreted by the epithelium cells and small molecules resulting from digestion have to pass the PM, this membrane is porous. Santos and Terra (1986) reported the presence of pores of 7–7.5 nm diameter in the PM of the caterpillar *Erinnyis ello*. Using the permeability of fluorescently labeled dextrans, Edwards and Jacobs-Lorena (2000) determined that the main part of the PM of larvae of two mosquito species was permeable to proteins of 148 kDa or smaller. These observations indicate that lectins can pass easily through the pores of the PM and exert their toxic action at the brush border region. At the surface of, and between the microvilli of the epithelial cells, a viscous secretion called the glycocalyx is present consisting of proteins and carbohydrates. This viscous glycocalyx traps and concentrates secreted enzymes and products of digestion. Different plant lectins have the potential to interfere with the physiological function of this glycocalyx by binding to the carbohydrate moiety. A toxic dose of GNA was able to induce morphological changes in the midgut region of planthoppers with disruption of the microvilli brush border region (Powell et al. 1998). Habibi et al. (2000) also showed that the gut epithelial cells of the Western tarnished plant bug (*Lygus hesperus*) were severely disrupted by PHA. PHA caused disorganization and elongation of the brush border microvilli, and swelling of the epithelial cells into the lumen of the gut leading to complete closure of the lumen.

Plant lectins can also destabilize insect metabolism by interfering with the gut enzymatic function by binding to glycosylated digestive enzymes. Sauvion et al. (2004) showed that ConA interacts with glycosylated receptors present at the cell surface or within the midgut epithelium cells (Fig. 5). One of these receptors was later identified as an aminopeptidase in *A. pisum* (Cristofoletti et al. 2006). Moreover, Majumder et al. (2004) showed clearly that ASAL binds on the epithelial membrane of the pea aphid (*Aphis craccivora*) midgut and that the receptors for ASAL must be glycosylated. Du et al. (2000) demonstrated that one of the major receptors for GNA in *N. lugens* was a subunit of ferritin, suggesting that this particular lectin may interfere with the insect's iron homeostasis. Similarly, Sadeghi et al. (2008b) identified ferritin in the midgut of *S. littoralis* as a receptor target for GNA.

Possibly, lectins also exert their toxic effect after crossing the midgut epithelium. For the homopteran *N. lugens* and lepidopteran *L. oleracea*, GNA has been reported to be transported across the midgut epithelium barrier into the circulatory system (Powell et al. 1998; Fitches et al. 2001). In *N. lugens* GNA was found to accumulate in the hemolymph, fat bodies and ovarioles (Powell et al. 1998), while this plant lectin was shown in the hemolymph and Malpighian tubules of *L. oleracea* after feeding (Fitches et al. 2001). This makes additional target sites inside the insect body likely (e.g. fat tissue, hemolymph, ovaries). Recently, Hogervorst et al. (2006)

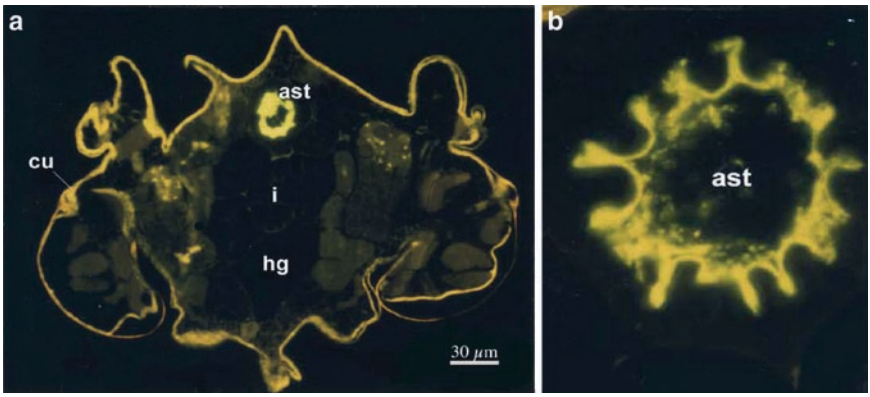

Fig. 5 Transverse sections of the first abdominal segment of an *Acyrthosiphon pisum* nymph (fourth instar) intoxicated with FITC-ConA. (**a**) Binding of the FITC-ConA lectin to the epithelium of the stomach (ast) and cuticula (cu) while no fluorescent was detected in the intestine (i) and hindgut (hg). (**b**) Enlarged image of the stomach with bound ConA. (Adapted from Sauvion et al. 2004)

studied the effect of GNA on three different aphid predators. Although no direct insecticidal effect was found on the insects, GNA was shown to move across the digestive system.

4.3 Using Plant Lectins as a Delivery Tool

Another application of lectins in insect control takes advantage of their capacity to move across the digestive system of insects. Using the mannose-specific GNA, researchers were successful in delivering other insecticidal proteins that normally cannot pass the midgut epithelium into the hemocoel (Fitches et al. 2002, 2004; Down et al. 2006; Trung et al. 2006).

Fitches et al. (2002) demonstrated for the first time that fusion proteins consisting of a lectin coupled to another small insecticidal protein can be successfully used to control pest insects. A fusion protein consisting of GNA coupled to the neuropeptide allatostatin, could inhibit the feeding and growth of the tomato moth *L. oleracea*. A similar construct with the spider venom *Segestria florentina* toxin I (SFI1) was shown to be lethal to first-instar larvae of the tomato moth (Fitches et al. 2004). In addition, Down et al. (2006) showed the insecticidal activity of the SFI11/GNA fusion protein towards the rice brown planthopper *N. lugens* and the peach-potato aphid *M. persicae* by incorporation into artificial diets. After 7-day feeding on the diet, 100% mortality was observed for *N. lugens*, while almost 50% of the aphids *M. persicae* were dead after 14 days. Also the development of *M. persicae* was slowed down and the reproductive capacity was severely reduced. Recently, a fusion protein consisting of GNA and a lepidopteran-specific toxin (ButalT) from the South Indian red scorpion (*Mesobuthus taumulus*) was shown to be highly toxic

to the larvae of the tomato moth (Trung et al. 2006). These experiments clearly show the utility of plant lectins as carriers to transport other toxic proteins across the insect gut. The fusion protein exerts an insecticidal action that is higher than that of the plant lectin alone.

5 Beneficial Insects

As discussed above, plant lectins can be used for the development of transgenic crops with enhanced resistance against pest insects. Therefore, plant lectins have the potential to play an important role in the development of integrated pest management strategies. However, by introducing genetically modified crops expressing insecticidal proteins like lectins into agriculture, beneficial insects can become an unwanted target for the transgenic crops. Beneficial insects may be exposed to lectins expressed in transgenic plants either directly, by consuming parts of the plant (e.g. honeybees) or indirectly, by consuming the target pest insect itself (e.g. predatory beetles) or by parasitizing the insects that have been feeding from these plants (e.g. parasitoid wasps). To address this concern several studies have been conducted to analyze the performance of these beneficial insects after being exposed to transgenic crops.

One type of beneficial insects are pollinators like honeybees (*Apis mellifera*) or bumblebees (*Bombus terrestris*). Because many food production crops heavily depend on pollination, these honey bees and bumblebees are considered to be key species for risk assessments of transgenic crops. If transgenic plants that express a lectin in their pollen influence the pollen choice of the worker bees, this can be a disaster for the pollination of that crop. On the other hand, pollen is the main protein source for adult bees as well as for the larval brood food. Therefore, a toxic effect of the collected pollen on the bee larvae can have a devastating effect on the bee population. In order to analyze the effect of an insecticidal lectin in transgenic pollen on the performance of bee larvae, pollen derived from two transgenic plant lines were mixed into a larval diet and compared to a control pollen diet (Lehrman 2007). The transgenic plants used in this study were oilseed rape which selectively expressed the pea lectin (PSA) in its anthers and pollen up to 1.2% of total soluble protein. After feeding the bee larvae, no negative effect could be detected on larval mortality, weight and developmental time. In another study, Babendreier et al. (2007) tested whether bumblebee (*B. terrestris*) workers are able to detect GNA dissolved in sucrose solution and whether consumption of this lectin affects survival and offspring production. No difference was found in the number of visits and the duration of the visits among the different GNA concentrations, indicating that bumblebees do not discriminate between GNA-containing and control solutions in a choice experiment. However, when GNA was fed to bumblebees that were kept in microcolonies, they appear to be strongly affected by ingestion of GNA. Mortality of both workerbees and drones increased and offspring production was significantly reduced (Babendreier et al. 2007). Recently, Konrad et al. (2008)

analyzed the potential effect of GNA on the larvae of the solitary bee *Osmia bicornis*. Low doses of GNA (0.01% of total protein) failed to affect overall development, while a high dose of GNA (0.1% of total protein) in the larval diet resulted in significantly increased development time and reduced efficiency in conversion of pollen food into larval body weight. However, it must be mentioned that in the field, bees are unlikely to be exposed to GNA doses that are ≥0.1% of the total protein content.

Predators of pest insects that can play an important role in controlling certain pest populations represent another type of beneficial insects. Well known examples of this type of tritrophic interaction are the larvae of lady beetles which are voracious predators of aphids. Although many studies have demonstrated deleterious effects of GNA towards different sap-sucking insects like aphids, Down et al. (2000, 2003) could not observe a significant effect on the larvae of the lady beetle *Adalia bipunctata* when fed on *M. persicae* containing GNA delivered though an artificial diet (0.1% GNA w/v). In contrast, a study by Birch et al. (1999) reported reduced fecundity, egg viability and a reduction in female longevity of *A. bipunctata* preying on *M. persicae* which was reared on GNA-expressing potatoes. From the latter study, however, it was not clear whether these sublethal effects were direct (caused by GNA toxicity) or indirect (caused by GNA effect on the aphids). The direct effect of GNA on larvae of different aphid predators, *Chrysoperla carnea*, *A. bipunctata* and *Coccinella septempunctata*, was evaluated by Hogervorst et al. (2006). In this study, no significant difference in weight gain was detected but the longevity was affected for all three predators after feeding on an artificial diet containing 1% GNA.

Parasitoids are a third type of beneficial insect that can be influenced by transgenic plants expressing a lectin. Parasitoids comprise endophagous insects that lay their eggs into the body of a pest insect host, which then is used as a food source for the developing larvae. Different parasitic wasps are known to lay eggs into aphids. Parasitoid larvae developing inside the aphid that is feeding with transgenic plants expressing a lectin, may be subjected to both direct and indirect effects of the lectin. Studies that have analyzed this topic used the aphid parasitoids *Aphidius ervi* (Couty et al. 2001b) or *Aphelinus abdominalis* (Couty et al. 2001a,c). When GNA was delivered to the target aphids by feeding on transgenic plants or artificial diets, sublethal effects were found on the larval development of *A. abdominalis*. However, this observation was probably an indirect host-quality-mediated effect by the parasitoid development in the smaller host aphids that were fed on a GNA-containing diet (Couty et al. 2001c). Moreover, it was found that GNA is mostly excreted by the parasitoid after its uptake. The adult stage of *A. abdominalis* in turn is also a predator of aphids, but it was shown that the adult females were not affected by feeding on GNA-dosed aphids (Couty and Poppy 2001). The performance of *Eulophus pennicornis*, a parasitoid of Lepidoptera, was studied under simulated field conditions with larvae of the tomato moth after feeding on GNA-expressing tomato/potato plants (Bell et al. 1999, 2001). The ability of the wasp to parasitize and subsequently develop on the pest larvae of *L. oleracea* was not altered by the presence of GNA in the diet of the host (Bell et al. 2001). *E. pennicornis* progeny

that developed on *L. oleracea* reared on GNA-expressing plants showed no significant alteration in fecundity when compared with wasps that had developed on hosts fed on control plants.

An alternative route through which non-target insects can be exposed to the transgenic product is by the excretion of honeydew. When honeydew-producing insects like aphids and other phloem-feeding insects feed on genetically modified plants, the honeydew can contain amounts of the transgenic product. To address whether different parasitic wasps (*Aphidius colemani*, *Trichogramma brassicae* and *Cotesia glomerata*) which are known to use honeydew as a carbohydrate source in the field are affected by GNA, Romeis et al. (2003) examined the toxicity of GNA on the wasps after feeding on a sucrose solution supplemented with the lectin. In all three species, GNA ingestion reduced parasitic wasp survival significantly. In contrast, for fecundity, negative effects were observed for *T. brassicae* but not for *A. colemani*. It remains unclear, however, if the GNA concentrations (0.1% and 1%) that exerted negative effects are representative for the GNA present in honeydew of aphids that were fed on transgenic plants. Moreover, Nagadhara et al. (2004) could not detect GNA in the honeydew of the whitebacked planthopper (*Sogatella furcifera*) after feeding on transgenic rice expressing GNA levels of 0.3% of total soluble protein, although the plant lectin exerted severe insecticidal effects on the planthoppers.

Based on the results described above, transgenic crops expressing insecticidal lectins can contribute to the development of integrated pest management strategies with minimal effect on beneficial insects. The use of the transgenic plants can help to significantly reduce the use of conventional pesticides worldwide.

6 Perspectives

During the last two decades, the insecticidal action of different plant lectins has been proven and substantial progress has been made in our understanding of the mode of action of plant lectins in insect tissues. It is generally accepted now that plant lectins definitely can play a role in plant defense, and several patents on the use of plant lectins in crop protection have been filed (Cavalieri et al. 1995; Gatehouse et al. 1996). The effect of different lectins is highly specific for each lectin and insect combination, even within the same insect order (Sétamou et al. 2002) and the mechanisms of action also seem to vary (Powell et al. 1998; Hopkins and Harper 2001). Most probably, the obvious differences in toxicity are somehow related to the fact that plant lectins are a complex composite of multiple families of evolutionarily related proteins with markedly different biochemical and physicochemical properties, carbohydrate-binding specificities and biological activities (Van Damme et al. 1998). Recent advances in molecular techniques to study sugar-protein interactions have contributed to recognition of the importance of these interactions in a lot of biological processes. Unfortunately, little is known about the overall glycome of insects today, but new molecular tools will help to detect differ-

ences between glycomes of different insect orders and give new insights in the mode of action of plant lectins towards insects.

We believe that the identification of target proteins for lectins in the insect body can be accelerated using some genomic approaches that were recently developed. For instance, the availability of whole genome sequences and EST databases of several pest insects, like caterpillars (*B. mori*, *B. Spodoptera* spp., *H. armigera*), beetles (*Tribolium castaneum*), aphids (*A. pisum*), grasshoppers (*Schistocerca gregaria*), as well as the use of new techniques of protein silencing such as RNAi will help to reveal the involvement and functionality of glycomics in insects. It will be fascinating to see if glycan structures are for example, involved in insect growth, development and reproduction, as seen in recent and fascinating studies carried out in the animal field.

In the past, especially the classical lectins that are expressed constitutively have been studied for their insecticidal properties. Since the inducible defense is thought to be more specifically directed to the kind of pest organism that is attacking the plant, defense proteins that are expressed after insect attack may have more specific insecticidal properties. Only few inducible plant lectins have been described until now, but advances in plant lectin research will undoubtedly result in the discovery of new interesting plant lectins in the near future. We believe that analysis of these proteins will reveal new sugar-binding motifs.

In the future, crop plants can be transformed to express several lectins that each show toxicity towards specific orders of pest insects. Lectins can also be combined with other proteins with known insecticidal effects like δ-endotoxins of *Bt* and even supplemented with other proteins like proteinase inhibitors or amylase inhibitors. The different classes of *Bt* endotoxins also show a wide range in variation of toxicity against lepidopteran, coleopteran and dipteran insects. In contrast, no *Bt* endotoxins are known that exert an insecticidal activity towards Hemipteran (Sharma et al. 2000). Unlike *Bt* endotoxins, several plant lectins are active against pest insects from different Hemipteran families. Bano-Maqbool et al. (2001) reported that triple transgenic rice plants expressing two Cry *Bt* proteins in combination with GNA were more resistant as compared to the binary counterparts, indicating that the simultaneous introduction of multiple resistance genes can be advantageous.

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Genetically Modified Insects as a Tool for Biorational Control

Luke Alphey, Kostas Bourtzis, and Thomas Miller

1 Introduction

Since the dawn of agriculture, pests and plant diseases have plagued crop production. The impact of pests is perhaps no better exemplified than by the desert locust, *Schistocerca gregaria*, whose image appears on stone monuments in Egypt dating around 2400 BC (Baron 1972). Similar records of plagues by other locusts appear in very early Chinese history as well (Lima 2007). The principal feature of locust outbreaks is their unpredictable nature, much like floods, drought, hurricanes and other "natural" disasters.

Not withstanding the Green Revolution in crop breeding of the 1960s, the latest breakthroughs in the history of agriculture are pesticides (ca 1940) and biotechnology (ca 1975). The former not only led to an increase in crop yield by reducing pre-harvest losses to pests, but allowed invasions to be treated and controlled. Indeed, the true value of neurotoxic insecticides is their ability to stop an insect or mite infestation immediately. This allows the grower to complete a production cycle and harvest a crop at a predictable time with a predictable yield. A dependable harvest also allows the commodity industries and government agencies to fund research and buys time for development of alternative methods of control that are more specific with fewer side effects.

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Having a predictable amount and quality of crop harvest is good for the agricultural production chain of shippers, commodity brokers, food and feed manufacturers and consumers making all of agriculture more efficient. The drawbacks of broad-spectrum insecticide use are well known. The more they are used, the greater is the chance to select pest strains that are resistant to the insecticide. Broad-spectrum insecticides can eliminate parasites and predators that help keep pest numbers low and can leave residues in the crop harvested. All of these drawbacks are related to the method of application; the wider the spray is broadcast, the less efficient the control and the greater the side effects. By contrast, the pharmaceutical industry has not suffered from the curse of inefficient application since doses are taken individually.

An insecticide that controls a larger number of pests will have a larger market; this justifies spending on research, development and registration that can be paid for from the projected potential income. An insecticide with a narrow spectrum of application that better fits the needs of environmental protection ironically has a smaller market niche and is less able to support research, development and registration costs. The biological control approach to crop protection, like narrow-spectrum insecticides, suffers from the very specificity that makes it so attractive. Also by their nature, purely biological methods are notoriously finicky and lack the power of immediate intervention that can save a crop (McFadyen 1998).

Purveyors of biopesticides have a slightly different product to offer. There are two types of biopesticide; a live organism, such as a fungus or virus that can attack an insect pest selectively and an organism that produces a toxic product, which is another form of chemical insecticide, and that is usually produced by a culture or fermentation process, often because the molecule is too complex to synthesis by an industrial process (Menn and Hall 1999). Although a biopesticide is fundamentally a biological method and therefore appears more environmentally friendly, the application method often uses equipment similar to agrichemicals with the same waste and inefficiency; and also, biopesticides are by nature narrow in spectrum of action and therefore have a small market.

The development of systemic insecticides changed the spray paradigm to one of selective application. An insecticide in the xylem fluid of a crop plant affects only xylem-feeding insects and may affect predators that feed on them.

The first direct application of biotechnology for crop protection came by inserting genes for insecticide and herbicide products in food and field crops (Menn and Hall 1999). Use of specific insecticidal precursor genes from *Bacillus thuringiensis* bacteria in corn and cotton combines the benefits of efficient delivery with the power of selective action.

The more recent types of biotechnology applied to crop protection include the use of transgenic insects with conditional lethal genes for area-wide insect suppression (Alphey 2007), symbiotic control of crop diseases and pests (Miller 2004; Miller et al. 2006) and conversion of symbionts into population suppression agents (Zabalou et al. 2004; Bourtzis and Robinson 2006). These more recent methods are reviewed in this chapter.

2 Population Suppression

Two major strategies have been described for using insect genetics to help in the control of pest insects. These are

(1) Population suppression – reducing the numbers of pests
(2) Population replacement or conversion – modifying the pest population to a less harmful form (i.e. reduced capacity to transmit one or more diseases)

Many agricultural pests cause harm directly, e.g. by larvae feeding on crops. Reducing this damage may only be achieved by reducing the number of pests. Reducing the number of transmitting insects (vectors) may also be the most attractive strategy for pests that cause harm indirectly, e.g. by transmitting diseases, but there may also be other possibilities, e.g. population replacement.

Several strategies have been proposed for population suppression through the use of modified insects. One possibility is to impose a genetic load on the population, so that deleterious genes accumulate in a population, with a severe effect on population size after some generations. One early suggestion was the use of cold-sensitive lethal genes, accumulating during the summer but leading to greatly increased mortality in the winter (Fryxell and Miller 1995; Schliekelman and Gould 2000). In colder conditions, the gene would not accumulate over time but lead to the death of each individual that inherited it (Fryxell and Miller 1995). This approach was named Autocidal Biological Control (ABC).

An alternative approach, more closely resembling the Sterile Insect Technique (SIT), involves the use of engineered lethal mutations which do not act after a multi-generation delay, but rather in the immediate progeny of released insects. This was termed the RIDL strategy (Thomas et al. 2000). In sufficiently cold weather, the cold-sensitive system of Fryxell and Miller (1995) might have a similar outcome, though temperature conditions in the field are likely to vary; the RIDL strategy might therefore be considered an advance within the general ABC concept. In this approach, pest insects homozygous for an engineered dominant lethal would be reared in large numbers and released to mate with the wild pest population. As in classical SIT, the released insects would compete for mates with wild insects; all progeny resulting from a wild insect mating a RIDL insect would die – in SIT because they have inherited radiation-induced lethal mutations from their irradiated parent; in RIDL because they have inherited one copy of the RIDL gene system from their RIDL parent. This type of system is known as ‘genetic sterilization.’

One complication for the actual construction of a RIDL strain is that the lethality of the RIDL gene system needs to be repressible so that, under defined conditions, the gene system does not exert its lethal effect. This is necessary in order that the strain can be reared in the laboratory. Suitable molecular systems have been devised, based on the ‘tet-off’ gene expression system (Gossen and Bujard 1992) and have been shown to work in several major insect pests including Mediterranean

fruit fly, Mexican fruit fly, the yellow fever mosquito *Aedes aegypti*, and pink bollworm (Gong et al. 2005; Fu et al. 2007; Phuc et al. 2007). Repression of the RIDL system, and hence survival of the insects, is then achieved simply by adding a suitable 'antidote' chemical to the larval diet.

Another version of RIDL has been developed in which the lethal effect is female-specific. Males carrying the RIDL gene system are unaffected, even if raised without the antidote. This system has some potential advantages. One is 'genetic sexing', allowing automated elimination of females to give a male-only population for release. For many species, it would be preferable to release only males. For example, only female mosquitoes bite – and therefore potentially transmit disease – not males. Therefore it would be preferable in the context of a control program to release only male mosquitoes, and not female ones. This issue, damage caused by adult females, also applies to some agricultural pests. For example, oviposition by female tephritid fruit flies tends to damage fruit. Furthermore, large-scale field tests with the Mediterranean fruit fly have shown that males are three- to fivefold more effective if released without females – it is inferred that sterile females 'distract' the sterile males, who tend to court and mate such co-released females, rather than seeking out wild females (Rendón et al. 2004). Some species have sufficient sexual dimorphism to allow rapid or automated separation of males from females, but many important pest species do not. Genetic sexing systems introduce such a sexual dimorphism. Several such systems have been developed using classical genetics, so the benefit is well established. However, classical systems depend on the use of special mutations and chromosome translocations that cannot be transferred from one species to another, so development starts from scratch in each new species and likely takes many years.

Other potential genetic improvements to SIT include the provision of a genetic marker. At present, sterile insects are marked, e.g. with a colored dye or fluorescent powder, in order to allow recaptured steriles to be distinguished from wild type – pest – insects. However, such markers have to be applied to every insect, and the dye may not be uniformly detectable, or recognizable in damaged specimens. In contrast, a heritable genetic marker, such as a gene encoding a fluorescent protein (Tsien 1998; Shagin et al. 2004), is present in every released insect and does not need to be applied (Handler and Harrell 2001; Horn et al. 2002; Gong et al. 2005). Furthermore, molecular methods, e.g. PCR, allow detection of the marker even in highly damaged specimens.

RIDL constructs and marker genes have been successfully introduced into several major pest species. These include the Mediterranean fruit fly *Ceratitis capitata*, Mexican fruit fly *Anastrepha ludens*, the dengue vector mosquito *A. aegypti*, *Aedes albopictus* and pink bollworm (*Pectinophora gossypiella*) (Gong et al. 2005; Fu et al. 2007; Phuc et al. 2007; Simmons et al. 2007 and Oxitec, unpublished, 2007). Multiple strains have been tested in confined field conditions; at time of writing one strain of pink bollworm (*P. gossypiella*) is in the third year of open field releases. Regulatory authorization to conduct these open field releases was preceded by a public Environmental Assessment (EA) and Finding of No Significant Impact (FONSI).

3 Population Replacement

This strategy is usually discussed in terms of mosquitoes and human disease, but could in principle also be applied to suitable vectors of plant or livestock diseases.

This strategy has two essential steps:

(1) Construct (in the laboratory) a modified strain of a disease vector carrying a gene or genetic system that renders it unable to transmit a specific pathogen, e.g. malaria or dengue. This gene or genetic system is termed the 'refractory' or 'refractoriness' gene, as it makes the insect refractory to transmission of the pathogen.
(2) Spread this gene or genetic system through a wild population to sufficient allele frequency and for a sufficient period of time to reduce or prevent pathogen transmission and human disease. Since the refractoriness gene likely imposes a fitness penalty on the insect, the gene will not spread of its own accord but rather needs an additional genetic system, a so-called 'gene drive system' or 'gene driver'.

Each step poses considerable technical problems. Some progress has been made, but fully-functional, deployable systems remain at least some years away. Regulatory and political issues around such self-propagating systems are also more challenging than for sterile-release methods. Several features of gene drive systems that may be seen as new and potentially concerning include:

- Population replacement systems aim to establish permanent, stable transgenic populations in the wild.
- Since the intention is generally to drive the transgene as close as possible to fixation, the original, non-transgenic population will essentially be eliminated.
- Gene drive systems have self-spreading, self-propagating characteristics that are unlikely to respect national boundaries.
- Though some form of reversal of spread may be possible for some gene drive systems, elimination of the transgene from the wild population by such means would likely take very many generations.

4 Refractoriness Genes

Proof-of-principle for malaria has been shown using rodent and chicken malaria models (Capurro et al. 2000; Ito et al. 2002; Moreira et al. 2002). It has also become clear that the mosquito mounts an immune response against pathogens such as *Plasmodium*. It is possible that manipulation of the mosquito immune system may strengthen this response sufficiently to confer refractoriness to mosquitoes that would otherwise be competent vectors (Shin et al. 2003; Nene et al. 2007). One potential problem with this strategy is evolution of resistance in the pathogen population to the refractoriness gene. Where the refractoriness gene has a single mode

or point of action, for example a single-chain antibody, it is likely that multiple effectors will need to be used simultaneously to reduce the risk of failure due to resistance. One proposed strategy that seems less prone to this risk is the use of long hairpin RNA molecules to confer refractoriness to viruses, especially RNA viruses such as dengue. Proof-of-principle has been achieved for one of the dengue viruses, DEN-2 (Franz et al. 2006). Because these molecules can target long essential regions of the virus genome, it is difficult to see how simple mutations in the virus can evade this form of refractoriness.

One interesting related system is engineered baculovirus resistance in the silkworm *Bombyx mori*. The BmNPV baculovirus is a serious pathogen of silkworm, causing major losses in sericulture. Engineered strains showing partial resistance have been produced (Kanginakudru et al. 2007). Silkworm is a farmed insect, so the question of driving the gene through a wild population does not arise, and no gene driver would be required. Deployment of this system may therefore be possible earlier than for refractoriness genes in human disease vectors. This system is in these respects more similar to engineered resistance to plant pathogens, for example resistance to papaya ringspot virus in engineered papaya (Fitch et al. 1992; Tripathi et al. 2006).

5 Gene Drive Systems

Several potential gene drive systems have been proposed. Essentially, these are selfish DNA systems, capable of spreading themselves through a target population through non-Mendelian inheritance, despite not conferring a direct fitness advantage (Gould and Schliekelman 2004; Sinkins and Gould 2006). Examples include transposons, Medea (Beeman et al. 1992; Salghetti et al. 2001), underdominance (Davis et al. 2001) and homing endonucleases (Burt 2003). Proof-of-principle has been achieved in *Drosophila melanogaster* for a small number of synthetic gene drive systems, but none yet in a pest insect, or in a wild population. However, most are based on a naturally-occurring selfish DNA system, which provides encouragement that these genetic systems can indeed spread themselves through wild populations under at least some circumstances; the exact circumstances can be further elucidated by mathematical modeling.

Environmental use of engineered gene drive systems would mean the deliberate release of a transgene designed and intended to spread and establish in the wild. This would be a considerable step further than any previous release of an LMO (live modified organism). There has consequently been considerable discussion about the desirable or acceptable characteristics of such a drive system (e.g. Braig and Yan 2001); no clear consensus has emerged. One specific technical issue is the problem of linkage – how to ensure that the refractoriness gene (“cargo”) remains associated with the gene driver as the latter spreads through the target population. If this linkage between cargo and driver breaks down, the driver may continue to spread through the target population, but without leading to the intended outcome of refractoriness (Curtis et al. 2006).

6 *Wolbachia*

Wolbachia pipientis (*Wolbachia* for the purpose of this chapter) is a group of obligatory intracellular and maternally transmitted bacteria (Werren 1997; Stouthamer et al. 1999). Phylogenetic studies based on 16S rDNA gene sequences indicated that these bacteria belong to the alpha-2 subdivision of the Proteobacteria (Breeuwer et al. 1992; O'Neill et al. 1992; Rousset et al. 1992). Close relatives of *Wolbachia* include intracellular bacteria of the genera *Ehrlichia*, *Anaplasma*, *Rickettsia* and *Cowdria* (O'Neill et al. 1992). More advanced phylogenomic studies have suggested that *Wolbachia* isolates can be classified into eight major clades (A-H), which have been named 'supergroups' (Lo et al. 2002; Rowley et al. 2004; Bordenstein and Rosengaus 2005). The development of "Multi Locus Sequencing Typing" (MLST) systems has allowed the genotyping of any *Wolbachia* strain (Baldo et al. 2006; Paraskevopoulos et al. 2006).

As yet, the distribution of these bacteria appears to be restricted to invertebrate species. In nematodes, *Wolbachia* have only been detected in filarial worms, where they establish a mutualistic association (Bandi et al. 1998), while these bacteria are widespread and abundant among arthropod species (Werren and O'Neill 1997; Bourtzis and Miller 2003, 2006). They have been so far detected in all major orders of insects, mites, isopods, scorpions, spiders and springtails (Werren et al. 1995; Jeyaprakash and Hoy 2000; Werren and Winsdor 2000; Lo et al. 2002; Rowley et al. 2004; Bordenstein and Rosengaus 2005). Several experimental and meta-analysis studies suggest that our planet has faced a *Wolbachia* pandemic since millions of insect species may be infected, rendering *Wolbachia* the most ubiquitous endosymbiotic organism on Earth (Werren et al. 1995; Jeyaprakash and Hoy 2000; Werren and Winsdor 2000; Hilgenboecker et al. 2008). Despite the widespread distribution of these endosymbiotic bacteria, many species of agricultural importance (e.g. the olive fly *Bactrocera oleae*), medical importance (e.g. the malarial vector *Anopheles gambiae*) and environmental importance (e.g. *Dendroctonus* spp.) are not infected with *Wolbachia*.

Although *Wolbachia* infections have been described in somatic tissues, these bacteria mainly reside within the reproductive tissues and organs of their insect hosts. Interestingly, *Wolbachia* have developed sophisticated mechanisms for the manipulation of host reproductive properties. Their presence in gonadal tissues and organs has been associated with the induction of reproductive alterations, namely feminization, parthenogenesis, male killing and cytoplasmic incompatibility (Stouthamer et al. 1999). All of the above phenotypes help *Wolbachia* to spread in nature since they favor the prevalence of *Wolbachia*-infected females. In all cases, normal reproduction can be restored by treating the infected hosts with antibiotics such as tetracycline. In the present chapter, we will focus on the mechanism of cytoplasmic incompatibility and how it can be used for applied purposes. The other *Wolbachia*-induced reproductive alterations have been reviewed elsewhere (O'Neill et al. 1997; Werren et al. 1997; Bourtzis and O'Neill 1998; Stouthamer et al. 1999; Bourtzis and Miller 2003, 2006).

7 *Wolbachia* and Cytoplasmic Incompatibility

Cytoplasmic incompatibility (CI) is a biological phenomenon that facilitates the spreading of the cytoplasmic factors inducing it. This is usually achieved by reducing (killing) the number of progeny that do not carry these factors (Bourtzis et al. 2003).

Cytoplasmic incompatibility is the most common and widespread reproductive alteration that *Wolbachia* endosymbionts induce in their arthropod host species (Hoffman and Turelli 1997; Bourtzis et al. 2003). Cytoplasmic incompatibility can be either unidirectional or bi-directional and is expressed as early embryonic mortality in diplo-diploid insect host species. Unidirectional cytoplasmic incompatibility is expressed when an infected male is crossed with a female of different infection status. The reciprocal cross is fully compatible, as are crosses between individuals infected with the same *Wolbachia* strain(s). Bidirectional cytoplasmic incompatibility is expressed in matings between individuals infected with different strains of *Wolbachia*. Cytoplasmic incompatibility favors the prevalence of infected females and consequently, *Wolbachia* infections are able to spread in nature by replacing populations of different *Wolbachia* infection status (Hoffman and Turelli 1997; Bourtzis and O'Neill 1998).

Despite the fact that the molecular mechanism of *Wolbachia*-induced cytoplasmic incompatibility is still unknown, research studies have conclusively documented the presence of *Wolbachia*-induced cytoplasmic incompatibility phenomena in insect species belonging to the orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera and Lepidoptera (Werren and O'Neill 1997; Bourtzis and O'Neill 1998; Tram et al. 2002; Bourtzis et al. 2003; Poinsot et al. 2003; Tram and Sullivan 2003). The expression levels of cytoplasmic incompatibility depend on the respective host–*Wolbachia* strain symbiotic association (Bourtzis et al. 2003). It has been shown that the bacterial density, particularly in the sperm cysts, is positively correlated with the penetrance of the cytoplasmic incompatibility phenotype (Boyle et al. 1993; Breeuwer and Werren 1993; Giordano et al. 1995; Mercot et al. 1995; Bourtzis et al. 1996, 1998; Poinsot et al. 1998; Rousset et al. 1999; Veneti et al. 2003). Factors affecting the expression of cytoplasmic incompatibility does not only depend on the genotype of a given *Wolbachia* strain but may also include the host nuclear genome, male age, repeated copulation of males, temperature, antibiotics and nutrition (Singh et al. 1976; Hoffmann et al. 1986; Stevens 1989; Boyle et al. 1993; Sinkins et al. 1995; Hoffmann 1988;1998 Clancy and Hoffmann 1998; Karr et al. ; Poinsot et al. 1998; Jamnogluk et al. 2000; Reynolds and Hoffmann 2002; Kittayapong et al. 2002; Reynolds et al. 2003; Champion de Crespigny and Wedell 2006; Zabalou et al. 2008). In addition, Bordenstein and colleagues suggested that the temperate bacteriophage WO-B of *Wolbachia* may also influence the expression levels of cytoplasmic incompatibility (Bordenstein et al. 2006). The WO-B phage can be either a lytic or a lysogenic status. Bordenstein and colleagues documented lytic phage development at a significant frequency in host–Wolbachia symbiotic associations suggesting an overall negative density relationship between bacteriophage and *Wolbachia*: reduced Wolbachia infection levels means reduced expression of cytoplasmic incompatibility (Bordenstein et al. 2006).

8 *Wolbachia* as a Tool for the Control of Insect Pests and Disease Vectors

Wolbachia endosymbionts can be used beneficially in many ways. For example, it has been proposed that *Wolbachia* infections can be used for the development of environmentally friendly strategies for the population control of insect pests and disease vectors (Beard et al. 1993; Sinkins et al. 1997; Ashburner et al. 1998; Bourtzis and O'Neill 1998; Bourtzis and Braig 1999; Sinkins and O'Neill 2000; Aksoy et al. 2001). Significant progress has been achieved in this field during the last years since research studies have shown that *Wolbachia*-induced cytoplasmic incompatibility can be used either as a tool for insect pest population control in a way analogous to the "Sterile Insect Technique" (SIT), namely the "Incompatible Insect Technique" (IIT) or as a drive mechanism to spread desirable genotypes in insect populations (Zabalou et al. 2004; Xi et al. 2005).

Zabalou and colleagues used embryonic cytoplasmic injections to transinfect *C. capitata*, the Mediterranean fruit fly, a major agricultural pest (Zabalou et al. 2004). Naturally *Wolbachia*-infected populations of *Rhagoletis cerasi*, the cherry fruit fly, were used as donors (Riegler and Stauffer 2002; Riegler et al. 2004). Two transinfected lines were established, namely WolMed 88.6 (singly infected with *w*Cer2) and WolMed S10.3 (singly infected with *w*Cer4), which transmission rates of 100% for over 70 generations. Crosses between uninfected females and *Wolbachia*-infected males from both transinfected lines resulted in 100% egg mortality, while the reciprocal crosses resulted in between 16% to 32% egg mortality. In addition, crosses between the two transinfected Mediterranean fruit fly lines WolMed 88.6 and WolMed also resulted in 100% egg mortality (Zabalou et al. 2004). Taken together, these data suggested that transinfected lines of *C. capitata* can express complete unidirectional and bidirectional cytoplasmic incompatibility. Next, Zabalou and colleagues performed laboratory cage population experiments in attempt to see whether *Wolbachia*-infected incompatible medfly males can be used in population suppression strategies. They set up laboratory cage populations using different ratios of incompatible males: uninfected males: uninfected females. These results clearly indicated that these populations were suppressed by these single "releases" of incompatible males in a ratio-dependent manner, reaching levels higher than 99% at "release" ratios of 50:1 (Zabalou et al. 2004).

Xi and colleagues performed a similar transinfection experiment in mosquitoes transferring the *Wolbachia* strain *w*AlbB from its natural host *Aedes albopictus* to a naïve host, *A. aegypti* (Xi et al. 2005). Crosses between uninfected females and *Wolbachia*-infected males resulted in 100% egg mortality suggesting complete expression of cytoplasmic incompatibility. Xi and colleagues set up laboratory cage population experiments and showed that *Wolbachia* infections can spread and replace uninfected *A. aegypti* population within seven generations (Xi et al. 2005). These results clearly indicated that *Wolbachia* is a promising tool for the development of novel strategies for driving desirable genotypes into natural populations of disease vectors.

Sinkins and O'Neill suggested that virulent strains of *Wolbachia* strains hold great promise as potential tools to control disease transmission by insect vector species (Sinkins and O'Neill 2000). Virulent *Wolbachia* strains, such as the "popcorn" strain, over-proliferate in the nervous system thus reducing the life span of their hosts (Min and Benzer 1997). Sinkins and O'Neill proposed that if the popcorn strain is transferred to *Anopheles* or *Aedes* species, the mosquito's life span may be significantly reduced ultimately resulting in the prevention of disease transmission (because only older mosquitoes transfer the pathogen in new hosts) with little effect on the biological (reproductive) life cycle of the insect vector species (Sinkins and O'Neill 2000).

9 Regulatory Aspects of Transgenic Insects

In the late 1980s Robert Staten, then Center Director of a USDA-APHIS (United States Department of Agriculture-Animal Plant Heath Inspection Service) laboratory in Phoenix, Arizona, called for the development of a transgenic pink bollworm, *P. gossypiella*, to supplement or replace sterile insects produced by radiation. The APHIS lab was the site of a mass-rearing facility for the pink bollworm sterile insect program (SIT) operated by APHIS and CDFA (California Department of Food and Agriculture) and the California Cotton Pest Control Board that supplied the bulk of the operational funding.

The need was very straight-forward; gamma radiation produces sterilization of the target insect in SIT programs, but has side-effects resulting in fitness costs (see for example, Bartlett and Lewis 1973). Developing a strain of pink bollworm with a conditional lethal gene would allow outcrossing and then back-crossing for possible fitness improvement, much like mass-reared insects are periodically improved by outcrossing with wild type insects (see for example, Franz 2002).

The first regulatory activity for the resulting transgenic pink bollworm was a permit to move the strain from University of California, Riverside, to Phoenix in 1998 (see Miller 2004; Miller et al. 2008). This was followed by successful permits to test competitiveness between the transgenic pink bollworm and laboratory strains from the Phoenix mass-rearing facilities. The key regulatory authority for transgenic pink bollworm is USDA Biotechnology Regulatory Services (BRS).

The requests for releases for field tests were accompanied by considerable publicity starting with an article on the front page of the Wall Street Journal by Scott Kilman, an agricultural reporter that appeared January 29, 2001. Some time later, the Pew Foundation did a study (Anonymous 2004) of the process for regulation of transgenic insects, which evoked further media commentary and attention.

Some of the resulting publicity was described in Miller (2004). After 2004 the publicity subsided and more permits were issued for transgenic pink bollworm, but only for the genetically marked insects, not for the intended strain with conditional lethal genes (Klassen et al. 1970; Fryxell and Miller 1995; Thomas et al. 2000) for use in a modification of the classic sterile insect technique (Staten et al. 1992;

Vreysen et al. 2007). However, a permit was issued for caged field testing of transgenic pink bollworms expressing conditional lethal genes in 2005. This strain has not yet been taken to open field release. In addition, permits were issued for open field releases of marked transgenic pink bollworm at half-radiation doses (G. Simmons, 2008, personal communication).

Many tests were done comparing the transgenic pink bollworm with lab reared strains by themselves and with radiation doses. Many more strains were produced as well with plasmids supplied by Oxitec, Oxford, UK. Some of these transformation experiments produced strains that were attracted to pheromone traps in larger percentages that irradiated lab strains (Table 1). When translated to field application, the implication is that these recombinant strains will be more competitive with wild types that irradiated strains.

Table 1 shows recapture rates of an EGFP transgenic strain in 2003 were 25% lower than the lab-reared strain, but when out-crossed and back-crossed (EGFP-OC) the difference dropped to 9% (methods described in Miller et al. 1984). One transgenic strain (DsRed S10) irradiated at 10 krad gamma radiation dose (^{60}Co source) was recaptured at significantly higher rates than the lab-reared strain with 20 krad radiation dose. These data clearly show quality improvement in competitiveness of transgenic strains and the value of outcrossing.

Table 1 Male adult pink bollworm, *Pectinophora gossypiella*, response to pheromone trap 24 h after release; various strains

Strain Type	Irad. Dose (krad)	Total Moths Released	% Recaptured	% Reduction from Control
			2002	
APHIS	20	300	22.7[a]	
EGFP	20	300	15.0[b]	33.9
			2003	
APHIS	20	796	41.3 a	
EGFP	20	797	30.9 bc	25.2
EGFP-OC	20	796	37.5 ac	9.2
			2004	
APHIS	20	540	45.0 a	
EGFP	20	540	33.5 bc	25.6
EGFP-OC2	10	540	39.0 ac	13.3
			2005	
APHIS	20	500	30.0 a	
DsRed S10	10	500	42.2 b	+28.9
EGFP-OC2	10	500	21.8 c	27.3

Taken from E. D. Miller et al., unpublished, 2005.
Numbers in same column (within each year of study) followed by the same letter are not significantly different >0.05: Tukey mean separation test. APHIS – lab-reared strain; EGFP, transgenic strain with green fluorescent protein gene marker; DsRed, transgenic strain with DsRed fluorescent protein gene; OC, out-crossed (and back-crossed) transgenic strains.

Further experiments, including cage and open field releases, have clearly demonstrated that strains of transgenic pink bollworm can be developed that possess valuable new traits (e.g. genetic marker, genetic sterility) without compromising other aspects of strain quality. Each significant step towards field use, release into field cages and open field releases, has been preceded by an Environmental Assessment (EA); in each case this led to a Finding of No Significant Impact (FONSI) for the proposed activity and the permit was granted.

A comprehensive Environmental Impact Statement (EIS) is being prepared by USDA, to examine the planned or potential use of genetic markers and autocidal control methods in their tephritid fruit fly and pink bollworm control programs. Following an extensive scoping period in 2007, during which public comment was solicited by internet, direct contact with interested parties, and a series of public meetings, a draft EIS was published in May 2008; further public comment on this draft was invited over a 45 day period. At time of writing, this period has finished, the comments are being considered and a final EIS prepared; publication is expected before the end of 2008.

10 Regulatory Aspects of Biopesticides

Regulatory activity surrounding symbiotic control approaches have met similar delays from the regulatory process; in this instance the key regulatory authority is the US Environmental Protection Agency (EPA). However, there is also resistance to the acceptance of these methods from scientific colleagues. For example, an article (Wang and St. Leger 2007) on efficacy of the *Metarhizium anisopliae* insecticidal fungus with a venom toxin gene inserted from scorpion, *Androctonus australis* was submitted to Nature Biotechnology. The original title of the paper included the word virulence (as increase virulence of the fungus that is selectively active on grasshoppers and locusts). This word alone raised concerns in the editor, Peter Hare who sent the manuscript out for a second "ethical" review resulting in the word virulence being changed to potency in the title. Hare mentioned a concern about giving the appearance of publishing irresponsible research in his journal.

This attitude on the part of the editor, who has the courage to bring it up in public, is exactly the point. Why would a fungal biopesticide that is selective for a narrow spectrum of grasshoppers be irresponsible when made more rapid acting by the addition of a scorpion toxin, which is itself also selective for insects? The public commentary on the paper (Thomas and Read 2007) that appeared in the same issue of Nature Biotechnology was largely positive ("… pave the way for innovative control strategies …"), but also cautionary ("… Analogous research to increase the virulence of insect viruses … has yet to be translated into practice, largely owing to commercial and political issues rather than technical limitations. Social resistance to genetically modified fungi may prove a bigger hurdle than the emergence of biological resistance.")

The theme of social acceptance was picked up in a later commentary article in the magazine of the American Society of Microbiology (Dixon 2008) who called

the work "… amply justified and entirely safe, yet which can sound dangerous, reckless and irresponsible?" Dixon went on to review the recombinant baculovirus work alluded to by Thomas and Read (2007) in their commentary. He implied that David Bishop was fired from his post as Director of the Institute of Virology in Oxford, UK because of a public outcry stemming from the publicity of the work.

In the application of symbiotic control of Pierce's disease in California, we were told by grape and wine industry representatives that a transgenic grapevine was unacceptable as a solution. Although this sounds like public wariness of recombinant methods, it has two aspects; one is the publicity could ruin the market for grape or wine and the other is the organic grower industry. One of the rules of certification of organic produce is restriction on recombinant organisms.

According to 7 CFR 205 (the organic standards) all genetically modified organisms are banned with the exception of recombinant vaccines in organic livestock production provided they have been approved according to CFR 205.600a – which then lists criteria, but in essence has to comply with the following three items:

(i) Would not be harmful to human health or the environment
(ii) Is necessary to the production or handling of the agricultural product because of the unavailability of wholly natural substitute products
(iii) Is consistent with organic farming and handling

Consequently it is rather unlikely that recombinant vaccines will be used in organic livestock production, although the door remains ajar.

Strategies from crop pest and disease control based on recombinant methods will be accepted when they prove their value and safety. If recombinant biotechnology offers significant improvement over existing methods, consumer demand may increase. It is in the public interest to allow testing to determine the ultimate value.

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Symbiosis Research as a Novel Strategy for Insect Pest Control

Alistair C. Darby

1 Good Insect Control Strategies

Insects cause problems in two ways they either physically damage or transmit disease. In both cases killing insects can be a very effective way of stopping the problem, but different strategies can be deployed to effect control. In some cases, the control of the insect problem needs to be immediate kill or "knock-down", but often more subtle methods are more advantageous in controlling the problem, i.e. decreasing longevity or modifying behaviour. Good pest management strategies seek to identify and exploit the vulnerable points of the insect's lifecycle or the disease they vector. This means that all control measures must be built on a solid understanding of the pest's biology and the economics of the problems they cause. This chapter will explore how microbial symbionts could be utilised in pest management. Although symbiont research may not provide a "silver bullet" it will provide important contributions to aid the control of insect pests within an integrated framework.

The potential of symbionts for pest control stems from two facts: (1) insect microbe interactions underpin a significant number of pest species biology and (2) these interactions are diverse in character and taxonomically varied.

2 Host–Microbe Interactions

Microbial associations with invertebrates are common, widespread and show large variation in their community diversity, fitness impacts/effects and routes of transmission (Buchner 1965; Dillon and Dillon 2004; Douglas 1994; O'Neill et al.

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1997). These characteristics are interlinked and fundamental to our understanding of host-microbe associations as these are the qualities that shape the ecology of microorganisms in association with their hosts. However, most of this diversity is not amenable to culture in the laboratory making the study and manipulation of these relationships difficult (Darby and Welburn 2005). The use of molecular techniques to identify non-culture viable microorganisms has allowed research to accurately classify organisms previously only described by drawings and micrographs (Buchner 1965). The ability to name and identify these bacteria has started to uncover a fascinating diversity of organisms that have a long and complex evolutionary history with their insect partners (Dale and Moran 2006; Ruby 2008).

An evolutionary perspective on microbial host interactions has aided our understanding of how important microorganisms are to insect biology. The ubiquitous presence of particular bacterial in association with an insect genus or group indicates that the bacteria may have coevolved with the insect ancestors for millions of years. The occurrence of very closely related bacteria in unrelated or distantly related host species may indicate that the bacterium is frequently transmitted between species and good coloniser of insects. This evolutionary perspective is very powerful, but it cannot tell us the details of the current relationship that a microbe has with its host. Pathogens (harmful or deleterious to the insect host), commensals (neutral to the host) and mutualists (beneficial to the host) all show parallel patterns of evolutionary association with their hosts so experimental work is often needed to determine the precise nature for the relationship (Bordenstein et al. 2009). In practice some microbes may possess different associations depending on the physiological state of the host. Importantly, an evolutionary overview can highlight long term and persistent relationships; but it is likely that a number of microbial relationships with the host are transient and/or opportunistic. Therefore, to understand the relationship and the role of these micro-organisms in the host we must study the impact they have on the insect's performance and fitness.

3 Symbiotic Relationships

Symbiont in this chapter is used to mean any microorganism that is in a persistent relationship with an insect "host", with no preconceived notion as to whether the relationship is mutualistic, commensal or pathogenic to the insect. Symbiotic taxon may display complex host relations that switches between being costly or valuable to the hosts (Clay 1988). The extremes of microbial relationships mutualistic (benefit) and pathogenic (costly) might seem to be mutually exclusive, but the similarities between mutualistic and pathogenic lifestyles have been the subject of many reviews (Dale and Moran 2006; Goebel and Gross 2001; Hacker and Kaper 2000; Hentschel et al. 2000; Herre et al. 1999; Ruby 2008)(see Section 5). The interaction between microbes and animals are often complex and the impact of microorganisms on the host can vary with both host factors (e.g. species, genotype and physiological condition) and environmental factor (Clay 1988).

This means that terms such as pathogenic and beneficial are relative terms depending on the host and context.

3.1 A Pathogenic Mutualist

A good example of how complex host microbe relationships can be is *Photorhabdus luminescens* (Enterobacteriaceae, gamma-proteobacteria), which is a pathogen of insects, but a mutualistic to entomopathogenic nematodes of the family Heterorhabditidae (Clarke 2008). In this relationship, the nematode carries the bacteria in its gut between insect hosts. The bacteria are released from the nematode's gut killing the insect. The growing population of bacteria then becomes the food source for the next generation of nematodes. Therefore, the same isolate of *P. luminescens* is both pathogenic and beneficial depending on the host species (Clarke 2008).

3.2 Friendly Bacteria

Many bacteria have co-evolved with insects to be exclusively mutualistic, typically these bacteria have long evolutionary histories with their host insect, e.g. *Buchnera* species in aphids (Tamas et al. 2002) and *Wigglesworthia* species in tsetse flies (Chen et al. 1999). In these relationships the symbiotic bacteria provides nutritional elements that are absent from the insects diet and the bacteria is essential to the fitness and reproductive success of the insect. This type of specialised mutalistic relationship is very important to insect performance, but the more common situation is likely to be that the insect's microbiota is generally commensal showing transient positive and negative effects of the insect's performance. One example is the mutual relationship of the desert locust with its resident gut microbiota. The beneficial effect of the microbiota is only evident either during periods of starvation or during the exhibition of certain behavioural traits by the insect host (reviewed in Dillon and Dillon 2004). These relationships can be very stable over evolutionary time, but can equally be short lived and ephemeral. This diversity of microbial interactions with hosts makes defining relationships between an insect and its microbiota difficult, as the outcome of the interactions may vary with circumstance and the length of their evolutionary association. However, we can define some types of symbiotic relationships quite precisely based on their evolutionary biology. Symbiont diversity can be summarised into three broad classes (Dale and Moran 2006): (1) the Mycetome or bacteriome-associated obligate symbionts (<10 million years); which have an ancient codiversification of host and symbiont, with no symbiont transmission between lineages. (2) Ancient reproductive parasites, which show evidence of ancient co-evolution with host, but occasional horizontal transfer between lineages. (3) Facultative symbionts, which show occasionally horizontally

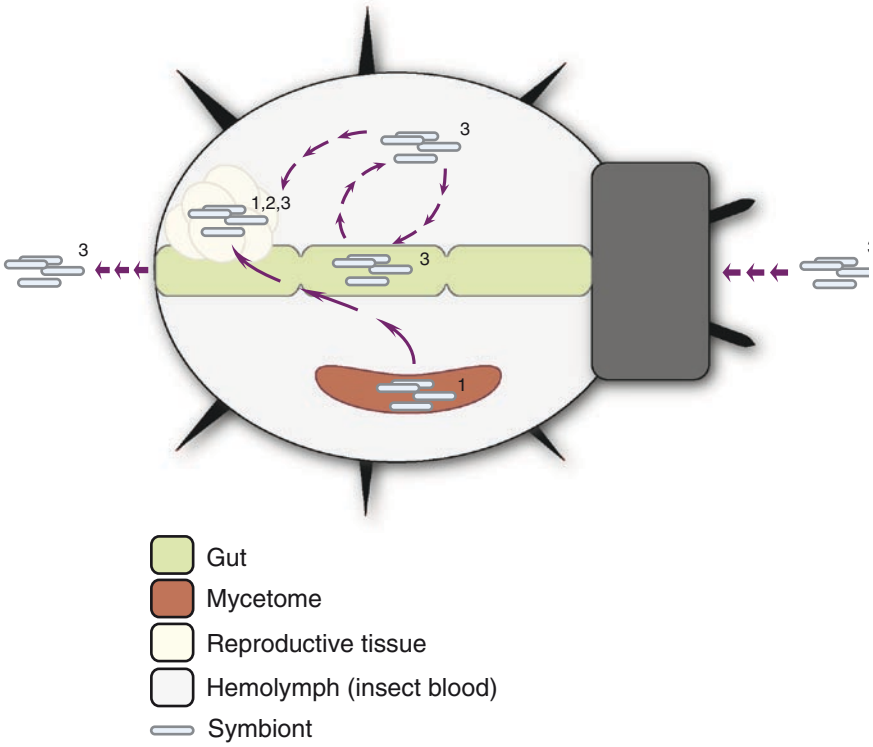

Fig. 1 A schematic representation of insect symbiosis showing the general relationship between symbiont evolutionary history and the site of symbiosis (tissue tropisms). (1) Ancient mycetce symbiont. (2) Ancient reproductive symbiont. (3) Recent facultative symbiont. *Arrows* indicate possible transmission routes for the symbiont

transferred, relatively resent origin of symbiosis (~2 million years) and clusters of closely related taxa across multiple hosts (Darby et al. 2001; Sandstrom et al. 2001). These different classes of symbiotic relationship lend themselves to different distinct and targeted applications, due to their importance to the insect biology, transmissibility and mode of interaction with the insect (Fig. 1).

4 Symbionts as Novel Targets – Mycetome-Associated Obligate Symbiont

The 'mycetocyte or bacteriosome symbioses' are a class of highly specialised symbionts that are required by the insect and often obligately vertically transmitted via the insect ovary. These symbioses are defined by the age of the association and location of the symbiotic microorganisms in insect cells, which have the sole

function of housing microorganisms (Buchner 1965; Douglas 1994). This class of symbiosis is universal in several insect groups, including various pest taxa, and involves a broad diversity of microorganisms, which includes bacteria and yeasts (Table 1).

It is apparent that mycetocyte structures have evolved multiple times in anatomically diverse locations in an insect's body (e.g. haemocoel, fat body and epithelium of gut) (Buchner 1965; Douglas 1989; Moran and Telang 1998). Mycetocyte symbioses have a number of common traits:

1. The microorganisms are housed within the mycetocyte cell, lying free in the cell cytoplasm or enclosed by an extra insect membrane.
2. The microorganisms are vertically transmitted, from mother to offspring, usually by transfer process known as transovarial transmission.
3. Both the insect and microorganism are dependant on each other. Bacterial mycetocyte symbionts have small reduced genomes and lack many genes needed for replication outside the mycetocyte (Akman et al. 2002; Degnan et al. 2005; Tamas et al. 2001).
4. Insects experimentally cured of their mycetocyte symbionts (e.g. by antibiotic treatment) grow poorly and are generally reproductively sterile (Douglas 1992).
5. These symbioses occur predominantly in insects feeding on nutritionally poor diets, for example vertebrate blood (deficient in B vitamins), plant sap (deficient in essential amino acids) and wood (deficient in vitamins, amino acids and sterols). The microbial symbionts synthesise and release these nutrients to the insect, and thus represent an essential supplementary source for these growth limiting nutrients. Therefore insects deprived of their symbionts will be unable to access an alternative supply of nutrients and will have their ability to reproduce severely impeded.

This last point suggests that symbiosis are an 'Achilles heel' to many pest species that live on blood, plant sap and wood (Table 1). The close link between symbiont and host biology makes this type of symbiosis ideal as a target for insect control. This target would not provide a rapid "knockdown" control measure, as insects without mycetocyte symbionts can often survive, but they will not be able to reproduce rapidly. Importantly the phylogenetic distance between bacteria and non-target organisms (e.g. humans, livestock and other insects) reduces the possibility of toxic side effects to non-target species.

4.1 Symbiont Based Control of Filarial Worms

Targeted killing or blocking of symbiont transmission is still a theoretical method for insect control, but symbionts have already been targeted by clinicians for a novel treatment for river blindness. The causal agent of river blindness is *Onchocerca volvulus* a parasitic worm with an obligate *Wolbachia* symbiont. These *Wolbachia* are phylogentically distinct from insect *Wolbachia* (Casiraghi et al. 2004)

Table 1 Arthropod pests and their symbionts (Adapted from (Douglas 2007))

Host arthopod	Pest status	Mycetome symbiont	Non mycetome symbiont	References
(a) Plant sap feeders				
Aphids[a]	All Homoptera are good vectors for plant disease and in some cases they can directly damage the crop	*Buchnera aphidicola* (γ-proteobacteria) or pyrenomycete fungi	A number of proteobacteria	(Munson et al. 1991a; Suh et al. 2001)
Auchenorrhyncha (including leafhoppers, plant hoppers, cicadas)		*Baumannia cicadellinicola* (γ-proteobacteria) and Sulcia muelleri (Bacteroidetes); Pyrenomycete fungi in some planthoppers	A number of proteobacteria+	(Suh et al. 2001; Wu et al. 2006)
Whitefly		*Portiera aleyrodidarum* (γ-proteobacteria)	A number of proteobacteria+	(Thao and Baumann 2004)
Psyllids (jumping lice)		*Carsonella ruddii* (γ-proteobacteria)	A number of proteobacteria+	(Thao et al. 2000)
Scale insects (and mealy bugs)		*Tremblaya princeps* (β-proteobacteria)	A number of proteobacteria+	(Baumann et al. 2002)
(b) Plant-cellose feeders				
Termites	Damage to crops and timber	Not present	Complex gut microbiota madeup of intracellular symbionts of various cellulolytic protists	(Ikeda-Ohtsubo et al. 2007)
Locusts	Large scale damage to crops	Not present	Complex gut microbiota	(Dillon and Charnley 2002)
Coleoptera (beetles)				
Anobiid timber beetles	Damage timber	Symbiotaphrina (yeasts)		(Noda and Koizumi 2003)
Weevils	Damage to crops and timber	Various γ-proteobacteria (including Nardonella)		(Lefevre et al. 2004)
(c) Vertebrate blood				
Cimicids (bedbugs)	Vectors of human disease	Present but unknown	*Wolbachia* and γ-proteobacteria described	(Hypsa and Aksoy 1997) (Buchner 1965)

Triatomine bugs	Vector for American trypanosomes	Present but unknown	Rhodococcus rhodnii, Nocardia and Arsenophonus species	(Beard et al. 2001; Buchner 1965; Hypsa and Dale 1997)
Anoplura (sucking lice)	Vectors of human disease	*Riesia pediculicola* (γ-proteobacteria) in human head louse and body louse		(Allen et al. 2007; Hypsa and Krizek 2007)
Tsetse flies	Vector for African trypanosomes	Wigglesworthia species	*Sodalis* glossindius, *Wolbachia* species	(Aksoy 1995, 2000; Dale and Maudlin 1999)
Mosquitoes	Vectors of Maleria and a number of viral disease	Not present	*Wolbachia* species, Asaia sp. (alpha-proteobacteria)	(Duron et al. 2007; Dutton and Sinkins 2004; Favia et al. 2008; Kampfer et al. 2006; Sinkins 2004)
Ticks	Vectors of bacterial and viral disease	Not present	Include: 'Candidatus Midichloria mitochondrii', Coxiella-like and Rickettsia species	(Epis et al. 2008; Mattila et al. 2007)
Fleas	Vectors of bacterial disease	Not present	Rickettsia species	(Adams et al. 1990)
(d) General feeders				
Blattidae (cockroaches)	Pest of stored foods, hygine problem	Blattabacterium (flavobacteria)		(Bandi et al. 1994)
Mallophaga (biting lice)	Pests of livestock and poltry	Presently identified as Legionella-like and γ-proteobacteria		(Hypsa and Krizek 2007)
Psocoptera (book lice)	Storage product pest	*Rickettsia* sp.		(Perotti et al. 2006)
Camponoti (carpenter ants)	Damage wood, but do not eat it	Blochmannia (γ-proteobacteria)	*Wolbachia* species	(Sauer et al. 2000)

[a] See Table 3 from more details +significant overlap with aphid taxa.

and are presumably required for provision of nutrients. Treating the patient with antibiotics kills the worm symbiont, stops disease progression and prevents reproduction and transmission of the worm. The treatment uses doxycycline, which does not kill the parasite but the infection is controlled and the cycle of transmission broken (Taylor et al. 2005). It is interesting to note that it is probably the mammalian immune systems response to the *Wolbachia* that causes the clinical symptoms for worm infection (Pearlman and Gillette-Ferguson 2007). The genome of this symbiont is now being used to direct research into new anti-filarial therapies (Foster et al. 2009). This example shows that with the correct compound symbiont targeting could be a very powerful tool in insect pest control.

4.2 Future Research

The filarial worm/symbiont example illustrates how targeted application using antimicrobial products specific to insect symbionts may also provide effective control of pest populations. Antibotics such as doxycycline will kill insect symbionts but the use of antibiotics on the scale required for pest control would have adverse implications for the treatment of bacterial diseases. As this practice would lead to problems with increased risk of antibiotic resistance in non-symbiotic species. It maybe possible to deliver the antibiotics in a very controlled and targeted way, but the use of traditional antibiotics may only be permissible for the control of arthropods where the alternative treatments are very toxic or environmentally damaging.

The challenge is to discover anti-symbiont compounds that will enter insects and kill the mycetome symbiont. The compounds need to pass into cells or kill symbionts when they briefly leave the mycetome for transmission to the next generation. Good candidates for novel anti-symbiont compounds are bacteriophage and insect antimicrobial peptides. Bacteriophages have an incredible environmental diversity and it is possible that they may provide a pool for new anti-microbial agents (Summers 2001). Their potential could be very specific and targeted to particular bacterial species, but maybe limited by the size of the virus particle being too large to reach bacteria in the mycetome.

Future strategies may seek to turn the insects immune system against the symbionts so curing the insect of the symbiont. The insect immune system can recognise pathogens using receptor molecules such as Toll and Imd, which induce the expression of small antimicrobial peptides (Lemaitre and Hoffmann 2007). These small peptides are very active against micro-organisms, but may not be effective against mycetome symbiont due to their protected intracellular location.

5 Symbionts for Novel Control

Symbionts have evolved a number of mechanisms for invasion, transmission and persistence in their hosts (Ruby 2008). The availability of complete pathogen and mutualist genomes has revealed their similar genetic content and function.

As discussed in Section 3 mutualists have genes that code for major 'virulence' factors classically thought to be exclusively associated with pathogenic islands in bacterial pathogens (Hacker and Kaper 2000). The fact that symbionts have these genes should come as no surprise as these genes are associated with cell adherence, iron uptake, cell invasion (Type III and Type IV secretion systems) (Hacker and Kaper 2000), quorum sensing and two compartment regulation systems (Hentschel et al. 2000) that are needed by both mutualistic and pathogenic taxa for maintenance in the host environment. Perhaps we can use the mechanisms symbionts have developed to disrupt pest populations and introduce "control" genes into pest populations.

The class of symbionts most relevant to these applications are not the mycetome symbionts, such as *Buchnera*, as they do not process genes encoding for 'virulence' factors and their transmission is organized by the host. The type of symbiont we need are those that can organize their own transmission. Therefore, the most relevant symbionts are the reproductive parasites and facultative symbionts. These microorganisms are not just passed form mother to offspring, but are capable of being transmitted between individuals within a population and ecosystem. This form of transmission requires genes for survival, invasion and persistence in the new host. These symbionts often contain genes classically associated with pathogenesis, but they generally use these genes to establish a stable commensal/parasitic relationship with the host (Dale and Moran 2006).

One of the strongest and best-defined symbiont phenotypes are generated by the reproductive parasites such as *Wolbachia*. These phenotypes have a great potential for control programs, but it is also possible that the toxins and genes used for other symbiosis may provide novel compounds.

6 Population Control Using Symbionts – Ancient Reproductive Parasites

There are a number of microbial agents that are transmitted during sex and affect the reproductive biology of insects (Table 2). The effects these bacteria have are (i) male killing - infected females sons die; (ii) feminisation - males become females; and (iii) cytoplasmic incompatibility (CI). The impact of these symbionts can be very dramatic and is well understood, but how they manipulate insect biology is still unresolved.

6.1 Cytoplasmic Incompatibility

Cytoplasmic incompatibility is perhaps the best known of these phenotypes and has been described for *Wolbachia* (Werren 1997; Yen and Barr 1971) and *Candidatus* "cardinium" species (Gotoh et al. 2007) (Table 2). The phenotype has currently

Table 2 The taxonomy of bacteria known to show reproductive parasitism, with notes on phenotypes observed, and the host range these are observed within

Taxon	Male-killing	Feminization	Parthenogensis induction	Cytoplasmic incompatibility	Notes	Reference
Wolbachia	Many arthropods	Isopoda, Lepidoptera; Hemiptera	Many haplodiploid arthropods	Many arthropods	One strains shows anti-viral resistance	(Hurst et al. 1999a; Stouthamer and Kazmer 1994; Werren 1997)
Rickettsia	Coleoptera		Hymenoptera		Some strains with horizontal transmission through plant/animal	(Werren et al. 1994)
Spiroplasma	Many arthropods				Many strains with horizontal transmission through plant/animal	(Hurst et al. 1999b, 2000; Jiggins et al. 2000)
Cardinium		Acari	Acari, Hymenoptera	Acari, Hymenoptera	Some strains likely not to be reproductive parasites	(Hunter et al. 2003; Weeks et al. 2001; Zchori-Fein et al. 2001)
Arsenophonus	Hymenoptera				Also has horizontal transmission. Relatives within the genus Arsenophonus are likely to be secondary symbionts.	(Gherna et al. 1991)

been reported in seven arthropod orders, including beetles, flies, wasps, planthoppers, moths, mites, and termites (Breeuwer 1997; Gotoh et al. 2007; Hoffmann and Turelli 1997; Hunter et al. 2003; Moret et al. 2001). The bacteria modify the host's sperm (in the testis) so that the same strain of bacteria must be present in the egg or the cross will be incompatible and the zygote will fail to develop (Poinsot et al. 2003; Werren 1997). Cytological studies suggest that CI is caused by an asynchrony between the male and female pronuclei due to delayed nuclear envelope breakdown and disruption of paternal chromosome condensation in CI embryos during the first mitotic division. This ultimately results in the loss of paternal chromosomes and the subsequent death of the egg (Callaini et al. 1997; Notomista et al. 2003; Tram and Sullivan 2002). There is natural variation in the strength of CI, which is probably due to interactions between the host and symbiont genotypes (Bourtzis et al. 1996; McGraw et al. 2002; Poinsot et al. 1998; Reynolds and Hoffmann 2002). The CI phenotype has great potential as a pest control (Bourtzis 2008). The following sections will provide a summary and highlights of some of the main themes and strategies.

6.1.1 Symbiont Sterile Insect Technique

The use of sterile insect technique (SIT) for insect control relies on the introduction of sterility to females in a wild population. This sterility is a result of the female having mated with males that have been sterilised using ionizing radiation. This standard sterile insect release technique has been used to control the screwworm fly (*Cochliomyia hominivorax*) and the Medfly (*Ceratitis capitata*), but has been proposed for use against *Anopheles*, *Aedes* and *Culex* mosquitoes, Tsetse fly (*Glossina* species), Painted Apple Moth (*Lymantriidae*) and various *Bactrocera* species. As well as radiation-induced sterility, a number of natural mechanisms such as hybrid sterility (tsetse flies) and symbiont induced cytoplasmic incompatibility have been explored. The problem with using radiation is that the doses needed to sterilize males have a negative impact on insect fitness, thus symbiont-induced sterility may have a number of advantages, producing males that show greater vigour and longevity then irradiated males (Robinson 2005).

Wolbachia can be experimentally transferred between insect hosts to produce the cytoplasmic incompatibility phenotype in new lineages. This opens the way to using symbionts to generate genetically sterile male lines due to novel cytoplasmic incompatibility (Bourtzis 2008; Townson 2002). These genetically sterile males can then be used for sterile male technique for population control.

Medfly Control Using *Wolbachia*

The symbiont sterile insect technique has been successful established, *Wolbachia*-infected lines of the medfly *Ceratitis capitata* were generated by injecting a *Wolbachia* from the cherry fruit fly *Rhagoletis cerasi* (Zabalou et al. 2004).

Complete CI was induced in the medfly by the introduction by the novel *Wolbachia* and subsequent laboratory experiments demonstrated that populations were completely suppressed by a single release of infected males. This is clear evidence that *Wolbachia*-induced CI could be used as a tool for the control of medfly populations. This result is encouraging and it is possible that the introduction of *Wolbachia* into other pest and vector species may lead to suppression or modification of natural populations (Zabalou et al. 2004). This method is only applicable when sterile male release is a correct and practical method for the control of the insect pest and this will be determined by the population biology of the insect.

6.1.2 Gene Drive

The mechanism of cytoplasmic incompatibility allows *Wolbachia* to spread quickly across a population and this ability could in principal be used to "drive" disease disruptive genes, which prevent disease transmission, into insect populations (Sinkins and Gould 2006). This method could be a very powerful way of distributing beneficial genes, but would be most successful if the genes were genetically engineered into *Wolbachia*, but there is currently no methodology for genetic transformation of *Wolbachia* or the subsequent the deployment of the transgenic bacteria into insect populations.

6.2 Male Killing

Male killing can also be caused by *Wolbachia*, but is a trait shown by a number of *Rickettsia*, *Arsenophonus*, and *Spiroplasma* species. One suggestion for a role for male-killing bacteria in pest management is in conjunction with sterile insect release (Hurst and Jiggins 2000). The success of sterile insect release depends on maintaining a high ratio of sterile to normal males in the population. The introduction of a male-killer in to the pest population would lower the number of fertile males and thus increaes the effectiveness of any release (Hurst and Jiggins 2000). However, the real value of male-killing bacteria to pest control maybe through the study of the virulence mechanisms of male-killers (see Section 7.1)

6.3 Future Research

It is clear that reproductive parasites have great potential for pest control. The use and development of these reproductive parasites as control agents is currently limited by our understanding of their biology. At the moment, we do not have a clear understanding of the host and symbiont genes responsible for cytoplasmic incompatibility,

so it is not possible to predict the strength of the phenotype. If the CI phenotype is not always strongly expressed, there is potential for males to be released that are fully compatible with the natural population. Therefore, the validity of this system would be increased by a full understanding of the bacterial and the corresponding host factors responsible for CI. The factors involved in CI are the focus of a number of research groups and once these gaps in our understanding are closed this knowledge can be used to develop robust pest control methods.

It is important to highlight that although effective pest control may rely on the genetic manipulation of symbionts such as *Wolbachia*, at present, we have no protocol for engineering these obligate intracellular bacteria. This coupled with the lack of methodology for deployment or dissemination means that most of the applications of reproductive parasites require much research and development before being realistically considered for applied control strategies.

7 Symbionts as Novel Products – Facultative Symbionts

7.1 Symbionts a Source of Novel Toxins

It is well understood that microorganisms are excellent providers of biologically active compounds. These compounds maybe the result of specialised metabolism or produced specifically as toxins and effectors to aid invasion, defence and competition. Bacterial toxins encoded by proteins are diverse in their mode of action and origin. Pathogenic species synthesise toxins that damage cell membranes, inhibit protein synthesis, inhibit neurotransmitters, activate host immunity and modulate apoptotic cell death. These toxin compounds are responsible for most of the primary virulence of bacterial pathogens.

7.1.1 The Example of *Bacillus thuringiensis*

The best example of how bacterial toxins can be applied to pest control come from the soil bacterium *Bacillus thuringiensis*, commonly know as Bt. It produces a potent insecticidal toxins which build up as proteinaceous crystals during sporulation (Schnepf et al. 1998). *Bacillus thuringiensis* has been commercialised in many forms, providing excellent biological control compounds for specific insect orders (e.g. *B. thuringiensis* serovar *kurstaki* - Lepidotera and *B. thuringiensis* serovar *israelensis* - Dipteran). The specificity of serovars is based on the mixture and types of Cry (crystal) toxins encoded in the genome. For example, cry I toxin genes show activity against lepidoteran larvae, cry II genes show activity against lepidoptera and diptera and cry III show specific activity to coleoptera. The genes encoding for these proteins are often plasmid borne and have already been genetically engineered into many crop species (De Cosa et al. 2001).

7.1.2 The Example of *Photorhabdus luminescens*

The nematode symbiont and broad-spectrum insect pathogen *Photorhabdus luminescens*, has a large genome of 5.7 Mb which is packed full of insecticidal toxins (Duchaud et al. 2003). The most interesting of these are a class known as Tc's (Toxin complexes). These proteins were first described in *Photorhabdus* and *Xenorhabdus*, but now have been described in other insect associated bacteria such as *Serratia entomophila* and *Yersinia enterocolitica*. The three components of the complex are all toxin to insect cells, but combined as a complex they have an additive effect. The Tc genes and other toxins have great potential as biological control agents and could be used in a similar way to the Cry toxins of *B. thuringiensis* (Nicholson 2007). They are currently being tested against the Formosan subterranean termite (*Coptotermes formosanus*) (Zhao et al. 2008).

7.1.3 Facultative Symbiont Toxins

Bacillus thuringiensis is not a symbiont, but as we study the genomes of symbionts like *Photorhabdus* we are beginning to understand that many symbiont genomes encode for novel toxins, these protein toxins have potential applications for biological control, as they are good candidates for insecticides and genetic engineering into plants.

A number of facultative symbionts have been shown to have Type Three Secretion Systems (TTSS) a structure used by pathogenic bacteria for injecting toxins into host cells. The TTSS is an important virulence component of pathogenic stains of *Salmonella typhimurium*, and the tsetse fly symbiont *Sodalis glossinidius*. The knockout of the invC gene in the *S. glossinidius* TTSS results in the bacterium being unable to invade insect cells and form the symbiosis with the tsetse fly (Dale et al. 2001). The symbiotic toxins injected via the TTSS have not been fully characterized for *S. glossinidius*, but facultative symbiont "*Candidatus Hamiltonella defensa"* has a TTSS and carries a number of putative toxins (Moran et al. 2005). The genome of *Hamiltonella* encodes for four potential toxins (Moran et al. 2005): (1) nuclease (CdtB), which has a putative action interfering with DNA replication during G2 phase of the eukaryotic (insect) cell cycle; (2) shiga toxin homolog, which are known to disrupt ribosome function inhibiting protein synthesis; (3) two RTX membrane pore-forming toxins genes and (4) YD-repeat containing ORFs (Toxin Complex-like genes).

These toxins show a broad overlap in function and homology with toxins found in *Photorhabdus* and other symbionts such as *Arsenophonus* (Wilkes, H.C. Darby and G.D.D. Hurst, 2009) and not all toxins will be TTSS associated. At present, it is unclear what role these proteins have in the forming of a symbiosis, but it demonstrates that symbionts potentially have toxins for effecting and influencing insect cells and their function. These toxins may have similar potential to Cry and the Tc toxins for insect control.

7.2 *Symbionts – Facilitators and Antagonists*

In a number of cases the pest status of the insect is not due to direct damage of a crop or resource, but due to the insect ability to carry and vector disease causing organisms. Diseases transmitted by arthropods cause severe morbidity and mortality especially in developing countries, causing an estimated 1.5 million human deaths per annum. Malaria, yellow fever, leishmaniasis and trypanosamiasis (African and American) are just a few examples of diseases whose vectors have at least one bacterial symbiont and similar lists can be made for plant disease vectors (Table 1). As discussed above, symbionts occupy an important position in insect biology, influencing mating structure and providing essential nutrition. In addition to these functions, symbionts can also modulate biologically important traits, such as host range, vector competency and disease resistance. The two examples below from aphids and tsetse demonstrate how symbiotic bacteria can modulate disease transmission and in some cases potentially work against control strategies.

7.2.1 Aphid Symbionts

Aphids are important vectors of plant viruses and it has been demonstrated that symbiont proteins such as GroEL can increase the aphids ability to transmit viruses (Filichkin et al. 1997; Hogenhout et al. 1998; Morin et al. 1999). In most agricultural situations aphid populations (and therefore virus transmission) are controlled by natural enemies (predators, parasitoids and fungal disease). However, symbionts also have an impact, as the facultative symbionts can make a significant contribution to aphid resistance to natural enemies such as parasitoid wasps and pathogenic fungi (Ferrari et al. 2004; Oliver et al. 2003, 2005) (Table 3). The common symbionts *Hamiltonella defensa* and *Serratia symbiotica* reduce the success of *Aphidius ervi* parasitism, while a third symbiont, *Regiella insecticola* is associated with resistance to infection by the fungus *Pandora neoaphidis* (Scarborough et al. 2005). The impact of resistance is not just to increase vector numbers, but increased stress caused by expose to natural enemies is likely to increase the production of wing forms (Muller et al. 2001) which disperse, carrying any virus they bear to new host plants spreading the disease.

7.2.2 Trypanosomes and Tsetse Flies

The tsetse fly (genus *Glossina*) is the sole vector of the zoonotic disease African trypanomiasis, which causes human sleeping sickness and nagana in animals. WHO estimate that there are currently 300,000–500,000 cases of trypanomiasis with 60 million people at risk of contracting the disease in Zimbabwe, Malawi, Zambia and 34 other African countries (WHO Committee 2001). For effective control of trypanomiasis it is essential to break the transmission cycle of these

Table 3 The symbiotic microflora of the pea aphid, *Acrythosiphum pisum*. All bacteria show maternal transmission. Synonyms given are those used in papers before taxonomic description. Note, whilst *Buchnera* is present in all populations and all individuals, secondary symbionts vary in frequency both geographically and temporally

Symbiosis type	Bacterium: division	Species	Fitness effect	Frequency and horizontal transmission	Synonym	References
Primary	γ-proteobacteria	*Buchnera aphidicola*	Provision of Essential Amino acids; essential for normal reproduction	Ubiquitous; No		(Douglas 1998; Moran et al. 1993; Munson et al. 1991a; Munson et al. 1991b)
Secondary	γ-proteobacteria	*Candidatus Hamiltonella defensa*	Increased parasitoid resistance	Common but variable; sex and oral/faecal	PABS, T type	(Darby et al. 2001; Degnan and Moran 2008; Ferrari et al. 2004; Moran et al. 2005; Moran and Dunbar 2006; Oliver et al. 2003; Sandstrom et al. 2001)
	γ-proteobacteria	*Candidatus Serratia symbiotica*	Increased parasitoid resistance	Common but variable; sex and oral/faecal	PASS, R type	(Chen et al. 2000; Chen and Purcell 1997; Oliver et al. 2006)
	γ-proteobacteria	*Candidatus Regiella insecticola*	Increased fungal resistance	Common but variable; sex and oral/faecal	PAUS, U type	(Ferrari et al. 2004; Scarborough et al. 2005)
	α-proteobacteria	*Rickettsia* sp.	Elevated Temperature tolerance	Yes, rarely, mechanism unknown	PAR, S type	(Chen et al. 1996; Chen et al. 2000)
	mollicutes	*Spiroplasma* sp.		Yes, rarely, mechanism unknown		(Fukatsu et al. 2001; Haynes et al. 2003)

protozoan. The trypanosome infection rates of tsetse are normally low (8–10%) as parasites entering the midgut die. This natural refractoriness of tsetse to trypanosomes is variable and has a number of contributing factors (e.g. age, sex and genetic background) (Welburn and Maudlin 1999). Tsetse fly susceptibility to trypanosome infection is also affected by the presence of the midgut symbiont *S. glossinidius* (Baker et al. 1990; Geiger et al. 2007). Genome data from *Sodalis* suggests that the symbiont utilizes similar genes to pathogens for host interaction (Dale et al. 2001; Toh et al. 2006). At present, the mechanisms underlining this interaction between bacteria, trypanosome and tsetse fly interaction are unknown.

The examples given of aphid and tsetse suggest that symbionts can promote disease transmission. This means that effective disease management needs to account for the presence of symbionts. In the case of tsetse flies, research into the mechanism of symbiosis and its link to trypanosome vectoring, may provide insights into how tsetse could be made more resistant to the parasite. In the case of aphids, symbiont monitoring maybe a useful parameter for predicting the likelihood of aphid populations escaping the control by their natural enemies.

7.2.3 Using Symbionts to Modify Vector Competency

Symbionts can also be used more proactively to control disease vectors. Novel Symbiont host combinations can negatively affect insect fitness (Bourtzis 2008). If novel symbiosis reduced insect longevity, this would have a significant impact on the transmission of diseases that require a significant period of development in the vector (e.g. malaria and dengue). This approach has been successfully tested by transferring a novel CI *Wolbachia* into the vector of dengue, *Aedes aegypti*. The adults of the *Wolbachia* infected line showed half the adult life span compared to the controls under laboratory conditions. In theory the CI phenotype should facilitate the symbionts invasion into natural field populations and its persistence over time (McMeniman et al. 2009).

Most of the important diseases transmitted by insects are exposed to insect gut microbiota and symbionts. In the case of tsetse, *Sodalis* is found in the same region of the gut where trypanosomes develop. This has led researchers to suggest that *Sodalis* could be genetically manipulated to carry genes that would kill the trypanosomes stopping the disease cycle (Cheng and Aksoy 1999). This approach has been developed for the control of *Trypanosoma cruzi*, the agent that causes Chagas disease (Beard et al. 1992; Durvasula et al. 1997). The streptomycete *Rhodococcus rhodnii*, has been transformed with an anti-trypanosome gene cecropin A. This gene when expressed in the insect gut by the bacterium, lyses *T. cruzi*. *Rhodococcus rhodnii* is the dominant bacteria in the insect's gut and is transmitted via faeces, this means that the bacteria can be formulated for spraying and used to treat areas infested with the insects (Durvasula et al. 1999, 2008). This approach may prove to be very effective in controlling the disease and could be tailored to other insect species where the gut microbiota play a significant role in vector competency or where the disease matures in the insect gut.

7.2.4 Future Research

As we learn more about how microbes and insects interact it is important that we investigate and understand the mechanisms involved. For effective control of vectored diseases, a greater understanding of how the gut microbiota affects the establishment, maturation and subsequent vectoring of the disease is essential. Without this knowledge we may be overlooking bacteria such as *R. rhodni* and *S. glossinidius* which could be used for control or enhance our understanding of the system. It is also possible that symbionts could be used to influence vector competency by modifying the insects' life history.

8 Developing Symbiosis Research for Pest Control

This chapter demonstrates that symbionts have great potential as novel pesticide targets, control agents and sources of new insecticidal compounds for pest control, but this potential is still to be realised. The main reason for the lack of uptake and use of symbionts in pest control is due to the problems of working with symbionts. Most symbionts are non-culture viable and this has led to problems in experimental protocols and design. Symbionts and their host are therefore difficult to study as we currently lack the molecular and genetic tools available in other systems.

8.1 New Tools for Symbiont Research

8.2 Genomics and Beyond

Recent advances in "next generation" sequencing remove a number of obstacles in symbiont research. The availability of low cost and high throughput sequencing provides the researcher access to the genome of host/symbiont, metagenomics and whole transcriptome of symbiosis. The new technology also simplifies research into the genetics of symbiont and host, as it is now possible to sequence whole genomes to find the genetic triggers of phenotypes (Darby and Hall 2008; Ruby 2008). This technology will change the way we work with non-model organisms such as symbionts.

8.3 Genetic Manipulation of Symbiont

One tool that is missing from many symbiont systems is the ability to culture and genetically manipulate symbionts. This is important for research and potentially essential for the use of symbionts in pest control. The use of transgenic symbionts

for targeted pest control provides a direct route for utilising symbionts, but do we need transgenic symbionts? In most cases the symbiont and symbiotic partnership with the host may not allow for this approach. The alternative is to use other symbionts that are culture viable (Darby and Welburn 2005). Where we understand little about the transmission and host range of symbionts, it may not be safe to release genetically modified symbionts. Symbionts released into the environment with control genes, may have unknown impact non-target species. If this approach is to be used then it is essential that research is used to provide realistic risk assessments of the impact and transmission of these genes beyond their intended targets. In the case of symbionts it should be possible to find combinations of microbes and hosts that are specific enough to permit the use of this technology.

9 Summary of Future Research

The basic requirement for the use of symbionts in pest control is that we understand their role in insects and how the symbiotic organism (i.e. host plus symbiont) works. How do symbiont and hosts interact? Even in well-studied systems, we still have much to comprehend about the mechanisms and genes used in symbiosis. For example, the mechanisms by which *Wolbachia* induce CI (Section 6.1) aphid symbionts increase resistance to natural enemies (Section 7.2.1) are only just been uncovered.

Of all the ideas and research discussed in this chapter, the use of symbionts as sources of novel compounds is the area most likely to rapidly produce products for pest control. New toxin discoveries can be funnelled into current technologies and formulations (e.g. sprays and transgenic plants). This shortens the distance between research and applied applications. Targeting the mycetome-associated symbionts may also be relatively easy, if appropriate anti-symbiont compounds can be identified.

The successful use of symbionts like *Wolbachia* in SIT and as a gene driver would be ground breaking technology, but both of these technologies need more research and it is not clear at this point how universal and scalable their application will be.

Symbionts have a major role to play in pest control, if we are to progress then symbiont research must move from observation and description to understanding mechanisms before the full potential for pest control can be realised.

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Novel Approaches for the Management of Mealybug Pests

José Carlos Franco, Anat Zada, and Zvi Mendel

1 Introduction

Mealybugs (Hemiptera: Pseudococcidae) are small, soft-bodied plant sap-sucking insects that constitute the second largest family of scale insects (Hemiptera: Coccoidea), with more than 2,000 described species and ca. 290 genera (Ben-Dov 2006; Downie and Gullan 2004). Their common name is derived from the mealy wax secretion that usually covers their bodies (Kosztarab 1996). A recent phylogenetic study, based on analysis of nucleotide sequence data, supported the existence of three subfamilies Pseudococcinae, Phenacoccinae and Rhizoecinae (Downie and Gullan 2005). This estimate was recently revised in light of integrated molecular and morphological data, and only two subfamilies emerged: Pseudococcinae and Phenacoccinae (Hardy et al. 2008).

Mealybugs are severe agricultural pests. According to Miller et al. (2002), 158 species of mealybugs are recognized as pests worldwide. These species most frequently originate from the Palearctic region (ca. 29%), followed by the Nearctic (17%), Neotropical (16%), Oriental (15%), Afrotropical (12%) and Australasian (11%) regions. Approximately 22% of the mealybug pests are polyphagous, 20% occur on grasses (e.g., sugar cane), 16% on citrus and tropical fruits, and 6% on coffee.

Chemical control is still the most common control tactic used against mealybug pests. However, the cryptic behavior of mealybugs, their typical waxy body cover, and clumped spatial distribution pattern render the use of many insecticides

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ineffective. Repeated insecticide use, especially of broad-spectrum chemicals, also adversely impacts mealybugs' natural enemies. Insecticide resistance has also caused the use of some chemicals to be unsustainable. Furthermore, many of these products are increasingly unacceptable because of their human toxicity and low selectivity; some are no longer available and others are targeted for reduction under national programs and regulations for sustainable use of pesticides, in light of their risk or hazard assessments (Charles et al. 2006; Franco et al. 2004b; Walton et al. 2006). Biological control of mealybugs, which is widely recommended, has also been constrained by different factors.

Because of the limitations and constraints imposed on the actual control tactics for mealybug pest management, there is a need to develop more effective, species-specific, and environmentally safe approaches. The objective of this chapter is to present the current knowledge regarding pest control tactics against economically important mealybug species, and to suggest new approaches to the practical management of this group of pests.

2 Economic Importance of Mealybugs

Pest mealybugs are often invasive species. In the USA, there are 350 species of mealybugs. Approximately 70% of the 66 mealybug species that are considered as pests are invasive (Miller et al. 2002). In New Zealand, most of the known 114 species of mealybugs are found only on native plants. Three cosmopolitan and invasive *Pseudococcus* species are frequently occurring pests of horticultural crops in the country, where they account for more than 99% of the mealybug fauna in orchards and vineyards (Charles 1993). In Israel, only one among 13 mealybug pests may be considered native (Z. Mendel, unpublished data, 2009). In France, scale insects, including mealybugs, represent 31% (Streito and Martinez 2005) of newly introduced species in recent years, although all mealybug pests on grapevine are native (Sforza 2008a, b).

Rather few narrow host-range plant mealybugs are considered major pests on an international scale, as in the case of the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Matile-Ferrero 1977; Neuenschwander 2001; Williams and Granara de Willink 1992; Zeddies et al. 2001). However, the most notorious species are polyphagous and have become serious pests of different crops under different environments. Many of them are cosmopolitan species belonging to the genera *Pseudococcus* and *Planococcus*; they spread between continents through international trade. Several members of the genus *Pseudococcus*, for example, *Ps. calceolariae* (Maskell), *Ps. longispinus* (Targioni-Tozzetti), and *Ps. viburni* (Signoret), are important pests of apple, pear and vineyards in New Zealand (Charles 1993), whereas around the Mediterranean they are considered mainly as pests of citrus, persimmon and several other subtropical fruit trees (Franco et al. 2004b). The citriculus mealybug, *Ps. cryptus* Hempel is a major pest of citrus in the east Mediterranean region and it attacks coffee roots in Asia and South America (Ben-Dov

1994; Blumberg et al. 1999; Williams and Granara de Willink 1992). Two members of the genus *Planococcus* are among the best known pests of the family: on an international scale, the vine mealybug, *Pl. ficus* (Signoret) damages mainly vineyards (Ben-Dov 1994; Daane et al. 2006a; Gutierrez et al. 2008a; Walton et al. 2004; Zada et al. 2008), whereas the citrus mealybug, *Pl. citri* (Risso), attacks mainly subtropical fruit trees under Mediterranean climate conditions and ornamental plants in interior landscapes in cooler zones (Ben-Dov 1994; Franco et al. 2004b). Polyphagous mealybugs pose a serious threat because of their tendency to adopt new host plants easily. The citrus mealybug has become a key pest in the mint and tarragon industry in Israel (Z. Mendel, unpublished). Another example that indicates the high economic importance of a polyphagous mealybug is the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green). This mealybug is indigenous to southern Asia, and actually is considered a potentially serious pest in the United States, because of its extremely broad range of economically important hosts, including citrus, ornamentals, vegetables, and the native American flora. It was first reported in the Western Hemisphere in Hawaii in 1984, and later in Grenada in 1994; subsequently it has spread rapidly through the Caribbean islands and to southern California (1999) and Florida (2002). Without control, the economic impact of the hibiscus mealybug to U.S. agriculture has been estimated at $750 million per year (Hall et al. 2008; Roltsch et al. 2006; Vitullo et al. 2007; Zhang et al. 2006). The solanum mealybugs, *Phenacoccus solani* Ferris and *Ph. solenopsis* Tinsley are examples of invasive pests of annual crops; they cause devastating damage to green pepper in Israel and cotton in the Indian sub-continent (Ben-Dov 2005b; Hodgson et al. 2008; Nakahira and Arakawa 2006).

The damage caused by mealybugs is linked to sap uptake, honeydew secretion and associated sooty mold development, toxin injection and virus transmission, although the presence of the insects may itself lead to economic losses (Franco et al. 2000; Gullan and Martin 2003; McKenzie 1967; Panis 1969). The typical injury includes: (a) leaf and fruit discoloration; (b) defoliation, flower and fruit drop; (c) reduction of fruit growth rate; (d) distortion of leaves, new shoots and fruits; (e) aborted plant shoots; (f) development of cork tissue on fruit peel; (g) soiling of fruits with mealybugs and honeydew; and (h) reduction of plant vigor (Franco et al. 2000, 2004b; Gullan and Martin 2003; Hodges and Hodges 2004; Sagarra et al. 2001). High densities or annually repeated infestations can even kill perennial plants (Hodges and Hodges 2004; Walton et al. 2004, 2006). Indirect damage can result from trophic interactions between mealybugs and other insect pests that are attracted by honeydew, such as Lepidoptera (Franco et al. 2000; Mittler and Douglas 2003; Silva and Mexia 1999b).

Several mealybug species are vectors of viral diseases of various crops: banana (Kubiriba et al. 2001; Thomson et al. 1996; Watson and Kubiriba 2005), black pepper (Bhat et al. 2003), cocoa (Dufour 1991; Entwistle and Longworth 1963; Hall 1945), grapevine (Cabaleiro and Segura 1997a, b; Cid et al. 2007; Garau et al. 1995; Minafra and Hadidi 1994; Petersen and Charles 1997; Sforza et al. 2003; Tsai et al. 2008; Zorloni et al. 2006), pineapple (Sether and Hu 2002a, b; Sether et al. 1998, 2005), rice (Abo and Sy 1998), and sugarcane (Lockhart et al. 1992).

In such cases, mealybugs may be economic pests even at low densities. For example, several mealybug species are responsible for GLRaV-3 (Grapevine LeafRoll associated Virus-3) transmission to grapevine, which has been shown by the strong positive correlations between mealybug numbers and infection levels in the following season. The virus infection was predicted to spread rapidly within the vineyard, with 50% infection occurring in years 6, 8 and 11 for high, intermediate and low infection rates, respectively. The economic impact of GLRaV-3 infection in sensitive varieties exceeded $10,000 per ha by years 7, 9 and 12 and profitability was sufficiently affected to justify replanting by year 11 (Walker et al. 2004).

Mealybugs have been also used as beneficial insects in biological control of weeds. For example, *Hypogeococcus pungens* Granara de Willink was successfully introduced from Argentina into Queensland (Australia) for the control of Harrisia cactus (*Eriocereus martini*) and related plants (Williams and Granara de Willink 1992). Some mealybug species may also be manipulated as beneficial insects in conservation biological control tactics. For example, the cypress mealybug, *Pl. vovae* (Nasonov), which occurs on cypress trees (*Cupressus* spp.) grown in windbreaks, serves as an alternative host for natural enemies of mealybug pests in surrounding citrus orchards and cocoa plantations (Cox 1989; Ho and Khoo 1997; Franco et al. 2004b). Mealybugs have also provided food for humans; the biblical manna, one of the food sources consumed by the Israelites during their wandering in the wilderness of Sinai, is believed to have been the honeydew excretion of the manna mealybug, *Trabutina mannipara* (Hemprich and Ehrenberg in Ehrenberg) (Ben-Dov 2006; Miller and Kosztarab 1979).

3 Understanding the Target Organism: a First Step to Sustainable Mealybug Management

3.1 Morphology and Life Cycle

Similarly to other scale insects, mealybugs exhibit sexual dimorphism. The wingless neotenic female is the larger gender (the adult female weighs about 100–200 times more than the adult male), ranging from about 0.4 to 0.8 mm in body length, and can continue feeding and growing until mating. Unmated females, as well as overwintering mated females, may live for several months. Mealybug females have five growth stages: egg, three nymphal instars, and the adult (imaginal stage). They lay their eggs (oviparity) in a waxy covering (the ovisac) or retain them in their reproductive tract until hatching (ovoviviparity). In contrast, adult males, if present, are delicate, ephemeral (they live a few days at most), neometabolic, dipterous insects, and therefore, are easily overlooked in the field. Males of some mealybug species (e.g., *Saccharicoccus sacchari* (Cockerell) exhibit wing polymorphism, including wingless forms. Mealybug males have six growth stages: egg, two nymphal instars, two pupa-like instars (prepupa and pupa), that develop inside a

waxy cocoon that is produced by the second-instar nymph, and the adult (Gullan and Martin 2003; Gullan and Kosztarab 1997; Kosztarab and Kozár 1988; McKenzie 1967). The adult male takes 1–2 days to complete sexual maturation and elongation of the "wax tail" in order to be able to fly, to respond to the sex pheromone emitted by virgin females, and to mate (Mendel et al. 2008; Silva et al. 2009). The male's wax tail consists of one or two pairs of caudal filaments that are secreted by glandular pouch setae located on segments VII and VIII of the abdomen (Afifi 1968; Kosztarab and Kozár 1988; McKenzie 1967); it is believed to assist in stabilizing flight (Duelli 1985).

3.2 Feeding Process and Endosymbionts

Mealybugs feed by inserting their stylets through the plant tissue to suck up sap from either phloem or mesophyll, or both. Males terminate their feeding towards the end of the second nymphal stage. Generally, stylet penetration is accomplished by secretion of solidified saliva that forms a sheath around the stylets. Similarly to other members of the suborder Sternorrhyncha, which includes scale insects, aphids, psyllids and whiteflies, mealybugs consume a diet containing mainly carbohydrates but also limited amounts of free amino acids and other nitrogen compounds (Franco et al. 2000; Gullan and Martin 2003; Silva and Mexia 1999a; Tonkyn and Whitcomb 1987). Thus, except for sucrose hydrolysis, food digestion is hardly necessary. However, organic compounds in phloem sap need to be concentrated before they can be absorbed, and this occurs in the filter chamber, a specialized component of the digestive system, which enables the direct passage of water from the anterior midgut to the Malpighian tubules, thereby concentrating food in the midgut (Terra and Ferreira 2003). The residue of ingested phloem sap, after digestion and assimilation in the insect gut, is released from the anus as a sugar-rich material, the honeydew. Up to 90% of the ingested sugars may be egested in this way (Mittler and Douglas 2003).

Mealybugs have an obligatory association with prokaryotic endosymbionts, probably because of the suboptimal nutrition furnished by phloem sap, which lacks essential nutrients. These endosymbionts are believed to be important to the nitrogen and sterol requirements of their hosts and may play a role in resistance to microbial pathogens or detoxification of plant secondary compounds (Baumann 2005; Gullan and Kosztarab 1997). Within their body cavities, mealybugs have a structure, the bacteriome, which comprises specialized cells, the bacteriocytes, which harbor the primary endosymbionts, that is, the P-endosymbionts (Kono et al. 2008; Thao et al. 2002). The P-endosymbionts have the unusual property of containing within their cytoplasm prokaryotic secondary endosymbionts, the S-endosymbionts (von Dohlen et al. 2001). During male development the bacteriome progressively degenerates in the prepupa and pupa and became almost unrecognizable in the adult male (Kono et al. 2008).

3.3 Reproductive Systems and Sex Determination

Most mealybug species reproduce sexually, and lay eggs (Gullan and Kosztarab 1997; Kosztarab and Kozár 1988). However, some, such as *Ph. solani*, (Nakahira and Arakawa 2006), *Ph. parvus* (Marohasy 2003), and *Ferrisia malvastra* (McDaniel) (Ben-Dov 2005a), reproduce parthenogenically, and others, for example, *Ps. longispinus* (Franco et al. 2000), and *Antonina graminis* (Maskell) (Ben-Dov 2006), are ovoviviparous.

Two different genetic systems may be found in mealybugs. The more common corresponds to a particular type of haplodiploidy, known as paternal genome elimination, in which both males and females develop from fertilized eggs; the male develops from a zygote containing one haploid genome from his mother and one haploid genome from his father, but only the maternal genome is transmitted to the offspring via the sperm, because the set of chromosomes of paternal origin becomes heterochromatic and genetically inactive (Normark 2003; Nur 1990). Male mealybugs are thus functionally haploid, owing to heterochromatization (parahaploidy) (Bongiorni et al. 2001). The other genetic system is thelytokous parthenogenesis, in which there are no males and therefore no mating occurs (Normark 2003).

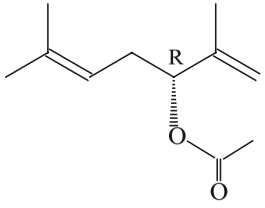
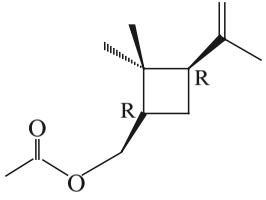
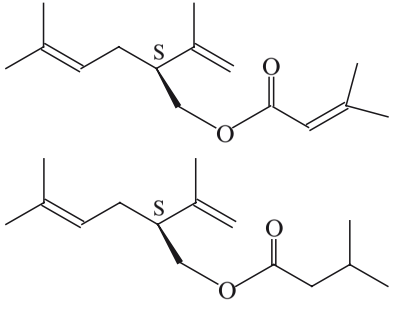
There are no sex chromosomes in mealybugs; sex is probably determined by a functional haploidy/diploidy mechanism, which seems to be dependent on the behavior of a set of chromosomes and not a single chromosome. If heterochromatization of an entire set of chromosomes takes place during the cleavage stage of embryogenesis, the embryo will develop into a male; otherwise it will develop into a female. Spermatogenesis is characterized by inverse meiosis and absence of chromosome pairing and genetic recombination (Khosla et al. 2006; Sánchez 2008). Recently Sánchez (2008) suggested that the genome of the mother determines the heterochromatization of the inherited paternal chromosomes in mealybug embryos. According to this model, heterochromatization is controlled by a maternal factor, with the maternally derived chromosomes imprinted so that they do not suffer the fate of the male chromosome.

Sex determination in mealybugs, and consequently the sex ratio, is known to be influenced by temperature and the age of the mother (Nelson-Rees 1960). The effect of the temperature or the age of the mother on the offspring sex ratio is attributed to a change in the ratio between the numbers of oocytes with and without the maternal factor (Sánchez 2008).

3.4 Sex Pheromones

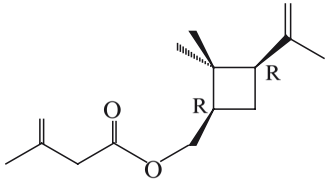
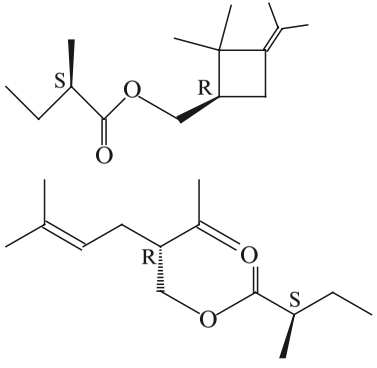
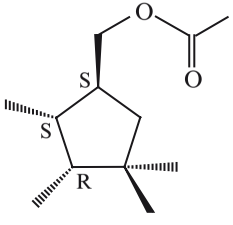
The site of production and release of sex pheromones in female mealybugs has not yet been determined, although suggested that it might be the translucent pores on the hind legs (Gullan and Kosztarab 1997). So far the sex pheromones of ten mealybug species, all of them of agricultural pests, have been identified. Table 1 summarizes the structures of these pheromones and presents references concerning

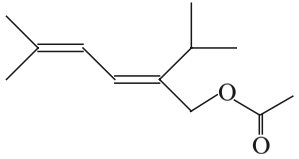
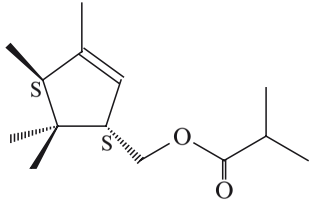
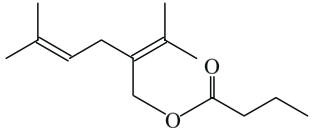
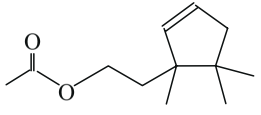
Table 1 Mealybug species whose sex pheromones have been identified or are under investigation

Species	Pheromone structure	Identification reference	Synthesis references
Comstock mealybug, *Pseudococcus comstocki* Kuwana		Bierl-Leonhardt et al. 1980, Negishi et al. 1980	McCullough et al. 1991; Baeckstroem and Li 1990; Kang and Park 1990; Skatteboel and Stenstroem 1989; Larcheveque and Petit 1989; Fall et al. 1986; Baeckstrom et al. 1984; Nakagawa and Mori 1984; Bierl-Leonhardt et al. 1982; Mori and Ueda 1981; Uchida et al. 1981
Citrus mealybug, *Planococcus citri* (Risso)		Bierl Leonhardt et al. 1981	Kukovinets et al. 2006; Zada et al. 2004; Passaro and Webster 2004; Chibiryaev et al. 1991; Odinokov et al. 1991; Serebryakov et al. 1986; Wolk et al. 1986; Odinokov et al. 1984; Carlsen and Odden 1984; Bierl-Leonhardt et al. 1981
Vine mealybug, *Planococcus ficus* (Signoret)		Hinkens et al. 2001; Zada et al. 2003	Ujita and Saeki 2008, Zada and Dunkelblum 2006, Zada and Harel 2004, Zada et al. 2003, Millar et al. 2002, Hinkens et al. 2001.

(continued)

Table 1 (continued)

Species	Pheromone structure	Identification reference	Synthesis references
Citriculus mealybug, *Pseudococcus cryptus* Hempel		Arai et al. 2003	Nakahata et al. 2003
Pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green)		Zhang et al. 2004a	Zhang and Nie 2005; Zhang et al. 2004a, b;
Obscure mealybug, *Pseudococcus viburni* (Signoret)		Millar et al. 2005a; Figadere et al. 2008	Hashimoto et al. 2008; Millar and Midland 2007

Passionvine mealybug, *Planococcus minor* (Maskell)		Ho et al. 2007	Millar 2008; Ho et al. 2007
Grape mealybug, *Pseudococcus maritimus* (Ehrhorn)		Figadere et al. 2007	Figadere et al. 2007
Japanese mealybug, *Planococcus kraunhiae* (Kuwana)		Sugie et al. (2008)	
Longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti)		Millar et al. (2009)	Millar et al. (2009)

their synthesis. All known mealybug pheromones are monoterpenoid esters, mostly of simple acids. However, most of them are irregular non-head-to-tail monoterpenoids, with unusual connections of two isoprene units (Millar and Midland 2007). The majority of naturally occurring isoprenoid compounds that have been identified have 1′–4, or head-to-tail, linkages between isoprenoid units, whereas most irregular terpenoids with non-head-to-tail linkages have been found in members of the plant family Asteraceae (Rivera et al. 2001). Non-head-to-tail isoprenoid compounds are produced in three biosynthetic reactions, that is, cyclopropanation, branching, and cyclobutanations (Thulasiram et al. 2008). Millar and Midland (2007) suggested that terpenoid biosynthetic pathways in mealybugs are distinctly different from the typical terpenoid pathways found in other organisms, representing a variety of enzymes that can catalyze novel cyclizations and rearrangements. On this basis, and considering that mealybugs endosymbionts are believed to be important to the nitrogen and sterol requirements of their hosts and may play a role in physiological processes such as resistance to microbial pathogens or detoxification of plant secondary compounds (see Section 3.2), we tent to speculate that these enzymes may originate, at least in part, from mealybug endosymbionts. Thus for example, a variety of symbionts associated with bark beetles are capable of producing compounds that are used as pheromones by their hosts (Hunt and Borden 1990).

3.5 Male Flight and Mate Location

In light of the pattern observed among the few species whose sex pheromones were identified (Table 1), mate location by mealybug males seems to rely mainly on chemical cues, that is, adult females of biparental mealybug species utilize sex pheromones to attract males (Dunkelblum 1999).

Few studies addressed the issue of male mealybug daily flight activity. Males of *Pl. citri*, *Pl. ficus* and *Ps. comstocki* (Kuwana) are morning fliers, whose flight begins just after sunrise (Moreno et al. 1972, 1984; Ortu and Delrio 1982; Silva et al. 2009; Tauber et al. 1985; Zada et al. 2008); males of *M. hirsutus* and *Nipaecoccus viridis* (Newstead) fly mainly around sunset (Francis et al. 2007; A. Protasov et al., unpublished data, 2007); and males of *Ps. calceolariae* fly both in early morning and late afternoon (Rotundo and Tremblay 1976). Moreno et al. (1984) suggested that the daily cycle of *Pl. citri* male flight activity is determined by the scotophase period and its onset in response to exposure to light. In light of the findings of Moreno et al. (1984) and O. Bar Shalom and Z. Mendel (unpublished data) an endogenous circadian rhythm, imprinted by the photoperiod, may also be involved.

Recently, Mendel et al. (2008) and Silva et al. (2009) studied seven mealybug species of the genera *Planococcus*, *Pseudococcus* and *Nipaecoccus*, to estimate for how long and at which physiological age the mealybug males are sexually active. Adult males take 30–40 h to achieve sexual maturity before being able to fly or to

mate. Most mature males live for 2–3 days, during which mating opportunities may be continuously available, but searching for a mate by flight is limited to 2–4 h per day. The finding, that mealybug males appear to fly only after exposure to daylight, suggests that visual cues also may be involved in male flight and mate location. Findings of recent experiments aimed at studying the effects of the color and design of sticky traps baited with sex pheromone on male captures support this hypothesis (Franco et al. 2008a; J.C. Franco and Z. Mendel, upublished data, 2008).

The lack of simple eyes in apterous males of polymorphic species of mealybugs, such as *S. sacchari* (Afifi 1968) is also indirect evidence that vision might be involved in male mealybug flight and mate location. As in other neococcoid families, mealybug males typically have a pair of dorsal and ventral simple eyes plus a pair of smaller lateral ocelli (Afifi 1968; Gullan and Kosztarab 1997). There is a lack of clear knowledge about the functional aspects of this bizarre visual system. Duelli (1985) suggested that the eyes in scale insect males are positioned in a horizontal ring around the head because the male's body axis is maintained almost vertical during flight. The main purpose of the eyes would be to monitor changes in the orientation of the body axis with respect to the horizon.

3.6 Defense System

Mealybugs developed several different defense mechanisms. Many of the species tend to establish themselves in protected sites, such as cracks and crevices in bark, leaf axils, root crowns, nodes of grass stems, under fruit sepals and within fruit navels, between touching fruits or fruits and leafs, and in tunnels bored by insect larvae in roots and stems (Franco et al. 2000; Kosztarab and Kozár 1988). This cryptic behavior of mealybugs may originate a spatial refuge from natural enemies and harsh environmental conditions (Berlinger and Golberg 1978; Gutierrez et al. 2008a). This type of plant colonization makes mealybugs practically invisible during the latent population phase. However, during outbreaks the population "boils over" from the refuge and becomes conspicuous.

The waxy secretion is the most common conspicuous trait of the mealybug family. It is a complex system that serves different functions, and which is produced by the epidermal wax glands and transported to the body surface via ducts, pores, and secretory setae of various types (Foldi 1983; Gullan and Kosztarab 1997). A. Zada et al. (upublished data, 2009) found that the main components of the wax of five mealybug species (*Pl. citri*, *Pl. ficus*, *Pl. vovae*, *Ps. cryptus*, and *N. viridis*) were trialkylglycerols and wax esters. The wax cover is believed to prevent water loss. The hydrophobic property of the wax enables the mealybugs to escape drowning or becoming swamped by water in their typical cryptic sites. The ovisac, which is also a wax secretion, is considered to be an adaptation that protects the offspring from both wet and dry conditions, and that may also provide an attachment to the host plant. Tubular ducts and multilocular disc pores, respectively, produce long hollow and shorter curled filaments, which

make up the ovisac and the male cocoon (Cox and Pearce 1983; Foldi 1983). The white wax of mealybugs is strongly light reflective, and may reduce desiccation. In some cases, the wax also serves to cover the honeydew droplets and to protect the mealybugs from contamination by their own honeydew and defensive exudates (Gullan and Kosztarab 1997).

The wax cover and the secretion process are involved in mealybug defense against natural enemies. It is hypothesized that the rarity of infestation by pathogens and nematodes is related to the wax shield. Stuart et al. (1997) found varied susceptibility of *Dysmicoccus vaccinii* Miller and Polavarapu to several nematode species; they showed that removal of the waxy coating from the mealybug did not influence their susceptibility to *Heterorhabditis bacteriophora* Poinar. The lateral wax protrusions protect the mealybugs from predators and facilitate spacing of individuals within the colony. The nymphs and adult females of most mealybugs possess two pairs of dorsal ostioles, located between the head and prothorax and on the sixth abdominal segment, that discharge a globule of liquid when the insect is disturbed. This waxy liquid solidifies quickly on contact with air and is believed to have a defensive function (Eisner and Silberglied 1988, Gullan and Kosztarab 1997). It was found, for example, that this discharge negatively affect *Sympherobius fallax* Navas (Neuroptera, Hemerobiidae) larvae (Gillani and Copland 1999), green lacewings (Neuroptera, Chrysopidae), and the parasitoid *Leptomastidea abnormis* (Girault) (Hymenoptera, Encyrtidae) (J.C. Franco, upublished data, 1999). Ostiolar secretions may have different functions in other mealybug species, for example, the highly developed condition of the dorsal ostioles in obligate ant-attended mealybugs suggests that the released fluid may attract the ants (Gullan and Kosztarab 1997).

Major parasitism in mealybugs involves members of the wasp family Encyrtidae. The encyrtids are koinobiont endoparasitoids, so that the parasitized mealybug continues to live for a few days, to grow and even to reproduce to some extent. This time gap between parasitization and deterioration of the physiological condition enables the mealybug to confront the immature individual parasitoid by encapsulation. The encapsulation is a common immune defense mechanism that involves the formation of a capsule around the parasitoid egg or larva; it is usually composed of host blood cells and the pigment melanin. The capsule may kill the parasitoid and thus prevent successful parasitism (Blumberg 1997). Various levels of encapsulation have been shown to occur in different mealybug species, in response to parasitism by encyrtids (Blumberg 1997; Blumberg et al. 1995, 2001; Blumberg and van Driesche 2001; Chong and Oetting 2007b; Giordanengo and Nenon 1990; Sagarra et al. 2000). Conversely, encyrtid parasitoids may use superparasitism as a strategy to overcome the immune response of unsuitable hosts (Blumberg et al. 2001). Besides superparasitism, other factors also affect the frequency of parasitoid encapsulation including: (a) host and parasitoid species; (b) the host's physiological age and condition; (c) the host and parasitoid origins (or strains); (d) the rearing and/or ambient temperature; and (e) the host plant species and stress conditions (Blumberg 1997; Blumberg et al. 2001; Calatayud et al. 2002).

3.7 Host Plants

The various species of plants and animals have characteristic climatic requirements for growth, survival and reproduction; requirements that limit their geographic distribution, abundance, and interactions with other species (Gutierrez et al. 2008b). Mealybugs feed on a variety of herbaceous and woody plants, including the angiosperm, gymnosperm and fern families. However, most of the species with known hosts develop on herbaceous plants, especially grasses (Poaceae) and composites (Asteraceae) (Ben-Dov 2006; Kosztarab and Kozár 1988).

As expected, information on the host ranges of mealybugs is mainly derived from observations of species of economic importance. Most species are apparently oligophagous or stenophagous (or monophagous); some are polyphagous (Ben-Dov 2006; Kosztarab and Kozár 1988). However, such a characterization is problematic. Most of the economically important species are known to be associated with long lists of hosts, and their performance varies widely, ranging from development of high population density, which eventually would kill the host plant, to poor development that renders the survival of the population for several generations questionable. Plant growth conditions may strongly affect the success of the population: under irrigation and fertilization plant species become favorable hosts of mealybugs, whereas in different environments the performance is usually poor. During laboratory studies many of the mealybug pest species easily could be reared on alternative hosts, such as potato sprouts or squashes, which are not colonized by mealybugs in the field. For example, the citrus mealybug has been found on plants from 70 botanical families, 60% of which are characterized as non-woody plants, whereas on the international scale this mealybug is a pest of subtropical and tropical crops, such as citrus (*Citrus* spp.), persimmon (*Diospyros kaki*), banana (*Musa paradisiaca*), and custard apple (*Annona* spp.), or it damages various types of plant species in interior landscapes, greenhouses in particular. Another example of the apparent contradiction between the long lists of host plants and the narrow ranges of damaged crops is the case of the citriculus mealybug, *Ps. cryptus*; although this mealybug is known from 35 host plant families (Ben-Dov 2006), in Israel it causes damage only to citrus trees. Under low pressure of natural enemies, for example, when they spread in new environments, mealybugs are observed on relatively large numbers of host plants, in contrast with the situation when there is effective biological control.

3.8 Overwintering

Many mealybugs overwinter as second-instar nymphs, although adult females, first-instar nymphs, and eggs also can fulfil this function (Miller 2005). For example, *Phenacoccus azaleae* Kuwana overwinters as a second-instar nymph within a wax cocoon (Xie et al. 1999); *Pl. vovae* as first and second instars (Francardi and

Covassi 1992); *Ps. viburni* as first instar in bark crevices, and rarely as second or third instars (Kosztarab 1996); and *Pseudococcus maritimus* (Ehrhorn) as eggs and first-instar under the bark (Geiger and Daane 2001).

3.9 Dispersal

Adult males and newly emerged first-instar nymphs, or crawlers, of most mealybug species display dispersal actively. Other nymphal stages and adult females may also move limited distances (Kosztarab and Kozár 1988) but, similarly to most scale insects, crawlers are the mealybugs' main dispersal agents. There is evidence that this developmental stage of scale insects is dispersed passively by the wind, and may be carried for distances of a few meters to several kilometers, or even more, from the natal plant–host, even though mortality is very high (Gullan and Kosztarab 1997). However, Williams and Granara de Willink (1992) reported that mealybugs were believed to be distributed by air currents over only short distances. As well as wind, water, bed-soil, humans, and domestic and wild animals may aid the passive dispersal of mealybugs (Kosztarab and Kozár 1988). Among arthropods, ants have also been reported to disperse some mealybug species (Gullan and Kosztarab 1997; Malsch et al. 2001; Ranjan 2006). Nevertheless, if conditions are favorable, crawlers usually settle on the natal host plant, often close to their mother, which leads to an aggregative distribution (Gullan and Kosztarab 1997; Nestel et al. 1995).

Many species of mealybugs have been widely distributed by commercial traffic, mostly carried on imported plant material (Williams and Granara de Willink 1992). Because of their cryptic habits and small size, mealybugs are difficult to detect at borders during quarantine inspections, especially if their population density on plants is low (Gullan and Martin 2003).

3.10 Population Trends and Seasonal Development

Most mealybug species are uni- or bivoltine, although some are reported to have as many as eight generations per annum in greenhouses. Despite having legs in all instars, most mealybugs remain relatively stationary throughout their life (Miller 2005). However, some species move to different areas of the host for overwintering, feeding, mating, ovipositing, and molting (Franco, 1994; McKenzie 1967; Miller 2005). The occurrence of seasonal movements within the host has been reported for various mealybug species, especially those associated with woody plants, such as: *Pl. citri, Ps. calceolariae, Ps. viburni*, and *Ps. longispinus* in citrus (Franco 1994; Nestel et al. 1995); *Pl. ficus* and *Ps. maritimus* in grapevine (Geiger and Daane 2001; Godfrey et al. 2003); *Pl. vovae* in *Juniperus* spp. (Francardi and Covassi 1992); and *Ph. azaleae* in bunge prickly

ash (Xie et al. 1999). Franco (1994) suggested that immature feeding stages of mealybugs on citrus tend to search for and to settle at the major "sinks", for example, growing fruits, of the host plant in each phenological period. We believe this hypothesis may also explain the migratory movements of other mealybug species within various hosts. For example, *Pl. kraunhiae* Kuwana was reported to feed on bacterium galls, because they are sinks for assimilates and have more nutritious phloem sap and parenchyma than do normal plant tissues (Yamazaki and Sugiura 2005).

3.11 Mealybug Relationships with Ants

Ants are often associated with mealybugs as honeydew consumers. Hemiptera-tending ants are mostly species of the subfamilies Myrmicinae, Dolichoderinae, and Formicinae (Degen and Gersani 1989; Mittler and Douglas 2003). Samways et al. (1982) reported that 11% of the 123 ant species identified in citrus orchards in South Africa were associated with mealybugs. Some mealybugs have an obligatory association with ants: all Southeast Asian myrmecophilous mealybugs have been collected only with ants of the genera *Acropyga*, *Dolichoderus*, or *Polyrhachis*, which attend the mealybugs either in subterranean nests or on aerial plant parts (Gullan and Kosztarab 1997). Above ground nests were also observed on grapevine in Europe in association with *Phenacoccus aceris* (Signoret) (Sforza 2008a).

Access to honeydew has been shown to enhance the rate of increase of ant colonies. Honeydew accounts for more than half of the diet of many temperate wood ants (*Formica* spp.), and is the dominant food source of some subterranean ants (Mittler and Douglas 2003). Some ant species may switch between tending and preying on mealybugs (Degen and Gersani 1989; Mittler and Douglas 2003; Way 1963). The tended mealybugs benefit from the protection against natural enemies, and the removal of honeydew prevents contamination, which may be especially detrimental to first-instar nymphs (Cudjoe et al. 1993; Daane et al. 2006b, 2007; Gullan and Kosztarab 1997; Moreno et al. 1987). The disruption of the activity of natural enemies by ants provides a temporal refuge for mealybugs (Gutierrez et al. 2008a), but some mealybug predators, such as coccinellids, apparently become tolerated by ants by mimicking the waxy body cover of the mealybugs (Daane et al. 2007). Besides the ants' impact on the mealybugs' natural enemies, tending by the ants may have other effects that alter mealybug densities: it may also improve the mealybugs' habitat or fitness (Daane et al. 2007); in the presence of ants, mealybugs are able to ingest larger quantities of sap (Degen and Gersani 1989); and some ant species actively construct shelters for mealybugs which provide some protection from unfavorable environments and natural enemies (Franco et al. 2000; Helms and Vinson 2002; McLeod et al. 2002).

Recent findings suggested that associations among invasive species of ants and mealybugs can be important in their success in new locations (Helms and Vinson

2003). The Argentine ant, *Linepithema humile* (Mayr) is an example of an invasive ant species that is a significant pest in both natural and managed habitats, and is commonly associated with mealybug outbreaks (Daane et al. 2006b, 2007; Silverman and Brightwell 2008).

3.12 Associated Pests

Published information about pests associated with mealybugs is rather limited; it mostly comes from studies and observation of three mealybug species that occur on fruits trees and vineyards in Mediterranean countries. All the associates are fruit moths attracted to the honeydew produced by the mealybugs; they lay their eggs in the vicinity of the mealybug colonies, near plant parts contaminated with sooty mold, and the larvae of some species bore into the peel of fruits whereas others excavate into the branch cortex or the wood. The honeydew moth, *Cryptoblabes gnidiella* Mill. (Lepidoptera: Phycitidae) is the most common one; it occurs in citrus orchards, on fruits infested by the citrus mealybug, *Pl. citri*, the citriculus mealybug, *Ps. cryptus* and, to a lesser extent, near infestations of the spherical mealybug, *N. viridis* (Avidov and Harpaz 1969, Franco et al. 2000, Silva and Mexia 1999a, b; Z. Mendel, unpublished data), with the long-tail mealybug, *Ps. longispinus* on avocado fruits (Swirski et al. 1980), and with the vine mealybug, *Pl. ficus* (Zehavi 2005). However, unlike the situation in citrus and avocado plantations, the occurrence of this moth is not obligated to the presence of the vine mealybug in the bunches. The carob moth *Spectrobates* (=*Ectomyelois*) *ceratoniae* (Zeller) (Lepidoptera: Pyralidae) is a polyphagous pest of various fruits. It usually injures citrus and persimmon fruit peels, under and near the sepals, and also the navel region in navel citrus varieties, in the presence of honeydew or sooty mold of the citrus mealybug (Franco et al. 2000; Gothilf 1964; Serghiou 1983; S. Ben Yehuda, personal communication, 2009), and has been found on pomegranate fruits infested with the vine mealybug in Turkey (N. Demirel, personal communication, 2009). *Lamoria* sp. (Lepidoptera: Pyralidae) (Harari et al. 2006) and *Paropta paradoxus* (H.-S.) (Lepidoptera: Cossidae) (Z. Mendel et al., unpublished data) are attracted to honeydew of the vine mealybug that infests the stem and arms of the vine: *Lamoria* sp. excavates the cortex, while *P. paradoxus* bores deep in the inner wood.

The population density of the associated moths is unrelated to that of the mealybug, although citrus or persimmon fruits which are not infested by the mealybug will not be colonized by the moth. However, the damage inflicted on citrus groves in Israel by the honeydew moth and the carob moth may be severe although the citrus mealybug infestation would be tolerated in the absence of the moths. Control of these associates presents problems because of lack of appropriate insecticides that suit the management practices applied to subtropical fruits. Because the outbreaks are sporadic the growers rarely monitor the occurrence of the fruit moths.

4 The Origin of Mealybug Pest Status

Similarly to other insect pests, mealybugs have diverse origins, including endemics, immigrants, and mutants (Kim 1993). An indigenous species may become a serious pest: when a susceptible crop species is introduced into the area; following environmental disturbance; or as a result of stress conditions. Invasive mealybug species may attain pest status as soon as they successfully colonize a new territory, and impact negatively crop yield, which may happen when they encounter a susceptible host, either local or exotic.

The citrus mealybug, *Pl. citri*, is an introduced pest in most citrus-growing areas of the globe. It may weaken young citrus saplings, but barely affects the growth of fruit-bearing trees; the damage is mainly due to fruit infestation. Two mealybug population trends were shown to occur in citrus orchards in the Mediterranean region (Franco et al. 2004b): (i) outbreak dynamics, whereby the percentage of infested fruits (mainly by *Pl. citri*) typically increases exponentially, with maximal values higher than 30% being recorded during mid to late summer; and (ii) non-outbreak dynamics, whereby the percentage of infested fruits does not increase significantly or, alternatively, exhibits only a small, relatively linear increase, with maximal values lower than 30%.

Three major causes may lead to mealybug outbreaks: (I) a recent invasion by an exotic mealybug species; (II) the application of non-selective pesticides, which disrupt the biological balance; and (III) the effect of environmental factors that might influence the tritrophic interactions among host–plant/mealybug/natural enemies.

These were subdivided by Franco et al. (2004b) as follows:

I. Recent invasion by exotic mealybug species
   i) Lack of control by natural enemies

II. Application of non-selective pesticides
   i) Mortality differences between pests and their natural enemies
   ii) Indirect effects of pesticides on natural enemies, for example, elimination of their prey
   iii) Effects on predator and parasitoid host interactions
   iv) *Trophobiosis* – positive indirect effects of pesticides on pests, mediated through changes in the host plant
   v) *Hormoligosis* – positive direct effect of pesticides on pests
   vi) Effects of pesticides on insect behavior
   vii) Effects of pesticides on interspecific competition among phytophagous species of different taxa

III. Effect of environmental factors (tritrophic interactions: host–plant/mealybug/natural enemy)
   i) Host–plant susceptibility and/or host–plant characteristics
   ii) Water stress
   iii) Nitrogen fertilization

iv) Weather
v) Mealybug defenses, for example, encapsulation
vi) Mealybug refuges from natural enemies
  – Spatial refuge (cryptic behavior), for example, under the bark and on roots
  – Temporal refuge: ant interactions
vii) Other factors that may affect natural enemies, for example intraguild predation and interference, hyperparasitoids

Cause I is well documented with regard to mealybug outbreaks, and is mainly driven by the combination of host susceptibility and absence of natural enemies in the invaded region (Ben-Dov 1994; Blumberg et al. 1999; Muniappan et al. 2006; Nakahira and Arakawa 2006; Roltsch et al. 2006; Williams and Granara de Willink 1992). The use of non-selective pesticides (Cause II) may lead to resurgence and secondary outbreaks. The mechanisms involved in these two types of outbreaks were discussed by Hardin et al. (1995), and studied by Franco et al. (2004b) with regard to the mealybug pests of citrus. Environmental factors (Cause III) may also directly and indirectly affect the tritrophic interactions that develop between mealybugs, their host plants and their natural enemies and thereby initiate mealybug outbreaks. Several mechanisms may be involved. Host–plant characteristics may favor or be detrimental to the development, reproduction and survival of mealybugs (Boavida and Neuenschwander 1995; Calatayud et al. 1994b; Leru and Tertuliano 1993; Nassar 2007; Tertuliano et al. 1993; Wysoki et al. 1977; Yang and Sadof 1995). The resistance mechanisms of the host plant may become involved in both the fixation (antixenosis) and the development of the mealybug (antibiosis) (Tertuliano et al. 1993). Tertuliano and Leru (1992) concluded that the different levels of resistance to the cassava mealybug, *P. manhioti*, that were observed in different varieties of cassava, were not associated with the concentrations of amino acids or sugars, with the ratios between these concentrations, or with the compositions of amino acids obtained from leaf extracts. The identification and assay of cyanogenic and phenolic compounds in the phloem sap of cassava and the honeydew of the cassava mealybug were carried out by Calatayud et al. (1994a). The best correlation between antibiotic, resistance and secondary compounds analyzed was observed for the rutin contents of infested plants. Yang and Sadof (1995) showed that variegation in *Coleus blumei* could increase the abundance of the citrus mealybug, *Pl. citri*. Sadof et al. (2003) found that the life history characteristics of *P. citri* on *Coleus blumei* were not correlated with total amino acids and sucrose contents in stem exudates, and were correlated negatively with the proportions of shikimic acid precursors and positively with those of other nonessential amino acids. Host–plant characteristics can also influence the performance of the natural enemies of mealybugs (Serrano and Lapointe 2002; Souissi 1999; Souissi and LeRu 1997; Yang and Sadof 1997).

Water stressed plants may favor population increases of mealybugs (Calatayud et al. 2002; Gutierrez et al. 1993; Lunderstadt 1998; Shrewsbury et al. 2004).

Mealybug life history parameters may also be influenced by the levels of nitrogen fertilization and leaf nitrogen concentration; high nitrogen concentrations were shown to lead to enhanced performance of the citrus mealybug, *P. citri* (Hogendorp et al. 2006). The antibiotic resistance of two varieties of cassava to the cassava mealybug increased with the addition of nitrogen (Leru et al. 1994). Survival of immature sugarcane mealybugs, *S. sacchari*, increased to a maximum at a soluble nitrogen concentration of 320 mg L^{-1} in sugarcane, and decreased at higher levels, whereas mealybug size increased with increasing nitrogen concentration over the whole tested range (Rae and Jones 1992).

Weather conditions, especially temperature and relative humidity, are major ecological factors that affect both mealybugs and their natural enemies (Chong and Oetting 2006; Chong et al. 2004, 2005; Daane et al. 2004; Gutierrez et al. 1993, 2008a; Kontodimas et al. 2004; Nakahira and Arakawa 2006; Walton and Pringle 2005).

Encapsulation may adversely affect the degree of biological control exerted by mealybug parasitoids, as it may either prevent the establishment of exotic parasitoids in new regions or reduce parasitoid efficacy (Blumberg 1997).

As already mentioned (see Section 3.6), the cryptic behavior of mealybugs, and tending of mealybugs by ants may, respectively, originate spatial and temporal refuges from natural enemies.

Several other factors may affect mealybugs' natural enemies; they include intraguild predation and interference (Chong and Oetting 2007a), and hyperparasitoids (Moore and Cross 1992), the latter factor was never proved to significantly impair biological control of mealybugs.

5 Actual Management Tactics

5.1 Biological Control

5.1.1 Natural Enemies of Mealybugs and Other Associated Arthropods

Mealybugs have many natural enemies, including parasitic wasps, arthropod predators and entomopathogenic fungi. However, parasitoid encyrtids (Hymenoptera, Encyrtidae) and predatory lady beetles (Coleoptera, Coccinellidae) are the most common natural enemies of mealybugs.

Mealybug-parasitizing encyrtids are primary endoparasitoids, most of them undergo solitary development. Their host specificity is not a clear issue. Many of the more studied parasitoids were collected from long lists of mealybug hosts (e.g., over 20 host species). This impression of the host range is derived from the ability of the parasitoids, in certain circumstances, to exploit unsuitable host species, and from misidentification of the true host in mixed-host colonies. Stable and effective parasitoid populations are usually restricted to a few congeneric mealybug species. For example, *Anagyrus pseudococci* (Girault) was obtained from mealybugs of

various genera (Noyes and Hayat 1994), however, only the vine mealybug and the citrus mealybug may be considered to be its principle hosts. Franco et al. (2000) compiled from published literature about 70 encyrtid parasitoid species that were reared from the citrus mealybug, whereas only four species are considered to be principle parasitoids of this mealybug. *Coccidoxenoides, Gyranusoidea, Leptomastidea, Leptomastix, Pseudaphycus*, and *Tetracnemoidea* are examples of encyrtid genera of mealybug parasitoids (Charles 1993; Franco et al. 2000; Noyes and Hayat 1994; Rosen 1981).

Coccinellids accept a wide range of food, but they complete larval development and produce viable progeny only if they consume their 'essential food'. Four genera of Chilocorinae (*Brumus*, *Aspidimerus*, *Stictobura* and *Orcus*) and five of Scymninae (*Diomus*, *Nephus*, *Sidis*, *Parasidis*, *Cryptolaemus* and *Pseudoscymnus*) prey preferentially on mealybugs (Iperti 1999). Other important groups of predators are brown lacewings (Neuroptera; Hemerobiidae) and predatory gallmidges (Diptera; Cecidomyiidae).

As sap feeders, mealybugs are not likely to be exposed to viral or bacterial infections (Moore 1988) and only a few species of entomopathogenic fungi were reported to be associated with mealybugs and confirmed to be pathogenic; they include *Aspergillus parasiticus* Speare, *Cladosporium oxysporum* Berk. and M.A. Curtis, *Hirsutella sphaerospora* H.C. Evans and Samson, and *Neozygites fumosa* (Speare) Remaudière and Keller (Browning 1994; Delalibera et al. 1997; Leru 1986; Moore 1988; Samways and Grech 1986).

During mealybug outbreaks, their abundant colonies serve as food sources for large numbers of predators and associates. Thus, for example, the arthropod fauna in Africa known to be directly and indirectly associated with the cassava mealybug *P. manihoti*, an invasive species from South America, consisted of about 130 species and 11 guilds (Neuenschwander et al. 1987). Usually, when the mealybug population is kept down only the few principle natural enemies and some species of attending ants are observed.

Four groups of associates deserve more attention: the associated pests (see Section 3.12), associated ants (see Section 3.11), associated general predators and hyperparasitoids. The role of the general predators in population trends of mealybug populations has hardly been investigated, although some of these predators may occur in high densities, for example *Dicrodiplosis* spp. (Cecidomyiidae) or *Nephus* spp. (Coccinellidae) in colonies of the citrus mealybug in Mediterranean citrus groves. Unlike specific parasitoids that continue to exist even when a rather low host density is maintained in a small area, predator populations need higher mealybug populations and, therefore, survive on relatively large areas by following the occurrence of mealybug "hot spots". The role of hyperparasitoids in the population dynamics of mealybugs is also not clear. Many of the mealybug hyperparasitoids belong to the Aphelinidae and many of the species may attack parasitized hosts of other scale insect families, and even hosts that belong to different insect orders. Thus, for example, local hyperparasitoids may easily attack exotic primary parasitoids that were introduced as biological control agents (e.g. Neuenschwander and Hammond 2006).

5.1.2 Classical Biological Control

Biological control of mealybugs has been practiced for many years; it involves three main tactics, that is, classical biological control, augmentative releases, and conservation biological control. However, since the major mealybug pests are invasive species, classical biological control has been the major control tactic.

Moore (1988) reviewed the natural enemies used against mealybugs in biological control programs worldwide. According to him, more than 70 species of parasitoids have been introduced against mealybugs, and at least 16% of the introductions were considered to initiate substantial or complete control. Most of the introduced parasitoid species were encyrtids, but species of Aphelinidae and Platygasteridae proved to be successful on several occasions. Often a single parasitoid was considered to be responsible for the success, even when more than one was introduced. Noyes and Hayat (1994) reviewed the use of encyrtids for biological control of pest mealybugs, and found that out of a total of 385 importations of encyrtids, targeting 22 mealybug species, about 24 and 7% were considered to give partial or successful control in the field and in greenhouses, respectively.

With regard to predators, Moore (1988) analyzed the use of *C. montrouzieri* separately from that of other mealybug predators. This lady beetle has been used many times against at least 10 different species of mealybugs and was considered to give substantial or partial control in about 19% of the introductions; on some occasions it has been regarded as an outstanding biological control success. Of the other 46 predator species – mostly coccinellids, but also cecidomyiids, chrysopids, hemerobiids and lycanids – used in biological control of mealybugs, only the cecidomyiid *Kalodiplosis pseudococci* Felt was regarded as having given significant control, when used against *Dysmicoccus brevipes* (Cockerell) in Hawaii in conjunction with two parasitoids.

Stiling (1993) in his analysis why in biological control campaigns, some introduced enemies fail to reduce pest populations substantially, showed that the major reason given for failure is related to climate (34.5%). Moore (1988) analyzed the reasons for the failures of both parasitoids and predators of mealybugs to become established in biological control programs. In the case of parasitoids he cites the following documented reasons: (i) incorrect identification of the target mealybug species; (ii) the target was a native species; (iii) hyperparasitism; (iv) failure of the parasitoid to adapt to unfavorable climates; and (v) other reasons, such as interference with ants, use of pesticides, and small numbers of individuals released. With regard to predators, Moore (1988) listed six main reasons for failure: (i) no adaptation of the released species to climate; (ii) effect of pesticides; (iii) density of the prey; (iv) effect of the host plant; (v) inability to reach the prey; and (vi) effects of other organisms.

The lack of adequate food resources for natural enemies within or near to agro-ecosystems may limit the performance of biological control agents against mealybugs. For example, Davies et al. (2004) observed that the survival and reproduction of *Coccidoxenoides perminutus* Girault, a parasitoid of the citrus mealybug, *Pl. citri*, was significantly influenced by the nature of the nectar on

which the parasitoid was fed. In light of these results, it was suggested that habitat management, for example, by providing suitable nectar sources for adult parasitoids, might be a means to conserve and enhance *C. perminutus* activity in the field.

In recent years successful classical biological control programs against mealybugs have targeted the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero, in Africa (Neuenschwander 2001), the mango mealybug, *Rastrococcus invadens* (Williams) in West Africa (Bokonon-Ganta et al. 2002), the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) in the Caribbean and California (Roltsch et al. 2006), and the papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink, in Palau (Muniappan et al. 2006). It is important to note that successes were mostly achieved in tropical regions where the target area for classical biological control and the area of origin of the introduced parasitoids displayed similar climatic conditions.

In a few cases modeling has been used as a tool to analyze actual systems and to identify major constraints, in order to improve biological control of mealybugs. For example, the model developed by Gutierrez et al. (2008a) predicted that the parasitoid *A. pseudococci* would have a larger impact on the vine mealybug, *P. ficus* than either *L. abnormis* or *C. montrouzieri*, and that biological control of the mealybug in California would require additional species of natural enemies and/or could be achieved by reducing the size of the spatial-temporal refuge. In another use of a modeling approach, Gutierrez et al. (2008b) concluded that biological control of the vine mealybug might be adversely affected by climate change. Gutierrez et al. (1993) developed a tritrophic model of the cassava system, and used it to explore the basis for the successful control of cassava mealybug, *P. manihoti* in Africa by the exotic parasitoid *Epidinocarsis lopezi* (DeSantis), and also to examine the causes for the failure of the related parasitoid *E. diversicornis* (Howard) to establish itself.

5.1.3 Augmentative Control Tactics

The first known case of an augmentative biological control program dates back to before 1917 and was aimed at controlling the citrophilus mealybug, *Ps. calceolariae*, a pest of citrus in Southern California, by using the coccinellid predator *C. montrouzieri* (Luck and Forster 2003; van Lenteren 2006). Since then, this Australian ladybird beetle has been commonly used in various countries on diverse crops (Copland et al. 1985; Franco et al. 2004b), and is actually one of the few species of natural enemies commercially available for biological control of mealybugs by means of augmentative tactics (Table 2). Augmentative releases of *L. dactylopii* and *C. montrouzieri* against *Pl. citri* have been reported to be effective in several Mediterranean countries, and in other citrus-growing areas, such as Australia and California. However, Mendel et al. (1999) released 5,000–10,000 individuals of *L. dactylopii* or 10,000–50,000 of *A. pseudococci* per hectare and obtained no significant impact on either the mealybug infestation or on fruit damage.

Table 2 Species of natural enemies commercially available (yr. 2008) for biological control of mealybugs using augmentative tactics, and respective number of producing companies (Biobest; Syngenta; Koppert; Bio-Bee; BCP-Certis; Bioplanet; BioPlant; WyeBugs; Entocare)

Family/species	Number of producing companies
Coccinellidae	
Cryptolaemus montrouzieri Mulsant	9
Encyrtidae	
Anagyrus pseudococci (Girault)	3
Anagyrus fusciventris (Girault)	2
Coccidoxenoides perminutus (Girault)	1
Leptomastix dactylopii (Howard)	6
Leptomastix epona (Walker)	4
Leptomastidea abnormis (Girault)	2
Pseudaphycus maculipennis (Mercet)	1

5.2 Pheromone-Based Management Tactics

Sex pheromones of insects, including mealybugs, are natural compounds emitted by virgin females in order to attract conspecific males for mating. The sex pheromones are effective in extremely small quantities; they are non-toxic and can be applied in various ways. Unlike pesticides, these chemicals are species-specific and do not affect beneficial insects. The behavioral impacts of the semiochemicals are limited to the target pest organisms.

The potential of mealybug sex pheromones as an alternative and ecologically friendly means for monitoring and control is important and promising.

Sex pheromones are used in lures for monitoring, for detection of outbreaks, and for population management. Monitoring systems provide vital information for the timing of insecticide applications. Population levels can be reduced or controlled by mass trapping, mating disruption, or lure and kill. The success of these methods depends on the availability of the pheromone, and on an appropriate formulation and deployment. In contrast to the extensive use of sex pheromones in controlling beetle and moth pests, sex pheromones are not yet employed in control operation of scale insects.

5.2.1 Pheromone Production

Pheromones must be isolated, identified and synthesized before any basic or practical studies can be performed. Entomologists and chemists must cooperate closely in order to achieve these goals.

Despite the availability of modern analytical equipment, the identification of natural mealybug sex pheromones remains a difficult and laborious task. Mealybugs are small or tiny insects that release minute quantities of pheromone,

therefore large numbers must be reared and often tedious separation of virgin females must be done to collect sufficient amounts of pheromone for isolation and identification. Males having a short lifespan of at most a few days are required for bioassay, either by attraction tests or by GC-EAG (gas-chromatography-electroantennography). All known mealybug pheromones are monoterpenoid esters, mostly of simple acids (Table 1). Unlike moth sex pheromones, the mealybug pheromones are not homologous compounds; their structures vary significantly (Table 1), and three types of structures have been found so far: open chain esters, cyclobutane derivatives, and cyclopentane rings. All the mealybug pheromones, except the *Pl. minor* and *Pl. kraunhiae* pheromones, are chiral compounds. Generally, enantioselective synthesis of chiral compounds is much more complicated and expensive than that of racemic compounds but, fortunately, racemic pheromones can be used because the unnatural stereoisomers have no behavioral effect and, therefore, are benign (see e.g., Zada et al. 2008). A unique case is the pheromone of *M. hirsutus*; it contains a chiral acid function that must have the correct chirality for biological activity (Zhang and Amalin 2005; Zhang et al. 2006). The passionvine mealybug is strongly inhibited by the (Z)-stereoisomer of its pheromone, suggesting that this compound may be the pheromone of a related sympatric species (Millar 2008).

Unlike moths and beetles, which are generally sensitive to isomers (structural and chemical) of their pheromone components, different species of mealybugs do not associate pheromone components neither stereoisomers of a component released by another species. In practice, this means that use a mixture of isomers of the pheromone will be effective for control of most of the mealybug pheromones. Moreover, mealybugs are responsive to small amounts (doses of about 1–dozens μg) of the pheromones (Millar at al. 2005b; Zhang and Amalin 2005, Sugie et al. 2008), so that potentially it is possible to achieve pheromone-based control at relatively low costs.

Neat mealybug sex pheromones are not commercially available. In fact, most of them, except for those of the citrus mealybug and the vine mealybug, are synthesized only for research in small (milligram) quantities. The citrus mealybug pheromone, for example, which has a rather complex structure, has been synthesized via a variety of routes (see Table 1), but it still is not available in the large quantities (hundreds of grams) required for mating disruption. At present, only commercial lures for monitoring are available. Because of the worldwide economic importance of the pest, there is a need to improve the efficiency of pheromone synthesis and to make the pheromone available for control application. A series of analogs of this pheromone was prepared, in order to find a less expensive attractant (Liu et al. 1995; Dunkelblum et al. 1987), but most of them were insufficiently attractive, except for a homolog in which a cyclobutaneethanol moiety replaced the cyclobutanemethanol moiety in the natural pheromone (Fig. 1). The homolog displayed about 40% attractiveness as compared with the pheromone, and in some field tests it was as active as the latter (Dunkelblum et al. 1987; A. Zada et al., unpublished data). The advantage of the homolog is that its synthesis is easier and less expensive than that of the pheromone.

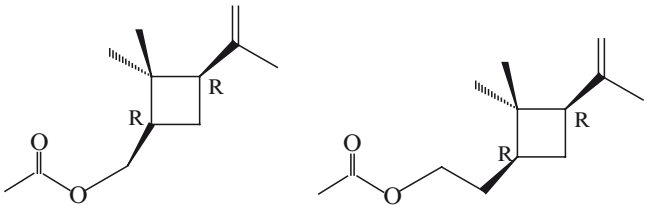


Fig. 1 *Planococcus citri* pheromone and its homolog

Some pheromone analogs of the Comstock mealybug, *Pseudococcus comstocki* Kuwana, were also synthesized and tested in the field (Uchida et al. 1981; Bierl-Leonhardt et al. 1982). In this case, the analogs were twice to seven times less active than the pheromone.

5.2.2 Pheromone Traps and Monitoring

Sampling is a key element of mealybug management, because of the need for real-time information on the mealybug population and the potential damage. Mealybug monitoring methods involve examination of specific plant parts for live individuals, and detection of honeydew, sooty mold, or ant activity (Franco et al. 2004b; Millar et al. 2005a). Sampling procedures have been developed for several mealybug species and various crops, such as the citrus mealybug, *Pl. citri* (Martinez-Ferrer et al. 2006), the grape mealybug, *Ps. maritimus* (Geiger and Daane 2001), or the sugarcane mealybug, *S. sacchari* (Allsopp 1991; Debarro 1991). However, the cryptic occurrences of mealybugs as well as their typical clumped spatial distribution (Allsopp 1991; Martinez-Ferrer et al. 2006; Nestel et al. 1995) make monitoring laborious and often impracticable. Population estimates based on the level of male capture in pheromone-baited traps is considered more convenient (Millar et al. 2005a). Much work has been done to optimize these sampling methods, especially in relation to trap design, trap color, type of dispenser, pheromone dose, and bait longevity and range (Francis et al. 2007; Franco et al. 2004a, 2008a; Millar et al. 2002; Vitullo et al. 2007; Walton et al. 2004; Zada et al. 2004, 2008). Nevertheless, the use of pheromone traps as a monitoring tool for mealybug damage risk assessment depends on the existence of a reasonable relationship between the number of males captured in pheromone-baited traps and other mealybug infestation parameters, as recorded by other means, usually, visual sampling. A linear relationship was found to exist for the vine mealybug, *Pl. ficus* (Walton et al. 2004), and the citrus mealybug, *Pl. citri* (Franco et al. 2001). But this correlation may be affected by different factors, including the weather, the activity of natural enemies and the phenological gap between male captures and infestation level (J.C. Franco et al. 2001, 2008, 2008a).

5.2.3 Mass Trapping

A 2-year study of mass trapping of *Pl. citri* males was recently carried out in small citrus plots in Israel and Portugal (Franco et al. 2004a). Sticky plate traps (30 cm × 30 cm) baited with 200 μg of pheromone were used at a rate of one per tree. The results indicate that a significant reduction of male numbers can be achieved by mass trapping, but the reduction obtained with the experimental design was not enough to reduce fruit infestation significantly. Therefore, since the pheromone trapping system used cannot reduce the number of attracted males effectively, it is most likely that many of them originated from outside the subplot. In fact, males are attracted to the pheromone source from ranges up to at least 100 m (Branco et al. 2006; Franco et al. 2004a). On the other hand, the higher level of mating observed in mass-trapping plots early in the spring, when the mealybug density is usually very low, suggested that mass-trapping led to a strong attraction of males from outside the subplots. In light of this finding, it was postulated that early in the season, when the male population is usually low, the attraction of males to the edge of the orchard by using attract-annihilate tactics combined creates a 'male vacuum' inside the plot and, consequently, reduces mating and infestation (Franco et al. 2004a).

5.2.4 Mating Disruption

Mating disruption seems to be more advantageous in mealybugs than in Lepidoptera since mealybug females are sessile and cannot migrate from one area to another as moths do. On the other hand, mating disruption of mealybug pests presents problems, especially because the complex structure of the pheromones prevents large scale synthesis. The vine mealybug pheromone is the only mealybug pheromone that can readily be synthesized in one step from two commercial starting materials, so that it can be prepared in large quantities, sufficient for field work, including mating disruption (Ujita and Saeki 2008; Walton et al. 2006). Walton et al. (2006) applied the pheromone to the leaves as a sprayable microcapsule formulation. Crop damage in that case was reduced from 9–11% in control plots to 3–4% in treated plots, but the effective life of the formulation presents a technical problem that needs to be solved; the efficiency of the pheromone formulation in the field declined after 3 weeks, so that more than four applications per season were needed. The proximity of the mealybug sexes on emergence may also impair the success of mating disruption.

5.2.5 Kairomonal Response

The sex pheromone of mealybugs may be used by their natural enemies as a kairomonal cue in host or prey selection. Millar et al. (2002) suggested that *A. pseudococci* (most likely *Anagyrus* spec. nov. near *pseudococci*) in California vineyards

was attracted to the pheromone of *Pl. ficus*. Franco et al. (2008c) showed, in light of both field and olfactometer experiments, that the females of the encyrtid *Anagyrus* spec. nov. near *pseudococci*, a major parasitoid of both the vine mealybug, *Pl. ficus* and the citrus mealybug, *Pl. citri* were attracted to the sex pheromone of *Pl. ficus* but not to that of *Pl. citri*, and that this kairomonal response was an innate behavior trait. This host–parasitoid relationship has been further investigated, and preliminary data from field trials in which sentinel mealybugs on sprouted potatoes were exposed in citrus orchards, suggested that the presence of *Pl. ficus* sex pheromone significantly increases the parasitization rate of *Pl. citri* colonies by *Anagyrus* spec. nov. near *pseudococci* (Franco et al. 2008b). Similar kairomonal responses have been suggested in a few other parasitoid species of mealybugs. Rotundo and Tremblay (1975) reported that traps baited with virgin females of *Ps. calceolariae* captured significant numbers of the encyrtid *Tetracnemoidea peregrina* (Compere) (=*Arhopoideus peregrinus*). A kairomonal response of the encyrtid *Pseudaphycus maculipennis* Mercet to the sex pheromone of the obscure mealybug, *Ps. viburni* was also observed in field experiments with pheromone traps (Bell et al. 2006 2008). Two species of mealybug parasitoids were caught in traps baited with the sex pheromone of *Ps. cryptus* in a citrus orchard in Japan (Arai 2002).

We postulate that the use of the host sex pheromone as a kairomonal cue in host location by mealybug parasitoids may reflect the general behavior of parasitoid species that attack third instar nymphs and young adult female mealybugs, and that are major natural enemies in the region of origin of their mealybug hosts. The sex pheromone emitted by mealybug virgin females provides reliable information on the location of a potential host for mealybug parasitoids, because of the sedentary nature of mealybugs. Furthermore, because of the typical clumped spatial pattern of mealybugs, the sex pheromone will also be a convenient chemical cue by which the parasitoid can efficiently locate aggregates (colonies) of hosts, which are expected to emit a stronger pheromonal signal than that of single virgin females (functional response). If our hypothesis is correct, then the sex pheromones of mealybugs could serve as a novel and efficient tool to support the classical biological control of invasive mealybug species, by identifying, in the region of origin of the target species, parasitoids that could be potential candidates for use in the biological control program.

The kairomonal response of a parasitoid to the sex pheromone of its host mealybug could impair its practical use for mealybug pest management by mass trapping and lure-and-kill tactics (Franco et al. 2008c). This side effect may be avoided by using pheromone analogs that lack kairomonal activity but that still preserve the pheromonal attractiveness to the males, as was successfully accomplished for other scale insects, for example, *Matsucoccus* spp. (Mendel et al. 2003). Optimizing the devices used in these tactics, for example, with regard to design and color, could also minimize the negative impact on natural enemies of the pests (Franco et al. 2008c). The results obtained by Walton et al. (2006) with pheromone-based mating disruption of vine mealybug indicated that the treatment had no negative effect on the level of parasitization of *Pl. ficus* by *A. pseudococci*. Franco et al. (2008c)

suggested that the kairomonal response of *Anagyrus* spec. nov. near *pseudococci* could be explored in connection with biological control tactics, by enhancing parasitization of *Pl. citri* as a component of integrated pest management strategies, by means of a similar approach to that used against aphid pests (Powell and Pickett 2003). The use of semiochemicals for enhancing the effectiveness of biological control tactics against pest mealybugs offers a potential novel approach that we think is worth further investigation and exploration.

5.3 Chemical Control

There are great similarities among the insecticide arsenals used to control mealybug species on different crops. In principal, three main modes of insecticide application are adopted: (i) foliage cover spraying for management of above-ground populations; (ii) application of insecticide solution to the soil to enable it to penetrate to the root zone, so as to combat subterranean colonies; and (iii) chemigation by application of systemic compounds via the irrigation system, for example, drip irrigation. Systemic insecticides are also used against mealybugs by smearing them on the stem or main branches. Two other, less common, techniques are fumigation, usually applied for eradication, for example, with methyl bromide, or slow-release strips to prevent colonization.

Organophosphates – such as chlorpyrifos, acephate, dichlorvos and diazinon – and, to a lesser extent, carbamates – such as aminocarb, carbaryl, thiodicarb or methomyl – are broad-spectrum nerve insecticides which have been used against mealybugs that colonize the plant canopy since the early 1960s (e.g., de Souza et al. 2007; Gonzalez et al. 2001; Shafqat et al. 2007). These insecticides when applied in high volume could successfully overcome the obstacles that make mealybugs hard to kill: (i) their hydrophobic wax cover, which repels hydrophilic insecticides; (ii) their tendency to feed in hidden and protected parts of the plant; (iii) their typically dense colonies; and (iv) the frequent overlapping of generations. Effective control is achieved when most of the mealybug population is in the dispersive crawler stage or the young nymphal instars, and when the host plant does not provide effective shelter. However, satisfactory control is often difficult to achieve over an extended period. These chemicals have detrimental effects on the environment as a whole, and on natural enemies in particular (Anand and Ayub 2000; Babu and Ramanamurthy 1998; Meyerdirk et al. 1982). The multivoltinous character of pest mealybugs, and the frequent application of inefficient control measures accelerate the development of insecticide resistance (Flaherty et al. 1982). Systemic organophosphates such as dimethoate could overcome some of these obstacles (Grout and Stephen 2005; Meyerdirk et al. 1982; Prasad et al. 1998). Pyrethrins and rotenone replaced these compounds in organic agriculture with limited effectiveness. Chlorpyrifos-impregnated strips are applied to protect banana bunches from mealybug infestation or as stem barriers for the control of ants (Addison 2002; Gross et al. 2001).

Oils have long been used for the control of scale insects but they have been ineffective against mealybugs. However, integration of narrow refined oils with other insecticides was suggested as a means to dissolve the insect's wax covering and thereby improve the insecticide efficacy (Cranshaw et al. 2000; Morishita 2005).

Insect growth regulators (IGRs), such as buprofezin, a chitin-synthesis inhibitor, or kinoprene, which mimics juvenile hormone, were sought as replacements for organophosphates and carbamates in controlling mealybugs; they have been considered a suitable alternative because they exhibit low human toxicity, they are more selective to many beneficial species, and they are specifically targeted at processes involved in particular stages of mealybug development. However, many of the IGRs are toxic to ladybeetles (James 2004; Cloyd and Dickinson 2006). Buprofezin is a commonly applied IGR against mealybugs (Muthukrishnan et al. 2005); however, its effectiveness is mainly limited to eggs and young stages, so that adult females may escape the consequences of the treatment. Buprofezin also suffers from the same limitations as other foliarly sprayed compounds.

More recently, an effective *group of compounds* has been found which combine toxicity to mealybugs with safety to other non-targeted organisms; they are the neonicotinoids. These compounds act on the central nervous system, and easily replace carbamates, organophosphates or pyrethroids, since there are no records of cross-resistance associated with them. These systemic compounds show high effectiveness against mealybugs. Examples include: dinotefuran applied to the canopy; acetamiprid applied by smearing on the stem or the branches (Gross et al. 2000; Larrain 1999); and imidacloprid and thiamethoxam that are introduced by watering the soil (Daane et al. 2006a, b; Fu Castillo et al. 2004; Grout and Stephen 2005; Martin and Workman 1999; Sazo et al. 2006). In organic agriculture, azadirachtin, an IGR chitin inhibitor derived from the Indian neem tree, may be used in similar modes (Irulandi et al. 2001).

6 Prognosis: Future Management Strategies Against Pest Mealybugs

Synthetic insecticides are still the most widely used tools to cope with mealybug problems. The current tendency to lessen chemical control has been little influenced by the limitations of current management strategies against mealybugs. Nevertheless, the change that has been mandated, mainly through legislation, aims to abandon the use of chemicals such as organophosphates, and there is reluctance to use neonicotinoids intensively, because of their widespread negative environmental effects.

In presenting the following outline of a new pest management strategy we visualize a plantation or a vineyard infested by biparental mealybug species. However, we believe that the components of the suggested management regime could fit other mealybug situations also.

Our suggestion of a new management strategy for mealybugs is based on several principles:

1. Integration of several tactics is needed to achieve sufficient control for a tolerated mealybug level that would be similar to that obtained with an effective insecticide;
2. An outbreak situation should be avoided; therefore constant monitoring is necessary;
3. Because of the significant role of general predators, such as coccinellids or gall midges, we suggest that the proposed management strategy should consider the entire crop-growing area, with attention to interactions between neighboring plots;
4. The management strategy should fit the requirements of organic farming, with respect to insecticide application.

The three major components of the suggested management strategy are:

1. Use of the female sex pheromone for monitoring and control;
2. Augmentation of the major natural enemy of the targeted mealybug in the managed ecosystem;
3. Use of environmentally safe insecticides to control mealybug "hot spots".

However, we estimate that none of the suggested tactics is sufficiently efficient to stand alone. The overall idea is that no negative interactions are expected between these three components or with other factors that play a positive role in the natural control of the mealybug.

6.1 The Management Tactics

6.1.1 Male Vacuum

The major tactic in coping with a mealybug pest is to promote a lack of males, in order to reduce the population natality. This may be achieved during periods when mealybug population is at low level, and the number of colonies in which males do not need to fly to reach females is minimal.

In such situations it is possible to prevent fertilization of most of the females. However, since many of the mealybug species overwinter as gravid females, prevention of male activity, either by mating disruption or by direct control, for example, in citrus orchards or vineyards, may be accomplished during the first generation in the spring. Later in the season male density may be too high to achieve this objective.

Mating disruption achieved some success against the vine mealybug in California (Walton et al. 2006). This method requires a large amount of sex pheromone, and, up to now, the vine mealybug pheromone is the only one with which

this tactic can be economically worth while. Another approach involves the use of baits for lure and kill. Male maturation involves the activation of the flight muscles (Mendel et al. 2008; Silva et al. 2009); and at low population densities, mature *Pl. citri* males, for example, may fly over 100 m to find suitable mates (Branco et al. 2006; Franco et al. 2004a), therefore bait stations may prove an appropriate tool. Whereas in the case of successful fruit fly feeding stations male annihilation is achieved through feeding, it is anticipated that in the case of mealybugs, males should be killed by contact. Males of *Pl. citri* were killed after walking on chlorpyrifos-impregnated ethylene sheets for 5 s (O. Bar Shalom et al., unpublished data, 2009). In such tactic, males would be lured to the bait by a sprayed, microencapsulated formulation of a sex pheromone or parapheromone (e.g. Daane et al. 2006a) combined with an insecticide. The major challenge, therefore, is presented by development of the formulation to be applied to the killing surface. This formulation should fit the requirements of organic agriculture; possibilities include botanical insecticides such as Limonene, Linalool, Neem, Pyrethrum, and Rotenone, or microbial toxins such as Spinosad. A less desirable possibility is application of sticky materials, such as non-drying glue, but these are harmful to non-target arthropods and even to small birds, and may soil the fruits.

Spraying these baits as spots, each 50–100 cm in diameter, on the tree crown, might succeed in killing the males while they walk on these treated surfaces. However, whereas the canopy of evergreen fruit trees is appropriate for such treated spots, in the case of deciduous fruit trees or vines, other objects will need to serve as the surface for the bait sprays. The distribution pattern of the treated spots and their density will be determined according to the mealybug density in the treated plot, and with consideration of the mealybug situation in neighboring plots.

6.1.2 Monitoring and Detection of Mealybug Hotspots

Whenever the sex pheromone of the mealybug pest is available, application of pheromone traps is expected to serve as a routine procedure. Therefore, simple correlations between male capture and mealybug density are expected to be available for a given crop area. Chronic hot spots of mealybug infestation should be treated in a prophylactic manner with an appropriate insecticide or by an inundating release of natural enemies (see Section 6.1.3). Randomly occurring hot spots may be discovered by a procedure named “targeted monitoring”, which enables the growers to pinpoint the mealybug hotspots in order to focus the control efforts on these small areas. Targeted monitoring is practiced by setting four to six pheromone traps, baited with dispensers impregnated with a low concentration of pheromone, and reducing the area covered by the traps with every round of trap setting. This procedure is successfully applied in herb greenhouses in Israel (Protasov et al. 2009).

6.1.3 Augmentation of Natural Enemies

When the mealybug population is low, the population densities of its specific natural enemies, especially the predators, are also low. Parasitoids, which are better fitted to survive at low mealybug densities, may find it difficult to reach their hosts in their most appropriate refuges, and these small colonies may also be well protected by ants. Furthermore, parasitoids of tropical or subtropical mealybug species do not tolerate Mediterranean climate winters very well. However, inoculative or inundating releases of parasitoids may compensate for their low survival. Augmentation of the parasitoid population in spring, when mealybugs leave their typical refuges for new colonization sites on the host plant, may improve the mealybug/parasitoid ratio (Mendel et al. 1999). Since the population density during this season is low, the released parasitoids tend to disperse over a rather large area in their search for mealybug colonies (Mendel et al. 1999). The kairomonal response of the parasitoids to the mealybug sex pheromone can be utilized to keep the released individuals in the targeted area. The parasitoids search for mealybugs in the vicinity of the pheromone release points (Franco et al. 2008c), therefore, we may increase the intensity of parasitization in the treated plots. Another tactic that may be considered involves measurement of the population of natural enemies in the managed area. Advance acquisition of information should be considered, in order to plan augmentation of natural enemies in the coming growing season. It is expected that if there was considerable mealybug mortality in a particular plot, it could be because of the activity of parasitoids and predators that had survived in this plot, and not because of migration of natural enemies from a long distance. Therefore, information about the natural enemy density late in the season may be achieved by setting up traps baited with mealybug colonies, with or without the sex pheromone (with respect to each individual case).

6.1.4 Chemical Control

An insecticide arsenal that is both suitable for organic farming and able to cope effectively with mealybug pests does not exist in practice. Since the growers will need to treat small hot spots of the mealybug, it is expected that some soft insecticides will be used and that more than one application may be needed, to selectively eliminate such hot spots (see above examples). When these hot spots are treated several points should be taken into account: (i) hot spots are expected to be in areas that are practically free of problematic mealybug populations; they actually constitute oases for parasitoids and predators; therefore, the ratio of mealybug to natural enemy populations in the hot spots should be considered before initiation of any control operation; (ii) an insecticide will be applied when augmentation with predators is not useful or cannot be implemented; (iii) a low-residue short-life insecticide is the most appropriate; (iv) augmentation of natural enemies will be needed if the hot spots are too numerous.

7 Conclusion

The five key elements ought to be considered in a decision-making system in the management of mealybug population are: (i) information on mealybug density, perhaps obtained late in the season, in case of overwintering population; (ii) awareness of the population distribution in the target area; (iii) information about the density of the relevant natural enemies; (iv) the density of associated insect species which may increase the damage or render the activity of the natural enemies; and (v) the risk of spread of mealybug transmitting viral disease. In this chapter we reviewed the current knowledge needed to take actions and to suggest solutions for different situations. On the basis of such knowledge, the grower may select the appropriate control tactics. While the current major tool to cope with mealybugs are synthetic insecticides, we consider that the combined use of sex pheromones and natural enemies will play the most important role in the coming decades. Our concern should be directed towards generating more information and practice in order to make those management approaches applicable. Hence, this is the point scientists should be more active in three main lines of research. We refer to (i) improve monitoring techniques and procedures for the mealybugs and their natural enemies, (ii) progress in pheromone synthesis thus making mealybug sex pheromones more cost-effective, and (iii) to generate the know-how using these components in tactics such as lure and kill technique, mating disruption, and augmentation of the natural enemies.

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Manipulation of Insect Signaling for Monitoring and Control of Pest Insects

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1 Introduction

Knowledge is obtained from inherited information, information gained from learning (experience), and by information received from others through communication (teaching). The importance of the role of communication in the life histories of living organisms has been recognized since ancient times, and there are many reasons to study communication in animals. For example, reproduction in sexual animals is not possible without communication, and communication is essential for the proper functioning of all types of social organisms that live together in groups or colonies. Furthermore, the study of animal signals can be used to understand general principles of evolution, and many practical applications of communication have their origins in basic studies of information exchange between animals. The manipulation of insect signaling for monitoring and control of pest species are just two examples that illustrate some of the applications that have been developed from basic studies of communication processes in animals.

Communication may be defined in a very restricted sense as the exchange of information between sender and receiver via signals transmitted through a medium, be it solid, gaseous, or liquid. "True" or "honest" communication, as determined by benefits for both sender and receiver, includes intraspecific sexual and social signals as well as some types of interspecific signals directed towards predators, competitors, or mutualist species. Signals emitted as modifications of the physical or chemical environment by the signaler's activity are received as raw data, and are translated by the receiver to provide information about such factors as the signaler's location, identity, and physiological or behavioral state. The information is processed

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and stored in the receiver's nervous system, and together with previous experiences and/or inherited knowledge, determines the response. Eavesdropping is the process whereby a receiver exploits signals intended for another individual, and which benefit the receiver with no benefit, or negative consequences, to the sender. Thus, predators and parasitoids frequently eavesdrop on the communications of their hosts as an effective method of locating potential hosts.

In this chapter, we briefly review the use of chemical and acoustic signals in insect communication, and their actual and potential applications for insect management. Although many different types of signals mediate a wide variety of behaviors, our discussion will focus on signals connected with reproduction, because these are probably the most well studied types of insect signals, and they have the broadest potential for exploitation. Signals of different modalities are used for communication associated with reproductive behaviors. The choice of which modality is used depends on a wide variety of factors, including the size of the insect, its habitat, its daily activity patterns, the transmission mediums available to it, and the distance over which the signal has to be effective. These in turn have been shaped by evolution. An insect also may use several different types of communication during a behavioral sequence, either simultaneously or sequentially. In general, most practical applications of insect signaling have focused on the exploitation of signals intended for long-range use, because these signals can be used to draw insects in to the source of the signal. It is more difficult to visualize methods of exploiting short-range or contact signals, and such types of signals have been less widely used in insect control, unless they have been combined with a longer-range attractant. Furthermore, most practical applications of long-range signals have focused on the manipulation of a single species, for example, by attraction of conspecifics with a species-specific pheromone.

Long-range communication in insects most commonly involves chemical and/or mechanical signals, although some insects do indeed use long-range visual signals (e.g., wing patterns of diurnal butterflies, or flashing of nocturnal fireflies). Chemical signals have a number of advantages for long-range communication, including the facts that they are relatively inexpensive to produce (pheromone glands are usually a small percentage of an insect's body mass, and the chemical structures of pheromone components are relatively simple), tiny quantities (nanograms or less) are effective in eliciting behaviors, and pheromones can be detected from hundreds of meters downwind. Furthermore, even a relatively small "vocabulary" of chemical "words", with simple manipulations (changing blend components and blend ratios), can provide a virtually unlimited variety of unique messages, so that species-specific signals are easy to produce.

Contrary to well established chemical signaling mechanisms in all living organisms, communication with mechanical signals is well developed only in arthropods and chordates. With this type of signaling, the sender uses some type of mechanical force to create a disturbance in the transmission medium, and this disturbance is detected by the receiver, for example, as airborne sound or substrate-borne vibrations. Whereas long-range communication with signals transmitted through air is relatively efficient, for reasons described below, airborne communication is much less widely used by

insects than substrate-borne communication, whether counted by species, families, or phylogenetic distribution (Cocroft et al. 2005). In the last few decades, the study of insect vibrational signaling on plants, the typical substrate employed by many insects, has accelerated following the development of sensitive and noninvasive methods for recording low intensity acoustic signals in relatively delicate plant structures. For some types of insects, substrate-borne communication is the most efficient method of identifying and locating a mate on a plant, as described below. Although our body of basic knowledge on such types of signaling is growing, practical applications, including exploitation for pest control, have remained elusive.

The aim of this chapter is to provide an overview of chemical and acoustic communication in insects, and how these signals are or might be exploited for insect management. Each of these signal modalities is presented separately, and we then describe how these signals might be used jointly for certain applications.

2 Chemical Signals

Chemical signals are widely used by all forms of life, from single-celled organisms such as bacteria through to the largest plants and animals. It is likely that chemical signaling was the first type of communication between organisms to evolve as early life forms developed (Wilson 1970). Such signals are highly versatile, because by adjustment of their chemical and physical properties during biosynthesis, a terrestrial organism can produce volatile molecules that transmit ephemeral messages over long distances downwind, or conversely, low-volatility signals that act over short ranges or on contact, but which can persist for long periods, and anything in between. Analogous types of signals are used by aquatic and marine organisms, generally using the alternate criterion of solubility rather than volatility to determine whether a particular signal acts on contact or over a distance. Thus, chemical signals have been shaped by evolution to suit the context in which they are used, and the types of information that they are intended to convey. The chemistry of signal molecules is very diverse, with all of the major classes of natural products being represented, including terpenoids, acetogenins, fatty acids, alkaloids, carbohydrates, and peptides.

Due to space limitations, our discussion of chemicals that mediate the behavior of insects generally will be restricted to pheromones, the subset of signal molecules (semiochemicals) that are used for communication between organisms of the same species (Nordlund and Lewis 1976). Furthermore, for the purposes of our discussion, we designate a chemical that is made by a producer for the specific purpose of communicating with a receiver as a signal, whereas compounds that are intercepted and exploited by a nontargeted receiver will be designated as cues (see discussion of these definitions in Greenfield 2002). It must be recognized that, in addition to pheromones, there are a wide variety of other types of semiochemicals that mediate interactions between organisms of different species. For example, chemical cues are widely used by insects to locate, recognize, and confirm the identification of suitable hosts or other food sources, and for location and recognition of suitable

oviposition sites (Miller and Miller 1986). Furthermore, compounds that are produced in the context of pheromones for signaling to a conspecific are frequently exploited by parasitoids or predators as a means of finding their hosts (e.g., Aldrich 1999). When a receiver benefits to the detriment of the producer, such chemicals function as kairomones (Brown et al. 1970). Thus, although a chemical signal may be produced for the specific purpose of communicating with a conspecific, it can have multiple functions within an ecological community, influencing the behaviors of several different species in different ways.

Naturalists were the first to document that some insects communicated with chemicals, but systematic attempts to understand the mechanisms and identify the signal molecules occurred only over the past 50 years. In one of the first experiments that conclusively showed that insects communicate with volatile chemicals, conducted in the late nineteenth century, the French naturalist Jean-Henri Fabre demonstrated that males of the moth *Saturnia pavonia* were strongly attracted to unmated females over long distances, and that the signal had to be a volatile chemical rather than an acoustic or visual signal. Analogous observations were made with other species, but the first insect pheromone was not identified until 1959, when Adolf Butenandt and coworkers culminated a heroic 20-year effort by identifying bombykol, the sex pheromone of the silk moth, *Bombyx mori* (L.) (described in Hecker and Butenandt 1984). This generated an explosion of interest in insect chemical ecology, to the extent that pheromones of various types now are known for hundreds if not thousands of insect species, particularly for species of economic importance (e.g., El-Sayed 2008). Furthermore, the tremendous advances made in both analytical and synthetic chemistry in the past few decades, coupled with increasing knowledge of the types of pheromone molecules to expect from different types of insects, has rendered the identification of many new pheromones routine. However, this is by no means always the case, because pheromones may be produced in extremely small quantities (femtograms per insect), they may be unstable and hard to isolate in pure form, and their structures are sometimes complex. Thus, in straightforward cases, pheromones can be identified from a few or even a single individual, whereas in other cases, powerful pheromones used by economically important species (e.g., the plant bugs *Lygus lineolaris* (Palisot de Beauvois) and other *Lygus* species, reviewed in McBrien and Millar 1999, or the Australian cane beetles in the tribe Melolonthini, Fletcher et al. 2008) have eluded identification, despite intensive research over several decades.

During the late 1960s, in conjunction with the first identifications and syntheses of insect pheromones, researchers began to focus attention on the potential for exploiting these compounds for managing insect populations by manipulation of insect behaviors. This potential can be graphically illustrated with a simple bioassay for any number of species, by the attraction of hundreds or even thousands of individuals to a trap baited with a milligram or less of synthesized pheromone, and the lure will remain attractive for periods of weeks, months, or even years. Furthermore, the attraction can be so focused and all-consuming that the responding animal will seriously injure or kill itself while attempting to respond. Thus, insect pheromones might superficially appear to be powerful tools with which to manage insect

populations, and major efforts have been devoted to developing reliable, practical, and economical methods of doing so. The use of pheromones for insect control, in place of insecticides, is particularly attractive for several reasons. For example, pheromones are nontoxic, highly species specific, biodegradable, and are used in relatively small amounts, so they result in no persistent environmental contamination or non-target effects. In fact, pheromones in current use are so benign that there is no reentry delay required after their application to a crop, in contrast to some pesticides, where reentry into a treated area may not be permitted for several weeks.

However, some of the major benefits of exploiting pheromones for insect control via behavioral manipulation, as opposed to using insecticides that kill the target pest (and which contaminate groundwater and affect non-target organisms including humans) also turn out to be some of their major limitations. For example, because pheromones do not kill insects and remove them from the system, the pheromone treatment usually must exert its effects continuously for the entire adult lifetime of the target pest, or the pheromone must be used in conjunction with another method of killing the insect (discussed further below). Furthermore, unlike most insecticides, insect pheromones are essentially species specific. Whereas this specificity prevents effects on non-target organisms, including bees and other beneficial insects, it has both practical and economic consequences. In practical terms, if a crop is attacked by more than one major pest, then other control measures may have to be used to manage those species whose behavior is not affected by the pheromone. In economic terms, the species specificity means that the pheromone can only be used to manage one or a few species, so that the market for a particular pheromone will be relatively small, in contrast to the market for an insecticide which can kill many insect species. Thus, commercial production of pheromones is a niche market, in contrast to the much larger markets for broad-spectrum insecticides, each of which can be used to control numerous insect species.

Before detailing the use of insect pheromones and related compounds for insect control, some brief descriptions of the major types of pheromones would be appropriate.

2.1 Types of Chemical Signals

Insects use pheromones in various contexts in all aspects of their life histories. Whereas adult insects have been the main focus of pheromone identification and development efforts, all life stages of insects (except possibly eggs) probably use pheromones in some form. Because of the enormous range of behaviors and effects that they mediate, pheromones have been categorized in several different ways. First, pheromones are designated as either releaser pheromones, whereby reception of the pheromone triggers an immediate behavioral response (e.g., a male insect being immediately attracted by a female's volatile sex pheromone), or primer pheromones, which induce a delayed response which may be manifested as a physiological change (e.g., caste determination in termites (Kaib 1999) or honeybees (Pettis et al. 1999; Keeling et al. 2004).

Pheromones are also further classified according to the types of behaviors or effects that they mediate. A short list of the main types of pheromones used by insects follows.

Sex pheromones, which mediate behavioral interactions between the sexes, have a multitude of functions in insect reproduction, which can be viewed as a cascade of sequential behavioral steps. The most well known types of pheromones are probably the sex attractant pheromones that are released by one sex (often but by no means always the female), and which mediate the initial steps of activation and directed upwind movement of the other sex towards the "calling" individual. Over shorter distances, other sex pheromones may come into play, such as courtship pheromones, or aphrodisiac pheromones that render an individual receptive to mating. At close range and on contact, individuals verify that they have found a mate of the right sex and species with contact sex pheromones, which constitute a subset of the hydrocarbons and other lipids that coat an insect's exoskeleton. These relatively non-volatile compounds can also be used as arrestants or trail-marking compounds, to alert a walking male that a female is close by, and to lead him to her (e.g., Kainoh 1999). During mating, males may transfer substances to females that inhibit further calling behavior, so that the females do not attract other males. After mating, males also may mark mated females with an anti-aphrodisiac pheromone that renders them unattractive to other males (Schulz et al. 2007). In both cases, these male-produced compounds serve to maximize the number of eggs that are fertilized by the first mate's sperm.

Aggregation pheromones are typically produced by one or both sexes, and elicit a response from both sexes, unlike sex pheromones which only elicit a response from one sex. They are often multifunctional, for example, serving to attract conspecific individuals to a host resource, while also serving to bring the sexes together for reproduction. Probably the best known and well studied uses of aggregation pheromones are those by some bark beetle species, in which aggregation pheromones are vitally important for bringing together the critical mass of conspecifics required to overwhelm the resin defenses of host trees (review, Schlyter and Birgersson 1999). Furthermore, there is a subsequent feedback step; once the host tree has been overwhelmed by a sufficient number of bark beetles, the occupants begin to produce anti-aggregation pheromones to divert latecomers away from the tree so that it does not become overcrowded, which would result in larval competition and cannibalism. Aggregation pheromones are also known for a variety of other insects, including other beetles and flies (Hardie and Minks 1999) and some arctiid moths (Conner and Jordan 2008).

Alarm pheromones are used both to warn conspecific individuals of danger, and to recruit conspecifics for defense. As early as 1609, Charles Butler described how the scent emanating from a detached bee sting attracted more bees and stimulated aggressive behaviors. Alarm pheromones are most common in insects that live in groups or colonies such as ants, bees, and aphids, but they are also produced by species such as true bugs. Because they need to be disseminated so that receivers can respond quickly, alarm pheromones are typically small and highly volatile compounds. Furthermore, to vertebrate noses, they often have pungent odors and may be toxic or irritating, so that they serve as direct defenses as well.

Trail pheromones are used primarily by social insects such as ants and termites, as a method of guiding conspecifics to food resources (e.g. Kaib 1999). They are also used by tent caterpillars for marking trails back to food resources as the caterpillars return to the communal tent (Fitzgerald 1995).

There are many other types of marking pheromones, that are used in contexts as varied as territory marking (e.g., African weaver ants, Hölldobler and Wilson 1978) to host-marking, whereby a female insect that has laid an egg in a host (including other insects, in the case of parasitoids) will mark that host to deter further oviposition by conspecific females. Thus, the offspring of the female making the mark can maximally utilize the host resource, without competition from conspecifics (Kainoh 1999; Landolt and Averill 1999).

Pheromones are also critically important in all aspects of the organization and functioning of social insect colonies. The cuticular lipids of each individual in the colony consist of a mixture of compounds that provide information to conspecifics about its species, caste, age, and gender, so that brief antennation of one individual by another provides instant recognition and categorization of that individual. Furthermore, the blend of compounds is subtly different between nests or colonies, even for colonies living adjacent to each other, and individuals from different colonies readily recognize each other as intruders. These chemical signatures consist of a mixture of an individual insect's cuticular compounds, and compounds that are associated with each nest. However, this is just the tip of the iceberg in terms of pheromone use by social insects, and we are still in the early stages of teasing out and understanding the multitude of signals that regulate their complex societies. For example, in honeybees, we now know that the queen's mandibular pheromone inhibits reproduction by workers, but also influences behaviors as diverse as swarming, foraging, and the regulation of tasks within the hive (Breed et al. 1998; Pettis et al. 1999; Keeling et al. 2004).

This is but a small sampling of the different types of insect pheromones and their uses. Clearly, pheromones are ubiquitous signals that mediate most aspects of insects' interactions with each other, for all life stages where interactions between individuals occur. For more in-depth surveys of known insect pheromones, the reader is referred to several online resources (Ando 2003; Witzgall et al. 2004; El-Sayed 2008) or books (e.g., Mayer and McLaughlin 1991; Howse et al. 1998; Hardie and Minks 1999; Schulz 2004a,b).

2.2 Exploitation and Manipulation of Chemical Signals

Given the number and variety of insect pheromones, there are many opportunities for manipulation or disruption of these signals, using several different strategies. For example, we can attempt to replicate the insect-produced signal and its intended effects by partial or complete reconstruction of the active blend of chemicals. It may also be possible to exploit the negative behavioral effects associated with naturally occurring behavioral antagonists that are produced as minor components of some pheromone blends, and that insects use to reinforce the specificity of their

pheromone channels (e.g., Bengtsson et al. 1994). Alternatively, interference with the reception or processing of chemical signals may provide opportunities for insect management (e.g., Ditzen et al. 2008). This is particularly true with the recent spectacular advances in molecular biology, whereby the genomes of a number of insect species have been sequenced, providing leads for manipulation of insect behavioral signals and mechanisms at the genetic level. To date, however, exploitation of insect pheromones has relied primarily on manipulation of conspecific attraction, although researchers are starting to explore whether other behavioral mechanisms might also be amenable to manipulation. Each of these is discussed in the following sections.

2.3 Pheromones for Detection and Sampling of Insect Populations

Volatile sex and aggregation pheromones are widely used in modern agriculture, and pheromone lures for several hundred insect species are commercially available. Pheromone-baited traps are fast and simple to use, and because of the species specificity of each pheromone, require no expertise to identify the insects caught. Pheromone-baited traps are particularly useful in providing early warning of the presence of a target species, and for following population cycles (e.g., flight phenology of adult insects) so that control measures can be accurately timed. However, it has proven more difficult to develop robust economic thresholds based on trap catches alone, for a variety of reasons (e.g., clumped rather than random distributions of target pests, and/or insufficient trap densities due to trapping costs), and so the detection of the target pest in pheromone traps often triggers secondary sampling of the crop to verify whether populations are indeed high enough to warrant control measures (e.g. Howse et al. 1998).

An equally important and rapidly growing use of pheromone-baited traps is for the detection and interception of invasive species, and the delineation of the current ranges of invasive species as populations spread outwards from the original site of introduction and establishment. Thus, semiochemically-baited traps form the cornerstone of detection efforts by regulatory agencies for notorious invasive pests such as the gypsy moth *Lymantria dispar* (L.), the Japanese beetle *Popillia japonica* Newman, the boll weevil *Anthonomus grandis* Boheman, the pink bollworm *Pectinophora gossypiella* (Saunders), and the Mediterranean (*Ceratitis capitata* (Wiedeman)) and related fruit flies (e.g., Ridgway et al. 1990; Howse et al. 1998). The importance of such sensitive trapping methods cannot be underestimated, because early detection has repeatedly enabled the eradication of small and localized populations of pests such as these, averting billions of dollars in crop and forestry losses, as well as avoiding the added pesticide load to the environment that would be required for ongoing control of invasive insects once they had become permanently established. Furthermore, insect pheromones and related compounds have often played a critical part in the eradication process, both in terms of being used to trap

or kill insects, and in terms of providing a sensitive method to determine when areas have been rendered pest-free. Because of the major expansion of world trade over the past couple of decades and the consequent rapid increase in the introduction of exotic pests into new areas of the world, the use of pheromones for detection of such pests will of necessity increase.

In addition to deploying pheromones for detection of pest insects, there is increasing interest in using pheromones for detection and sampling of beneficial insects, particularly for programs in which natural enemies have been deliberately introduced into a new country for biological control of an introduced pest (e.g., Suckling et al. 1999, 2000, 2006; Stanley et al. 2000; Cossé et al. 2004). There are obvious parallels between the use of pheromones for detecting introduced beneficial insects and undesired exotic pests, i.e., in both cases, the purpose is to use the most sensitive method possible to detect the target insect at low densities.

2.4 Management of Insects by Pheromone-Based Mating Disruption

Because many insect species are entirely dependent on the use of sex pheromones to bring the sexes together for mating, the potential for controlling insects by manipulation of this fundamentally important aspect of their life histories has been recognized for a long time. In 1967, Harry Shorey and his coworkers conducted the first successful trial of this concept, demonstrating disruption of mating of pink bollworm moths with a synthetic insect pheromone (Gaston et al. 1967). Since that landmark trial, enormous efforts have been devoted to developing mating disruption into an effective, reliable, and economical insect control tactic, with results varying from spectacular success to complete failure. Currently, mating disruption is used for insect control on approximately 660,000 ha worldwide, with the main target pests being gypsy moth *L. dispar* (forestry), codling moth *Cydia pomonella* (L.) (pome fruit), grapevine and grape berry moths, *Lobesia botrana* (Denis et Schiffermüller) and *Eupoecilia ambiguella* Hübner respectively (vineyards), oriental fruit moth *Grapholita molesta* (Busck) (peach, apple), pink bollworm *P. gossypiella* (cotton), and leafrollers (orchard crops and tea) (Witzgall et al. 2008). Development of mating disruption in various crops still constitutes a very active area of research; the types of pests and crops under investigation and implementation continually increase. For example, mating disruption of the western poplar clearwing moth *Paranthrene robiniae* (Hy. Edwards) in poplar plantations in the northwestern United States recently proved to be spectacularly successful, completely controlling a devastating problem that could not be managed effectively with insecticides (Brown et al. 2006). Mating disruption is also being attempted with insects other than moths: for example, a pheromone formulation for mating disruption of vine mealybug *Planococcus ficus* (Signoret) in vineyards came on the market in 2008 (Suterra LLC., Bend, Oregon, USA).

Overall, the history and description of research on mating disruption have been covered in a number of reviews (themselves summarized in Cardé 2008), and so only a few main points will be touched upon here. The main premise underlying pheromone-based mating disruption is that male insects are prevented from finding females by blanketing a field with the synthesized pheromone, either by releasing it from multiple point sources per hectare (e.g., microencapsulated pheromone or small, discrete dispensers dispersed throughout the crop), or by dispensing pheromone from a much smaller number of dispensers that release a correspondingly larger amount of pheromone per dispenser (e.g., plastic bags that passively release pheromone, or mechanical devices that release metered puffs of pheromone at regular time intervals). One of the points about mating disruption that must be borne in mind is its fundamental difference from management of insects with insecticides. That is, with mating disruption, the pheromone is not toxic and only interferes with an insect's behavior. Thus, to be effective, the pheromone must completely blanket a crop, with no holes or gaps, for the entire duration of the male insect's lifetime, or more widely, for the entire period that the adult stages of the target insect are present. For insects that have multiple generations per year, this can translate into a period of at least several months. In sharp contrast, with insecticides, a single contact with a lethal dose of the toxicant removes the insect from the system (although several applications may be required for season-long control). Thus, mating disruption may be inherently more difficult to use effectively than insecticides. However, this possible limitation is counterbalanced by a number of distinct advantages, not least of which is the decrease or elimination of insecticide use in crops protected with mating disruption. Furthermore, in some 40 years of use, there has only been one documented case of development of behavioral resistance to mating disruption, caused by the use of an incomplete pheromone blend (Mochizuki et al. 2002). Full efficacy was restored by simply reformulating the pheromone to include the minor components.

One of the continuing problems with implementation of mating disruption is our incomplete understanding of the mechanisms by which it works, further compounded by the fact that it is likely that different possible mechanisms are more or less important for different species, i.e., there is no "one size fits all" model applicable to all species. Three principal mechanisms have been invoked: sensory adaptation of the pheromone receptors and/or habituation of the central nervous system to the synthetic pheromone, competition between the point sources of synthetic pheromone and calling females (also known as false trail following), and camouflage of the pheromone plumes produced by calling females by the blanket of synthetic pheromone. Given good coverage of a crop with dispensers releasing quantities of synthetic pheromone equivalent to many thousand or even millions of female insects (e.g., a fraction of a gram to several grams per hectare per day), logic would seem to dictate that any one or any combination of these mechanisms should be sufficient to achieve disruption. The fact that effective control of some species has not been achieved demonstrates that successful implementation of mating disruption may not be straightforward. However, recent attempts to mathematically model mating disruption have provided insight into which mechanisms may be most important (Miller et al. 2006a,b), and may help to resolve difficulties that have been encountered in attempted mating disruption of some species.

2.5 Insect Control by Pheromone-Based Mass Trapping

Although the concept of insect control through mass trapping may be technically feasible, practical and economic considerations have limited its general use; the costs of purchasing, deploying, and servicing a sufficient number and density of traps are often prohibitive. However, under some circumstances, pheromone-based mass trapping can be both highly effective and economically competitive, and it is currently used for control of a number of species in diverse systems. For example, mass trapping of ambrosia beetles around log sorting areas and sawmills in northwestern North America has been used effectively for several decades to minimize damage to saw logs from the beetles and the blue-stain fungus that they vector (McClean and Borden 1977). In a somewhat different application, baiting of lodgepole pine trees with a combination of aggregation pheromones and host tree volatiles is used to concentrate mountain pine beetles *Dendroctonus ponderosae* Hopkins into the baited trees, which are then removed or treated with insecticide to destroy the developing brood (Borden 1989). Mass trapping for control of a lepidopteran, the brinjal borer *Leucinodes orbonalis* Guenée, was effective in India, in part due to low labor costs (Cork et al. 2003). However, the most effective and largest scale use of pheromone-based mass trapping has been in the management of large tropical weevils such as the palm weevils *Rhynchophorus palmarum* Linnaeus and *R. ferrugineus* Olivier (Oehlschlager et al. 2002; Faliero 2006) and related species, and the banana weevil *Cosmopilites sordidus* Chevrolat. One of the key factors in the success of these programs is that the weevils reproduce slowly, so that their reproduction cannot keep pace with their removal by mass trapping, and continuous mass trapping drives their populations steadily downward. Because most of the research and development on mass trapping these weevils has been done by industry rather than academic researchers, spearheaded by Chemtica International in Costa Rica, much of this work has not been published in the scientific literature, and it is difficult to estimate the total area that is mass trapped. It is clearly many thousand hectares, because pheromone-based mass trapping is a major component of IPM programs for coconuts, dates, oil palms, and bananas in South and Central America, the Mediterranean basin, and Asia. Thus, given the right sets of biological and economic circumstances, pheromone-based mass trapping can be both highly effective and economical, and in the case of the tropical weevils described above, it provided a solution to a problem that was otherwise almost intractable.

2.6 Semiochemically Based Attract and Kill Methodologies

Pheromone-based attract and kill methods involve combining point sources of pheromone with a killing agent. In theory, attract and kill may have some advantages over mating disruption; for example, the responding insect is killed or intoxicated by contact with the attracticide source, and so is effectively removed from the system. The attract and kill technique may be more economical when expensive pheromones

are used, because it requires a lot less pheromone than mating disruption, i.e., it is only necessary to attract the target insects to the toxic bait, rather than overwhelming their sensory systems with pheromone. A disadvantage to attracticide technology is that it does use a toxicant as part of the formulation, and consequently, it may face more regulatory hurdles than mating disruption or mass trapping. In practice, the number of current applications of pheromone-based attracticides is relatively small, but will probably grow.

To date, attracticide technology has been most widely applied for the control and eradication of invasive tephritid fruit flies such as the Mediterranean fruit fly *C. capitata* (reviewed in Millar 1995), the oriental fruit fly *Dacus dorsalis* Hendel, the melon fly *D. cucurbitae* Coquillet, the Mexican fruit fly *Anestrapha ludens* (Loew), and the olive fruit fly *D. oleae* (Gmelin) (Metcalf and Metcalf 1992). For most of these species, the best formulations consist of food baits combined with a toxicant, but pheromone-based attracticides have now been developed for control of olive fruit fly (e.g., Mazomenos et al. 2002). Pheromone-based attracticides also form a cornerstone of effective efforts to eradicate boll weevil *A. grandis* from the United States (reviewed in Prokopy and Roitberg 2008). Whereas pheromone-based attract and kill methods have been tested for a number of insect species, such as leafrollers (Curkovic et al. 2008), diamondback moth *Plutella xylostella* (Linnaeus) and cabbage looper *Trichoplusia ni* (Hübner) (Maxwell et al. 2006), and sap beetles *Carpophilus* spp. (Hossain et al. 2007), this strategy has not been as widely adopted as mating disruption. Unfortunately, pheromone-based attracticide research has not yet been summarized in a review article, so it is difficult to obtain an accurate estimate of how widely this technology has been adopted, and how effective it is.

2.7 Exploitation of Alarm Pheromones

To date, the only well documented attempts to exploit alarm pheromones for insect control have been with aphids. The aphid alarm pheromone (*E*)-ß-farnesene induces movement and dispersal in aphids. The general concept was to use these increased activity levels to induce aphids to pick up a lethal dose of a co-applied insecticide or fungal pathogen more quickly, thus allowing lower application rates of the toxicants to be used (Pickett et al. 1992). This idea has been commercialized, but the overall efficacy and cost effectiveness for aphid control remains unclear.

There may be potential for using alarm pheromones for other types of insects that avoid or move away from sources of alarm pheromone. For example, insects such as leaf-footed bugs *Leptoglossus* spp., which can cause extensive damage in seed orchards and nut crops in western North America, disperse rapidly from a tree upon release of alarm pheromone by a conspecific (J. Nay, personal communication 2007). It might be possible to exploit this behavior to prevent infestation of nut crops, particularly as the alarm compounds have simple structures and are cheap and readily available.

2.8 *Practical Considerations for Exploitation of Pheromones for Insect Management*

A number of insect pheromones and related substances are now in common use for detection, sampling, and management of insect populations, but this represents only a tiny fraction of the total number of insect pheromones known, for reasons that vary from basic biology to economics. Furthermore, the total acreage of crops in which pheromones are used as a significant or primary method of controlling pests (as opposed to detection and monitoring), is small (Witzgall et al. 2008) in comparison to the acreage on which insecticides or some other pest control method, such as transgenic crops, are used. Nevertheless, there is continuing strong interest in exploitation of pheromones, and steady growth in total acreage treated and the number of different crops in which pheromones are deployed. This interest is driven in part by the joint desires to minimize use of toxic insecticides, and to delay development of resistance to insecticides. To continue this growth trend, we need to be pragmatic about our choice of pheromones to develop. First and foremost, we need to ensure that a target pest is sufficiently economically important to warrant both the initial investment in research, and to ensure a large enough market for commercial pheromone products to be profitable. If these initial caveats can be satisfied, then there are a series of more detailed questions that need to be asked to assess whether a pheromone project is both biologically and economically feasible.

2.8.1 Biology of the Target Insect

A detailed knowledge of the target insect's biology is essential to assess whether there is a reasonable possibility that it can be controlled with pheromones. For example, an assessment of the strength of behavioral responses to the pheromone is critical. For insects such as lepidoptera, which are generally accepted to be strongly attracted to pheromones, this may seem obvious, but this is not always the case: in our experience, for some lepidoptera with long-lived adults that feed, the males may only be attracted to pheromones during a particular period in their adult lives. For other insects for which pheromone chemistry and biology is less well known, a critical and conservative examination of the pheromone-mediated behaviors (using live insects as producers and responders) should be undertaken to verify that responses are both strong and reliable.

Having determined that there is a strong response to pheromone, further aspects of the insect's biology must be considered. For example, multivoltine insects may require control for much longer periods than univoltine insects, keeping in mind that the pheromone does not remove the insect from the system, it just disrupts behavior at a critical period in the reproductive cycle. Thus, multiple generations are both more difficult to control, and may require two or more applications of pheromone. There may also be seasonal differences in response, or the adult insect may have a long sexual maturation period during which it is unresponsive to pheromones.

For some types of insects (e.g., weevils and the *Carpophilus* beetle species mentioned above), responses to pheromones are strongly synergized by food volatiles. In short, a detailed assessment of the biology of a target pest is an essential prerequisite to a pheromone development project.

2.8.2 Crop Characteristics

In theory, pheromone-based methods of insect control might be most amenable to high-value crops grown on a limited acreage, with a single major insect pest, largely due to economic reasons. That is, application of pheromone dispensers, attracticides, or traps for mass trapping is often done by hand, which becomes costly and logistically difficult for crops grown on large acreages. Thus, high-value crops grown in nurseries or greenhouses might constitute an ideal venue for pheromone use. This ideal mix of characteristics is seldom realized, and most current applications of pheromones for insect control are in orchards, vineyards, and plantations. In terms of other desirable characteristics, with other things being equal, a pheromone-based method of control will probably be less effective with tall trees versus shorter trees, because of the difficulties in getting good pheromone coverage of the much larger airspace of a crop with tall trees (it should be noted that obtaining good coverage of tall trees with insecticides also may be problematic). Similarly, pheromone control methods might be expected to be more effective when foliage is present during the main part of the growing season, to help disperse and hold the pheromone in the airspace of the crop, rather than during early spring when there is minimal foliage. Experience also has shown that pheromone-based control strategies work best when large contiguous areas are treated (so-called area-wide programs, e.g., Witzgall et al. 2008) rather than treating isolated small blocks, where immigration of mated females may be a significant problem.

2.8.3 Pheromone Chemistry

Although it is straightforward to synthesize many insect pheromones in small quantities for research purposes, only a small fraction of the known insect pheromones are amenable to synthesis in large scale at a cost that will make them economically competitive with insecticides or other control measures. To provide an idea of the scale required, in 2006 the ~160,000 ha of fruit crops that were treated worldwide for control of codling moth (*C. pomonella*) by mating disruption required 25 t of pheromone (Witzgall et al. 2008). The chemistry of pheromones may provide additional constraints. For example, the chemical and isomeric purity of pheromones can be critically important for good efficacy of monitoring lures, with trace impurities producing strongly antagonistic effects (e.g., Millar and Rice 1996). However, the effects of isomers and other impurities on the efficacy of pheromone used for mating disruption are largely unknown, and will probably vary from species to species, and with the types and proportions of impurities in the formulations. In fact,

attempts have been made to exploit these strongly antagonistic effects by including known antagonists in mating disruption blends, with the aim of deterring male moth flight (Bengtsson et al. 1994). In contrast, the efficacy of insecticides generally is unaffected by chemical purity; as long as the toxicant is present, the material will be efficacious. Furthermore, some insect pheromones are rather unstable, rendering it difficult or even impossible to formulate effective blends with the required shelf and field lifetimes (e.g. Grant et al. 2003).

2.8.4 Economic and Regulatory Issues

In addition to the cost of pheromone synthesis, a series of other economic factors must be considered. One of the most intimidating may be the costs of registration of a pheromone for insect control. Although all pheromones are classified as insecticides in the United States, under the relatively relaxed requirements for toxicology and related testing of most lepidopteran pheromones, a registration for application to food crops may be obtained for a few thousand dollars. However, this may not be the case for pheromones of insects other than lepidoptera, and other types of semiochemicals. For these materials, full toxicological screening costing tens of thousands of dollars per compound may be required, although waivers of toxicological and ecotoxicological data may be granted if the materials can be shown to be identical or substantially similar to components of flavors and fragrances. Given the fact that many current pheromone manufacturers are small to medium-sized specialty chemical companies, the costs of registration alone may become prohibitive. In the United States, further costs are incurred because each product must be registered in each of the states in which it will be used. Costs are further compounded because registrations are required for each country in which the pheromone will be used, and the registration requirements have not quite been unified, although the Organization for Economic Cooperation and Development (OECD), the U.S. Environmental Protection Agency, and Canada's Pesticide Management Regulatory Agency are working towards this goal.

There are also other economic factors. For example, most insect pheromones are actually blends of compounds, rather than single components, and it is inherently more expensive to synthesize and formulate a mixture of compounds than a single component. If the components of the blend have widely differing volatilities and other properties, this may provide further complications in developing a formulation and dispenser that will release a constant blend for long periods of time. Third, depending on the crop, the application, and the insect, deployment and maintenance of pheromone devices or traps may constitute a substantial labor cost, particularly if hundreds of release devices have to be deployed by hand (mating disruption and attracticides), or large numbers of traps have to cleared and serviced (mass trapping) for extended periods of time. A fourth cost, which is generally under-appreciated, is a consequence of the fact that pheromone-based control methods may be more complex to use than insecticides, because the target pests are not killed by the pheromone. Thus, crops protected with pheromones require careful and repeated

monitoring, so that if pheromone control starts to break down, for whatever reason, alternative control treatments can be applied.

Thus, there are significant biological, chemical, and economic hurdles that singly or in combination might deter the development of semiochemicals for insect control. Nevertheless, the major advantages of insect control with semiochemicals, such as the low toxicity and minimal effects of insect pheromones on nontarget organisms including humans, their biodegradability, and their expected long (and possibly indefinite) effective lifetimes before resistance develops, provide strong incentives to overcome these hurdles. Furthermore, semiochemicals will play an increasingly important role in the detection and eradication or management of exotic invasive pests, worldwide. That is, we have in general learned to live with the insect pests that are already present in our countries. Thus, the pests that represent the biggest threats may be those that we have not yet seen in our respective countries, particularly when they are released from the pressure of the natural enemies that normally hold them in check. Some examples where semiochemicals have been critically important tools in the detection and eradication of exotic pests would include the Japanese beetle, the Mediterranean fruit fly, the boll weevil, and the pink bollworm. In total, several decades of research on and implementation of insect pheromones have demonstrated that insect pheromones are and will continue to be valuable tools for the detection and management of insect pests, despite the number of hurdles that have be overcome in their successful implementation.

3 Mechanical Signals in the Insect World

Communication is a complex process of information exchange by signals transmitted through different media. Chemical, visual, and mechanically generated signals produced by arthropods travel between the signaler and receiver via defined communication channels. Mechanical and visual signals are emitted as temporally discrete packets of energy, whereas chemical signals are often persistent. Generally, the amount of information that can be conveyed by the signaler's message increases with increasing signal-to-noise ratio.

Communication with mechanical signals is only known to have developed in the phyla Chordata and Arthropoda. Such signals include contact (tactile) signals, and signals transmitted through air (airborne sound), water (waterborne sound), or solids (substrate-borne sound). Tactile signals are involved in courtship and social interactions at short distances, and are not really amenable to manipulation for insect control purposes.

Until recently, amongst the various other types of mechanical signals, airborne sounds were most intensively studied because they are mostly audible to the human sense of hearing. Waterborne signals and signals transmitted over water surfaces are restricted to relatively few and economically unimportant insect species. In contrast, counted by species, family, or phylogenetic distribution, substrate-borne sound communication is common in arthropods, using a variety of different modes

of mechanical signaling (Cocroft and Rodriguez 2005). Development of sensitive devices for recording substrate-borne sounds, such as accelerometers and laser vibrometers, has greatly increased the number of species for which communication through substrates has been described (Drosopoulos and Claridge 2006). In the sections that follow, we will describe vibrational signaling in insects, and then discuss the potential for manipulation of mechanical signals transmitted through plants, because there has been virtually no research on how to use this channel as a tool for insect pest control.

3.1 Characteristics of Mechanical Signals Transmitted Through Plants, Air, or on Water Surfaces

The transmission characteristics of mechanical signals through different media are described by the physics of acoustics, the details of which are beyond the scope of this chapter. A brief overview is presented to provide a basis for understanding phenomena related to the interactions between a signaling insect and the plant substrate upon which it sits while producing signals.

Sound is described as a mechanical disturbance created in a compressible medium (air or water) and is transmitted from the source as longitudinal waves. In water, sound travels about four times faster than in air, and thus travels over longer distances: the velocity of sound waves in air at 20°C is 343.3 m/s and increases by 5.8 m/s for every 10°C rise in temperature. In contrast, the velocity of sound at the same temperature is 1,521.5 m/s in seawater and 1,482.3 m/s in fresh water. The amplitude of sound waves is measured as sound pressure level (SPL) or intensity level (ITL). Both measures are usually given in units (decibels, dB) relative to a standard value, which in air is 20 μPa (20×10^{-6} N/m^2) and at 20°C is equal to an intensity of 10^{-12} W/m^2. The decibel is a logarithmic measure, with each tenfold increase in intensity being equivalent to an increase of 20 dB. Air particle movements are described by displacement (velocity) and pressure. Close to the source (in the near field) the air particle velocity aspect is more pronounced than pressure, but with increasing distance its importance decreases rapidly. The magnitude of near field sound depends on the frequency of the sound, and the geometry and size of the sound source. Airborne sound communication in near field conditions occurs in insects that possess organs sensitive to sound in the frequency range from a few hertz to more than 100 kHz. The medium (fluid) particles absorb sound energy and scatter the sound waves. Both phenomena result in > 6 dB SPL decrease with each doubling of distance from the source. Higher frequency sound is more attenuated by absorption and scattering than low frequency sound. Near-field sound is further attenuated by diffraction by solid objects like vegetation: this phenomenon is again stronger for higher frequency signals. Consequently the time and spectral characteristics of the input signal are changed with increasing distance from the source.

Plants are the usual substrate for the production of substrate-borne signals of most insects and different kinds of waves are propagated through plant tissues.

In the first detailed introduction to the physics of structure-borne sound, Michelsen and co-workers (1982) demonstrated that insects communicate through plants with bending waves. Bending waves include longitudinal and transverse components and propagate with velocities that increase with the frequency, with only minor dependence on the mechanical properties of the structure through which they are transmitted. Low propagation velocity is the main characteristic by which dispersive bending waves are identified. For example, Barth (1985) measured group velocities below 50 m/s for 100–500 Hz signals transmitted through banana pseudostems and leaves. Thirty Hertz signals have been reported to propagate at 4.4 m/s through the apical third of *Agave americana* L. leaves, and at 35.7 m/s through the basal end where the leaf is thicker (Barth 1993). Group velocity for 2 kHz signals was 120 m/s when measured in bean plant stems (Michelsen et al. 1982) and 220 m/s in the reed *Phragmites communis* (Trinius).

Insects do not use longitudinal and transverse waves for vibrational communication. These types of waves occur in structures in which the dimensions of the structures are much larger than the wavelength of the signals. Highly damped Rayleigh waves combine longitudinal and transverse characteristics; their velocity is slightly lower than that of transverse or longitudinal waves but well above the value of bending waves. Rayleigh waves may be relevant for larger arthropods communicating through sand (Aicher and Tautz 1990).

In general, insect-produced substrate vibrations are propagated as non-dispersive bending waves (Casas et al. 2007) characterized by no correlation between propagation velocity, signal frequency, and stem diameter size; such types of waves were demonstrated in rush stems (*Juncus effuses* L., radius from 0.85 to 2.1 mm) for signals of frequencies above 5 kHz. The propagation velocity of this type of wave approaches the value of Rayleigh waves (Graff 1975). Although non-dispersive bending waves maintain their temporal and frequency structures when transmitted through plants, most plant-dwelling insects communicate with low frequency vibratory signals because green plants act as low-pass filters, efficiently transmitting signals below 500 Hz and significantly attenuating those above this value (Čokl et al. 2006, 2007). Burrower bugs of the species *Scaptocoris castanea* Perty and *S. carvalhoi* Becker (Heteroptera: Cydnidae) are an instructive example of this phenomenon. Both species feed and mate on roots of soybean plants (Lis et al. 2000) where they stridulate (Čokl et al. 2006). During transmission through the surrounding soil, signals with about 0.07 mm/s velocity (a measure of intensity of the signal, measured as a vector quantity that specifies time rate of change of displacement) at the source are attenuated approximately 3–9 dB/cm. In contrast, when transmitted from the roots to the stem and leaves of the plant, signal attenuation did not exceed 1 dB/cm. Spectra of signals recorded at the source had harmonic frequencies up to 10 kHz, with the dominant frequency at 500 Hz. Spectral units of signals above 500 Hz that were recorded from leaves and stems were significantly attenuated. In this respect, the spectra of signals recorded from above-ground plant parts resemble those characteristic of low frequency vibratory signals emitted by stink bugs such as *N. viridula* and *Murgantia histrionica* Hahn (Čokl et al. 2007).

The resonant properties of green plants also determine the spectral and amplitude characteristics of the transmitted signals. Spectra of *N. viridula* female calling songs recorded on a non-resonant substrate were characterized by a narrow dominant frequency peak around 100 Hz with harmonics not exceeding 500 Hz (Čokl et al. 2000). Spectra of the same signals recorded on a plant had new peaks below and above the dominant frequency shifted to about 20 Hz higher value (Čokl et al. 2005). Resonant spectra of signals measured from several green plants have distinct peaks below 500 Hz, with the dominant frequency between 160 and 220 Hz. This peak corresponds to the range of the first harmonic values determined in all stink bug vibratory signals recorded to date (Čokl and Virant-Doberlet 2003; Moraes et al. 2005; Gogala 2006; Bagwell et al. 2008) and corresponds to the maximum sensitivity of the middle frequency subgenual organ receptor cell in these insects (Čokl 1983).

Vibratory signals travel through green plants with low attenuation (Barth 1998). For example, signals of 75 or 5,000 Hz frequency were attenuated during transmission through monocotyledonous plants (banana, agave) by only 0.3 and 0.35 dB/cm respectively. Still less attenuation was measured for 30 Hz signals (Barth 1985). Michelsen and co-workers (1982) vibrated bean stems with short pulses and recorded significantly prolonged vibrations at some distance from the source. This means that vibration pulses reflect from the roots and apices of the plant and travel up and down the plant's rod-like structures such as stems, stalks, and side branches with low attenuation, thus creating standing wave conditions. Consequently, the amplitudes of the vibratory signals do not decay linearly with distance (Michelsen et al. 1982; Barth 1998) but are characterized by regularly repeated peaks of minimal and maximal values at nodes and internodes (Čokl 1988; Čokl et al. 2007). Regular variation of signal velocity with distance also has been demonstrated under natural conditions (Čokl et al. 2007): the distance between peaks for *N. viridula* female calling song transmitted through a *Cyperus alternifolius* L. stem ranged between 10 and 15 cm and the maximum difference between neighboring velocity peaks at nodes and internodes was 19.4 dB. The overall attenuation of naturally emitted stink bug signals of ~100 Hz was about 0.1 dB/cm at a distance of 1 m.

The distance between nodes (and internodes) decreases with increasing frequency of the signal. Spectra of signals with different frequency components have different ratios of spectral peak amplitudes at different points on the plant. The distance between nodes of the dominant frequency is double the analogous distance for its first harmonic, and at the position of the dominant frequency node (lowest dominant frequency spectral peak amplitude value) one can get the highest amplitude of the first harmonic (Čokl et al. 2007). Although the amplitude of the signal varies in phase with the amplitude of the dominant frequency, the amplitude pattern of frequency modulated signals, or those composed of different frequency components, can vary significantly at different distances from the source on the plant.

Several insects communicate with signals transmitted over water surfaces. At wavelengths above 1.7 cm, most wave motion is maintained by gravity, whereas with oscillations with frequencies above 15 Hz wave motion is mainly maintained by capillary forces (Greenfield 2002). For example, a small aquatic insect emits 25

Hz capillary waves of 10 mm wavelength traveling outward with 0.25 m/s velocity. Vibratory signals (the modulation envelope of the wave group) are transmitted over the water surface either more slowly for pure gravity waves or faster for pure capillary waves. The damping coefficient in deep water is about 0.11 dB/cm (Lighthill 1978). The coefficient between the energy and area of capillary waves is proportional to the square of frequency, and high frequency signals, like airborne sound, are attenuated to a higher degree than low frequency ones. Thus, the effective communication range over water surfaces is restricted to distances between 1 and 2 m for insects emitting vibrational signals of frequencies above 20 Hz (Wilcox 1995). A sinusoidal wave is dispersed by water into a range of wavelengths, and surface waves of different wavelengths travel at different velocities.

Only four orders of insects (Odonata, Hemiptera, Trichoptera and Coleoptera are known to communicate with specialized sounds underwater (Aiken 1985). Water-borne sounds are strongly reflected from solids and from the water surface; because of the longer wavelengths used in these signals, most communication of this type occurs in the acoustic near field.

3.2 The Use of Different Mechanical Signal Modalities

Several analogies can be drawn between chemical signaling and communication with mechanical signals. For example, mechanically produced signals are transmitted with high velocity over relatively long distances, analogous to wind-borne volatile chemicals. Because mechanical signals can be emitted in temporally defined bursts in distinct patterns, they can be highly species and sex specific, in parallel with many chemical signals. Furthermore, insects detect the position of the signaler by homing in on the signal transmitted through the air or through a substrate. Airborne acoustic signals are less dispersed into different wavelengths and velocities than substrate-borne signals when traveling through acoustic free-field. Nevertheless, compared with lower frequency sound, higher frequency signals are more attenuated by absorption and scattering, and more attenuated by diffraction by solid objects like vegetation. Thus, low frequency airborne signals should be more effective for long distance communication by plant-dwelling insects, but insects cannot efficiently emit low frequency airborne sound because of their small body sizes. An object oscillating in an elastic medium (air or water) radiates compressional sound waves efficiently when the vibrating body is large compared with the wavelength of sound in the transmission medium (Markl 1983). Thus, insects can communicate efficiently with airborne sound in the frequency range in which their body size (radiator's diameter) equals or is larger than one third of the radiated wavelength. For example, a one cm insect can efficiently radiate sound waves only above 10 kHz in air and above 50 kHz in water. This is one of the major reasons why most small plant-dwelling insects communicate through the substrate. Furthermore, higher frequency sound and ultrasound signals that are transmitted through air in the acoustic free field expose the signalers to discovery by flying predators and parasitoids. In contrast,

substrate-borne signals can only be exploited by predators and parasitoids if they are on the same substrate as the signaler. The same restriction applies to intended receivers (i.e., conspecifics), but may be overcome by using the vibrational signal in tandem with other, longer-range signaling modalities such as volatile pheromones.

Contact vibration and near field medium motion are widely used in insects but because they are only effective over very short ranges, they are not discussed here. Instead, we will focus on possible manipulation of long range calling signals as a method for insect management.

3.3 Signal Types and Behavior

Signal features are designed according to the communication range, the positions of the signal sender or receiver, the duty cycle of the signal (the ratio between the signal being "on" and "off"), the sender identification level, the modulation level (the amount of inter-individual variation measured by the position of the signal along a scale from stereotyped to graded), and the degree of form-content linkage, defined as the degree to which signal form is dependent upon signal content (Bradbury and Vehrencamp 1998). The design rules have been generally examined for signals emitted in the context of mate attraction, courtship, territorial defense, threat, and alarm.

In the mate attraction process, questions about species and sex identification, receptivity, and location are important (Bradbury and Vehrencamp 1998). In most insects, mate attraction is relatively simple, with one sex producing calling signals when it is receptive. In this case, the signal only needs to carry information about species and location of the signaler. The signal has to be readily repeated from one place, has to carry the information over long distances, needs to be complex enough to retain its species specificity, and has to have a high duty cycle (long "on" time). In contrast, courtship signals are emitted when conspecifics are in close proximity, and function during mate assessment and mating synchronization (Bradbury and Vehrencamp 1998). Because of the short distances over which they act, signals used in courtship are different in structure than those used in long distance communication; they mediate a specific sequence of events, each triggered by specific signals carrying different information. Usually males are less choosy and more motivated to mate than females, and use courtship to induce the female to mate. In addition, errors in species recognition (and mate choice) are more costly for females, and thus females evaluate males' signals carefully. Acoustic courtship signals are generally more species- and sex-specific than long-range signals, and have a high duty cycle. Thus, insect courtship signals are characterized by long pulse trains with complex temporal patterns, and often have frequency modulated subunits. Because of the short distance over which the signals are used, these characteristics are not significantly changed during transmission through the medium. They also do not need to carry directional information because at that point, prospective mates are usually in visual and/or tactile contact.

Threat signals are emitted during direct competition of two individuals at close range for resources like food, mates, or territory (Bradbury and Vehrencamp 1998).

Threats are produced when a sender and receiver are competing for a resource, and serve as a way of minimizing actual combat which can result in damage to both competitors. Threat signals are in some ways similar to courtship signals because they are intended for short range use, the threats are directed at a specific individual, and the signals need to convey honest information as to the quality of the signaler. On the other hand, the duty cycle is usually shorter, with threat signals usually being short and of high intensity.

Bradbury and Vehrencamp (1998) also describe territorial defense and alarm signals. The territorial defense signals are known only from insects and the alarm signals are found primarily in some social species. Alarm signals can be differentiated into those that stimulate flight (if a group is in immediate danger), assembly signals (dispersed individuals are called to the signaler), and alerting signals, when a threat is perceived but danger is not imminent. The design of each type of alarm signal is different. Signals that stimulate flight do not have to be species specific, their duty cycle is low, and they should carry as little information as possible about the position of the signaler. In contrast, assembly alarm signals can be longer, are usually species specific, and should allow location of the sender. Although alarm signals of different modalities have been described, auditory alarm signals show the greatest complexity, employing repetition rate, duration, frequency and intensity characteristics of the signals to encode information about danger, urgency, or other important details.

3.4 Sound Communication in Stink Bugs

Stink bugs, particularly those in the subfamily Pentatominae, represent one of the insect groups that has been most intensively studied, in large part because of their economic importance as agricultural pests. They can be difficult to control, and in the search for novel methods of managing these pests, researchers have investigated numerous aspects of their biology, ecology, and behavior (Panizzi et al. 2000). Pentatominae, and particularly the southern green stink bug *N. viridula* (L.), form useful models for studies of chemical and substrate-borne sound communication because these insects make use of both of these methods of communication, providing an opportunity to understand how interruption of basic communication processes can be used as a tool for insect pest control. The general use of chemical signals for insect control are described in sections 2.2 to 2.8. Because olfactory and substrate-borne sound signals are both involved in communication in *N. viridula*, these types of signals will be described in tandem, in the context of stink bug mating behavior.

3.4.1 Biology and General Statement of Stink Bug Economic Importance

Stink bugs, family Pentatomidae, with about 4,100 described species, form the third largest heteropteran family, after Miridae and Lygaeidae (Panizzi et al. 2000). The family Pentatomidae comprises eight subfamilies: Asopinae, Cyrtocorinae, Discocephalinae, Edessinae, Pentatominae, Phyllocephalinae, Podopinae, and Serbaninae

(Schuh and Slater 1995). Many stink bugs are herbivorous, with some species in Edessinae and Pentatominae being regarded as major pests worldwide. They feed by piercing plant tissues with their maxilliary and mandibulary stylets, then sucking up plant fluids with nutrients from the food source. Thus, they cause loss of fluids and decrease in turgor pressure, and they also inject destructive digestive enzymes, transmit plant pathogens, and delay plant maturation (McPherson and McPherson 2000). The edessine species *Edessa meditabunda* (F.) is a pest of many Solanaceae and Leguminosae, feeding also on cotton, tobacco, sunflower, papaya, and grapes (Panizzi et al. 2000). Major pests among the Pentatominae include *N. viridula*, *Oebalus pugnax* (F.), *Acrosternum hilare* (Say), *Euchistus servus* (Say), and *Euchistus variolarius* (Palisot de Beauvois) (McPherson and McPherson 2000).

The southern green stink bug, *N. viridula*, originated from eastern Africa but it has been introduced into tropical and subtropical regions of Europe, Asia, Australia, Africa, and the Americas by global commerce (Hokkanen 1986; Jones 1988; Kavar et al. 2006). The recent expansion of this species in South America is the result of increased acreage for soybean production (Panizzi and Slansky 1985), and climatic change is the probable cause of this invasive species becoming established on the Galapagos islands (Henry and Wilson 2004), in Hungary (Redei and Torma 2003), and in Britain (Barclay 2004; Shardlow and Taylor 2004). The northern border of the range of this species has moved 70 km in Japan within the past few decades, related to the movement of the 5°C isotherm of the average temperature in January (Musolin and Numata 2003). Because of its economic importance, the southern green stink bug is one of the most intensively investigated pentatomid species in the world (Todd 1989).

N. viridula is highly polyphagous, feeding on both monocotyledonous and dicotyledonous plants from more than 30 families, with a distinct preference for legumes (Todd and Herzog 1980; Todd 1989; Panizzi and Slansky 1991; Panizzi 2000; Panizzi et al. 2000). As with other multivoltine stink bugs, *N. viridula* switches from one host to another, taking advantage of differences in temporal patterns of fruiting in their various hosts (Panizzi 1997). In addition to being one of the major global pests of soybean (Kogan and Turnipseed 1987), *N. viridula* also damages many other economically important crops including cowpea, pecan, macadamia, rice, wheat, sorghum, corn, tomato, tobacco, and cotton (Hoffmann 1935; McPherson and McPherson 2000). Plants with developing fruits or pods appear to be more attractive than those with mature ones (McPherson and McPherson 2000).

Damage to soybeans is of particular current importance because of recent increases in acreage of this crop in many areas of the world. Southern green stink bugs quickly adapted to soybean, and the damage from increasing numbers of this species has spilled over into other crops like cowpea and pecan.

3.4.2 Signals Involved in Communication During Mating Behavior of Stink Bugs

The mating behavior of *N. viridula* and many other pentatomine bugs consists of two phases, long range mate location, followed by short range courtship interactions

(Borges et al. 1987). These authors confirmed the presence of a sex attractant pheromone in *N. viridula* that had been reported earlier by Mitchell and Mau (1971), and demonstrated that odors from males elicited long range mate location behaviors from females. Thus, the male-produced pheromone appears to attract females to the vicinity of the odor source (Aldrich et al. 1987), at which point shorter range acoustic signals that are transmitted through the plant substrate take over. The stereotyped short range courtship phase of stink bug mating behavior was described first for *Chlorochroa ligata* (Say) and *Cosmopepla bimaculata* (Thomas) (Fish and Alcock 1973), and in *N. viridula* it includes male–female antennation, abdominal vibration by the male, and head-butting of the female to induce her to lift her abdomen to the position required for coupling of the genitalia, genital coupling, and copulation (Kon et al. 1988). Courtship is preceded by the medium range calling phase which serves to bring the two sexes together. This phase starts with the emission of the female calling song (FCS) (Čokl et al. 2000). The signals or cues that stimulate the female to begin calling are not yet fully known, but preliminary experiments suggest that the male pheromone may be involved. FCS emission activates a male to move towards the female, mediates directionality at branch points on the stem (Ota and Čokl 1991; Čokl et al. 1999), triggers male emission of the calling (MCS) and courtship (MCrS) songs (Čokl et al. 2000), and increases the rate of pheromone emission by the male (Miklas et al. 2003).

Male rivalry singing has been recorded and described for *N. viridula* and other phytophagous stink bugs (Čokl et al. 2000; Čokl and Virant-Doberlet 2003; Moraes et al. 2005a; Bagwell et al. 2008). A pair of male rivals alternate singing in an a-b-a-b-a-… fashion with short signals of high intensity and low species specificity, which inhibit singing of the competing male. The song is produced when two (or more) males are courting the same female. Female *N. viridula* are silent during male rivalry. A male emits the rivalry song to silence the rival and afterwards resumes calling and courting the female, who in turn resumes emitting her calling and courtship songs.

A broad-band repelling song emitted by females (FRS) has been recorded only in *N. viridula* as a vibration of several seconds duration, without a pulsed pattern (Čokl et al. 2000). The song was recorded within the courtship phase when a female rejected a courting male, and the song inhibits male singing.

The *N. viridula* male pheromone (Aldrich et al. 1989) and vibratory songs (Čokl and Virant-Doberlet 2003) are species specific. Nevertheless, they do not completely prevent interspecific copulations. In areas of sympatry in Japan, interspecific copulations frequently have been observed between *N. antennata* Scott and *N. viridula* (Kiritani et al. 1994; Kon et al. 1994), although no viable offspring result (Kiritani et al. 1963).

To date, calling and courtship songs have been described in more than 20 pentatomine species (Gogala 2006). Mating behavior, as described for *N. viridula*, is similar in all these species except for stink bugs of smaller body size such as *M. histrionica* (Hahn) (Čokl et al. 2004) and *Holcostethus strictus* (Fabricius) (Pavlovčič and Čokl 2001). In the latter two species, males initiate communication with vibratory signals, and females do not emit a long calling song with steady repeated pulses or pulse trains. The female courtship song is triggered by male courting and

no regularity in the exchange of courtship songs has been observed. Differences in the calling phase between these species and *N. viridula* and the other larger pentatomids may be a consequence of shorter distances between the legs of the smaller species, which do not enable the smaller individuals to determine the direction of a signal from the time difference between the moving vibration hitting the first and subsequent sets of legs; for these smaller bugs, the time interval may be too short for the individual to be able to discriminate which legs were vibrated first (Virant-Doberlet et al. 2006).

Contrary to the well-expressed temporal structure of the species and sex specific pentatomine songs, the spectral characteristics of these signals are similar between species, reflecting the shared mode of sound production. Vibratory signals are produced by synchronous contraction of the tergal longitudinal (TL) and lateral compressor (LCr) muscles attached on one end to the side of the tergal plate formed by fusion of the first and second abdominal tergites, and on the other to the thoracic (TLI) or abdominal wall (TLII, LcrI, and LCrII) (Maluf 1932; Kuštor 1989). Muscular contractions vibrate the abdomen versus thorax in a 1:1 fashion according to electromyiogram potentials recorded by implanted electrodes (Kuštor 1989). The dominant (fundamental) frequency of all known songs of pentatomine bugs ranges between 80 and 150 Hz. Spectra differ in the number of higher harmonics, which do not exceed 1 kHz, and in the presence (or absence) of frequency modulated units. As described above, such spectral properties are well tuned to the resonant frequency of green plant hosts as the medium for transmission of vibratory communication signals.

3.4.3 Insect–Plant Interactions During Substrate-Borne Communication

Vibrational signals are characterized by their time (pulse duration, repetition time, number of pulses per pulse train, etc.), frequency (fundamental frequency, higher harmonics, frequency modulation), and amplitude characteristics. The amplitude variation with distance depends on the signal's frequency characteristics. According to the dispersive nature of bending waves transmitted through a substrate under standing wave conditions, the amplitude of vibratory signals transmitted through green plant stems varies, with regularly repeated peaks of minimal and maximal values at nodes and internodes. The distance between peaks decreases with increasing signal frequency. The velocity of signals measured at the body of a singing *N. viridula* ranged between 0.3 and 0.7 mm/s (Čokl et al. 2007) and decreased by less than 0.5 dB/cm. Because the threshold sensitivity of leg vibrational receptors lies 20–40 dB below this value (Čokl 1983), bugs can efficiently communicate over distances greater than 1 m when on the same plant. The fundamental frequency remains stable, but at different distances from the source, different ratios of spectral peak amplitudes determine the amplitude of frequency modulated or spectrally different signal subunits.

Michelsen and coworkers (1982) demonstrated that sine wave pulses of a few milliseconds, when transduced into a plant's stem, persisted for more than 20 ms.

Signal duration is prolonged because the vibration pulse reflects from apices and roots, and due to low attenuation, the signal may travel up and down the stem several times, creating standing wave conditions in which the signal pattern is frequency dependent and often complicated. The repetition rate of vibratory signals or of their subunits remains the parameter that generally is not changed during transmission. Thus, this characteristic of the signal may transmit the most reliable information about sex, species, and location within the calling phase of mating behavior.

Recently, Cocroft and coworkers (2006) measured the influence of the substrate on frequency and temporal characteristics of vibratory signals of male treehoppers, *Umbonia crassicornis* (Hemiptera: Membracidae), on two woody plants, *Albizia julibrissin* Durazz. (Mimosaceae) and *Viburnum lentago* L. (Adoxaceae). At a distance of 10 cm between the measuring points (5 and 15 cm from the source) the authors found that the influence of the substrate on temporal parameters was relatively small. The distance influenced signal duration, but the effect varied among individual plants and the difference was slight. As expected, the signal repetition rate was not influenced by the plant species, individual plant, or distance. The dominant frequency was not different for different plant species but varied among different plants of the same species. The authors found no overall effect of distance on dominant frequency.

Miklas and coworkers (2001) demonstrated that the inner time structure of a pulse train may change during transmission when prolonged pulses are repeated with such high repetition rates that they fuse. This is the case in *N. viridula* male–female calling duets (Miklas et al. 2001). The *N. viridula* female calling song sequence is composed of pulse trains with two or more pulses (Čokl et al. 2000). The non-pulsed type (FCS-np) is characterized by a short prepulse followed by a long pulse of about 1 s. The pulsed type (FCS-p) contains three or more pulses that are quickly repeated. The duration and repetition rate of pulse trains of both types are similar. On a non-resonant substrate, males respond only to the FCS-np pulse trains. When stimulated with these signals on a plant, males responded to pulse trains of both types. The analyses of plant recorded FCS-p signals has shown that short FCS-p pulses become prolonged by transmission through a plant and fuse to such an extent that males cannot differentiate them from the FCS-np pulse trains and respond to them. Results of investigations with *U. crassicornis* cannot be directly compared with those with *N. viridula* because experiments with the two species were conducted on different substrates (woody and green plants respectively).

Acoustic communication by plant-dwelling insects over longer distances through a plant occurs predominantly or exclusively through the substrate. Determination of the informational value of calling songs is easier than similar determinations with songs involved in the courtship phase, where signals of different modalities are involved. According to expected signal parameter changes during transmission through a plant, signal design follows general rules including steady repetition rate of simple and less amplitude modulated units, the spectral properties of which are tuned to the mechanical properties of the transmission medium. The necessity to provide information about the signaler's position seems to be more important than information about the signaler's identity. For example, *N. viridula* males responded

when stimulated with calling songs of females of the sympatric pentatomine species *Palomena prasina* (L.) and *P. viridissima* (Poda) (Čokl et al. 1978). Results of this early study revealed that *N. viridula* males recognized and responded to synthesized female calling songs in which the changes in spectral and temporal characteristics that would be expected during transmission through a green plant had been simulated.

3.5 Disruption or Manipulation of Acoustic Signals as a Potential Method for Insect Management

The study of vibrational communication in insects is relatively recent, following development of recording instruments that are sensitive enough to detect and measure signals of low intensity from mechanically delicate biological substrates such as plants. Although substrate-borne acoustic communication is widespread in insects, data about basic phenomena are still lacking. With the accumulating body of basic research in this field, scientists can begin to think about practical applications of substrate-borne communication for management of insect pests.

A good basic knowledge of the biology of the target species is critically important in order to be able to exploit or manipulate aspects of the insect's life history for our own purposes. In this context, investigations should be focused on species of sufficient economic importance to warrant the costs of basic research and development. A number of stink bugs fit this criterion, and both the economic importance and the extensive knowledge of the basic biology of *N. viridula* renders it a good model species for such studies. The reliance on both chemical and acoustic communication during mate location in this species provides opportunities for disruption of these communications to achieve pest control. We shall discuss the following phenomena: (Section 3.5.1) attraction of parasitoids and predators, (Section 3.5.2) interruption with induced vibrations, (Section 3.5.3) combination of signals with sub-lethal doses of insecticides, and (Section 3.5.4) combination of acoustic signals with chemical or other signals.

3.5.1 Attraction of Parasitoids and Predators

All life stages of *N. viridula* and other phytophagous stink bugs are subject to attack by a variety of parasitoids, predators, and entomopathogens (Panizzi and Slansky 1985; Todd 1989). For example, Jones (1988) listed 57 parasitoids attacking *N. viridula*. The most important are the wasps *Telenomus podisi* Ashmead and *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae), *Trichopoda pennipes* F. (De Groot et al. 2007), and *T. giacomelli* (Blanchard) (Diptera: Tachinidae). Predatory stink bugs of the pentatomid subfamily Asopinae, including *Picromerus bidens* L., *Podisus maculiventris* Say (De Clerq 2000; De Clercq et al. 2002; Vandekerkhove and De Clercq 2004), and *P. nigrispinus* (Dallas) (Saini 1994) are

among numerous arthropod predators that feed on *N. viridula*, and the fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae) is a significant predator of eggs (Kryspin and Todd 1982; Stam et al. 1987).

It has been demonstrated recently that females of the egg parasitoid *T. podisi* responded with oriented movements towards vibratory signals of the Neotropical brown stink bug, *Euschistus heros* (F.) (Laumann et al. 2007). The reaction was sex specific, and was triggered by female but not male songs. The *E. heros* female song is a typical pentatomine calling song characterized by repeated pulses of the fundamental frequency around 145 Hz (Moraes et al. 2005a), and is similar to the FCS of *N. viridula* (Čokl et al. 2000). We can expect that female calling songs of different species with similar time and frequency characteristics will also attract this (and possibly other) parasitoids, but further experiments are needed to determine the specificity of the reaction. Overall, the parasitoids exploit these signals for medium to short range location of hosts; the initial host finding steps consist of longer range orientation to a plant that might be infested with hosts, mediated by chemical signals from the plant (Colazza et al. 2004; Moraes et al. 2005b). Host-produced kairomones are also involved in longer range orientation (Mattiacci et al. 1993; Medeiros et al. 1997; Borges et al. 1998; Colazza et al. 1999; Conti et al. 2003). Over shorter ranges, parasitoids then locate and recognize their hosts using both chemical and visual cues (Sales et al. 1980; Bin et al. 1993; Borges et al. 1999, 2003; Colazza et al. 1999; Conti et al. 2003).

Thus, one potential method of exploiting stink bug acoustic signals would be to vibrate a plant with a stink bug female calling song, with the aim of stimulating parasitoids to search for hosts more intensively. Although such experiments could be conducted relatively easily in the laboratory, it is difficult to visualize how this might be used in the field as a crop protection strategy. There are at least three problems. First and most obvious is the problem of how to vibrate a whole field of plants. The second problem is that artificial vibration of the whole plant will probably disrupt the ability of parasitoids to locate real calling hosts, so that parasitoids would find hosts only by chance. Finally, vibration of a plant may silence calling host females. Thus, it is difficult to see how the the oriented movement of parasitoids in response to vibrational signals from their hosts might be manipulated for practical purposes.

3.5.2 Interruption with Induced Vibrations

The emission of different songs in a male-female *N. viridula* duet follows defined rules. All the songs are triggered by songs emitted by the opposite sex, except for the female calling song which is triggered by the presence of a male (unknown triggering signal), and also emitted in the absence of male responses. The response of males with the courtship song stabilizes the repetition rate of female calling signals. Increasing ambient temperature up to 28°C increases the repetition rate of the FCS pulse train, but further temperature increase results in a decreased repetition rate (Čokl and Bogataj 1982). This early experiment also pointed to the important

role of chemical communication in the courtship phase of mating behavior: antennectomized males readily courted and copulated with normal females but females without antennae ignored male courting, rarely responded with the courtship song, and never copulated.

Recently Polajnar and Čokl (2008) studied the possible disrupting effect of a 100 Hz signal on vibrational communication between male and female *N. viridula*. The artificial signal significantly decreased the number of males responding to female calling songs, and significantly fewer males responded with the courtship song. However, the disturbance signal did not change the time that males needed to locate calling females. Females still produced calling songs during disturbance with the continuous 100 Hz signal, but some of them changed the rhythm by skipping one or more signal intervals or emitted the repelling song. Females did not change the time characteristics of their calling signals but varied the dominant frequency when the disturbing frequency was similar; the number of females which changed the dominant frequency increased with decreasing difference between the dominant frequencies of their own and the disturbing signal.

These experiments need confirmation under field conditions. As stated above, a major practical problem is how to vibrate plants in the field. If this can be worked out, there are several possible ways in which communication could be interrupted. For example, played back female repelling song might be used to silence males and inhibit their courting, although further experiments are needed to confirm the hypothesis that the repelling song causes males to leave the plant. Another possibility is to disorientate males by reproducing female calling songs with unnatural signal repetition rates, to give stochastic and interfering signals at branch points, with the aim of disrupting males' ability to orient to calling females. Finally, stimulation with the male courtship or rivalry songs may induce rivalry and inhibit courtship.

Thus, use of artificial signals to disrupt normal acoustic communication may have some promise from a theoretical viewpoint. However, its potential only can be tested properly if we can surmount the technical challenges posed by the problem of vibrating large numbers of plants under field conditions.

3.5.3 Communication and Insecticides

Sublethal doses of neurotoxic insecticides directly affect the peripheral and central nervous systems of insects, which may result in indirect effects on their behaviors. The neuronal basis of behavior has been studied in insects at several levels, mainly in the context of a single neuron's responses to stimulation with signals with parameters that mimic those characteristic of natural communication signals. To our knowledge, the effect of insecticides on the activity of neuronal nets underlying communication have not been explored thoroughly. Nevertheless, we can expect that sublethal doses of neurotoxic agents will affect insect communication by their direct action on receptors and the underlying neuronal networks. This hypothesis has been confirmed in *N. viridula* treated with sub-lethal doses of imidacloprid, which has been shown to act on synapses as an acetylcholine agonist (A. Žunič, personal

communication 2008). Doses 10–100 times below the lethal level significantly decreased calling, resulting in fewer successful mate locations and copulations. Thus, insecticides may have some potential for insect control by disruption of behavior when applied at sublethal levels. However, this potential benefit must be weighed against the increased risk of enhancing the development of insecticide resistance by challenging the population with sublethal doses of insecticide.

3.5.4 Calling Signals in Combination with Pheromone Traps

One of the most important factors in effective management of stink bugs is knowing when and where bugs are present, and in what numbers. Because many species are oligophagous and highly mobile as adults, they can migrate from crop to crop in response to senescence or harvesting of one crop, or they can migrate into crops from surrounding uncultivated land as the natural vegetation senesces. Thus, efficient monitoring is crucial for stink bug management. Unlike most insects, which use a single communication mode for long range attraction of mates or conspecifics, stink bug communication is bimodal, with chemical signals probably acting over longer ranges to bring individuals together on the same plant, and vibrational signals becoming predominant to actually bring the individuals together. Thus, for many phytophagous stink bug species, including *N. viridula* (Aldrich et al. 1987), *Acrosternum hilare* (JGM, unpublished data 2001), *Thyanta* spp. (McBrien et al. 2002), *Chlorochroa* spp. (Ho and Millar 2001a,b), and *Biprorulus bibax* (James et al. 1996), monitoring traps baited with pheromones caught few bugs. However, all of these authors noted that the plants in which traps were placed had many more bugs on them than control plants. Thus, as expected, the pheromones did indeed act as long range attractants, but once attracted to the vicinity of the pheromone lure, bugs require the secondary vibrational signals to locate and enter the trap. Thus, a combination trap that incorporates both pheromonal and vibrational signals may provide the solution, and this concept is under active investigation (JGM, unpublished results). What is required is a small playback device with bug songs prerecorded on a chip, a power source to drive it, and a transducer to transmit the signal into the trap substrate. All of these components are cheap and readily available, and we are currently working with prototype devices to obtain proof of concept. In short, a vibrating pheromone trap may provide an effective method of monitoring stink bug populations. Furthermore, it should be possible to extend this concept to other insects that communicate with both acoustic and pheromonal signals, such as clothes moths (Takács et al. 2003) and the peach twig borer moth (Hart 2006).

4 Summary

Insect pheromones and related semiochemicals are much more than scientific curiosities. Because of their critical role as essential intraspecific signals, without which insect species cannot live and reproduce, pheromones have tremendous

potential for exploitation for insect control, and they can and should be developed in our ongoing struggle to protect crops and forests from insects. The identification of the first pheromones some 50 years ago resulted in an initial period of great excitement and tremendous expectations for pheromones, followed by disillusionment and some loss of interest in pheromones when practical applications were slow to develop. However, steady progress in recognizing and understanding the complexities of semiochemically-based insect management, coupled with the development of reliable formulation and dispenser technology, has positioned us for continuing growth in practical applications of semiochemicals. This growth will be enhanced by the increasingly restrictive regulatory climate for insecticides, and by increasing public pressure for foodstuffs that have not been treated with insecticides. Semiochemically-based insect control methods cannot and will not be developed for all crops, for reasons described above. However, for those crops and insect pests for which both the biology is favorable and the economics are at least competitive with current management practices, semiochemicals may have a strong future.

Furthermore, acoustic signals transmitted through substrates have now been described in numerous groups of insects, and these signals are essential mediators of insect behaviors. The signals are relatively short range, being restricted to one plant or to two or more plants that are in direct contact with each other. These acoustic signals, alone or in concert with signals of other modalities, enable efficient and precise mate location and recognition in a complex environment over distances of several meters. The use of laser vibrometry technology for recording and measuring low intensity vibratory signals from delicate biological substrates has enabled deeper insight into the basic phenomena underlying the interactions between the calling insect and the plant substrate during communication. Stink bugs form an instructive model system for illustration of efficient bimodal communication systems that use both pheromones and substrate-borne signals. Whereas no methods of exploiting or manipulating vibratory signals for insect pest control have been commercially developed to date, this is currently an area of active investigation, for several types of insects. With our expanding knowledge of this widespread mode of communication in insects, it is certain that new possibilities will arise. To date, the most promising use of vibrations may be in combination with pheromone traps for pest insects, but as discussed, there also may be possibilities for using vibratory signals to manipulate the behaviors of beneficial insects such as parasitoids. Thus, substrate-borne acoustic communication in insects represents a virtually untapped field for both basic research and for practical applications in insect control.

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Physical Control: An Important Tool in Pest Management Programs

Phyllis G. Weintraub

1 Introduction

Various methods of physically controlling arthropod pests are simultaneously both the oldest techniques and a current source of innovation. Unlike pesticides, there is no need for governmental regulation/registration with the concomitant need to spend millions of dollars satisfying environmental and animal toxicology, food safety and efficacy requirements. The focus of this discussion will be on pre-harvest agricultural practices (Table 1) in North America and Europe; there are two recent extensive reviews of pre- and post-harvest physical control methods (Vincent et al. 2008 and Vincent et al. 2009). Physical control methods work well with either pesticide-centered, genetic resistance or biological control-centered integrated management strategies.

2 Insect Exclusion Screens

The first greenhouse was erected for the Roman emperor Tiberius and it produced cucumbers out of season (Woodes and Warren 1988). Since then, a range of structures have been built to cater to the epicurean cravings and horticultural demands of various august personages down the ages. At the beginning of the 20th century the use of greenhouses to grow flowers and vegetables out of season – basically in winter months – began expanding exponentially. However the idea of erecting a structure as a physical barrier per se – to deny insect pests access to plants – dates back to just three decades ago. Following the invasion of virus-bearing whiteflies (*Bemisia tabaci* (Gennadius)), it was impossible to grow tomato crops in open fields anywhere in the Mediterranean region from late spring through autumn (Berlinger et al. 1996). The development of insect exclusion screens (IES) – fine mesh screening, not a solid plastic

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Table 1 Types of pre-harvest physical control methods and exemplary pests

Method	Target pests	Comments
Exclusion screening	Aphids, leafhoppers, leafminers, moths, thrips, whiteflies	Primarily in greenhouse and tunnels, includes UV absorbing screening
Fences	Beetles, flies, leafhoppers	Good for flying insects moving close to the ground
Floating row covers	Aphids, leafhoppers	Especially important for controlling plant pathogen vectors as costly to use
Mulching	Aphids, beetles, flies, whiteflies	Includes natural products and plastics
Particle films	Leafhoppers, mites, moths, psyllids	Primarily used in orchards during times of low rainfall
Pneumatic	Beetles, leafminers, mirids, moths, whiteflies	Open field (tractor driven) or greenhouse
Rogueing/pruning	Vector species	Used primarily in orchard or perennials
Sanitation	All pests	Open fields and greenhouses
Trapping	All flying pests	May be used in conjunction with pheromones

barrier – enables the cost effective production of tomatoes and other vegetables (Taylor et al. 2001; Berlinger et al. 2002) even under significant pest pressure. The advantage of mesh screening, as opposed to solid plastic sheets, is that it permits movement of air and help reduce humidity, which enhances plant pathogen development. The various forms of IES (woven, knitted and microperforated) have been reviewed extensively (Weintraub and Berlinger 2004). The IES is probably the single most important physical control method developed in the last century.

To illustrate just how important this physical control method is to agriculture today, we can look to Spain, whereas in 2006 there were almost 77,000 ha of covered crops (Anonymous 2008). However, distribution throughout the country was far from even – about 1% was located in the northern region (Oceanic climatic zone) and 6% in the central region (Continental climatic zone), both of which require greenhouse heating in the winter months. Some 93% of the crops covered with IES were grown in the coastal, Mediterranean climatic zones, which do not require heating in the winter. Almost 50% of the major vegetable crops: cucumber, eggplant, tomato, pepper and strawberry, were grown under IES (50,400 uncovered versus 48,700 covered hectares). This demonstrates just how important physical control measures are in pest management.

During the last decade there has been innovative research to develop specialized agricultural plastics, which include: agents to alter spectral properties (Ashkenazi 1996); additives to augment diffusion and the penetration of light deeper into the plant canopy (Pollet et al. 2000); additives that reduce energy losses by blocking infrared radiation (Edser 2002); and additives to modify the transmitted light spectrum (Edser 2002).

It has long been known that some species of insects use ultraviolet light for orientation (Kring 1972) and all insect species examined to date have UV receptors (Briscoe and Chittka 2001). The first report on the effects of UV-blocking plastic films was

published in 1982 (Nakagaki et al. 1982), but the emphasis on modifying the characteristics of plastics for agriculture is a recent development (see review, Diaz and Fereres 2007). The effects of UV-absorbing films and nets on reducing the populations of different species of insect pests (aphids, leafhoppers, thrips, and whiteflies) have been well documented (Antignus et al. 1996; Costa and Robb 1999; Costa et al. 2002; Chyzik et al. 2003; Kumar and Poehling 2006; Doukas and Payne 2007b; Weintraub et al. 2008). Furthermore, these UV-absorbing materials have proven to be very effective in reducing the spread of insect-transmitted plant viruses (Antignus et al. 1996; Diaz et al. 2006). The effects of these plastic films on biological control agents have also been studied but reports are conflicting. Doukas and Payne (2007a) found that UV-absorbing films had no effect on the dispersal of *Encarsia formosa* Gahan, a parasitoid of the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood). Conversely, Chiel et al. (2006) found that the whitefly parasitoid, *Eretmocerus mundus* Mercet, was adversely affected by UV-absorbing plastics, losing the ability to locate *Bemisia tabaci*-infested plants. However, they also found that the aphid parasitoid, *Aphidius colemani* Viereck, and the leafminer parasitoid, *Diglyphus isaea* Walker, were not adversely affected by UV-absorbing plastics. Chyzik et al. (2003) found that in the aphid parasitoid, *A. matricariae* (Haliday), whereas UV-absorbing films did affect flight activity, they did not interfere with host finding and fecundity. Clearly, more research is needed to investigate the searching behavior of parasitoids and predators under UV-absorbing films.

In addition to the effects of plastics with altered spectral properties on insect pests, plant pathogens are also affected. *Botrytis cinerea* (Pers.: Fr) (West et al. 2000; Paul and Gwynn-Jones 2003), *Pseudoperonospora cubensis* (Berk and Kurtis) (Reuveni and Raviv 1997), and *Alternaria solani* (Sorauer) (Fourtouni et al. 1998) have all been shown to be inhibited by UV-absorbing plastics. Thus, modified IES serve double duty as and plant pathogen inhibitors.

The initial successes of IES and advances in plastics technology were enough to enable researchers to consider covering field crops. Insect vectors of plant pathogens present a unique set of problems; regardless of whether the pathogen is circulative/propagative in the insect or not, transmission occurs faster than the action of any insecticide. Furthermore, in some instances, such as in the case of aphid vectors of the noncirculative potato virus Y (PVY), insecticides actually enhance the spread of the virus because the aphids are irritated by the insecticide and tend to move more frequently (Radcliffe et al. 1993).

Various forms of floating row covers (FRC) or fleeces were tested in the 1980s (Harrewijn et al. 1991). FRCs are made from spun-bonded polyester or polypropylene and are cloth-like in appearance. They are loosely applied after seeding or transplanting to ensure there will be sufficient material volume to float up as plants grow and they are removed just prior to harvesting. Studies on the protection afforded to field crops against pathogen-bearing insects causing direct damage have been conducted in: Cantaloupe (Vaissiere and Froissart 1996), carrots (Rekika et al. 2008), peppers (Avilla et al. 1997; Waterer 2003), potatoes (Lachman et al. 2003; Chatzivassiliou et al. 2008), squash (Webb and Linda 1992; Qureshi et al. 2007). Additionally, these covers have been shown to enhance leaf and root vegetable productivity in a number of plants (Gimenez et al. 2002; Hernandez et al. 2004; Wadas and Kosterna 2007).

3 Colored Shade Netting

Most recently, colored shade netting has been under evaluation as a promoter of plant growth and yield (Shahak et al. 2004; Rajapakse and Shahak 2007). Different colored nets were shown to have effects on tree growth and fruit yield. In conjunction with the studies on plants, entomologists have started looking at the effects of the colored shade nets on insect pest populations. Ben-Yakir et al. (2008) tested the landing of thrips and whiteflies on black, red, yellow and blue nets covering chives or cotton plants. They found that covering plants with yellow netting protected plants from whitefly infestation; the pest landed on the net but did not penetrate the nets to reach the plants. Similarly, thrips demonstrated an arrest response on the yellow and blue nets and were less likely to penetrate the netting.

4 Fencing

IES have also been used recently as fence barriers. Cole and crucifer crops are adversely affected by root-feeding pests, which fly close to the soil when moving from crop to crop. Vernon and Mackenzie (1998) showed that there was an inverse linear relationship between the number of cabbage flies (*Delia radicum* (L.)) entering a rutabaga plot and fence height. Bomford et al. 2000 demonstrated that proper construction of the fence is an important factor in achieving maximum catch and retention of flies; an overhang at a 45° angle at the top of the screening significantly improved fly catches. Blua et al. (2005) studied the effect of a 5 m high barrier screen used to prevent the movement of the sharpshooter *Homalodisca coagulate* (Say), which is a vector of the plant pathogen *Xylella fastidiosa*, into high value vineyards and nursery stock. They found that the leafhopper behavior changed; the leafhoppers moved away from the barrier towards surrounding plants and only small numbers actually flew over the barrier. These results demonstrated that a barrier could make a significant contribution to vector control. In the author's laboratory, work currently underway examines the efficacy of vertical screening with a 45° overhang, when used to protect peanut crops from *Maladera matrida* Argaman beetles. First field results indicated a reduction in infestation rates, but more work is needed. Promising results have been shown with insecticide-impregnated nets to control cabbage pests (Martin et al. 2006).

5 Soil Solarization

Solar-heating of soil requires the capture of incoming solar radiation beneath clear plastic sheeting laid out on the soil surface and it is usually enhanced by moistening the soil either before the plastic sheets are rolled out or by using drip irrigation lines beneath the plastic. This method was first developed in Israel (see

review, Katan 1981) and California and in areas of abundant sunshine it is an economically viable method for the control of plant-parasitic nematodes, soil borne fungal pathogens, soil-inhabiting insect pests, and some weed seeds. While this is not a new technology, with the gradual elimination of methyl bromide (from 2005–2015, revised Montreal Protocol, Anonymous 2006) the frequency and scale of its use is growing.

6 Mulching

Natural or synthetic mulches are used to control weeds, retain soil water and to deflect insect pests. Natural materials include straw, compost (including decomposed manure), peat moss, bark chips, sawdust, etc. Synthetic materials include various colored plastics and aluminum. Mulch effectiveness varies considerably according to type and has been reviewed (Weintraub and Berliner 2004). Mulches are frequently used, but there is no uniform application with regard to materials, crops or pests.

7 Pneumatic Removal

Hand picking pests, which was perhaps the first physical control method is still used today in many poor areas (Morales 2002 and references therein), but it is also the inspiration behind the modern idea, which uses a vacuum to remove pests. The year 1965 saw the first published report (Stern et al. 1965) on tractor-driven vacuum machines, used to collect *Lygus hesperus* Knight for mark-release studies. Ellington et al. (1984) tested a high clearance, single-row vacuum machine, which sampled large acreages of cotton in an effort to determine population densities accurately. They demonstrated mean catch rates, of 14–64% on mature cotton. Since then, a number of studies have been conducted on various pests which have been reviewed (Weintraub and Horowitz 2001; Vincent 2002). The crucial problem is soil compaction resulting from frequent passes through moist, open fields. In some European greenhouses, vacuum machines have been installed that run along tracks similar to those used for mobile watering systems. These automated systems move from one end of the greenhouse to the other effectively removing pests without applying chemicals.

8 Conclusions

Good physical control methods adapted to different circumstances are sometimes so pervasive that growers and researchers no longer recognize them as a control method. The most common physical control method is the use of an insect exclusion screen as a barrier or fence and modern plastics technologies and innovations have

much more to offer in this field. Whereas insect exclusion screens are now standard agricultural practice, growers relying entirely upon chemical-based control measures in screened greenhouses often fail to recognize that first and foremost, they are using a physical barrier to deter arthropod pests. Physical control measures are very important tools in pest management, but more research on these relatively simple techniques could have vast consequences, similar to the revolution in agriculture created by the exclusion screens. The beauty of physical control measures is that there is no need for the monetary expenditures on the order of those necessary for the development of new pesticides or genetically modified control measures, nor the worry about the concomitant development of resistance to those measures.

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A Systems Approach to IPM Integration, Ecological Assessment and Resistance Management in Tree Fruit Orchards

John Wise and Mark Whalon

1 Introduction

1.1 Twentieth Century IPM

Twentieth century Integrated Pest Management (IPM) was indisputably marked by the dominance of organophosphate (OP) insecticides for pest control in U.S., European, and Australasia specialty crop production (Perry et al. 1998, Ware and Whitecre 2004). Even though this early IPM period brought forth the concepts of economic thresholds, and robust pest monitoring and modeling (i.e. synthetic pheromones, traps, computers, systems engineering, etc.), once a control action was deemed necessary, the application of a lethal agent to kill the target pest followed before injury could occur (Metcalf 1980). This approach was successful in part because most conventional broad-spectrum insecticides, regardless of chemical class, carried a similar set of performance attributes, as all were fast-acting contact nerve poisons. Thus, the success of the organophosphates, carbamates and synthetic pyrethroids led to a narrow concept of pest control, and reduced the perceived need for scientific investigation for anything beyond the determination of acute toxic effects of insecticides on the target pest and beneficials. One notable exception to this concept was the emergence of pheromone-mediated control, which provided important new avenues in specialty crop IPM beyond chemical control tactics, and is likely to have expanded application in the twenty-first century (Gut et al. 2004).

Early IPM authors envisioned a progression of IPM levels (I, II, III), whereby managed agro-ecosystems would gradually ascend to higher degrees of sustainability (Kogan 1998). Level I IPM represents the integration of methods for the control of a single species or species complex. Level II IPM incorporates the impacts of multiple pest categories (insect, disease, weed) and non-target effects at a bio-community level. Prokopy and Croft (1994) added a third level that integrated social, political,

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and legal dimensions of IPM. Though much progress has been made in the scientific and educational aspects of IPM, Kogan (1998) admits that current levels of IPM adoption have in most cases reached only level I or II integration.

2 Impact of FQPA and the Global Food System on the IPM Paradigm

There are several key factors in the global food system that will influence the way that the current IPM paradigm will evolve in the twenty-first century. In the late twentieth century, consumer preferences, engineering advancements, and economics of global markets resulted in the concentration of food processors and distributors (Wise 1999). Historically, local markets could separate minimally blemished fruit from the highest quality product by directing them to different food channels, but increasingly there is one highly restrictive standard for all raw products (Wise 1999). One unfortunate consequence of this in specialty crop production is a more limited application of economic thresholds in IPM decision-making. A low level of pest infestation at harvest may not be economically important in terms of its impact on crop yield, but the risk of load rejection on the basis of a zero-tolerance supersedes the traditional IPM decision-making process. The IPM paradigm will therefore be continually refined by the "real-world" pressures of intensely contested markets.

A second factor driving IPM evolution is the increasing societal and regulatory focus on dietary, worker and environmental safety (USEPA 1996). The twentieth century closed with the passing of the 1996 Food Quality Protection Act (FQPA) in the U.S. (Public Law 104-170) (USEPA 1996). This law fundamentally changed the basis on which the United States Environmental Protection Agency (USEPA) registers and regulates pesticides. The USEPA is currently implementing new standards related to dietary risk, worker exposure, and environmental impacts for the use of agricultural chemicals, and as a result has begun the process of eliminating or severely restricting many of the conventional pesticides traditionally relied upon for agricultural production. Thus, as consumer cosmetic standards and supplier innovation previously drove the markets towards blemish-free products, now concerns over harmful microbial and pesticide residues will further reduce consumer acceptance of pesticides. These two seemingly opposing forces are placing tremendous pressure on specialty crop producers, and IPM practitioners are being looked to for solutions.

Even though the USEPA (1997) has provided an expedited process for registering new insecticide chemistries that meet their guidelines for being "reduced-risk" or "OP-alternative", scientists are finding that these compounds are uniquely different from conventional insecticides in many ways. The current evidence suggests that even though many of these new insecticides are encouraging in terms of field performance against specific pests, the means by which they provide crop protection is more complex, and agro-ecosystem-level impacts are not well understood. Whereas conventional insecticides rely upon a singular lethal mode of activity to

achieve control, many of the new chemistries encompass a suite of mechanisms that work in concert to achieve the prescribed level of crop protection. In addition, whereas conventional insecticides provide a largely similar "surface residue" profile on the plant over time, the new chemistries exhibit a wide range of plant–chemical interactions, which will inevitably influence the exposure and response characteristics of the target organism (Wise et al. 2006). These factors have serious implications on how the concepts of pest control and product performance are perceived within the current IPM paradigm, and what changes must occur for a successful transition to a twenty-first century model.

When new insecticide tools are promoted as "OP-alternatives", there is a great risk that growers will reflexively substitute them into their spray programs without considering the diversity of performance characteristics they exhibit. This over-simplification will, at best, result in minimally sufficient performance at a higher cost to the grower, and at worst, will end in field failures with compounds that could have provided excellent crop protection if used optimally (Whalon et al. 2008). If specialty crop production systems are going to remain globally sustainable, bold efforts in research and education must be initiated to resolve the real gap that remains in appreciating their complexities. The twentieth century IPM paradigm will fail, biologically and economically, to function under the constraints of the modern global food system and will not effectively exploit the inherent strengths of the new tools available for pest management. There is a need to return to a "systems approach" in pest management to meet the demands of global markets, economics, and human and environmental safety expectations.

3 A New Paradigm for IPM Integration

Optimal integration of IPM tools for effective and sustainable twenty-first century pest management can be achieved by employing a "systems theory" approach to problem solving (Boulding 1956). A systems approach fundamentally takes into account the components of a given system as well as the relationships between the components. Therefore, the first task in a systems approach, whether the scale of the focus is large or small, is to identify the components of the system. For a landscape-scale focus, the components might include a cherry orchard, areas of natural habitat, populations of insects (pests and beneficials), and major human production factors (i.e., farm infrastucture, spraying equipment, agrichemical inputs). A smaller scale focus might be limited to a single insect pest, the plant, and a chemical. Once the components of the system are identified, the next step is to describe the interactions among the individual components of the system. The scale of the system will determine what means are most appropriate for capturing the relationships between the elements of the system. Combining knowledge of the key elements of the system and the relationships between each will provide an optimal data set for predicting how human actions or combinations of actions will influence the whole system.

4 Employing the PIC Triad for IPM Integration

The principles of the systems approach can be demonstrated with the simple model called the PIC Triad (Fig. 1). The PIC Triad represents three key components of a tree fruit pest management system, the Plant, the Insect, the Chemical, and the relationships among them. Granted, the twentieth century produced vast quantities of entomological research on plant-insect interactions, as well as a wealth of studies focused on the response of specific insects to chemical insecticides. However, the entomological community has given much less attention to chemical-plant interactions. Without all of the PIC-Triad elements, we lack vital information on how a specific IPM tactic will perform against a pest on a particular crop. In relation to the *Insect* element of the model, the historical focus of IPM practitioners has been primarily on the most economically important pest behaviors, such as fruit feeding and ovipostion. A broader understanding of pest biology is needed, including behaviors of all life stages inside and outside of the orchard, to effectively exploit the weakness of the pest. Relative to the *Plant* element of the model, the crop cannot be understood as simply the host for a pest, but also as the substrate that holds and delivers the poison. The plant is also actively growing, and its changes in phenology, physiology, and surface area ultimately influence its relationship with the other two elements. In relation to the *Chemical* element of the model, there is a need to understand not only direct lethal effects, but also other modes of insecticidal activity that may contribute to overall plant protection. It is important to recognize the term "mode of activity" as being distinct from "mode of action". Whereas insecticide toxicologists may describe "mode of action" as the mechanism by which an insecticide controls the target organism, we refer to "mode of activity" as the field-assessable symptoms of an insecticide's action on an organism that are responsible for control. For example, the neonicotinoid insecticide, imidacloprid, is an agonist of the nicotinic acetylcholine receptor (i.e.; mode of action) (IRAC 2008), but when used for white fly, *Bemisia tabaci,* control is known to have acute lethal activity as well as sub-lethal antifeedant activity on the pest, each with distinct field symptoms (Nauen et al. 1998). Fully describing all the modes of activity that contribute to control serves IPM practitioners in their role of field assessment of an insecticide's performance and crop protection status. The PIC-Triad approach focuses on describing

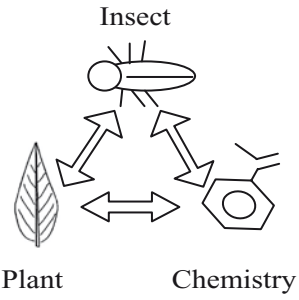

Fig. 1 The PIC-Triad (By Eric Hoffmann and John Wise)

both the spatial and temporal dimensions of the interface between plant, insect and chemical within an ever-changing environment. To test this model, a series of research tactics are employed, including laboratory-based bioassays to determine a compound's acute and sub-acute effects on a pest, field-based residual bioassays to measure the temporal dimension of insecticidal activity as residues age and plants grow, and residue profile analysis to understand the contribution of surface and sub-surface residues to the observed insecticidal activity on the pest. In some cases, population effects of a compound require an "area-wide" spatial perspective in order to be understood. No single component provides sufficient information to fully describe the natural interactions between pest, plant, and chemical, but the combination forms a powerful systems-based data set that provides key insights for optimizing insecticide performance, and direction for whole-system IPM integration.

5 The PIC Triad Applied to IPM Integration in Tree Fruits

Control of plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), in Michigan (U.S.A.) tree fruits is a real-world example of how the PIC Triad can be employed for IPM integration. The plum curculio (PC) is a native beetle that occurs east of the Rocky Mountains (Chapman 1938) and is a key pest of cultivated pome and stone fruits in eastern and central North America (Racette et al. 1992, Yonce et al. 1995). In the spring, female plum curculio migrate from overwintering sites (i.e., woodlots, fence rows, etc.) into orchards and oviposit into the developing fruit, and larvae feed internally on the fruit flesh. Internal feeding damage can render the fruit unmarketable, as can the scar tissue resulting from oviposition and adult feeding. Left unchecked, the plum curculio can cause significant damage in orchards in a short amount of time. PC control has been historically based on organophosphate insecticides, primarily azinphosmethyl (Guthion®), aimed at killing adult PC before they oviposit in fruit. In the last 10 years, a series of new reduced-risk and OP-alternative insecticides have been registered that show promise for PC control. The neonicotinoid compounds thiamethoxam (Actara®) and thiacloprid (Calypso™), and the oxidiazine indoxacarb (Avaunt®) have all demonstrated significant levels of control in field efficacy trials on apple (Wise and Gut 2000). The chitin synthesis inhibitor benzoylurea chitin synthesis inhibitor (CSI) insecticide, novaluron (Rimon®), and juvenoid hormone mimic, pyriproxyfen (Esteem®) are also registered in tree fruits for use on lepidopteran and/or homopteran pests. To date, research on PC insecticide control has focused on the adult life stage of this pest, with the assumption that once eggs are laid, they and the subsequent larvae inside the fruit are out of reach of a toxicant (Howitt 1993).

To use the PIC Triad model as a guide for developing an integrated strategy for PC control, we first studied the plant–insect interaction. The economically important behaviors like fruit feeding and oviposition were well documented, but their temporal patterns in relation to non-economic feeding behavior were not well known. We conducted field-based observational bioassays at key crop phenology periods (pre-bloom, bloom, petal fall, early-ripe fruit, post-harvest), and documented

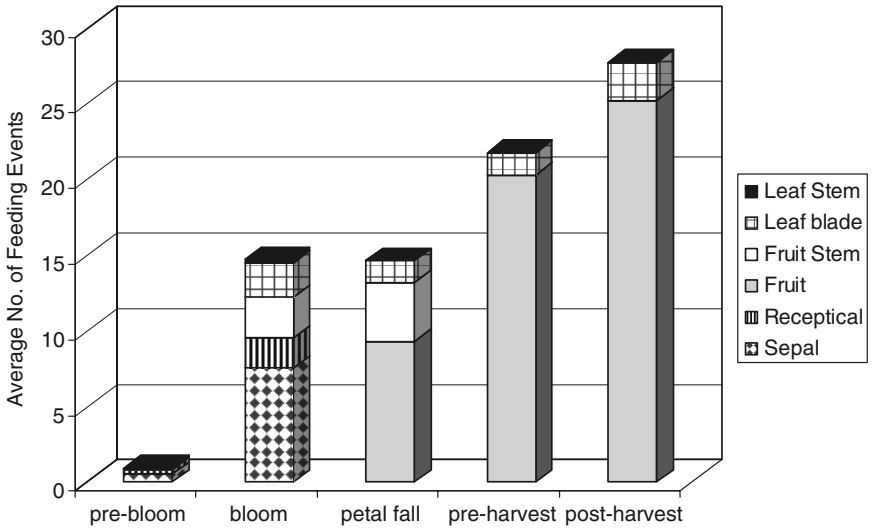

Fig. 2 Plum curculio adult feeding behaviors at various cherry tree phenology stages

the relative patterns of adult PC feeding and oviposition (Hoffmann 2008). We learned that adult PC readily feed on cherry leaf and flower parts during the bloom period, and then on leaf and fruit parts after petal fall. The non-economic feeding gradually declines as oviposition activity commences, and the fruit feeding reaches its highest level in the post-harvest time period (Fig. 2). Next, we focused on the insect–chemical interactions by first conducting traditional laboratory topical bioassays to document the acute toxicity and lethal time of these compounds on PC adults (Wise et al. 2006). The results showed that, with topical laboratory methods, azinphosmethyl, indoxacarb and the neonicotinoids were all lethal to PC adults. Azinphosmethyl had the shortest lethal time, followed by the neonicotinoids, and indoxacarb took the longest time to kill adults. Field-based residual bioassays were then conducted to describe the temporal dimension of insecticidal activity and fruit protection (Wise et al. 2006). These field-based bioassays showed that, while azinphosmethyl relied upon a singular *lethal mode of activity* over the 14-day control period, the neonicotinoids were lethal to PC adults for only the first several days, thereafter protecting the plant through *antifeedant* and *oviposition deterrent* modes of activity. Indoxacarb relied primarily on a *lethal mode of activity*, but ingestion was seen as an important delivery mechanism to attain optimal toxicity. To describe the plant-chemical interactions, parallel fruit and leaf samples were taken from the same field plots, and analysis of the surface and sub-surface residue concentrations were made for every compound at each time period. The residue analysis showed that, while azinphosmethyl and indoxacarb had primarily surface residue profiles on fruit and leaves over the 14 days of the study, large proportions of the neonicotinoid residues moved into the plant tissues, and surface residues

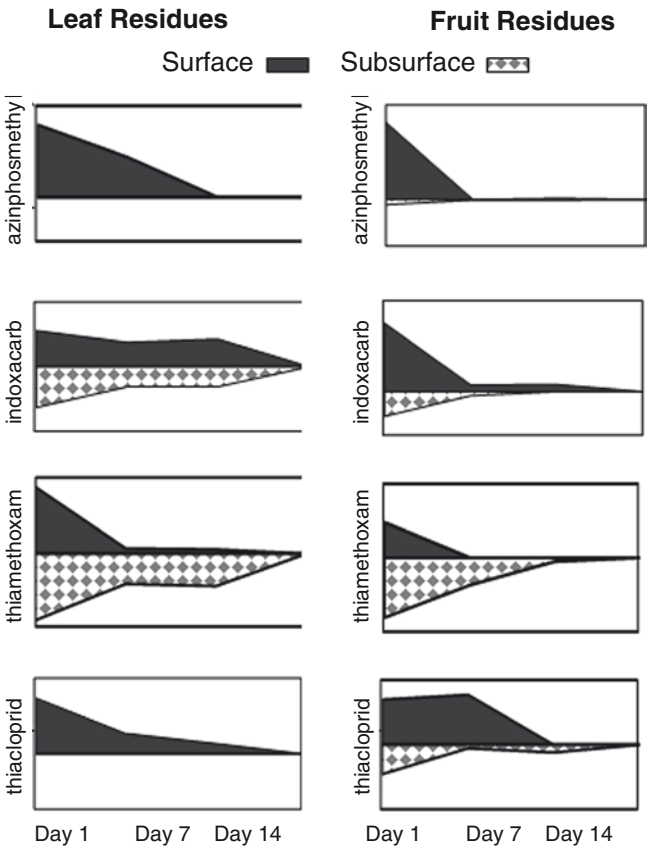

Fig. 3 Residue profiles for various insecticides on apple fruit and leaves. Surface and subsurface residues measured in μg/g of active ingredient per leaf and fruit tissue taken at time periods post field application. Further investigations suggest that indoxacarb subsurface residues bind deep into the leaf cuticle, but do not have translaminar movement like thiamethoxam. For complete description of residue profile methods and data see Wise et al. 2006

diminished more rapidly after application (Fig. 3). For the neonicotinoid insecticides, this dynamic interaction with fruit and leaf tissues serves to regulate the mode of activity. Lethal activity persists as long as sufficient residues are present on fruit and foliage surfaces. As these surface residues diminish, a range of sub-lethal behavioral effects, like oviposition deterrence and anti-feedance, are generated for the remaining period of time. The temporal sequence of lethal and sub-lethal modes is responsible for the overall crop protection seen in the field. Regression analyses were also applied to residue and mortality data to determine which sources of exposure (i.e.; leaf versus fruit, surface versus sub-surface residues) contributed to the observed lethal activity of a compound (Wise et al. 2006). While lethal activity of an insecticide results in direct mortality of the pest, antifeedant and oviposition

deterrent activities reduce the desirability of the crop as a food source or as a host for oviposition. With antifeedance and oviposition deterrence operating, the pest may remain present in the canopy of the plant, but does not damage or further infest the crop. This is distinct from repellency, for which the pest actively avoids the treated substrate.

Sub-lethal activity laboratory studies were conducted by exposing adult PC to the CSI novaluron, via contact, ingestion, and topical spray. Exposed adults were then transferred to clean fruit for oviposition. These studies showed that, although novaluron lacked acute toxicity to adult PC, eggs subsequently laid in the fruit were non-viable, suggesting a possible transovarial activity from the adult exposure (Wise et al. 2007). Field-based residual bioassays, however, showed that these sub-lethal effects declined after 7 days when southern strain PC where exposed to field-aged novaluron residues on leaves, whereas the effects persisted for 14 days for PC adults exposed to fruit (Kim 2009). Since residue profile analysis showed that the proportions of novaluron on surfaces and subsurfaces were similar between leaves and fruit, it is likely that the post-petal fall feeding preference of PC adults for fruit served as an enhanced delivery mechanism for the poison (Fig. 2). Similar laboratory studies were conducted with the juvenoid hormone analog, pyriproxyfen, where exposure of pre-diapause PC adults to treated fruit induced oocyte development and reproductive maturation in females (Hoffmann et al. 2007). Subsequent field studies showed that pyriproxyfen exposure reduces cold-hardiness of diapause-bound female PC and lowers their chance of overwintering survival (Kim 2009). These studies showed that pyriproxyfen and novaluron are not acutely toxic to the life stage targeted for exposure, but the subsequent generation of the pest is diminished as a result of compromised health of the adult or reduced viability of eggs.

To investigate the potential impact of these compounds on alternative life-stage targets (i.e., PC eggs and larvae), a similar sequence of steps related to the PIC Triad model were followed. In the first study, pre-infested fruit were treated topically with field rates of insecticides and successful larval emergence from fruit was evaluated over time (Wise et al. 2007). This field-based study showed, contrary to published reports (Howitt 1993), that azinphosmethyl and the neonicotinoids (thiamethoxam and thiacloprid) provided significant lethal activity on PC eggs and/or larvae within fruit. Residue profile analysis was conducted for each compound, documenting the concentrations of residues in the apple fruit skin, outer 2 mm of flesh, center 3 mm, and inner 2 mm of flesh to the core, and cherry fruit skin, outer 1 mm of flesh, and inner 1 mm of flesh, 24 h after the application (Figs. 4 and 5). For azinphosmethyl and each of the neonicotinoids, active ingredient was found either in the outer portion of flesh where the eggs are laid, and/or in the center/inner flesh regions where larvae eventually feed. Next, laboratory bioassays were conducted to determine whether the post-infestation lethal activity seen in field bioassays was based on ovicidal or larvicidal toxicity for each compound. The results showed that azinphosmethyl and thiacloprid had significant ovicidal activity at concentrations similar to that found in fruit flesh (Hoffmann et al. 2008). While thiamethoxam did not show direct ovicidal activity in the laboratory study, a related metabolite, clothianidin, was highly toxic to PC eggs. Interestingly, novaluron did show ovicidal activity on PC,

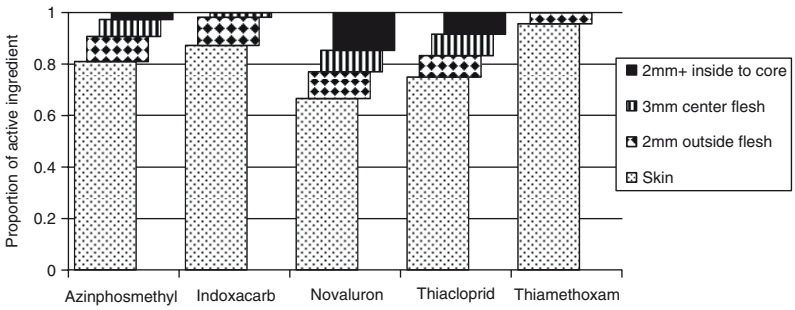

Fig. 4 Penetration of insecticides into apple fruit, graphically presented as the proportion of mean active ingredient recovery in the skin, outside 2 mm of flesh, center 3 mm flesh, and the remaining section to the core. The actual mean values of total active ingredient recovered (μg AI per g of apple substrate) for each compound were as follows; 18.10 g of azinphosmethyl, 1.73 g of indoxacarb, 0.48 g of novaluron, 0.12 g of thiacloprid, 2.34 g of thiamethoxam

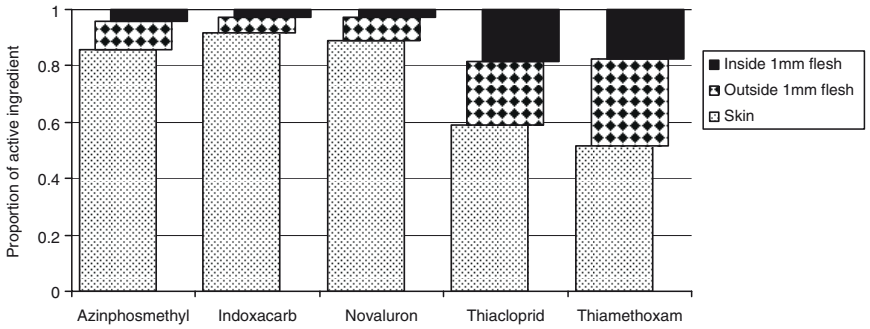

Fig. 5 Penetration of insecticides into cherry fruit, graphically presented as the proportion of mean active ingredient recovery in the skin, outside 1 mm of flesh, and the inside 1 mm flesh to the pit. The actual mean values of total active ingredient recovered (μg AI per g of cherry substrate) for each compound were as follows; 28.3 g of azinphosmethyl, 4.79 g of indoxacarb, 5.05 g of novaluron, 0.71 g of thiacloprid, 6.8 g of thiamethoxam

but at concentrations higher than what was documented in fruit flesh (Wise et al. 2007). Indoxacarb was not toxic to PC eggs. Azinphosmethyl and the neonicotinoids all had significant larvacidal activity at concentrations similar to that found in fruit flesh, substantiating the bioassay results (Hoffmann 2008). Indoxacarb showed significant larvacidal activity on PC, but at concentrations higher than what was documented in fruit flesh. Thus, this unique mode of insecticidal activity can be called "curative", and defined as the lethal action of an insecticide on a pest post-infestation, from the transitory penetration of the compound into plant tissue. As in the case of plant pathology (Schwabe et al. 1984), curative activity does not claim to un-do the injury symptoms incurred before the point of intervention, but does prevent further harm to the host by killing or inhibiting development of the

Table 1 PIC Triad data base for plum curculio management in apple and cherry

Compound Trade Name	Chemical Class	Life Stage Toxicity	Modes of Activity on Adult PC	Curative Activity on PC Larvae/ Eggs in Fruit
Azinphosmethyl	Organophosphate	Egg, larva, adult	Lethal via contact	Curative
Indoxacarb	Oxadiazine	Larva, adult	Lethal via ingestion	Minimally curative on larvae
Thiamethoxam	Neonicotinoid	Larva, adult	Lethal, Oviposition deterrent, Antifeedant	Curative
Thiacloprid	Neonicotinoid	Egg, larva, adult	Lethal, Oviposition deterrent, Antifeedant	Curative
Novaluron	Benzoylurea	Egg	Sub-lethal (Transovarial)	None
Pyriproxyfen	Juvenile Hormone Analog	Adult	Physiological on diapausing female	None

infective agent. These curative activity studies demonstrate the principle that, to accurately predict the outcome, all elements of the PIC Triad must be addressed.

Fulfilling the research elements of the PIC Triad results in a comprehensive database with which to guide the optimal integration of available tools into a PC management program (Table 1). The following is an example of how a PIC Triad data set can be applied to a seasonal pest management program in cherry and apple systems (Fig. 6). The PIC Triad database for indoxacarb suggests that this compound is lethal to PC adults, but that the lethal time is long and ingestion enhances its insecticidal activity. Based on these performance characteristics, the optimal timing for indoxacarb is petal fall for both cherry and apple. In this phenological period, adult PC are still feeding on non-economic plant parts (bloom would be an ideal application timing, except that indoxacarb is toxic to pollinators), and there is sufficient time for this slow-acting compound to work before oviposition begins. In apple production, novaluron is often used at the petal fall timing to control the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). The PIC Triad data set indicates that this timing will also effectively induce sub-lethal insecticide activity on PC, significantly reducing the viability of the subsequent cohort of PC eggs laid in fruit. In order to optimize the activity of neonicotinoid insecticides on adult PC, applications should be delayed until after shuck-split on cherries and 10 mm diameter fruit in apples. With this delayed timing, the initial lethal action on PC adults is maintained, and residue coverage of fruit surfaces is maximized, thus assuring the antifeedant and oviposition deterrent modes of activity that provide long term protection of fruit from injury. At approximately 350 GDD base 50°F after the initiation of PC oviposition, most of the eggs will have been laid and hatch will have begun to occur inside the fruit. For growers who either have low populations of PC or have failed to prevent oviposition in fruit by this time of the season, this

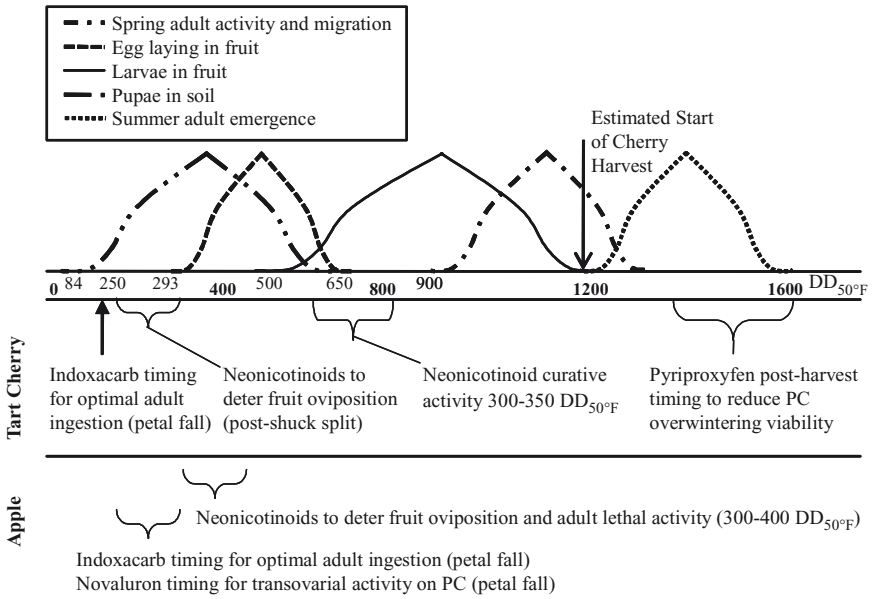

Fig. 6 Phenology model for optimal timing of new insecticide chemistries for plum curculio based on PIC Triad data base. (by Mark E. Whalon)

is optimal timing for a curative spray with neonicotinoids. A curative spray at this timing will not result in unblemished fruit, but will prevent the survival of eggs and larvae in fruit. For cherry growers who farm under the zero-tolerance mandate (USDA 1941), this could save them from a load rejection at harvest, and for apple growers, this tactic can reduce resident PC populations. Lastly, for cherry growers with evidence of PC populations post-harvest, a pyriproxyfen application will reduce the viability of overwintering female PC, which will reduce resident populations. This tactic is not recommended for apple growers, because late season female PC may have the capacity of laying fresh eggs in apples just prior to harvest (Hoffmann et al. 2007).

6 Influence of the Environmental or GREEN Social Culture in the US on IPM

To accelerate the environmental or green movement in the US (see http://www.ecotopia.org/ehof/timeline.html for a US history), various political figures, cinema personalities and famous authors have used their influence to direct the public toward more environmentally friendly products, which in turn has further heightened the need to change the way food and fiber is produced. Yet, by whatever mechanism (e.g., government fiat, marketing strategy, advertising, public personality endorsement

or government or non-government environmental organization education efforts), agriculture is moving toward greater conservation of key ecosystem components, and this includes a movement toward environmentally and ecologically 'friendly' means of managing pests, to produce abundant *ecosystem services* which include conservation and enhancing biological control, native pollinators, accelerated decomposition, carbon sequestration, water filtration, etc. (Naylor and Ehrlich 1997).

In essence, these converging social, non-governmental, governmental and special interest groups' focus on the environment are moving agriculture toward environmentally and ecologically produced food and fibers that do not devalue the land, pollute water or reduce biodiversity. These production and marketing systems are the future of most developed and many developing nation's agricultural production systems throughout the world. As a result, a number of environmental and ecological certification systems have been promulgated to assure consumers of "ecologically or environmentally grown" produce and products in Europe, the U.S., Asia and Australasia. Essentially all of these systems utilize toxicity indexes or certified practices e.g. Global and US Good Agricultural Practices (GAP), to assure customers that their produce, grains, fiber, meats, building materials, etc., meet high standards for environmental and biodiversity protection.

7 Integrating Functional Ecology into the IPM Paradigm

Recognizing these emerging properties of 'responsible' agriculture, we as a team at Michigan State University have invested considerable effort to develop a reliable, efficient, economic and timely ecosystem assessment system within an existing IPM system that measures a production system's impact on key taxa. Our ecosystem services data are generated and analyzed within an individual farm as well as across an area-wide grouping of farms. This approach offers some unique indicators of the health of the overall production system and its impacts on biodiversity at a very reasonable cost within an IPM monitoring system. We are combining the PIC Triad/IPM approach with this inexpensive but effective system for assessing the diversity, evenness and richness of native pollinators, key indicator species and arthropod natural enemies. To date this system has been referred to as "functional ecology measures," so as not to confuse it with a larger effort among basic and research ecologists who are interested in looking at how ecosystems "function" in total, but instead to communicate to producers, purchasing agents, and consumers, the details of an agricultural system that is in "working order" (Whalon and Croft 1984). We have now developed a number of these systems and deployed them in several U.S. Upper Midwest crops including apples, cherries, peaches, corn and soybeans. This biodiversity assessment within an IPM system is part of a larger monitoring system that encompasses direct and indirect sampling of soil, yield, quality and surrounding habitat.

The PIC Triad, as noted above, is a model that can have extensive impact on current and future IPM systems research because it begins to approach a systems-level understanding of pest management. By "systems-level," we refer to a discipline

that approaches pest management in the context of an entire ecosystem that can be understood and therefore managed. In this context, IPM properly should measure and modify an agricultural system's output of not only high quality produce, but also ecosystem services. Thus IPM, emphasizing its 'integrated' and 'management' components, should monitor and manage specific ecosystem services. The development of this aspect of IPM already has, as we have demonstrated above, scientifically derived *measures* which support the development of sustainable production practices from not only an economic standpoint, but also from the perspective of such considerations as biodiversity, energy flow, soil quality, carbon sequestration and a host of other critical attributes necessary for sustainability (Fig. 7).

Thus IPM must expand its monitoring goals from just pest control decisions to a broader understanding of pest management within a functioning ecosystem not only for environmental benefits but also for market assurance (e.g. GAP Standards). The authors (particularly Whalon) have essentially placed IPM into an ecosystem framework in order to obtain an understanding, through monitoring, of whether a given production system is headed in a sustainable direction. Monitoring only pests as a goal of IPM is altogether insufficient for this purpose. IPM needs to wholeheartedly enter the ecological *measurement just as it has always measured pest* pressure for economic management decision making. Today, agriculture needs sustainability *measures that are solidly based upon sound ecosystem assessments*. Not only are these measures needed for government programs aimed at supporting and expanding sustainability, biodiversity and market access, but also for the producer's own

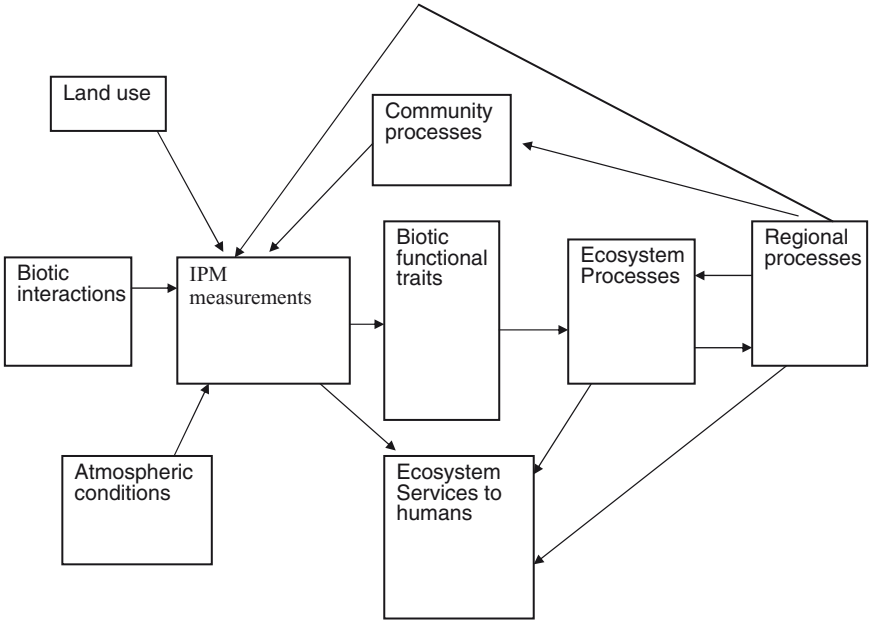

Fig. 7 A generalized ecology model for integrating biodiversity, energy flows, soil quality, carbon sequestration into IPM systems. (By Mark E. Whalon)

understanding of how one set of production practices compare to another from field to field, or orchard to orchard. Since most growers are constantly "experimenting" with pest management programs to find systems that improve the economics, yield and quality, IPM monitoring information since its inception has contributed to producer's continuous quality improvements in pest management decision-making. With the added power of measuring ecosystem services, a similar process of agroecosystem improvement will follow. Essentially, what gets measured gets managed. In our experience, this is more forcefully demonstrated if economic services can be converted into a monetary economic returns to the producer. This can be achieved by simply assigning a monetary value (e.g. $0.01 or 0.05/predator or parasite) and expanding the measuring device's or sampling system's effective trapping or monitoring area to a management unit like an orchard or a field. We maintain that this viewpoint is particularly true where insecticide-based pest management systems relying on older chemistries are transitioning to new classes of pesticides. These new insecticides are being marketed aggressively, while the older classes of products, for reasons including higher risk, and patent termination, are less economical and perhaps under scrutiny, as in the U.S., where the use of organophosphates, carbamates and pyrethroids is being curtailed as a result of the Food Quality Protection Act of 1996.

8 Integrating Ecosystem Assessment into the IPM Paradigm

This ecosystem assessment is particularly needed in the transition from the old to the new, where the integration of new pest management tools like the neonicotinoids, spinosyns, pheromones, oxadiazines, insect growth regulators (IGRs), diamides, biopesticides and augmentative biological controls are supplanting historically important insecticide classes such as those mentioned above. These older chemistries have been used reliably for 20, 30, or 50 years in some U.S. production systems such as apples, cherries, blueberries, brambles, potatoes and many different vegetables. However, a legislated change to new chemistries, which from a human toxicological standpoint appear to be "reduced risk" (USEPA 1997), does not necessarily mean better or safer ecosystem services. This is particularly true for arthropod natural enemies, which may have evolved resistance or been "selected" through time at the ecosystem level through indirect and subtle processes. In this way, ecosystems are able to adjust or adapt to pest management regimes over time. In the case of tart cherries and azinphosmethyl in Michigan, this selection process has been ongoing for almost 50 years.

Thus, subtle yet significant ecosystem-wide changes can result from the introduction of an insecticide or other pest management tool, which in turn can lead to secondary pest outbreaks in orchards (Croft and Hoyt 1978), and rapid resistance development (McGaughey and Whalon 1992). These types of ecosystem impacts almost always occur when a newly introduced class of chemistry does not control the complex of pest species (e.g., plum curculio, *Conotrachelus nenuphar* (Herbst); cherry fruit fly, *Rhagoletis cingulata* (*Loew*); green fruitworm, *Orthosia hibisci*

(Guenée); and obliquebanded leafroller, *Choristoneura rosaceana* (Harris) in a tart cherry orchard system) that a former class (i.e., organophosphates) once did. IPM managers and growers must then introduce several new insecticide classes together in order to manage the whole pest complex, which in turn can lead to more significant ecological perturbations not present in the original IPM system.

For instance, a recent 4-year USDA-supported tart cherry research project provided paired comparisons of ecosystem services between nine organophosphate-based (i.e., conventional) management systems and nine USEPA (1997)-defined "Reduced Risk" insecticide-based (i.e., new) management systems. Each pair consisted of a new management system in a ~4.5-ha orchard, with a conventional management system immediately adjacent to it (Whalon et al. 2008). All other horticultural and disease management practices were identical and temporally synchronous, including equipment use, horticultural amendments, and disease and ground cover management. In some instances, the orchards were actually one, large contiguous orchard divided in half, with buffer rows separating the different treatments. The 'Reduced Risk' compounds included neonicotinoid, oxadiazine and spinosyn insecticides, while the organophosphate blocks received only azinphosmethyl and phosmet.

Shannon–Weaver indices (Shannon 1948) were used to compare 24 taxa (Table 2) of natural enemies captured on yellow sticky traps (GL IPM Inc., Vestaburg, MI, U.S.A.) from each orchard, and were employed as a means of assessing ecosystem services or as a measure of the orchard's "health" as it underwent transition from one insecticide regime to another. Standard species diversity, richness and evenness measures were used across four years of the transition from old to new insecticides. Natural enemy sampling was conducted at phenologically synchronous intervals across orchards using 120, 18.5 × 32 cm yellow sticky cards placed spatially in a systematic array, 10 per sample period in each orchard over the four growing seasons. Each card was assessed to determine natural enemy numbers and taxa collected, then recoded for analysis. Historically, these sticky cards have been used to assess the densities of true bugs, fruit flies, and some aphids in orchards (Whalon and Croft 1984). During the study, species diversity was similarly assessed along the border rows and across adjacent habitats out to 100 m, but these data are not reported here.

Surprisingly, arthropod natural enemies as an indicator of ecosystem services were significantly reduced ($P < 0.05$) in the "reduced risk" orchards (Table 3) (Nortman 2009). A number of factors probably contributed to this outcome, but this study illustrates that lower toxicity to mammals (which is the basis of USEPA's definition of "Reduced Risk") or even low adult arthropod toxicity as observed in preliminary tests does not necessarily represent a pesticide's true ecosystem effects. These effects are likely to be more subtle and complex, and apparent only when examined on a long-term and intergenerational basis. Ecosystem- or landscape-level testing is almost never employed in standard pesticide evaluations world-wide, as best as can be determined by the authors. Although USEPA's registration testing is rigorous and expensive, it does not yield sound measures of the ecosystem impacts for societally mandated changes in pest management regimes (USEPA 1996), which may have far-reaching and long-term ecosystem and economic consequences on production agriculture.

Table 2 Survey template for natural enemies across 24 taxa, captured on yellow stick traps in orchards

Taxa	Common Name
Araneae	
Thomisidae	Crab spider
Araneidae	Orb Weaver
Pholcidae *Pholcus phalangioides*	Daddy longlegs
Tetragnathidae *Tetragnatha nitens*	Long-jawed spider
Coleoptera	
Coccinellidae	Ladybeetle
Carabidae	Ground beetle
Cantharidae	Soldier beetle
Lampyridae	Lightningbug (or firefly)
Dermaptera	Earwig
Diptera	
Syrphidae	Syrphid
Tachinidae	Tachinid
Hemiptera	
Miridae *Campylomma verbasci*	Mullein plant bug
Nabidae: Nabis spp.	Damsel bug
Reduviidae	Assassin bug
Anthocoridae	Minute pirate bug
Pentatomidae	Stink bug
Hymenoptera	
Colletidae: Colletes spp.	Solitary bee
Megachilidae: Osmia cornifrons	Hornfaced bee
Apidae	Honeybee
Halictidae *Agapostemon splendens*	Sweat bee
Braconidae	Braconid wasp
Ichneumonidae	Ichneumonid wasp
Neuroptera	
Hemerobiidae	Brown lacewing
Chrysopidae	Green lacewing

Table 3 Average Shannon-Weaver Indexes for the natural enemy taxa represented in Table 2. Taxa diversity, richness and evenness were from paired tart cherry orchards (nine conventional and nine reduced risk) in Michigan, USA from 2006 and 2007

Average	Reduced Risk	Conventional
H′	0.86	1.41
Richness	2.72	4.06
Evenness	0.78	1.03

Even though pesticide toxicity has long been understood from a laboratory or small-plot standpoint (Johansen 1977, Theiling and Croft 1988, Johansen and Mayer 1990, Naylor and Ehrlich 1997, Altieri and Nicholls 2004), larger landscape-level costs have never been documented over the transition period of a management

Table 4 Average financial benefit ($) per acre to growers from the presence of natural enemies in nine paired reduced risk and nine conventional (organophosphate based program) orchards from 2006 and 2007. Figures based on a calculation of $0.05 per natural enemy

	Reduced Risk	Conventional
Sum ($)	233.51	426.71
Average ($)	15.57	25.10

strategy from one class of chemistries to another. To demonstrate the value in knowing these costs to regulators and society in general, and to communicate their ecological consequences to the grower community, we assigned a $0.05 value to each natural enemy counted, regardless of taxon, behavior, or predation/parasitism rate. By tallying these economic estimates of natural enemy contributions over a 2-year period in each of the two types of orchard management systems, we ranked the value of ecosystem services delivered by natural enemies (Table 4). These measures grossly underestimate the true value to growers of these 24 taxa, as each trap has a calling area of less than a 1 m^3 for most of the taxa studied (Whalon et al. 1982). Annual value of ecosystem services varied somewhat between treatment regimes (conventional and reduced-risk) across growers. Surprisingly, the organophosphate-based system yielded more valuable ecosystem services than did the "reduced-risk" regime, which was more ecologically disruptive and included neonicotinoid, oxadiazine and spinosyn chemistries as replacements needed to control the pest complex formerly controlled by two organophosphate insecticides (Nortman 2009). There are many potential reasons for this counterintuitive outcome, particularly the USEPA's mostly mammal-based toxicity risk assessment pesticide registration system. The central lesson is that natural enemy ecosystems have definite value in orchard settings, and they warrant IPM assessment because "what gets measured gets managed", which is one of the central tenets of systems science and management theory.

Using this simple economic example of ecosystem services assessment, nine growers were apprised of the considerable per-ha value of the natural enemy taxa in each of their orchard operations. They were also made aware of an almost 62% decrease in these services (Table 4) and a 48% increase (USDA RAMP 2008) in the cost of their pest management insecticide program, which was implemented to compensate for the loss of organophosphates resulting from the U.S. Food Quality Protection Act. These ecosystem costs may subside over time as the cherry ecosystem's natural enemies adapt. With this study, perhaps for the first time, tree fruit extension specialists in Michigan were able to present the economic value (in dollars) of one aspect of ecosystem services—natural enemies—directly to the grower community in tangible terms. All of these producers now understand how IPM management practices from a systems and area-wide perspective can impact their operation's economic bottom line. If the effective calling range of a single yellow sticky trap is estimated at less than 1 m^3, all of the traps combined in this study represented less than perhaps 1×10^{-5} of the natural enemy activity in each of the orchards. No wonder ecosystem services have been estimated at approximately $8 billion in the U.S. annually (Losey and Vaughn 2006).

9 Ecosystem Friendly IPM in the Twenty-First Century

For many reasons, producers, pest managers, marketers and governments are increasingly demanding to know the 'impact' of agricultural practices on natural ecosystems, including ambient water and the atmosphere. Therefore, significant effort has been made within our IPM programs in developing both toxicity indices and the aforementioned ecology-based indicator systems in an attempt to address concerns over agriculture's impacts on the environment and biodiversity (http://ipcm.wisc.edu/ProgramInfo/ EcoPotato/tabid/87/Default.aspx). As seen in this example, we believe producers have both economic as well as ecological incentives to implement this new IPM perspective.

Society's overall movement toward "ecosystem-friendly" or green production systems is widely apparent, particularly in developed nations in which non-government environmental movements have arisen and educated the public about agriculture's environmental and ecological impacts. Within some of the more radical of these movements, agriculture has been targeted as a primary cause of declining biodiversity. Certainly, governments have moved toward greater environmental and ecological scrutiny, and regulation will likely continue to escalate (1973 establishment of the US Environmental Protection Agency, 1996 US passage of the Food Quality Protection Act, 2001 US passage of the Pesticide Re-registration Improvement Act, etc.). Perhaps too often, agriculture has been depicted as one principal activity whereby mankind is devastating the planet with pesticides and fertilizers, and thereby polluting the land and waters of the earth. Yet this characterization is probably, one would hope, far from the truth when ecosystem services on agricultural lands are considered together with the food and fiber produced. However, without a growing sensitivity and understanding within the grower community, these environmental and ecological concerns will mount and impact many producers and their suppliers in negative ways, unless the agricultural community begins to practice actual ecosystem measurement. What better way to accomplish this critical step forward than through a slight expansion of a producer's existing IPM system?

Faulty interpretation of biodiversity and a focus on endangered species have further exacerbated the public's view of agriculture, and many are convinced that agriculture has been the major factor in the decimation of plant and animal species around the world. Large companies have hired environmentally-minded marketers who now want the public to be aware that their products are grown sustainably, but they also want to appeal to the public to recognize ecologically healthier production systems as well. Thus major food marketing chains, processors and 'super stores' have made boardroom decisions aimed at developing "ecologically friendly" products and labels to attract environmentally educated consumers who in turn purchase "greener" products. But to achieve any number of green labels, producers must comply with an array of measures, practices and restrictions. This process has progressed to the point where a number of food chain marketing companies now use documentation of agricultural production practices as a means of assuring environmentally

and ecologically friendly produce (http://ipcm.wisc.edu/ProgramInfo/EcoPotato/tabid/87/Default.aspx). Our example of an IPM-based ecosystem assessment system could be a logical means of not only keeping growers well informed, but of informing the public, markets and government what is actually occurring to ecosystem services within a production system.

10 Implications of the Twenty-First Century IPM Paradigm on Resistance Management

Pesticide resistance management is a critical part of any long-term IPM program in specialty crops where insecticides, acaricides and fungicides are used frequently. Recent developments in area-wide IPM programs rely on resistance management by means of synchronized planting dates, insecticide treatment windows and active ingredient rotations (e.g. Carriere et al. 2001). Certainly this approach has a place in high value crops and may become more commonplace even in moderately valuable cropping systems (Whalon et al. 2008b). Moreover, the incorporation of more diverse mortality mechanisms, especially through ecosystem services like biological and natural control, is becoming an increasingly important part of resistance management in robust IPM programs. Without actually estimating natural enemy contributions to the management of key and secondary pests, how can IPM managers actually adapt and optimize advanced IPM programs to different crops?

In our view, a concerted and broad-based IPM program in the era of ecosystems services and resistance management entails extensive planning and monitoring to determine natural enemy contributions, as well as discriminating dosage studies or alternate means of assessing susceptibility changes of key pests to frequently used pesticides. Our sense of this process has been fortified by the number of cases of resistance cited in the Arthropod Pest Resistance Database (Whalon et al. 2008c) and recent abstracts published in the Resistant Pest Management Newsletter (http://whalonlab.msu.edu/rpmnews/vol.7_no.1/rpm_coordinator.htm). Future resistance management research must adapt to the range of life stage-specific chemistries coming into the marketplace, and will need to determine how the development of resistance impacts observed modes of pesticide activity and, ultimately, real-world IPM practices (Mota-Sanchez et al. 2008).

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Mechanisms of Acaricide Resistance in the Two-Spotted Spider Mite *Tetranychus urticae*

Thomas Van Leeuwen, John Vontas, Anastasia Tsagkarakou, and Luc Tirry

1 *Tetranychus urticae* – The Two-Spotted Spider Mite

The subclass Acari, comprising mites and ticks, is one of the largest and biologically most diverse groups within the class of the Arachnida, which also includes scorpions, spiders and harvestmen. They are distributed worldwide and have successfully colonised a wide range of terrestrial and aquatic habitats. The majority of mite species living on the aerial parts of higher plants feed mainly on microflora or predate on other small arthropods living on plants. However, mite species within the order of the Prostigmata, belonging to the families of the *Tetranychidae* (spider mites), *Tenuipalpidae* (false spider mites), *Tarsonemidae* (tarsonemid mites) and the superfamily of the *Eriophyoidea* (gall and rust mites) are able to use their specialized needle-like mouthparts to feed on the plant cells and tissues. Their feeding activity can lead to severe losses in field and protected crops (Walter and Proctor 1999; Evans 1992).

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Tetranychus urticae Koch, the two-spotted spider mite, is probably the most important pest mite in glasshouses throughout the world, and may also severely damage field crops when temperatures are sufficiently high. It is one of the most polyphagous species of the tetranychid family. Nearly 200 plant species have been recorded as hosts of *T. urticae*, including a number of economically crops such as cotton, maize, tomatoes, sweet pepper, fruits and a wide range of ornamentals (Jeppson et al. 1975).

Tetranychus urticae is mainly active on the underside of the leaves, where it punctures the leaf cells with its cheliceral stylets and feeds on the leaking cell content. The cells immediately adjacent to these punctures collapse and dehydrate. Initially, mite activity results in a fine mottling of the upper leaf surface. As feeding progresses, the leaves become hard and almost parchment-like. During this process, mites are spinning fine webbings on and between leaves. The infestation thus can significantly reduce the photosynthetic capacity of plants and their production of nutrients, often resulting in the destruction of the plants.

Upon emergence from overwintering places in spring, fertilised female mites start to feed and lay up to a 100 eggs in a period of 2–4 weeks on the lower leaf surface, attached to fine silk webbing. Eggs appear as pearl-like spheres, 0.1 mm in diameter, which are deposited singly, though commonly close together. As the eggs develop, they become reddish. The development period of the eggs varies from 3 days at 32°C to 28 days at 10°C. The six-legged flesh-coloured larva hatches from the egg and, when food supplies are adequate, remains near the oviposition site all its active life. At 21°C it is active for only 1 day before entering a quiescent, immobile non-feeding stage, which lasts about a day. This resting stage is firmly attached to the leaf surface by silken fibres, usually near a vein. The next eight-legged stages, the protonymph and deutonymph, also have active and quiescent stages. In the deutonymphal stage, the sexes can be distinguished since the males are more elongate. Adult males (0.3 mm long) are more active than females (0.45 mm long), searching for and waiting (called "guarding behaviour") beside a female deutonymph in its resting stage (teleochrysalis), in order to copulate immediately after her final molt. Oviposition starts after a pre-oviposition period of 24–48 h after fertilisation. The developmental time from egg to adult varies greatly with temperature, but in optimal conditions (20–30°C), spider mites complete their development in 7–20 days. There are many overlapping generations per year (Helle and Sabelis 1985).

Spider mites belong to a group of species known to be arrhenotokous, i.e. unfertilised eggs are haploid and develop into males, while fertilised eggs develop into females. Unfertilised females lay eggs that only develop into males, while fertilised females produce offspring of both sexes, with a strong preponderance of females. The first eggs of a fertilised female often develop into males, because male gametes can not penetrate these eggs in time before oviposition (Helle and Sabelis 1985).

When conditions are less favourable, *T. urticae* can enter diapause. The factors inducing this phenomenon are photoperiod, temperature and nutrition, and they interact in a complex way on the different developmental stages (Vaz et al. 1990). In practice, if any two of the factors tend to be diapause-inducing, most of the mites become dormant. No change is induced during nymphal development but resulting

adult females become brightly red, do not feed and are negatively photokinetic, which leads to hiding in cracks. Diapausing mites are also less affected by acaricides and they do not resume activity until they have experienced a period below 6°C (Hussey et al. 1969; Helle and Sabelis 1985)

2 Control of *Tetranychus urticae*

Biological control of *T. urticae*, mainly based on the use of predatory phytoseid mites, has proven to be successful in many greenhouse crops (Bale et al. 2008). However, acaricides continue to play an essential role in integrated management of spider mites in these crops, mainly as corrective measures where biological control fails. In field crops, and in greenhouse ornamentals, spider mite control is still predominantly dependant on acaricides.

One of the interesting features of acaricides is the variety in chemical structure and modes of action found among the many types of compounds that are toxic to phytophagous mites. The mode of action of acaricides and the identification of their target site was reviewed by Knowles (1997), and more recently by Dekeyser (2005). Table 1 gives an overview of the acaricides, listed according their primary site of action and the chemical (sub)–group, based on the IRAC (Insecticide Resistance Action Committee) Mode of Action Classification (http://www.irac-online.org; version 6.1, August 2008). Although the number of compounds available for mite control looks impressive, in practice, the number of acaricides that is registered for use in a certain crop is rather limited. For example, in Belgium, only 12 compounds are registered specifically for spider mite control, half of them are METI (Mitochondrial Electron Transport Inhibitors) and organotin acaricides. For the control of spidermites in greenhouse tomatoes, only six compounds are registered, mainly for corrective applications after failing biocontrol (Fytoweb 2008, http://www.fytoweb.fgov.be/).

3 Resistance Development and Mechanisms

According to reports in literature, more than 550 species of insects and mites have developed resistance to one or more classes of insecticides/acaricides. The number of effective chemicals for the control of crop pests and disease vectors is rapidly decreasing (Georghiou 1990). Meanwhile, fewer new insecticides/acaricides are being introduced to the market, largely because of the high costs associated with research, development and registration, together with the prognosis of a limited effective lifespan of the new molecules (Metcalf 1980). In comparison with the production of DDT, which is the product of a one-step synthesis process from readily available precursors, the production of some modern compounds sometimes involves up to 13 steps, resulting in a 100-fold increase of production costs. This increased degree of

Table 1 List of acaricides and their mode of action/target site, based on the IRAC Mode of Action Classification (version 6.1, August 2008) (http://www.irac-online.org)

Acetylcholinesterase inhibitors (Group 1)
Carbamates (Aldicarb, carbarl, carbofuran, fenobucarb, formetanate, methiocarb, oxamyl, propoxur)
Organophosphates (Azinphos-ethyl, azinphos-methyl, chlorfenvinfos, chlorpyrifos, coumaphos, demeton-S-methyl, diazinon, dichlorvos, dimethoate, disulfoton, ethion, heptenophos, malathion, mecarbam, methamidophos, mevinphos, monocrotophos, omethoate, parathion, phorate, phosalone, phosmet, phoxim, pirimiphos-ethyl, quinalphos, sulfotep, tetrachlorvinphos, triazophos, trichlorphon, vamodothion
GABA-gated chloride channel antagonists (Group 2)
Cyclodiene organochlorines (Endosulfan)
Sodium channel modulators (Group 3)
Pyrethroids (Acrinathrin, bifenthrin, cyhalothrin, cypermethrin, fenpropathrin, fenvalerate, flucythrinate, flumethrin, tau-fluvalinate, halfenprox, lubrocytrhinate, permethrin)
Chloride channel activators (Group 6)
Abamectin, milbemectin
Mite growth inhibitors (Group 10)
Clofentezine, hexythiazox, etoxazole
Inhibitors of mitochondrial ATP synthase (Group 12)
Diafenthiuron, organotin miticides (azocyclotin, cyhexatin, fenbutatin oxide), propargite
Uncoupler of oxidative phosphorlylation via disruption of the proton gradient (Group 13)
Chlorfenapyr
Chitin synthesis inhibitors (Group 15)
Benzoylureas (Flucyloxuron, flufenoxuron)
Octopamine receptor agonists (Group 19)
Amitraz
Mitochondrial complex III electron transport inhibitors (Group 20)
Acequinocyl, fluacrypyrim
Mitochondrial complex I electron transport inhibitors (Group 21)
METI-acaricides (Fenazaquin, fenpyroximate, pyrimidifen, pyridaben, tebufenpyrad)
Inhibitors of acetyl Coenzyme A carboxylase (Group 23)
Spirodiclofen, spiromesifen
Compounds of unknown or uncertain mode of action
Dicofol, bifenazate, tetradifon

sophistication is typical for most of the molecules recently introduced, which include also fermentation products requiring costly purification (Metcalf 1980), e.g. avermectin. On top of that, an increasing consumer concern about the environment and food safety has put the availability of agro-chemicals under additional pressure.

With regard to sustainable agriculture and food production, it is therefore of the utmost importance to safeguard those compounds still providing effective mite control. Avoiding resistance development is one major factor to succeed in this intention. Studying resistance aims at a better understanding of the genetic, biochemical and molecular mechanisms involved in resistance development, and helps to implement sustainable resistance management programs.

There are two ways in which organisms can become resistant to xenobiotics such as pesticides: either by reducing the effective dose of the pesticide available at

the target site or by modifying the target site itself. Well known mechanisms of resistance such as behavioural resistance, reduced penetration or adsorption, sequestration and detoxification all contribute to decrease the amount of the pesticide that finally reaches the target site, whereas target site modifications will directly reduce the binding of the pesticide to its target protein. In practise, probably more than 90% of all resistance cases in insects and mites are caused by a less sensitive target site (target site resistance) and/or an enhanced pesticide detoxification (metabolic resistance) (Roush and Tabashnik 1990; Feyereisen 1995).

3.1 Metabolic Resistance

The enzymes involved in the detoxification process of pesticides may be quantitatively and/or qualitatively altered. There are three main enzyme groups known to be involved in metabolic resistance to insecticides and acaricides: esterases, glutathione S-transferases (GST) and mono-oxygenases. Esterases and mono-oxygenases are referred to as phase I detoxification enzymes. Esterases hydrolyse compounds by cleaving ester bonds and monooxygenases introduce electrophilic or nucleophilic centres in a toxicant, rendering it more hydrophilic and reactive. Subsequently phase 2 detoxification enzymes such as glutathione S-transferases can catalyze the formation of glutathione-conjugated adducts, thus facilitating excretion (Devorshak and Roe 1998; Hemingway et al. 1998; Feyereisen 1999; Li et al. 2007).

Esterases are a large and heterogenous group of enzymes metabolizing a variety of exogenous and endogenous substrates with ester linkages. They have been associated with insecticide resistance in over 50 species of insects, ticks and mites (Devorshak and Roe 1998). While many organophosphates, particularly the oxons, are irreversible inhibitors of carboxylesterases, some organophosphates, but also carbamates, pyrethroids, organochlorines, benzoylphenylureas and juvenile hormone analogues, are susceptible to esterase hydrolysis. Some esterases conferring insecticide resistance have only limited catalytic properties but are produced in such large quantities that they effectively remove the insecticide from its target by binding to it, thereby reducing its availability (Field et al. 1988). This process is called sequestration. In some cases, as with E4 and FE4 in the green peach aphid *Myzus persicae*, esterases may account for as much as 3% of the total protein in the insect (Devorshak and Roe 1998; Sogorb and Vilanova 2002).

Amplification of esterase genes is the most common up-regulation mechanism and is well documented in organophosphate resistant aphid and mosquito strains (Devonshire and Field 1991; Hemingway et al. 1998).

Glutathione S-transferases are a family of proteins that conjugate glutathione (the sulphur atom of cysteine) to various electrophiles. The polarity of glutathione plays an important role in crossing membranes and thus the removal of conjugated toxicants from the cell and organism by excretion. A typical example is the conjugation of organochlorine and organophosphate compounds with glutathione (Pickett and Lu 1989; Prapanthadara et al. 1993).

It was hypothesised that gene amplification might be responsible for increases in glutathione S-transferases by Oppenoorth (1985), but contradictory findings

have also been published. Hemingway et al. (1998) consider up-regulation of transcription by trans acting factors the most likely molecular mechanism of glutathione S-transferase based DDT resistance in the mosquito *Anopheles gambiae*.

Monooxygenases (also referred to as P450-monooxygenases, P450's or mixed function oxidases) are a functionally diverse class of enzymes found in virtually all organisms. These enzymes play a critical role in the metabolism of endogenous and exogenous chemicals. The diversity in function is achieved by a diversity in structure, as insect genomes can carry about a 100 P450 genes, sometimes arranged in clusters. It is now well established that many cases of metabolic resistance are the result of elevated levels of P450, but because of the multiplicity of P450 genes, correlations between resistance and activity of monooxygenases measured by model substrates are rarely demonstrative (Feyereisen 1999).

In contrast to the gene amplification of esterases, increased gene expression is the primary molecular basis of P_{450} monooxygenase based resistance. P450 overexpression has been documented in DDT and malathion resistant *D. melanogaster* (Daborn et al. 2002). No amplification of the gene was observed in the resistant strain, and a post-transcriptional mechanism involving mRNA stability was proposed. P450 overexpression was also observed in a multi-resistant strain of the house fly *Musca domestica* and was probably caused by a trans acting factor (Feyereisen 1999).

3.2 Target Site Resistance

Structural changes by point mutations in the target site of insecticides, leading to reduced toxicant affinity, has been often demonstrated, and results in high resistance. A single common point mutation of Ala 302 to Ser (A302S) in the *rdl*-encoded subunit in GABA gated chloride channels of *D. melanogaster* resulted in dieldrin resistance (ffrench-Constant 1994). Resistance to pyrethroids can be caused by point mutations in the para-type sodium channel gene as demonstrated in many insects (Davis et al. 2008). Point mutations in the acetylcholinesterase genes are known to provide insensitivity of the enzyme in organophosphate and carbamate resistant insect strains (Fournier et al. 1993). Up to date, limited studies have aimed at understanding the importance of an altered target-site in *T. urticae* or other mites. This is mainly caused by the lack of molecular sequence data of acaricide target sites. In addition, the molecular target site of many frequently used acaricides is not completely understood.

4 Acaricide Resistance in *T. urticae*

T. urticae is capable of rapidly developing resistance to many acaricides used for its control. A very high level of resistance to many compounds has developed after 1–4 years of use and often also induces a high degree of cross-resistance. Resistance development is accelerated by their high fecundity, inbreeding, and very short life-cycle resulting in many generations per year, especially in warmer

conditions. Spider mites also appear to have a high mutation rate, which favours the development of resistance alleles. In addition, the phenomenon of arrhenotoky, resulting in haploid males, leads to a faster fixation of possible resistance alleles, and recessive traits become immediately visible in the population (Cranham and Helle 1985; Helle 1985).

Although some progress has been made in understanding acaricide resistance in mites, especially tetranychids, knowledge on the mechanisms associated with acaricide resistance has not kept pace with that of insecticide resistance studies in insects. Limited in depth studies have been published concerning acaricide resistance in *T. urticae*, with the exception of the genetics of acaricide resistance and data on cross resistance.

4.1 Genetics of Resistance in T. urticae

Determination of the genetics of resistance is necessary for resistance risk assessments (McKenzie and Batterham 1998). Conventional methods of genetic analysis, adapted for the arrhenotokous reproduction of spider mites, revealed that often single major genes control specific types of resistance, although important exceptions have also been published. A survey of genetic factors (number of genes, dominance) involved in acaricide resistance in *T. urticae* is presented in Table 2.

Table 2 Genetics of resistance as determined by crossing experiments in *Tetranychus urticae*

Acaricide	Genes	Dominance	Reference
Formetanate	1	Dominant	Croft et al. 1984
Dicofol	1	Incompletely recessive	Dennehy and Granett 1984
Propargite	>1	–	Keena and Granett 1990
Dicofol	1	Incompletely dominant	Martinson et al. 1991
Dicofol	1	Recessive	Martinson et al. 1991
Hexythiazox	1	Incompletely dominant	Herron et al. 1993
Dicofol	1	Incompletely recessive	Kim et al. 1994
Abamectin	>1	Recessive	Clark et al. 1995
Tetradifon	1	Dominant	Park et al. 1996
Tetradifon	1	Incompletely dominant	Park et al. 1996
Etoxazole	1	Recessive	Kobayashi et al. 2001
Fenpyroximate	–	Dominant	Stumpf and Nauen 2001
Pyridaben	–	Dominant	Stumpf and Nauen 2001
Etoxazole	1	Recessive	Uesugi et al. 2002
Chlorfenapyr	1	Dominant	Uesugi et al. 2002
Chlorfenapyr	>1	Intermediate	Van Leeuwen et al. 2004
Pyridaben	1	Dominant	Van Pottelberge et al. 2008
Fenpyroximate	1	Dominant	Van Pottelberge et al. 2008
Tebufenpyrad	>1	Dominant	Van Pottelberge et al. 2008
Bifenthrin	–	Incompletely recessive	Tsagkarakou et al. 2009
Spirodiclofen	>1	Incompletely dominant	Van Pottelberge et al. 2009a

Dominance refers to the phenotypic expression of a resistance trait in heterozygotes, relative to the expression in homozygous parents (Roush and McKenzie 1987). This is essential for predicting the spreading of resistance in the field. If applied rates can effectively kill the heterozygotes, resistance acts as a recessive trait, and spreads slowly. In contrast, when dominance is high and heterozygotes survive the toxicant, resistance spreads rapidly through the alleles present in heterozygotes. The number of genes involved is equally important for the management of resistance in the field. Monogenic resistance is more likely to spread then polygenic resistance, particularly when pesticide exposure varies across time or space. If a polygenic resistant individual immigrates to a susceptible population, the resistance alleles are likely to be diluted by hybridization if the population is not treated for a few generations. When monogenic resistance is introduced into a susceptible population by immigrants, resistance is more likely to stay in the population, even after several untreated generations (Roush and McKenzie 1987). This hypothesis was first supported by experiments using pyrethroid resistant predatory mites (Hoy et al. 1980).

4.2 Cross-Resistance

Cross-resistance occurs when a mite strain with resistance to one acaricide shows resistance to other acaricides without prior contact. The term is correctly used in relation to the spectrum of responses to a range of pesticides influenced by a single resistance mechanism. For example, selection with one organophosphate can result in resistance to many other organophosphate compounds which have not been used.

However, reports on cross-resistance between different chemical groups with different mode of action have also been published. Negative cross-resistance occurs when resistance to one compound results in a greater susceptibility to another. Sound evidence for the spectrum of cross-resistance can be obtained only when using a mite strain that contains just one type of resistance factor, a condition best assured by repeated back-crossing with a susceptible strain under selection. However, such studies were rarely undertaken. Table 3 summarizes some important published cross-resistance patterns in *T. urticae*, reported during the past 20 years

5 Investigated Cases of Resistance in *T. urticae* to Some Major Acaricide Groups

5.1 Organophosphates

Organophosphates (OPs) are the most widely used insecticides to control a broad range of arthropod pests with medical and agricultural importance, including *T. urticae*. Together with carbamates, they account for more than 35% of total global insecticide

Table 3 Survey of detected cross-resistance in *T. urticae* resistant to important acaricides (+ : positive cross-resistance)

Resistance	Cross-resistance	References
Dicofol	+Organophosphates +Amitraz +Bromopropylate +Chlorobenzilate +Bifenthrin +Chlorpyrifos	Kono 1985; Fergusson-Kolmes et al. 1991; Kim et al. 1994
Cyhexatin	+Azocyclotin +Fenbutatin oxide	Edge and James 1986; Tian et al. 1992
Organophosphates	+Carbamates +Formamidines +Organophosphates	Khajehali et al. 2009
Clofentezine	+Hexythiazox	Herron et al. 1993
Organotins	+Hexythiazox	Flexner et al. 1988
Tetradifon	+Clofentezine +Benzoximate +Chlorfenson	Park et al. 1996
Tebufenpyrad	+Pyridaben +Fenpyroximate	Herron and Rophail 1998; Van Pottelberge et al. 2008
Pyridaben, fenpyroximate, tebufenpyrad	+Dicofol +Dimethoate +Pyridaben	Stumpf and Nauen 2001; Sato et al. 2004
Dimethoate	+Bifenthrin	Yang et al. 2002
Chlorfenapyr	+Clofentezine +Amitraz +Dimethoate +Bromopropylate	Van Leeuwen et al. 2004
Bifenazate	+Acequinocyl	Van Nieuwenhuyse et al. 2009
Spirodiclofen	+Spiromesifen	Van Pottelberge et al. 2009a
Bifenthrin	+Fluvalinate	Tsagkarakou et al. 2009

sales (McCaffery and Nauen 2006). Both chemical groups target the enzyme acetylcholinesterase (Ace, AChE; EC 3.1.1.7), which plays a key role in nervous impulse transmission by hydrolyzing the neurotransmitter acetylcholine. OPs are analogous to the substrate acetylcholine, bind to the active site of AChE, and block the enzyme by phosphorylating the serine at the active site (Aldridge 1950).

5.1.1 OP Resistance Reports

OPs are among the first chemical groups used to control *T. urticae* and to which the species developed resistance. The early historical use and appearance of resistance is given by Helle in 1962. The first reported cases of failure of OPs to control *T. urticae* go back to the late 1940s. In many cases, especially in greenhouses, resistance appeared quickly after the first contact of *T. urticae* with the chemical. Within a period of only 4 years resistance appeared in different greenhouse crop areas of the

USA and Europe. Some years later resistance appeared in *T. urticae* populations infesting orchards and other open field crops in many countries. Since these first cases of resistance to OPs, *T. urticae* has developed resistance to more than 30 OPs and carbamates in 40 countries spread all over the world. (Georghiou and Lagunes-Tejeda 1991 and Arthropod Pesticide Resistance Database http://www.pesticideresistance.org/DB).

As early as the first reports of control failure appeared, OP resistance could be confirmed by laboratory bioassays. *T. urticae* populations were often resistant to several acetylcholinesterase inhibitors, with variable levels. For example, two field strains collected on hop plants in the **UK** which displayed a similar pattern of resistance, were found to be highly resistant to parathion (Resistance Ratio, RR > 100), much less resistant to azinphos ethyl (RR = 7), and intermediately resistant to demeton-S-methyl (RR = 21–27) (Cranham 1974). One of these strains displayed high levels of resistance (RR = 78–112) to triazophos, amiphos, dioxathion, dialifos and phosalone, and lower (RR < 31) to methidathion, aziphos-methyl and phosphamidon.

Evidence on cross-resistance within the OP group was reported in **New Caledonia**. Three field populations collected from strawberries, roses and *Bauhinia* were highly resistant to the OPs dimethoate, monocrotophos and methidathion (RRs ranged from 150 to 240, 110 to 340 and 85 to 200, respectively). These populations were highly resistant to profenofos, (RR = 110–150) even though this compound was never used in the field (Brun et al. 1983).

Similarly, in **Australia**, profenofos resistance was already detected before its release in 1978 in a *T. urticae* population collected from cotton, which proved to be already highly resistant to other OPs (Herron et al. 1998). *T. urticae* field populations collected on cotton from 1976 to 1995 displayed high resistance to all OPs tested. Resistance ratios were found to be up to 650 for demeton-S-methyl, up to 750 for dimethoate and up to 78 for parathion. Profenofos and monocrotophos resistance remained stable from 1976 until 1988 (RR of approximately 15 and 40 respectively) but finally the resistance rose and reached RRs of 100 for profenofos and 400 for monocrotophos in 1995, rendering both chemicals ineffective.

T. urticae collected from cotton in **Florida** displayed high resistance against chlorpyrifos (RR = 78), but moderate resistance against demeton-S-methyl and ethyl paraoxon (RR < 5) (Stumpf et al. 2001).

In **Belgium**, a *T. urticae* population collected in a greenhouse nursery at Ghent University, where poplar cuttings, beans and ornamentals were grown, was highly resistant to dimethoate (RR = 250) and displayed cross resistance to parathion-ethyl (RR = 24) (Van Leeuwen et al. 2005; Khajehali et al. 2009).

In **Greece**, resistance to two OPs (methyl-parathion and methidathion) was found in a number of *T. urticae* strains derived from mites collected on different host plant species in greenhouse and open field crops (Tsagkarakou et al. 1996). With methidathion, resistance ratios between strains varied considerably, from 5 to 63. On the contrary, with methyl-parathion the strains displayed a striking similarity with a resistance ratio of approximately 50 (Tsagkarakou et al. 1996). More recent investigations on Greek *T. urticae* populations resistant to OPs from different locations in Crete and the mainland displayed RRs ranging from 10 to 54 to pirimiphos

methyl (A. Tsagkarakou, unpublished data, 2008). One pirimiphos methyl selected (RR = 89) Greek strain collected from rose in a greenhouse near Athens displayed high cross-resistance to dimethoate (RR = 145) and methomyl (RR = 250), but a low resistance to chlorpyrifos (RR = 2.7) (Khajehali et al. 2009).

5.1.2 OP Resistance Mechanisms

Target Site Insensitivity-Based Resistance

The fact that cross resistance within the OP group is found as a rule in *T. urticae* is in accordance with the main mechanism involved in the resistance to OPs, i.e. the reduced sensitivity of the target enzyme acetylcholinesterase (AChE). AChE terminates transmission at cholinergic synapses by hydrolyzing acetylcholine (ACh) released from the presynaptic membrane. The inhibition of AChE by organophosphate and carbamate insecticides results in an accumulation of ACh at the postsynaptic membrane and causes a desensitization of the ACh receptor, leading to a blockage of the signal transmission. The insensitivity of AChE to OPs was first reported in *T. urticae*, associated with OP resistance (Smissaert 1964). This was the first demonstration of an altered target site in an arthropod pest. Biochemical properties of the insensitive AChE suggested that some modification occurred at the active site of the enzyme (Voss and Matsumura 1964).

Biochemical Studies on the AChE Insensitivity

So far, AChE insensitivity to OPs in *T. urticae* has been reported in strains from the Netherlands, Germany, the USA, New Zealand, Israel, Egypt and Greece (Zahavi and Tahori 1970; Helle 1984; Cranham and Helle 1985; Tag El-Din 1990; Stumpf et al. 2001; Tsagkarakou et al. 2002). In these studies the kinetics of the AChE ki (the bimolecular rate constant) and I_{50} (the inhibitor concentration causing 50% decrease of AChE activity) in many OP resistant strains clearly showed that AChE is less sensitive in the resistant strains compared to the susceptible ones (Table 4). For example, the AChE of a German *T. urticae* strain (LRR) resistant to parathion (RR = 10) displayed ki values that were ~ 600 and 150 times lower, which means that it was 600 and 150 times less sensitive than those of the susceptible strain, when treated with paraoxon and diazoxon, respectively. For this strain the I_{50} for paraoxon and sevin (a carbamate) was 12- and 6.5-fold higher compared to the susceptible strain respectively (Smissaert 1964; Voss and Matsumura 1964; Smissaert et al. 1970). In a *T. urticae* strain from Greece the I_{50} was higher (119 and 50 times higher with paraoxon and methomyl respectively) and the bimolecular rate constant ki was lower (39 and 47 times lower with paraoxon and methomyl respectively) in the resistant strain, compared to the susceptible strain (Tsagkarakou et al. 2002). The AChE of one strain may display variable levels of insensitivity to different inhibitors (Table 4 and Smissaert et al. 1970) indicating that mutations at different positions or combination of different mutations are involved in AChE insensitivity in *T. urticae*.

Table 4 Degrees of insensitivity of AChE in *Tetranychus urticae* with variable levels of resistance to OPs. Resistance Ratio (RR), bimolecular rate constant (ki) ratio and ratio of I_{50} are given

Insecticide	Strain	RR	I_{50} ratio	ki ratio	Reference
Parathion	LRR (Germany)	10	12	1,000	Smissaert 1964
	LR (Germany)	10	2.5		Voss and Matsumura 1964
	BR (USA)	10	1		
Parathion	HB-R	1200		1,000	Ballantyne and Harrison, 1967
	CH-R	1150		1,000	
Malathion	Wadi Ara (Israel)	1.2	1.6		Zahavi and Tahori 1970
	Kfar Sattaf (Israel)	1.4	1.1		
	Neve Ur III (Israel)	43	2.7		
	Par'od (Israel)	143	270		
	Ayeletch Hashahar I (Israel)	127	330		
	Ayeleth Hashahar III (Israel)	190	400		
	Neve Ur I (Israel)	250	650		
Parathion	R (Egypt)	102	30	34	Tag El-Din 1990
	KR (Egypt)	36	28		
	BR (Egypt)	17	13		
Chlorpyrifos	VB (USA)	78	120	110	Stumpf et al. 2001
	WI (Germany)	9.5	130		
Ethyl paraoxon	VB	4.9	290	340	
	WI	2.8	380		
Demeton-S-methyl	VB	2.8	34		
	WI	3.9	38		
Carbofuran	VB		12		
	WI		18		
Parathion	RLAB (Greece)	44	119	39	Tsagkarakou et al. 2002
Methomyl	RLAB	8	50	47	
Chlorpyrifos	ATHRos-Pm (Greece)	2.7	29		Khajehali et al. 2009
Pirimiphos methyl	ATHRos-Pm	89	>10		

ki = bimolecular rate constant
I_{50} = the inhibitor concentration inducing 50% decrease of acetylcholinesterase activity
ki ratio = ki susceptible/ki resistant
I_{50} ratio = I_{50} resistant/I_{50} susceptible

Molecular Basis of the AchE Insensitivity

As mentioned above *T. urticae* was the first arthropod in which target site insensitivity was proven to be the resistance mechanism (Voss and Matsumura 1964). Since then biochemical studies led to the conclusion that AChE insensitivity is the most common type of OP resistance in *T. urticae.* However, the exploration of the molecular basis of this insensitivity in this species only started quite recently. In insects, several studies have already identified the molecular changes in the target site of OP and carbamate insecticides that are responsible for resistance to these compounds.

A number of point mutations in the AChE gene (AChE2 for higher diptera and AChE1 for other insects) have been identified as being responsible for insecticide resistance in *D. melanogaster* (Mutero et al. 1994), *M. domestica* (Kozaki et al. 2001; Walsh et al. 2001), *Bactrocera oleae* (Vontas et al. 2002), mosquitos (Weill et al. 2002, 2003, 2004; Nabeshima et al. 2004), *Bemisia tabaci* B and Q biotype (Alon et al. 2008) and aphids (Nabeshima et al. 2003; Andrews et al. 2004; Benting and Nauen 2004; Toda et al. 2004).

Anazawa et al. (2003) have first published the molecular structure of AChE of *T. urticae*. The AChE1 cDNA sequence was determined, and the 687 amino acids of the primary structure were deduced from an OP susceptible (TKD) and an OP resistant strain (NCN). Six replacements of amino acid residues were found in TKD and two in NCN. F331C (numbering according to *Torpedo californica* sequence) was the only substitution unique to NCN, however, this mutation existed homozygously in only two out of nine mites. At the same position, a F331W substitution was found in resistant *T. kanzawai* (Aiki et al. 2005), a species for which insensitive AChE was considered as a major factor conferring OP resistance since 1982 (Kuwahara 1982). Recently the F331W mutation was found in OP resistant strains of *T. urticae* from Greece, Belgium and Germany (Khajehali et al. 2009). In insects the F331W substitution at AChE1 was initially characterized in *Culex tritaeniorhynchus* (Nabeshima et al. 2004) and recently found in the whitefly *B. tabaci* (Alon et al. 2008; Tsagkarakou et al. 2009). The F331 position in AChE1 is located in the acyl pocket neighboring the active center in the active site gorge of AChE. By comparing the catalytic properties and insecticide sensitivity of the wild-type and the mutant recombinant AChE1 expressed in a baculovirus–insect cultured cell system, Oh et al. (2006) demonstrated the extremely reduced sensitivity to OP compounds conferred by F331W in AChE1.

In line with the variable degrees of susceptibility to different OPs and carbamates revealed by *in vivo* toxicity and *in vitro* inhibition studies, other mutations were also found in the AChE1 of resistant *T. urticae* strains (Khajehali et al. 2009). In some strains or individuals of the same strain, the F331W was found together with A201S (a substitution found also with S331F in AChE1 of OP resistant *Aphis gossypii*, Andrews et al. 2004) and/or with G328A (a substitution already found in AChE2 of OP resistant *M. domestica* Walsh et al. 2001, and of OP resistant *D. melanogaster* Menozzi et al. 2004). It is known that the different mutations as well as the combination of these mutations in the same protein result in different levels of resistance (Fournier and Mutero 1994; Oh et al. 2006). However, the role of F331W, A201S and G328A in the resistance of *T. urticae* is still unexplored. By site directed mutagenesis, the role of each substitution or combination of substitutions in OP and carbamate resistance of *T. urticae* should be studied.

Metabolic Resistance

In most of the studied cases there is a correlation between *in vivo* toxicity of OPs and *in vitro* insensitivity of AChE in the presence of inhibitors (Table 4). However, some discrepancies suggest the involvement of other resistance mechanisms in OP

resistance. For example, although most of the mite strains from Israel (Table 4) showed a good correlation between *in vivo* resistance to malathion and insensitivity of their AChE to malaoxon (Zahavi and Tahori 1970), the strain Neve Ur III did not. This strain was 43-fold resistant to malathion, but its AChE was only 2.7 times less sensitive to malaoxon. Another example comes from the study of Stumpf et al. (2001) where two strains (VB and WI) with similarly insensitive AChE to chlorpyrifos oxon (I_{50} ratio = 120 and 130 respectively) displayed different *in vivo* responses to chlorpyrifos. In an other study three resistant strains (LRR, LR and BR), although having the same RRs, had a different *in vitro* response of their AChE to the inhibitory action of paraoxon and displayed different cross resistance to a carbamate (Table 4) (Voss and Matsumura 1964). All together these results show that although insensitive AChE is the main resistance mechanism to OPs, other mechanisms may also be involved in some cases. This was first suggested and documented by Voss and Matsumura (1964) who showed that enhanced detoxification by carboxyesterases and phosphatases was involved in the degradation of malathion, malaoxon, and ethyl-parathion to non toxic products in OP resistant strains from Germany and USA.

Since then, evidence of increased detoxification as a mechanism of resistance to OPs has been found in several cases, although often in association with a modified AChE. For example the activity of 7-ethoxycoumarin-O-deethylase (ECOD) was twofold higher in the resistant RLAB Greek strain. The absence of synergism of parathion or methomyl toxicity by PBO *in vivo* suggests that P450s are not involved in resistance. However, the RLAB strain also possessed an insensitive AChE, and the absence of synergism may be explained by the masking effect of the presence of target resistance on a detoxification mechanism rather than absence of such a mechanism (Tsagkarakou et al. 2002).

More examples of the possible involvement of detoxifying enzymes in OP resistance are derived from cases of positive or negative cross resistance. In 1960 the strain Baardse originating from a rose greenhouse in Aalsmeer (the Netherlands) was found to be OP and Kelthane (dicofol) resistant (Helle 1961). In New Caledonia some OP resistant field strains were also highly resistant to dicofol. Cross resistance between dimethoate and bifenthrin was observed in a dimethoate-selected *T. urticae* population from U.S. corn fields. After 10 cycles of exposure, the dimethoate-exposed *T. urticae* strain had a 15.9-fold cross-resistance to bifenthrin (Yang et al. 2002). The absence of differences in esterase activities between the strains possibly indicates another detoxification mechanism (P450s or GSTs). In Belgium, an OP resistant population (MR-VL, RR = 250 and 24 to dimethoate and parathion ethyl respectively) collected in a greenhouse nursery at Ghent University, was cross-resistant to several currently used acaricides (clofentezine, chlorfenapyr, bromopropylate, amitraz, flucycloxuron and azocyclotin) (Van Leeuwen et al. 2005). This population was for many years treated with fenbutatin oxide, dicofol and bifenthrin, until these products failed. In this population, enhanced detoxification by increased activity of monooxygenases and esterases was observed which can at least partially explain the broad cross-resistance spectrum (Van Leeuwen et al. 2005).

An indication that P450s may modify the tolerance of *T. urticae* towards acaricides comes from a case of negative cross resistance with dicofol. Fergusson-Kolmes et al. (1991) observed that a dicofol resistant strain became more susceptible to chlorpyrifos and concluded that dicofol resistance was most probably P450 related. Hatano et al. (1992) found that a dicofol resistant strain more rapidly oxidised chlorpyrifos to chlorpyrifos-oxon, compared to susceptible strains. The lack of differences in AChE properties suggests that the increased oxidation responsible for dicofol resistance renders this strain more susceptible to chlorpyrifos.

5.1.3 Genetics and Evolution of OP Target Resistance in *T. urticae*

Studies from the 1960s and 1970s investigating the mode of inheritance of OP resistance suggested that resistance is dominant and controlled by one major gene. OP resistance was attributed to a single dominant or semi dominant gene in an American malathion resistant strain of *T. urticae*, a demeton selected German laboratory strain, several ethyl parathion resistant strains from the Netherlands and New Zealand and in a parathion resistant strain from Greece (Taylor and Smith 1956; Helle 1962; Schulten 1968; Ballantyne and Harrison 1967; Tsagkarakou 1997). Furthermore, in *T. urticae,* the gene coding for an altered AChE and the gene coding for resistance were shown to be allelic suggesting that the same gene was involved (Schulten 1968).

Resistance mutations will be selected only if the AChE keeps its capacity to hydrolyze its natural substrate acetylcholine. Comparing the relative tolerances of a susceptible strain, a resistant strain and their hybrid, Smissaert et al. (1975) concluded that the minimum non-lethal fraction of AChE activity is 2.3%. This is much less than the decrease in AChE activity of the resistant strains compared to the "wild-type" enzyme. The dominant nature of the altered AChE is in accordance with the low fraction of activity that is necessary in *T. urticae* as well as in insects (Fournier and Mutero 1994). The low fraction necessary for viability accounts also in part for the low fitness cost associated with altered AChE in *T. urticae*. Absence of fitness cost was reported by Smissaert (1964) and Helle (1962) based on their observations that no decreased viability occurred in their resistant strain. Evidence for a low fitness cost comes from investigating the stability of resistance even after a relaxation of the selection pressure. Voss and Matsumura (1964) showed that similar resistance levels were observed in a resistant strain with and without selection pressure. Periodic tests with the Leverkusen-R strain and two New Zealand resistant strains, highly resistant to oxydemeton-methyl, have shown that in spite of the absence of selection pressure, no significant reduction in resistance had occurred (Ballantyne and Harrison 1967). Although the decline of OP resistance in a strain from Israel (Neve Ur III) indicates possible fitness costs, this was not associated with a modified AChE, since a resistance mechanism other than target site resistance seems to be involved in this particular case (Zahavi and Tahori 1970).

More recently Stumpf et al. (2001) developed a microfluorometric assay which was used to genotype individual mites and to examine the frequency of sensitive

and insensitive AChE in two resistant strains. Resistance to OPs has been shown to be very stable, since the high frequency of resistance alleles (0.91) which was initially observed was maintained after several years. Progress in the understanding of the molecular basis of resistance will permit the development of molecular tools for monitoring resistant genotype frequency and their spreading. In addition, it will be possible to determine whether the resistance has a single or several origins. An important question is whether the resistance genes were derived from mutation events occurring in one region or if they were introduced. Gene flow is high between *T. urticae* populations which are only separated by short distance meters but gene flow decreases sharply as geographic distances increase (Tsagkarakou et al. 1997, 1998, 1999). These observations, and the fact that resistance in *T. urticae* appeared rapidly and simultaneously in different regions of the world after the first use of insecticides (Cranham and Helle 1985), suggest that mutations conferring resistance may have appeared independently in the different localities. Comparisons of the sequences including introns of resistant and susceptible AChE of *T. urticae* from various regions worldwide is needed to clarify this issue.

5.2 Pyrethroids

Pyrethroids have become a widely favored class of insecticides accounting for approximately 20% of the world insecticide market. They have a number of distinct advantages compared to other chemical classes, such as great selectivity and potency for insect/mite sodium channels, low mammalian-toxicity, low persistence in the environment, and rapid killing effect on the insect/mite (Khambay and Jewess 2005). Their efficacy varies between product and target pest. The pyrethroids bifenthrin, fluvalinate, and fenpropathrin have been more often registered and used for the control of *T. urticae* worldwide. They were more recently introduced compared to other classes of insecticides and proved particularly useful in cotton, where their contact activity and good efficacy enabled the growers to regain control of mites and other pest species that had become resistant to previously used insecticides (Herron et al. 2001).

The global decrease of the effectiveness of pyrethroids has provoked research efforts to understand the nature of resistance in several insects (Khambay and Jewess 2006). Several *T. urticae* pyrethroid resistance cases have been reported worldwide. However, only a limited number of studies were conducted to analyze pyrethroid resistance mechanisms in this species.

5.2.1 Pyrethroid Resistance Reports

T. urticae resistance against pyrethroids used for its control is widespread, but the magnitude and pattern of resistance and cross-resistance varies considerably among countries and cropping systems. Bifenthrin resistance was first reported by

Farnham et al. (1992) in *T. urticae* collected from an intensively sprayed New York State apple orchard in 1985.

Pyrethroid resistance is well documented in cotton crops in **Australia**, where bifenthrin was a popular option when both *Helicoverpa armigera* and spider mites required control (Herron et al. 2001). The molecule was used experimentally on a small scale for several years before full registration in 1993. Despite the implementation of resistance management strategies, bifenthrin resistance evolved by the 1996/97 cotton-growing season, as verified by bioassays in a number of field-collected strains from cotton in the valleys of New South Wales (Herron et al. 2001). By 1999, bifenthrin became unreliable for *T. urticae* control in Australian cotton, although resistance ratios were relatively low (up to eightfold). During the 1999/2000 cotton season, resistance was detected in nine of the 10 strains tested (Herron et al. 2001). Resistance levels peaked at 109-fold in a population containing 80% resistant mites. Two strains (Col-A and Col-B) contained mites which survived 12,800 mg a.i./l bifenthrin, the highest rate that could be applied without severe phytotoxicity (Herron et al. 2001).

Pyrethroid resistance was also observed in *T. urticae* populations from cotton fields in **Turkey** (Ay and Gurkan 2005). In most cases Turkish farmers used broad-spectrum insecticides that were effective against all cotton pests. Such insecticides eliminated major pests, as well as their natural enemies, and as a result secondary pests such as two-spotted spider mites became a major problem. Bifenthrin was used intensively in Turkey against spider mites from the late 1980s to early 1990s. It became ineffective in the field, due to resistance development in the mid 1990s and as a result its use was stopped in 2000 (Ay and Gurkan 2005). Three resistant strains (URF, PAM and DEN) isolated from cotton plants from Adana and Urfa in 1998–1999 were extremely resistant to bifenthrin, with resistance ratios up to 669 (Ay and Gurkan 2005).

A resistant strain (MR-VL) was more recently isolated from a greenhouse nursery at Ghent University (**Belgium**) where poplar cuttings, beans and ornamentals were grown (Van Leeuwen et al. 2005). Spider mites in this greenhouse nursery were controlled in previous years by sequentially spraying bifenthrin, fenbutatin oxide and dicofol in a rotational spray program, until all three products failed. The MR-VL strain exhibited strong resistance to bifenthrin (RR >10,000) in comparison with a susceptible laboratory strain (Van Leeuwen et al. 2005).

Beside the first report of bifenthrin resistance in a *T. urticae* population from New York (**USA**) that had never been exposed to bifenthrin (Farnham et al. 1992), resistance to bifenthrin and λ-cyhalothrin was also found in a *T. urticae* laboratory strain originating from USA corn fields, repeatedly exposed to three insecticides. After 10 cycles of exposure, susceptibility to the corresponding insecticides, bifenthrin and λ-cyhalothrin, decreased 14.8- and 5.7-fold relative to the reference strain, respectively (Yang et al. 2002).

In the most recent study, bifenthrin resistance levels were monitored in 11 *T. urticae* populations from outdoor and indoor vegetable and ornamental crops from **Greece**. Resistance factors varied from 1 (absence of resistance) to very high levels. Two highly resistant strains were derived after mild laboratory selection of

the heterogeneous resistant populations identified in the field: the ATHRos-Bf derived from greenhouse roses from Athens, and the CREVeg-Bf, derived from vegetable crops from Crete (Tsagkarakou et al. 2009). Resistance levels to bifenthrin were 2,495- and 1,026-fold for the ATHRos-Bf and the CREVeg-Bf strains respectively (Fig. 1a).

5.2.2 Mechanisms of Resistance to Pyrethroids

The pyrethroid class of insecticides can be subdivided into two groups. Type I pyrethroids (e.g. permethrin) lack an alpha cyano group, whereas type II pyrethroids (e.g. deltamethrin) contain a cyano group in the alpha-benzylic position (Khambay and Jewess 2005). An interesting development in pyrethroid chemistry has been the replacement of 3-phenoxybenzyl alcohol by 3-phenylbenzyl alcohol, which is sterically more restricted. Activity in this series is enhanced by the introduction of a methyl group at position 2 as in bifenthrin, which forces noncoplanarity of the phenyl rings presumably so matching the conformation of the target site (Khambay and Jewess 2005). This chemistry has broad activity and is particularly effective against some mite species, such as *T. urticae*. Although bifenthrin has one of the highest levels of mammalian and fish toxicity it is commercially very successful.

The principal site of action of pyrethroids is the voltage-gated sodium channel of nerve cells (Soderlund & Bloomquist 1989; Bloomquist, 1996; Zlotkin 1999) (Fig. 1). Pyrethroids modify the gating kinetics by slowing both the activation and inactivation of the channel. This action causes the repetitive firing of neurons that is typically found in pyrethroid-poisoned insects and mites (Zlotkin 1999).

On the basis of electrophysiological properties there are two distinct binding sites for pyrethroids on the sodium channel (O'Reilly et al. 2006). A combination of protein-ligand modeling and docking procedures, in conjunction with experimental data on pyrethroids sodium channel interactions has recently produced a hypothesis as to how different types of pyrethroids interact with insect sodium channels (O'Reilly et al. 2006). The model predicts a key role for the IIS5 and IIIS6 helices in pyrethroid binding. Some of the residues on the helices that form the putative binding contacts are not conserved between arthropod and non-arthropod species, which is consistent with their contribution to insecticide species selectivity (O'Reilly et al. 2006). The model suggests that the exceptionally high activity of halogenated compounds, such as bifenthrin, may be due to their enhanced interaction with aromatic residues on the domain IIIS6 (O'Reilly et al. 2006).

The majority of reports implicate either enzymatic hydrolysis by carboxylesterases (COEs), or oxidation by microsomal monooxygenases in *T. urticae* populations resistant to pyrethroids (Ay and Gurkan 2005; Van Leeuwen et al. 2005, 2007). One report has identified behavioral changes as a possible resistance component, while changes in target site sensitivity have only recently been investigated at the molecular level (Tsagkarakou et al. 2009).

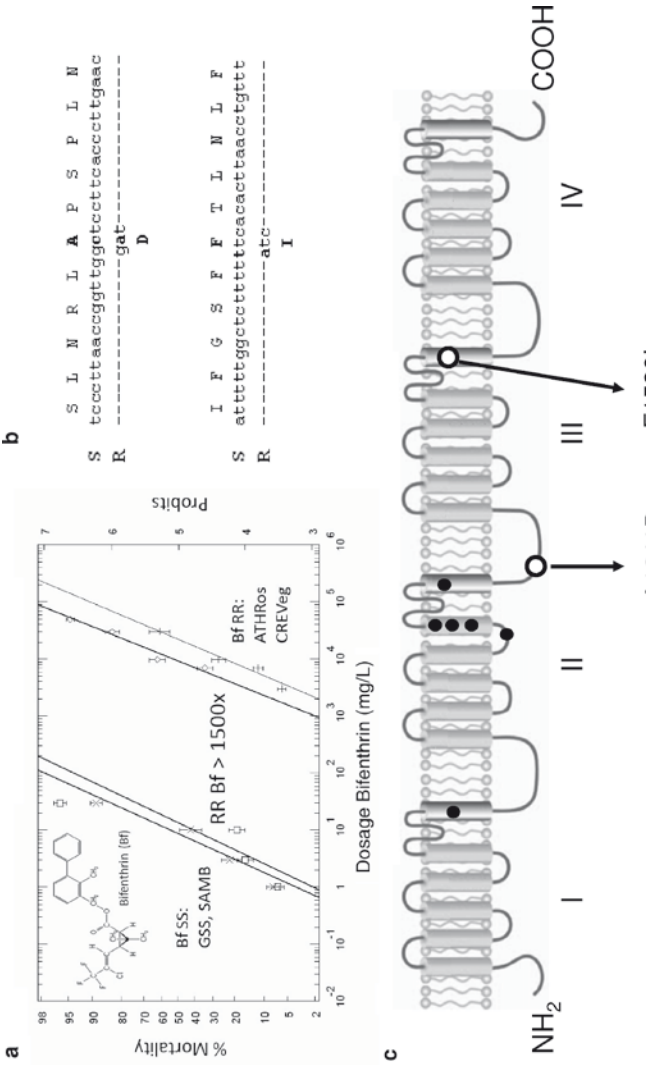

Fig. 1 Resistance to pyrethroids and a schematic representation of a voltage-gated sodium channel. **(a)** Toxicity data obtained from Greek susceptible and bifenthrin resistant strains as described in the text, indicating high resistance. **(b)** Partial nucleotide sequence alignment of *T. urticae* sodium channel cDNAs. Mutated positions in bifenthrin resistant strains are indicated. **(c)** Sodium channel protein structure. The pore-forming a-subunit consists of a single polypeptide chain with four domains (I-IV), each having six transmembrane helices. (Adapted from Davies et al. 2007). *Solid circles* show the sites for amino acid substitution polymorphisms known to affect insecticide sensitivity in insects, and *open circles* the position where resistance mutations have been found in *T. urticae* (Tsagkarakou et al. 2009), with residues numbered according to the sequence of the housefly (*Musca domestica*) voltage-gated sodium channel (EMBL accession: X96668)

Behavioral Response

Behavioral changes of insects and mites have been studied less and have generally been considered of lower importance when compared with enhanced detoxification and target site resistance. A reduced agitated and milder locomotory excitation was observed in a bifenthrin resistant strain originating from a New York State apple orchard in Ontario Country (NY) (Kolmes et al. 1994). This change in behavior resulted in reduced exposure of the mites to insecticides, which however mostly affected the susceptibility of the bifethrin resistance strain to dicofol (Kolmes et al. 1994).

Metabolic Resistance

Most studies provided direct or indirect evidence that pyrethroid resistance in *T. urticae* is conferred by carboxylesterase (COE)-mediated ester hydrolysis (Van Leeuwen et al. 2007). Cytochrome P450-based cleavage of the ester bond or hydroxylation of aromatic rings or methyl groups might also contribute to pyrethroid metabolism (Van Leeuwen et al. 2005), although no direct evidence has been provided yet. Glutathione S-transferases do not appear to have a primary role in resistance to pyrethroids in *T. urticae*.

The Use of Synergists and Cross Resistance Studies

The metabolic inhibitors DEF (S,S,S-tributyl phosphorotrithioate) and PBO (piperonyl butoxide), which inhibit COE and cytochrome P450 detoxification enzymes, significantly synergized pyrethroid toxicity in *T. urticae* populations from corn fields in the USA (Bynum et al. 1997). The synergist triphenyl phosphate (TPP), another inhibitor of esterases, enhanced the toxicity of bifenthrin and λ-cyhalothrin against mites by 6.2- and 1.9-fold, respectively (Yang et al. 2001). The synergist diethyl maleate (DEM), which depletes glutathione levels, enhanced the toxicity of bifenthrin by 4.1-fold (Yang et al. 2001).

The toxicity of bifenthrin was synergized at least ninefold after treatment of resistant mites of the highly resistant MR-VL strain isolated from Belgium with DEF (Van Leeuwen et al. 2007). Although this is a strong synergism, mites did not become fully susceptible after pretreatment with DEF (Van Leeuwen and Tirry 2007).

In contrast to earlier studies, the metabolic inhibitors PBO and DEF only partially synergized the high levels of pyrethroid resistance in the ATHRos-Bf and CREVeg-Bf resistant strains isolated from Greece (Tsagkarakou et al. 2009), indicating that metabolic resistance is probably not the primary mechanism in these particular strains.

In contrast to an earlier undefined negative cross-resistance between azinphos-methyl and fenvalerate in a *T. urticae* strain from New Zealand (Chapman and Penman 1979), remarkable cross-resistance levels between dimethoate and bifenthrin were observed in *T. urticae* populations from corn fields in the USA, repeatedly exposed to these insecticides. After 10 cycles of exposure, the dimethoate-exposed *T. urticae* strain was 15.9-fold cross-resistant to bifenthrin (Yang et al. 2002).

Given the difference in dimethoate and bifenthrin target sites, this cross resistance pattern possibly implicates the role of COEs or other detoxification enzymes in resistance.

Cross-resistance to several insecticides and acaricides was also reported for the bifethrin resistant MR-VL strain from Belgium. Cross-resistance was detected to clofentezine (RR = 2631), dimethoate (RR = 250), chlorfenapyr (RR = 154), bromopropylate (RR = 25), amitraz (RR = 17), flucycloxuron (RR = 15) and azocyclotin (RR = 7), while no signs of cross-resistance were observed for abamectin, acequinocyl, bifenazate, tebufenpyrad and spirodiclofen (Van Leeuwen et al. 2005). The observed cross-resistance pattern includes a number of different chemical classes of acaricides and thus possibly indicates several mechanisms of resistance being involved in the MR-VL strain and/or broad spectrum metabolic resistance.

The bifenthrin resistant strains ATHRo-Bf and CREVeg-Bf isolated from Greece displayed high levels of cross-resistance to the pyrethroid fluvalinate (RR = 1132 and 876 for ATHRo-Bf and CREVeg-Bf, respectively), but relatively low with fenpropathrin (RR = 83 and 47 for ATHRo-Bf and CRETVeg-Bf, respectively). Both strains showed similar low levels of resistance to the organochlorine DDT (RR = 6.55 and 2.25 for ATHRo-Bf and CREVeg-Bf, respectively), while cross-resistance to dicofol was high in ATHRos-Bf (RR = 200) but much lower in CREVeg-Bf (RR = 2).

Biochemical Studies

Elevated esterase activities were associated with bifenthrin and λ-cyhalothrin resistance in *T. urticae* from corn fields in the USA (Yang et al. 2002). Reduced glutathione *S*-transferase 1-chloro-2, 4-dinitrobenzene conjugation activity was also associated with pyrethroid tolerance, but this did not appear to be related to changes in insecticide susceptibility (Yang et al. 2002).

Biochemical assays with the model COE substrates 4-nitrophenyl acetate and 1-naphthyl acetate, indicated that increased COE levels were present in the MR-VL resistant strain from Belgium. Acetyl-, aryl- and carboxyl-esterase isoenzymes were detected in *T. urticae*, by using inhibitors after separation by native PAGE (Van Leeuwen et al. 2005; Van Leeuwen et al. 2007).

A correlation between COE enzyme activities and bifenthrin resistance was also reported in bifenthrin resistant strains isolated from Turkey (Ay and Gurkan 2005). Polyacrylamide gel electrophoresis analysis revealed differences in esterase banding patterns in the bifenthrin resistant strains PAM and URF and the moderately resistant strain DEN. In general, COE activity was high for these strains in the microtiter plate enzyme assay (Ay and Gurkan 2005).

The role of COEs in *T. urticae* bifenthrin resistance was further postulated in a comprehensive analysis of the metabolic resistance mechanisms of the MR-VL strain from Belgium (Van Leeuwen et al. 2007). Kinetic analysis and competition experiments showed that the elevated COEs had significantly higher affinity for bifenthrin. In addition, bifenthrin was hydrolyzed 7.2-fold faster in the resistant strain, compared to the susceptible strain (Van Leeuwen and Tirry 2007).

While COE levels were not higher in the Greek ATHRos-Bf and CREVeg-Bf bifenthrin resistant strains (Tsagkarakou et al. 2009), P450 mono-oxygenase activities

determined with the substrate 7-ethoxy-4-trifluoromethylcoumarin were elevated in the two resistant strains, compared to the susceptible strain GSS. However, increased monooxygenase activities were also detected in the MR-VL strain from Belgium (Van Leeuwen et al. 2007). The putative role of P450s in *T. urticae* pyrethroid resistance remains to be resolved, since variable P450 activity levels have been often observed in *T. urticae* populations, which may not necessarily be associated with insecticide resistance traits (T. Van Leeuwen, unpublished).

In summary, the majority of the studies suggest that metabolic resistance mediated by COEs is probably the most common mechanism involved in pyrethroid/bifenthrin resistance of *T. urticae*. The presence of additional detoxification mechanisms has been also indicated. Target site insensitivity was implicated in a few cases, such as in the highly resistant strains from Greece, where changes in detoxification enzymes could not, or only partially, explain resistance.

Target Site Resistance

Target site resistance occurs due to a change in affinity between the insecticide and its binding site on the sodium channel, caused by single or multiple amino acid substitutions. For selection of the mutations to occur, the change must reduce the binding of the insecticide without causing a loss of primary function of the target site. Hence, identical resistance-associated mutations are commonly found across highly diverged taxa and the number of possible amino acid substitutions is very limited (Hemingway and Ranson 2000).

Sodium channel genes have been cloned in several insect species (Soderlund 2005; Davies et al. 2007). The *para* sodium channel of houseflies contains 2108 amino acids, which fold into four hydrophobic repeat domains (I-IV, each containing six subunits) separated by hydrophilic linkers (Soderlund and Knipple 2003) (Fig. 1c). Changes associated with pyrethroid resistance are limited to a small number of regions on this large channel protein, such as the domain II region where five different residues have been implicated to date: Met918 in the IIS4-IIS5 linker, Leu925, Thr929 and Leu932 in IIS5 and Leu1014 in IIS6 (Soderlund and Knipple 2003) (Fig. 1c). The most common mutation is L1014F, originally found in houseflies, but now reported in at least seven additional pest species. This mutation is commonly referred to as the "*kdr*" mutation and is associated with moderate resistance levels (10–30-fold) (Soderlund and Knipple 2003).

Pyrethroid resistance associated mutations have also been reported outside domain II, and are often located in the corresponding S4, S5 and S6 segments of domains I and III. Of these, only two, V410M in IS6 and F1538I in IIIS6 (He et al. 1999; Tan et al. 2005), have been shown to confer insensitivity to pyrethroids in functional assays.

Target site insensitivity to bifenthrin has been very recently analyzed in the ATHRos-Bf and CREVeg-Bf strains from Greece, where combined bioassay and biochemical analysis showed that metabolic detoxification pathways were not major resistance components (Tsagkarakou et al. 2009). A 3.3 kb cDNA fragment of the *T. urticae* para sodium channel gene encompassing domains IIS4 to IVS6,

where the majority of pyrethroid resistance mutations has been found across insect and mite species (Davies et al. 2007), was isolated. The gene was found to possess some distinct characteristics, which possibly explain the occurrence of pharmacologically different voltage gated sodium channels in this mite compared to insects (Tsagkarakou et al. 2009). A number of alternative transcripts were also recorded (Tsagkarakou et al. 2009).

The mutation F1538I in domain III segment 6 (IIIS6) was found to be associated with extremely high bifenthrin resistance levels in both the ATHRos-Bf and CREVeg-Bf strain (Tsagkarakou et al. 2009) (Fig. 1B). The same F-to-I mutation was previously identified in the sodium channel of pyrethroid-resistant southern cattle tick (*Boophilus microplus*) (He et al. 1999). This mutation has been functionally characterized in the cockroach channel expressed in *Xenopus laevis* oocytes and conferred resistance to eight pyrethroid molecules (Tan et al. 2005). It is considered as one of the most highly effective resistance loci for the synthetic pyrethroids found to date (Tan et al. 2005). The F1538 is one of several aromatic residues in the IIIS6 helix that form part of a putative hydrophobic binding site for pyrethroids (O'Reilly et al. 2006; Davies et al. 2008). In this model, a high-affinity site for pyrethroid binding is formed during channel activation, with the bound pyrethroid then acting as a molecular 'wedge' to hold the channel in its open conformation, thereby delaying channel de-activation. F1538 is predicted to interact with the alcohol groups of double-ring cyclic pyrethroids (such as bifenthrin) and so mutations at this residue will destabilise pyrethroid binding to give resistance (O'Reilly et al. 2006; Davies et al. 2008; Tsagkarakou et al. 2009).

Comparison of the cDNA sequences between the resistant ATHRos-Bf and CREVeg-Bf and the susceptible SAMB and GSS *T. urticae* strains revealed one additional substitution consistent with the phenotype mutation: the alanine to aspartic acid substitution at amino acid residue 467 of the *T. urticae* sequence (Tsagkarakou et al. 2009). This residue corresponds to the amino acid D1215 in housefly, which is located in the intracellular linker between domains I and II and has not been previously reported to be associated with resistance in any species. It is not possible to predict whether this substitution (A1215D) is contributing to the resistance phenotype, as the residue lies within the domain II-III intracellular linker, a non conserved protein region among species with unknown role in determining channel structure and function. The possibility that the A1215D mutation has an additive effect in resistance, in line to I/II linker substitutions, which have been shown to strongly synergise the kdr (1014F) resistance mutation (Tan et al. 2005), cannot be excluded. However, it is also likely that this polymorphism is away from the binding site and just fixed in the R allele.

Similar to genetic studies in insects (Hemingway and Ranson 2000), mortality data with bifenthrin in the F1 progeny from crosses between SAMB and CREVeg-Bf indicated that the mode of inheritance of the target insensitivity-based bifenthrin resistance was almost completely recessive, which means the trait will be mainly expressed in homozygous individuals. Therefore, the ability to detect heterozygotes is of paramount importance for the early detection of resistance. The development of molecular diagnostic tests, to discriminate between homozygous-susceptible

resistant and heterozygous individuals with the F to I mutation is a prerequisite for resistance detection and the application of resistance management strategies. This type of monitoring can be based on rapid high-throughput low-cost tech molecular diagnostic assays accessible to laboratories with a limited budget and infrastructure (reviewed in Black and Vontas 2007). By applying these assays to field collected individuals, the gain of useful information on the relative frequencies and spatial distributions of the resistance alleles is envisaged, which can be used to prevent ineffective insecticide applications, and for early identification of the spreading of resistant alleles into new regions.

5.3 METI-Acaricides

In the early 1990s, four new compounds were launched for spider mite control in quick succession: pyridaben, fenpyroximate, tebufenpyrad and fenazaquin (Hirata et al. 1995; Konno et al. 1990; Kyomura et al. 1990; Longhurst et al. 1992). At a later point, pyrimidifen, which was only registered in Japan, was added to the list. Although these compounds belong to different chemical families (quinazolines, pyrimidinamines, pyrazoles and pyridazinones), their mode of action is similar, hence their classification in the group of mitochondrial electron transport inhibitors (METI's) inhibiting complex I of the respiratory chain (Fig. 2)

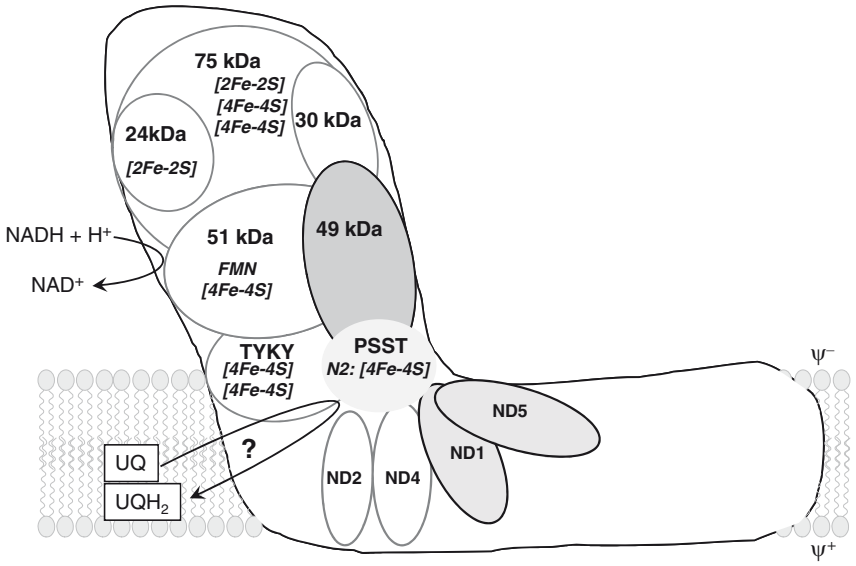

Fig. 2 Complex I structure. (Redrafted from Lümmen 2007)**.** Structural model of complex I, indicating the positions of several subunits possibly involved in the binding of inhibitors (METI-acaricides) as discussed in the text. FMN, flavin mononucleotide; 2Fe-2S, binuclear iron–sulphur cluster; 4Fe-4S, tetranuclear iron–sulphur cluster; N2, iron–sulphur cluster

(Hollingworth and Ahammadsahib 1995; Wood et al. 1996). The four major compounds became very popular and globally used due to their high efficacy, quick knockdown effect and long residual activity. In addition, they are effective on all life stages of spider mites (with the exception of pyridaben, which lacks ovicidal activity) (Bylemans and Meurrens 1997; Stumpf and Nauen 2001). Although their frequent application has resulted in control failure due to resistance, METIs are currently still used in many crops for *T. urticae* control worldwide.

5.3.1 METI-Resistance Reports

METI resistance in *T. urticae* was first reported in field strains from **Korea** (Cho et al. 1995). In Europe, it was first reported in **Belgium** in 1997. Unacceptable field control of *T. urticae* with pyridaben in strawberry cultures was detected as early as 1995, only 2 years after the introduction of this compound to the Belgian market, and cross-resistance to tebufenpyrad and fenpyroximate was already suspected (Bylemans and Meurrens 1997).

Soon after, tebufenpyrad resistance was discovered in Western **Australia** (Herron and Rophail 1998). Control failure occurred in apple orchards after only five applications of tebufenpyrad spread over four seasons. Resistance was confirmed in the laboratory and the RR ranged from 63 for tebufenpyrad to >210 for pyridaben. It was hypothesised that the rapid manifestation of resistance resulted from an undetermined cross-resistance, since clear regional differences were observed in resistance development, possibly linked to the difference in acaricide use between regions.

The Japanese AKITA (AK) strain was collected in 1996 from greenhouses with ornamentals, illustrating early resistance development to METIs in **Japan**. This strain exhibited high resistance levels to pyridaben (RR = 2,000) and fenpyroximate (RR >4,000) (Nauen et al. 2001).

After the approval of tebufenpyrad in 1993, it soon became the most frequently used acaricide in hops in the **UK**. Devine et al. (2001) reported on a resistant strain (TUK4) collected from hop gardens after only a short history of tebufenpyrad use. This strain (RR = 64) showed remarkably high cross-resistance to pyridaben (RR = 346), fenazaquin (RR = 168) and fenpyroximate (RR = 77). In the same study, a second strain (TUK5) was identified from a chrysanthemum nursery exhibiting resistance to tebufenpyrad.

Sato et al. (2004) reported on a fenpyroximate resistant Brazilian strain collected from strawberries. It was the first report on METI-resistance in **South America**. After five laboratory selections, the partially resistant field collected strain exhibited very high resistance (RR = 2,910).

In a large monitoring study in commercial greenhouses and apple orchards located in different regions in **Korea**, Suh et al. (2006) found most *T. urticae* populations moderately resistant to fenpyroximate and pyridaben, while some populations exhibited high resistance. In general RRs were higher in strains originating from commercial greenhouses, indicating that greenhouse populations were more strongly selected.

In a recent report, Van Pottelberge et al. (2008) investigated a field strain collected from bean plants in a greenhouse at the Belgium national botanical garden, exhibiting high resistance to tebufenpyrad, fenpyroximate, pyridaben and fenazaquin.

Some other studies have reported on METI resistance after selection in the laboratory (Kim et al. 2004, 2006).

5.3.2 Mechanisms of Resistance to METIs

Metabolic Resistance

Cross-resistance between METIs has been observed in all strains under study, originating from different regions throughout the world, suggesting a common resistance mechanism. (Stumpf and Nauen 2001; Van Pottelberge et al. 2008). For example, field selection with tebufenpyrad led to high cross-resistance to pyridaben, fenazaquin and fenpyroximate, even when the latter were never used in the field (Devine et al. 2001; Stumpf and Nauen 2001; Van Pottelberge et al. 2008). Laboratory selections with pyridaben and fenpyroximate equally conferred cross-resistance between METIs (Kim et al. 2004, 2006; Sato et al. 2004).

Although METIs belong to different chemical families, all METI compounds contain heterocyclic rings with two nitrogen atoms associated with long hydrophobic tail structures and at least one tertiary butylgroup (Stumpf and Nauen 1989). Consequently, hydroxylation of this tertiary butylgroup could be a common mechanism of oxidative detoxification (Stumpf and Nauen 2001). Hollingworth and Ahammadsahib (1995) also concluded that the main metabolic pathway for pyrazoles (including tebufenpyrad) in spider mites is oxidative degradation, and Motoba et al. (2000) found oxidative degradation products of fenpyroximate in *T. urticae* preparations.

Studies with the synergist PBO and direct measurements of P450 monooxygenase activity in enzyme assays on crude mite preparations have further supported this oxidative detoxification theory. Stumpf and Nauen (2001) found strong synergism of pyridaben toxicity after PBO treatment in the AKITA strain, and although synergism was much lower with other METIs, a strong correlation was found between METI toxicity (LC_{50} values) and P450 activity measured with model substrates. Strong synergism after pre-treatment with PBO, linked to an increase in P450 activity, was also observed in other studies analysing laboratory selected strains (Kim et al. 2004; 2006). However, most studies only revealed a moderate (twofold) increase in P450 activity. Only Van Pottelberge et al. (2008) reported on a METI resistant strain with a remarkable 23-fold increase in P450 activity. Pre-treatment with PBO reduced pyridaben toxicity 96-fold in this study, compared to only six-fold in the susceptible strain, which possibly rules out additional mechanisms like increased penetration for enhanced toxicity in the resistant strain. While the other studies have used 7-ethoxycoumarin and *p*-nitroanisole as model substrates (Stumpf and Nauen 2001; Kim et al. 2004, 2006), Van Pottelberge et al. (2008) quantified O-deethylation activity with 7-ethoxy-4-trifluoromethylcoumarin.

It could be hypothesised that this substrate is more specific for the P450s involved in oxidative breakdown in METI resistant strains. Low cross-resistance to chemically not related compounds with different modes of action have also been reported, including pyrethroids and dicofol (Devine et al. 2001; Kim et al. 2004; Stumpf and Nauen 2001).

Although there seems to be a consensus on the involvement of oxidative metabolism in METI (cross) resistance, there are also arguments indicating the involvement of other mechanisms.

First of all, if a common mechanism is involved, one would expect that the genetics of resistance towards different compounds are similar. Goka (1998) was the first to notice clear differences in the inheritance pattern of METIs in *T. kanzawai*. The inheritance of tebufenpyrad, fenpyroximate and pyridaben resistance was intermediate, incompletely dominant and recessive, respectively. The author concluded that the mechanisms conferring pyridaben resistance were almost certainly different from that conferring tebufenpyrad resistance. An in depth study into the inheritance of METI acaricide resistance by Van Pottelberge et al. (2008) revealed a dominant trait for tebufenpyrad, fenpyroximate and pyridaben resistance. However, backcrosses with susceptible mites showed a different response. While pyridaben and fenpyroximate were inherited as a single major gene, tebufenpyrad resistance was not under monogenic control. In addition, this study revealed clear differences in the effect of synergists on toxicity: pyridaben resistance was highly synergised by both DEF and PBO, while tebufenpyrad resistance was only synergised by PBO and no synergism was detected for fenpyroximate resistance. This lack of synergism in fenpyroximate resistance, together with a clearly monogenic inheritance of the resistance trait, could suggest an altered target site.

The conclusion in several studies on the involvement of metabolic resistance provided by elevated P450 activity was mainly deduced from PBO synergism experiments. However, this may divert the attention from other possible factors which are involved in resistance. A detoxification mechanism that is comparable in a sensitive and a resistant strain can have a much greater impact on the degradation of the compounds if an altered site of action in the resistant strain retards the intoxication. The effect of a synergist that blocks the detoxification will then be much larger, which could lead to the false conclusions that the detoxification is the major resistance mechanism (Oppenoorth 1984, 1985). Several studies clearly indicate that P450s also metabolise METIs in susceptible strains (Kim et al. 2004, 2006; Van Pottelberge et al. 2008).

These examples clearly illustrate the complexity of METI resistance and cross-resistance, and factors other than oxidative metabolism conferring a resistant phenotype cannot be ruled out at this point.

Target-Site Based Resistance

METI acaricides target the proton translocating NADH:ubiquinone oxidoreductase (Complex I, EC 1.6.5.3), one of the most complex structures of all

respiratory chain enzymes, with a total molecular mass of nearly 1,000 kDa (Fig. 2) (Hollingworth and Ahammadsahib 1995; Wood et al. 1996; Lümmen 1998, 2007).

Based on kinetic analysis of membrane-bound complex I activity, two inhibitor classes have been distinguished. The first class, represented by piericidin A, is a competitive inhibitor of ubiquinone while the latter, represented by rotenone, was characterised as a non-competitive inhibitor class (Degli Esposti 1998; Lümmen 2007). However, recent advances in the understanding of the complex and inhibitor binding have suggested that both classes share a common binding domain (Okun et al. 1999; Lümmen 2007).

It is believed that the catalytic core of the quinone reduction site of the enzyme consists of subunits TYKY, PSST, ND1, ND5 and the 49 kDa subunit (Fig. 2) (Schuler and Casida 2001; Lümmen 2007). However, ND5 is also suspected to be involved in proton pumping together with ND4 as indicated by the homology with the Na^+/H^+ antiporter. Interestingly, subunits ND1 and ND5 are mitochondrially encoded and target-site resistance by mutations in these proteins should therefore inherit only maternally.

Crossing experiments performed by Stumpf and Nauen (2001) revealed the presence of a small maternal effect in the inheritance of METI resistance: only the maternal trait was fully dominant, and this effect could be caused by a resistance gene in the mitochondria. To our knowledge, no follow-up report was published on the importance of this effect. Target site resistance caused by mutations encoded by the mitochondrial genome can in theory be easily detected by crossing experiments, but with the exception of Devine et al. (2001), Stumpf and Nausen (2001) and Van Pottelberge et al. (2008), this has been neglected. Also, the mitochondrial genome of *T. urticae* was recently sequenced completely, which allows easy screening for mutations in ND1 and ND5 (Van Leeuwen et al. 2008) (Fig. 3).

Genetic studies using insecticidal complex I inhibitors as selective agents have already resulted in inhibitor resistant complex I mutants in model organisms (Lümmen 2007). A point mutation in the proteobacterium *Rhodobacter capsulatus* homologue of the nuclear encoded 49 kDa subunit revealed the involvement of this subunit in binding (Darrouzet and Dupuis 1997). In addition, site-directed mutagenesis of the nuclear encoded 49 kDa subunit of the yeast *Yarrowia lipolytica* showed decreased inhibitor binding (Kashani-Poor et al. 2001). Mutations in the PSST homologue from *Y. lipolytica* also slightly decreased inhibitor binding (Ahlers et al. 2000). These examples illustrate that single point mutations in catalytic core subunits are likely to seriously affect the binding of METIs to complex I in *T. urticae*, and resulting in target site based resistance. Especially METI resistance in strains that lack any synergism and where resistance is inherited as a single major gene should be examined in depth for the occurrence of target site based resistance (Van Pottelberge et al. 2008). However, quantifying inhibitor binding on complex I preparations from *T. urticae* is not easy (T. Van Leeuwen, unpublished results, 2008), and genomic sequence information on the nuclear encoded complex I subunits is not yet available.

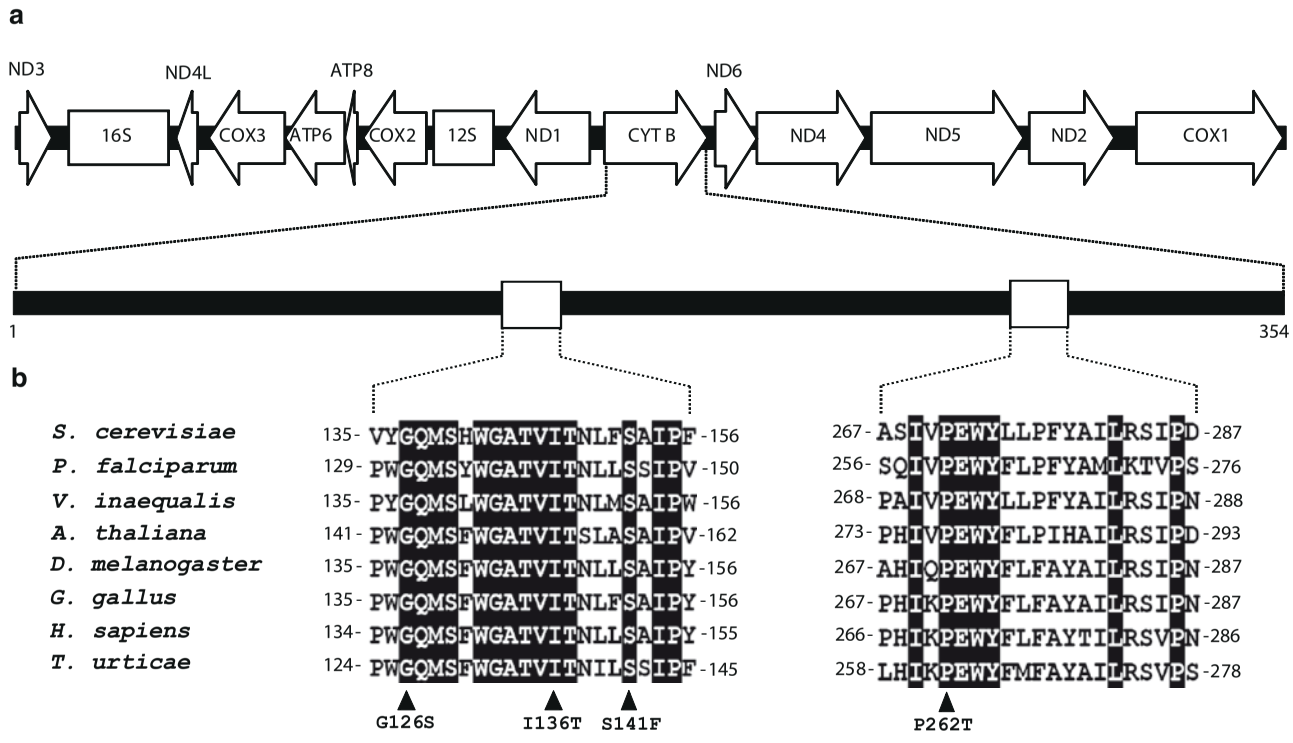

Fig. 3 *Cytb* resistance mutations in the mitochondrial genome (redrafted from Van Leeuwen et al. 2008). **(a)** A linear representation of the mitochondrial genome of *T. urticae* (13103 bp) showing the position and orientation of the protein-encoding genes (n = 13) and the ribosomal RNA genes (n = 2). **(b)** Sequence alignment of conserved Q_o pocket residues positioned on the cytochrome b of *T. urticae* with those of *Saccharomyces cerevisiae* (ABS28693), *Plasmodium falciparum* (NP_059668), *Venturia inaequalis* (AAC03553), *Arabidopsis thaliana* (CAA47966), *Drosophila melanogaster* (CAB91062), *Gallus gallus* (AAO44995) and *Homosapiens* (AAX15094). Fully conserved residues in the alignment are marked in black. Point mutations linked to bifenazate resistance in *T. urticae* are indicated by triangles

5.4 *Bifenazate*

Bifenazate (D2341, N'-(4-methoxy-bipheny-3-yl) hydrazinecarboxylic acid isopropyl ester) is a novel hydrazine carbazate compound, belonging to the recently discovered acaricidal group of hydrazine derivatives. It was discovered in 1990 by Uniroyal Chemical and first commercialised in 1999 by Crompton Corporation (Dekeyser 1996; Grosscurt and Avella 2005; Dekeyser 2005). Bifenazate is nowadays used worldwide for the control of spider mites (*Tetranychus* spp., *Panonychus* spp. and *Oligonychus* spp.) in several crops such as greenhouse ornamentals, apple, pear, citrus and cotton. It is registered for commercial use in Europe, Africa, Asia, Japan, South-America and USA (Dekeyser 1996; Grosscurt and Avella 2005). It provides quick knockdown through contact activity and exhibits long residual control.

From experiments illustrating how organophosphates can antagonise bifenazate toxicity, it has been suggested that bifenazate is a pro-acaricide, which needs conversion to its active component, and ester hydrolysis is considered to be the first step in this process (Van Leeuwen et al. 2006; 2007). It has been reported that the diazene structural analogue of bifenazate is equally toxic to spider mites (Ochiai et al. 2007).

Bifenazate was first thought to be a neurotoxin (Dekeyser 2005), since preliminary studies on the mode of action indicated that, at high concentrations, bifenazate acts on the post-synaptic inhibitory GABA receptor in the insect nervous system (Dekeyser 2005; Grosscurt and Avella 2005). However, this information was never supported by results of mechanistic studies providing proof for this assumption. Therefore IRAC initially listed bifenazate in group 25 of their Mode of Action Classification (www.irac-online.org) as a neuronal inhibitor, but with unknown mode of action. However, recent studies (Van Leeuwen et al. 2006, 2008) have questioned this interference with a neuronal target and have reported on an alternative target site: the Qo-site of the mitochondrial encoded cytochrome b in Complex III of the electron transport chain. In the latest version of the IRAC classification scheme (V. 6.1, August 2008), bifenazate was removed from group 25 and placed in the special group 'un' (compounds of unknown or uncertain mode of action).

5.4.1 Mode of Action and the Discovery of Putative Target-Site Point Mutations

Resistance to bifenazate was first studied in a laboratory selected *T. urticae* strain (Van Leeuwen et al. 2006a). Artificial selection for bifenazate resistance was carried out by successive applications of bifenazate that killed 90% of the population. This selection scheme generated a more than 100,000-fold resistance after 22 selection cycles. Synergism studies and enzyme assays revealed that known detoxification routes were most likely not involved in resistance. Reciprocal crosses between susceptible and resistant mites showed that resistance was inherited only maternally:

offspring of resistant females were strongly resistant but those of susceptible females remained fully susceptible. Such complete uniparental (non-Mendelian) inheritance suggested mitochondrial control as one possible explanation, a hypothesis supported by experiments showing that bifenazate treatment caused a rapid decline in ATP content and formation (Van Leeuwen et al. 2006). To check this hypothesis of mitochondrial control, Van Leeuwen et al. (2007) amplified, sequenced and compared complete mitochondrial genomes of susceptible and resistant *T. urticae* mites, revealing only three nucleotide substitutions between genomes. The detected point mutations resulted in amino acid substitutions in the mitochondrial cytochrome b (cytb) (G134S, S149F and D169G) (Fig. 3). Cytochrome b alignment of translated sequences from the susceptible and laboratory bifenazate resistant strains with those of several model organisms, revealed that mutations were located in the highly conserved Q_o-site of the protein, more precisely in the cd1 helix aligning the enzyme pocket (Fig. 3). These results suggest that bifenazate acts as a Qo inhibitor in spider mites (Esser et al. 2004; Giessler et al. 1994; Lümmen 2007; Van Leeuwen et al. 2008).

Resistance Mutations in Field Collected Bifenazate Resistant Strains

In all the former experiments, a laboratory selected bifenazate resistant strain was used. In order to check whether cytb mutations are also involved in bifenazate resistance in the field, several *T. urticae* strains from commercial rose greenhouses with suspected decreased field efficacy of bifenazate in different regions in the Netherlands were screened for resistance (Van Leeuwen et al. 2008). Two strains were identified containing a G134S mutation, identical to that found in the laboratory selected strain. In one strain this mutation was accompanied by a second point mutation (I144T), also located in the cd1 helix of the Qo pocket. The combination of these mutations gave extreme high resistance to bifenazate in the field (Fig. 3). Interestingly, the strain with only a G134S mutation is still affected by bifenazate, suggesting that a combined effect of at least two mutations in the cd1 helix is necessary to confer high resistance towards bifenazate. This was also observed in the laboratory selected strain (G134S and S149S).

Interestingly, a field strain with a new type of mutation was also found: a P270T substitution in the highly conserved 'PEWY motif' at the ef helix, also aligning the Qo pocket of cytb. This single mutation caused an extremely high resistance to bifenazate (RR ≥ 10,000) (Van Leeuwen et al. 2008) (Fig. 3). Recently, the P270T substitution was also found in a Belgian strain, originating from rose, again leading to a highly resistant phenotype (Van Nieuwenhuyse et al. 2009).

In conclusion, field selection pressure has resulted in similar and identical mutations as those found in the original laboratory selected strain (Van Leeuwen et al. 2006, 2008). To confer high resistance, a combination of mutations was observed in the cd1-helix, while a single mutation in the ef-helix can also result in a highly resistant phenotype.

Heteroplasmy and the Inheritance of Mitochondrial Encoded Resistance

The mode of inheritance of mitochondrial genes such as cytochrome b is essentially non-Mendelian. This has some important consequences, e.g. recombination through meiosis is lacking and transmission of these genes to progeny is predominantly uniparental. In contrast to nuclear DNA, of which there are only two copies per cell, the mitochondrial genome (mtDNA) copy number in somatic cells is usually in the range of 10^3–10^4 per cell (Legros et al. 2004). The emergence of any new mtDNA type (such as the bifenazate resistant haplotype) within a population implies a mutation in one female germ cell, followed by a heteroplasmic stage, bearing both wild-type and mutated mtDNA in a single individual. In this initial stage, the mutated mtDNA molecule is inside a mitochondrion together with thousands of mitochondria with wild-type mtDNA, in the same cell. However, this stage is generally believed to be short, due to rapid sorting out by random drift through a bottleneck in the female germ line or early embryo (Ashley et al. 1989; Jenuth et al. 1996). It is clear that investigation into this heteroplasmic stage is essential for studying the evolution of this new type of resistance.

Van Leeuwen et al. (2008) identified a partially bifenazate resistant strain consisting of heteroplasmic individuals with a C to A nucleotide substitution at position 808 in cytb, resulting in the previously described P270T mutation. Mutation frequency in single mites varied between 30% and 98% and was found to be continuously spread in the sampled populations. The authors subsequently investigated the inheritance of heteroplasmy of single fertilised mothers bearing a different and gradually increasing mutation frequency: 37%, 53%, 68%, 81% and 96%. The mutation frequency varied significantly between mothers and their offspring, especially when the mutation percentage in the founding mother was low, and it was spread around the initial value in the mother. Even at the lowest initial mutation frequency (37%), mites in the progeny were found to almost reach the discriminating level of 60%, which was shown to confer a resistant phenotype when treated with the recommended field dose of bifenazate (Van Leeuwen et al. 2008).

Using the observed variance distributions of the resistant haplotype in the progeny, Van Leeuwen et al. (2008) estimated the magnitude of random processes influencing the segregation and partitioning of mitochondria during reproduction and embryogenesis. Modelling revealed that only a small proportion of the total number of mtDNA molecules likely to occur in mature oocytes were transferred to the next generation, and that this was most likely a random process (Van Leeuwen et al. 2008; Solignac et al. 1984).

It was concluded that a combination of heteroplasmy in early stage, a genetic bottleneck and sustained selection can lead quickly to the emergence of a resistance phenotype, and drive populations rapidly towards fixation of the resistance trait. This is consistent with observations that most bifenazate resistant strains investigated so far have been homoplasmic for mutations conferring bifenazate resistance (Van Leeuwen et al. 2008; T. Van Leeuwen, unpublished).

Complex III as Target-Site and Bifenazate Cross-Resistance

Cytb is part of the ubihydroquinone:cytochrome c oxidoreductase enzyme complex (bc1 complex or complex III) (Fig. 4) which catalyses the electron transfer from reduced ubiquinone to cytochrome c, coupled to the vectorial translocation of protons. In eukaryotes, the catalytic core of the enzyme is formed by cytb, cytochrome c_1 (cyt c_1) and the Rieske iron-sulfur protein (ISP). Only cytb is mitochondrially encoded. The fundamental mechanism of complex III activity is the protonmotive ubiquinone (Q) cycle, requiring two distinct quinone binding sites (Q_o and Q_i) (Lümmen 2007) (Fig. 4).

A number of Q_o-site inhibitors (Q_oI) are long known and are used as pesticides or therapeutic agents in agriculture and medicine. They belong to two chemical classes. The first class, β-methoxy-acrylates (MOAs) were developed from the strobilurins, i.e. antibiotics produced by certain species of Basidiomycetes, into a commercially successful family of fungicides (Gisi et al. 2002). Fluacrypyrim, a recently developed acaricide was the first non-fungicide member developed from

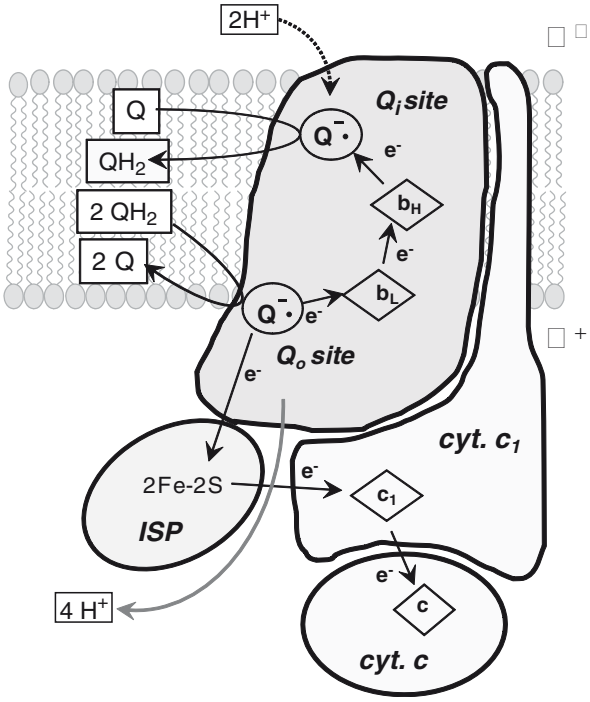

Fig. 4 Complex III structure (redrafted from Lümmen 2007) Schematic presentation of the bc1 complex and protonmotive Q-cycle (redrafted from Lümmen 2007). Q ubiquinone, QH2 ubihydroquinone, b_L low-potential cytochrome b, b_H high potential cytochrome b, ISP iron-sulfur protein (Rieske protein), membrane anchor subunits are omitted. The arrows denote the flow of electrons according to the protonmotive Q cycle. Mutations resulting in bifenazate resistance are located in the Q_o site

this family of compounds, and is registered in Japan (Dekeyser 2005). The second class, the 2-hydroxy-naphthoquinones date back to research from the 1940s and comprises the anti-malaria drug atovaquone (Hyde 2007) and the recently developed acaricide acequinocyl (Kinoshita et al. 1999). They are thought to act as structural analogues of ubiquinone (Lümmen 2007; Dekeyser 2005).

It could be expected that bifenazate resistance mutations confer to some extent cross-resistance to acequinocyl and fluacrypyrim. Van Nieuwenhuyse et al. (2009) screened several field and laboratory strains with known cytb genotype for cross-resistance to fluacrypyrim and acequinocyl. Two strains were identified that showed cross-resistance to acequinocyl. These strains (BR-VL and BEL1) carried the G126S + S141F and P262T mutation. Inheritance of acequinocyl cross-resistance in these strains was clearly maternal, implicating the mitochondrial mutations in resistance. Other field strains bearing the single G126S mutation and the combination G126S + I136T were not cross-resistant to acequinocyl. Although most investigated strains from different origin were resistant to fluacrypyrim, there was no link between resistance and cytb genotype. Inheritance of fluacrypyrim resistance was investigated in BR-VL and BEL1 and proved to be dominant and monogenic. Experiments with synergists and direct measurement of detoxifying enzymes (esterases, P450s and glutathione-S-transferases) have not provided any evidence of enhanced metabolism as a cause of resistance (Van Nieuwenhuyse et al. 2009).

Hydroxynaphthoquinones like acequinocyl are thought to interact with the Q_o site of cytb in a similar way as quinol does. In the current model used to explain interactions of Q_o inhibitors and complex III (Kim et al. 1998; Palsdottir et al. 2003; Esser et al. 2004) the cd1- and ef-helices represent a conformational barrier between the b- and the c1-positions of the ISP. According to this model the quinol as well as the quinoid inhibitors displace cd1 and ef residues to allow the oxidized 2Fe-2S centre to be captured in the b-position. Cross-resistance between bifenazate and acequinocyl hence suggest that the bifenazate active metabolite should act in a similar manner by "broadening" the ISP docking site, thereby locking the ISP protein and preventing its re-oxidation by c1. Binding of quinoid inhibitors also involves hydrogen bridge formation to a His ligand of the ISP (Kessl et al. 2003), but it is not clear if such a functionality is present in bifenazate. However, this does not necessarily contradict the hypothesis, since famoxadone binding and inhibition also does not involve direct ISP interaction, as the compound binds deeper in the Q_o pocket, which makes an H-bridge to the ISP-His highly unlikely (Gao et al. 2002).

The methoxyacrylates like fluacrypyrim have a different binding mode in comparison to hydroxynaphthoquinones, i.e. they do not restrict the movement of the Rieske ISP and its re-oxidation by c1 (Esser et al. 2004). Methoxyacrylates are non-competitive inhibitors with respect to the quinol binding site (Brandt et al. 1991; Lümmen 2007). Bifenazate resistance mutations seem to have no effect on fluacrypyrim toxicity. But since the observed fluacrypyrim resistance in field strains is not yet elucidated, sound conclusions cannot be drawn.

It can be concluded that the observed cross-resistance between acequinocyl and bifenazate is linked to mutations in the cd1-and ef-helices of the mitochondrial cytb. These findings provide further evidence that the active metabolite of bifenazate acts as a Q_o inhibitor. The observed cross-resistance is also of high practical importance,

since acequinocyl is recently registered for use in Europe against spider mites and possible control failure in the field might be linked to prior selection with bifenazate. However, not all bifenazate resistance mutations seem to cause cross-resistance, and it remains to be seen how serious the problem will develop under field conditions.

5.5 Avermectins and Milbemycins

Avermectins (abamectin) and milbemycins (milbemectin) belong to the class of the macrocyclic lactones (IRAC group 6), derived from the soil micro-organisms *Streptomyces avermitilis* and *Streptomyces hygroscopicus*, respectively. They are chemically characterised by the presence of a 16 membered lactone ring. The difference between avermectins and milbemycins is a disaccharide substitution at carbon 13, present in the avermectins but not in milbemycins (Clark et al. 1995; Shoop et al. 1995). Avermectins and milbemycins possess excellent insecticidal, acaricidal and anthelmintic properties. They act on γ-aminobutyric acid (GABA) and glutamate-gated chloride channels, leading to the activation of the chloride ion channel, thereby causing paralysis in the target pest (Fritz et al. 1979; Bloomquist 1993, 2000). Their biochemical mode of action, biological activity and applications in agriculture, have been extensively reviewed in the past (Clark et al. 1995; Jansson and Dybas 1997; Bloomquist 2000). Abamectin and milbemectin are currently still frequently used to control spider mites in many crops, but reports on resistance are scarce.

5.5.1 Abamectin Resistance Reports

Campos et al. (1995) first reported a decreased activity of abamectin in a monitoring study in ornamental nurseries in **California**. Resistance ratios at LC_{95} level ranged from 1 to 658. Resistance was clearly correlated with the number of applications per year and the total time (number of years) of abamectin use. Although resistance was detected in the laboratory using a leaf residual assay, no field failures had occurred in these nurseries. In a second study, abamectin susceptibility was investigated in strains originating from nurseries in **Florida**, **the Netherlands** and **the Canary Islands** (Campos et al. 1996). Although decreased abamectin susceptibility was again positively correlated with the frequency of treatments in the field, the authors concluded that resistance development differed between regions, and the highest resistance levels were detected in populations originating from rose nurseries in The Netherlands. Beers et al. (1998) reported on decreased susceptibility in *T. urticae* populations collected from pear orchards in **Washington**. Stumpf and Nauen (2002) screened populations from different host plants and geographical regions (Germany, Australia, Japan, the Netherlands, France, Greece, Brazil, Columbia and South Africa) and identified two resistant populations originating from rose cultures in **the Netherlands** and **Columbia**. Sato et al. (2005) report on decreased efficacy and shortened residual control of abamectin used in strawberry and ornamental plants.

5.5.2 Abamectin Resistance Mechanism

Only a few authors have studied how spider mites develop resistance to abamectin. Clark et al. (1995) used [^{3}H]avermectin B_{1a} to investigate the role of pharmacokinetics in abamectin resistance. Resistant mites excreted more [^{3}H]avermectin B_{1a} after ingestion than their susceptible counterparts. In addition, metabolism studies showed that susceptible mites retained a considerable higher total percentage of the unaltered parent compound. The authors hypothesized that abamectin resistance could result from an increase in excretion and decrease in absorption, combined with high metabolism or conjugation of the compound. The effect of synergists on abamectin resistance was determined in a subsequent study, using the same resistant populations (Campos et al. 1996). Relatively low synergistic ratios were observed for PBO (between 0.7 and 2.7) and although abamectin could be synergised 7.9-fold by DEF in one population, inconsistent patterns were observed for all strains, so no clear conclusions could be drawn.

Abamectin resistance in a Dutch strain studied by Stumpf and Nauen (2002) was strongly synergised by PBO (4.4-fold) and DEM (6.1-fold). The involvement of metabolic detoxification as a major mechanism in the abamectin resistant strains was also inferred from direct measurement of the enzymatic activities of P450s and GSTs, revealing a 13- and 11-fold increase respectively. Similar values were detected in an abamectin resistant strain from Columbia. The authors finally concluded that abamectin resistance is probably polyfactorial, involving both oxidative metabolism and increased conjugation to glutathione. Higher oxidative breakdown, followed by conjugation to glutathione could lead to an increased excretion, which was also detected by Clark et al. (1995).

Cross-resistance between milbemectin and abamectin has been observed by Sato et al. (2005) and might be linked to a common detoxification route. However, in contrast to the oxidative pathway in abamectin proposed by Stumpf and Nauen (2002), resistance to milbemycins has been associated with increased esterase metabolism in a study by Yamamoto and Nishida (1981).

Insensitivity of the GABA or glutamate-gated chloride channels, the target-site of avermectins and milbemycins, has not been reported in spider mites, although some studies on insects have suggested a decrease in abamectin binding sites as a possible resistance mechanism (Clark et al. 1995; Scott 1995; Blackhall et al. 1998).

5.6 Tetronic Acid Derivatives

Spirodiclofen is a new selective, non systemic acaricide belonging to the chemical group of the spirocyclic tetronic acid derivatives. It is commercialised for the control of economically important phytophagous mite species including *Tetranychus*, *Panonychus*, *Brevipalpus, Phyllocoptruta* and *Aculus* (Rauch and Nauen 2002). It acts on mite development and is active against all developmental stages, including eggs, and additionally strongly affects the fecundity and fertility of female adults

after tarsal contact (Wachendorff et al. 2002; Nauen 2005). It was observed that the lipid content in treated female adults of *T. urticae* was significantly decreased, suggesting an interference with lipid biosynthesis, through selective and potent inhibition of acetyl-CoA carboxylase (ACCase) (Bretschneider et al. 2007). It was classified in IRAC group 23, together with spiromesifen and spirotetramat, showing the same mode of action. Spirodiclofen is registered worldwide for use on a variety of crops including citrus, pome fruits, stone fruits, grapes and ornamentals.

5.6.1 Resistance to Spirodiclofen

Since the compound has been introduced only recently, there are no reports available on field resistance against spirodiclofen. To assess the risk of spirodiclofen resistance development in *T. urticae*, Rauch and Nauen (2002) selected a laboratory strain by successively spraying *T. urticae* infested French bean plants with increasing concentrations of spirodiclofen. After 21 months, the selected strain proved 13-fold less susceptible compared to the initial susceptible strain. Synergists inhibiting P450s and esterases could significantly synergize spirodiclofen toxicity in the resistant strain. In addition, measurements of enzyme activities revealed a twofold increase in P450 mono-oxygenase activity. No significant differences were observed in esterase activities with the common model substrate 1-naphtyl acetate. However, with a specially designed 1-naphthyl derivative mimicking the acylated side chain of spirodiclofen (1-naphthyl 2,2-dimethylbutyrate), significant differences were found between strains. These results suggest an increased metabolism as a cause for decreased spirodiclofen toxicity, which could be further confirmed by metabolism studies with [^{14}C]spirodiclofen (Rauch and Nauen 2002). Analysis of *in vivo* formed metabolites revealed that metabolism was more rapidly in the laboratory selected resistant strain, suggesting a faster hydrolytic and oxidative degradation of spirodiclofen. Due to the low resistance ratios obtained after prolonged selection, the authors concluded that spirodiclofen has a low tendency to select for resistance in *T. urticae*. However, in a study by Van Pottelberge et al. (2009a, b), a similar selection experiment yielded a much more resistant strain (274-fold), exhibiting an LC_{50} value of more than 1,000 mg a.i./l determined on larvae, which is far above the registered field dose of spirodiclofen. High synergism ratios were found with PBO (3.5) and DEF (3.3) and a direct measurement of P450 monooxygenase activity on larvae and adults revealed a 6- and 11-fold increase respectively. In an experiment determining the stability of resistance, the monthly determined toxicity and P450 activities were correlated. The same spirodiclofen specific 1-naphthyl derivative (1-naphthyl 2,2-dimethylbutyrate) as used by Rauch and Nauen (2002), revealed a twofold increase in esterase activity. The spirodiclofen resistant strain showed moderate cross-resistance to spiromesifen (18-fold). Since resistance ratios were remarkably lower when spirodiclofen toxicity was determined on eggs, an altered target site is most likely not the reason for the observed cross-resistance. Giving the data on synergists and detoxifying enzyme activities, it seems more likely that the chemicals share a common detoxification route.

This route is obviously less active in eggs. Van Pottelberge et al. (2009a) extensively studied the genetics of spirodiclofen resistance and found evidence that more than one gene is involved in resistance. This supports the hypothesis of higher metabolism in resistant strains, as concluded by both studies.

5.7 Miscellaneous Compounds

Organotin compounds (cyhexatin,azocyclotin, fenbutatin oxide) have been used for almost 40 years around the world. Fenbutatin oxide is still an important acaricide in IPM programs due to its selectivity towards predatory mites (Tian et al. 1992; Jacobson et al. 1999; Van Leeuwen et al. 2004). Although resistance against organotin compounds has been reported in Australia (Edge and James 1982, Herron et al. 1997), the USA (Croft et al. 1984) and Europe (Van Leeuwen et al. 2004), the number of cases is very limited. Cross-resistance between organotins has been observed, but not in all cases (Edge and James 1986). Organotin resistance is usually unstable in the field (Flexner et al. 1988). In a study on the mechanisms of cyhexatin resistance, Carbonaro et al. (1986) found no differences in penetration and degradation between a susceptible and resistant strain. However, oligomycin-sensitive magnesium ATPase activity in the resistant strain was 6.5-fold less sensitive to inhibition by cyhexatin than in the susceptible strain. This suggested that a modified target site was involved.

Resistance to **chlorfenapyr,** an N-substituted halogenated pyrrole working as a mitochondrial uncoupler (Black et al. 1994) was simultaneously reported in a nectarine orchard in Australia and in eggplant farms in Japan (Herron and Rophail 2003; Uesugi et al. 2002), and later occurred in cotton (Herron et al. 2004). The biochemical and genetic mechanisms of resistance have been extensively studied by Van Leeuwen et al. (2004, 2006b). In this study, resistance is correlated with two esterase isozymes and altered activities of general oxidases and P450s.

Resistance to **dicofol** was found in New Zealand, USA, Japan and Europe (Baker 1985; Fergusson-Kolmes et al. 1991; Martinson et al. 1991; Dennehy and Granett 1984; Kim et al. 1994; Van Leeuwen et al. 2005). Resistance was reported to be due to increased degradation to unidentified water-soluble metabolites and was accompanied with an increased conversion of chorpyrifos to its oxon analogue, suggesting that increased metabolism was the likely resistance mechanism (Fergusson-Kolmes et al. 1991). Resistance to dicofol was incompletely dominant under field conditions, in contrast to its recessive inheritance in laboratory assays (Martinson et al. 1991).

Mite growth inhibitors are a chemically diverse class comprising of the compounds **clofentezine**, **hexythiazox** and **etoxazole**. Clofentezine resistance has been detected in Europe and Australia (Van Leeuwen et al. 2005; Herron 1994; Nauen et al. 2001). Hexythiazox resistance proved to be dominant and conferred by a single gene in Australian populations (Herron et al. 1993). The genetic basis for resistance to etoxazole has been studied in Japanese strains (Uesugi et al. 2002; Kobayashi et al.

2001) and was completely recessive and conferred by a single major gene. Resistance to **propargite** is not well documented. Keena and Granett (1990) reported that propargite resistance is inherited intermediately and is probably under the control of more than one gene.

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