A. Rami Horowitz · Isaac Ishaaya *Editors*

Advances in Insect Control and Resistance Management

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Preface

This book covers advanced concepts and creative ideas with regard to insect biorational control and insecticide resistance management. Some chapters present and summarize general strategies or tactics for managing insect pests such as the principles of IPM in various crop systems and biorational control of insect pests, advances in organic farming, and alternative strategies for controlling orchard and field-crop pests. Other chapters cover alternative methods for controlling pests such as disruption of insect reproductive systems, utilization of semiochemicals and diatomaceous earth formulations, and developing bioacoustic methods for mating disruption. Another part is devoted to insecticide resistance: mechanisms and novel approaches for managing insect resistance in agriculture and in public health.

The authors of the various chapters have a wealth of experience and are considered world leaders specializing in novel approaches of insect pest control.

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Chapter 1
Advances in Insect Control and Resistance Management: An Overview

Isaac Ishaaya and A. Rami Horowitz

Abstract The present book covers different approaches regarding advances in insect control and in resistance management: some chapters present and summarize general strategies or tactics for managing insect pests, while others cover alternative and nonchemical methods for controlling pests. Another part is devoted to different aspects of insecticide resistance: mechanisms and novel approaches for managing insect resistance in agriculture and in public health.

1.1 Introduction

Various pest control methods have been used during the past five decades, in which the common synthetic pesticides have been mostly exploited. Significant progress in the synthesis of new chemicals has resulted in the discovery of new structures with novel biological activities reducing thereby losses in agricultural yield. However, the severe adverse effects of these pesticides on the environment along with problems of insecticide resistance reach crisis proportions of public health and agricultural pests. In addition, public protests led to stricter regulations and legislations aimed at reducing the use of pesticides. Since the late century, an implementation of integrated pest management (IPM) principles has resulted in the development of novel insecticides with selective properties acting on biochemical sites or on physiological processes present in a specific insect group but differ in their properties from those present in the mammalian system (Casida and Quistad 1998). This process

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has led to the formation of insecticides which affect the hormonal regulation of molting and developmental processes and exploiting new insects' sites such as the insect growth regulators (IGRs), neonicotinoids, and diamides. The second advance is the utilization of alternative methods such as biological and cultural controls using pheromone and biopesticides (Horowitz and Ishaaya 2004a, b; Rosell et al. 2008) along with substantial advancement in genetically modified crops (James 2014). The nowadays advanced technologies have been focused on safer and on environmentally friendly (biorational) approaches.

The current book is updating our previous ones relating to biochemical sites of insecticide action and resistance (Ishaaya 2001), insect pest management in field and protected crops (Horowitz and Ishaaya 2004a, insecticide design using advanced technologies (Ishaaya et al. 2007), biorational control of arthropod pests, application and resistance management (Ishaaya and Horowitz 2009), and advanced technologies for managing insect pests (Ishaaya et al. 2012).

The present book covers different approaches regarding advances in insect control and in resistance management: some chapters present and summarize general strategies or tactics for managing insect pests (Chaps. 2, 3, 4, 5, and 6), while others cover alternative and nonchemical methods for controlling pests (Chaps. 7, 8, and 9). Another part is devoted to different aspects of insecticide resistance: mechanisms and novel approaches for managing insect resistance in agriculture and in public health (Chaps. 10, 11, 12, 13, 14, 15, and 16).

1.2 Short Descriptions of the Book Chapters

According to Gross and Gundermann (Chap. 2), the concept of IPM is based on an ecosystem approach that combines different management strategies. IPM emphasizes the growth of healthy crops with the least possible disruption to the agroecosystem. The concept is based on the use of integrated plant protection guidelines (Hommel et al. 2014). In Denmark, an extensive monitoring system is operating and has an impact in reducing pesticide use (Kudsk and Jensen 2014). In Germany, training details and education topics have to be specified by region and crops (Frier and Zornbach 2008). Pesticides applied on crops should be specific for the target pests and have the least side effect on human health, on nontarget organisms, and on the environment. Integrating several alternative methods could generate synergies in pest control (Barzman et al. 2014). Anti-resistance strategies, using multiple pesticides with different modes of action, should be applied to maintain the effectiveness of the products (Barzman et al. 2014). Various approaches should be taken to reduce the use of pesticides (Welter et al. 2005; Fadamiro and Baker 2002; Millar et al. 2002).

Bloom and Crowder (Chap. 3) report on the importance of natural enemies and pollinators in organic farming. In general, insects perform important functions such

as organic decomposition, biological control, and pollination (Losey and Vaughan 2006). Biological control is a central tenet of organic farming aiming at controlling insect pests while reducing pesticide input, allowing thereby pollinator activity (Paoletti 1999; Gabriel et al. 2013).

Nestel et al. (Chap. 4) attempted to highlight the complexity of novel approaches in controlling the olive fly in southern Europe. They indicate the complexity involved in the management of the agroecosystem and the effect of the global and local processes involved in plant protection activities. They demonstrate the need for a holistic approach for controlling this important pest. The sterile insect technique (SIT) based on the release of sterile males which can compete with their wild counterparts for inseminating mature females (Robinson 2005) along with the use of mass-trapping methodologies has been suggested (Tabic et al. 2011; Yokoyama 2014).

Wise (Chap. 5) suggests an alternative delivery system in tree fruits using trunk-injected insecticides. This system shows that vascular delivery is predominantly accumulated in foliage with fruit residues below US-EPA limits. Field and laboratory studies demonstrate the effectiveness of this system against insect pests, suggesting that it is a promising delivery system for tree fruit IPM and resistance management (Wise et al. 2014).

Manipulation of the insect reproductive system as a tool in pest control was thoroughly demonstrated by Harari et al. (Chap. 6). Attract-and-kill by using sex pheromone along with a suitable control agent result in a male annihilation (Brockerhoff and Suckling 1999; El-Sayed et al. 2009). Mating disruption strategy has led to a decrease in pesticide use and increase in natural enemies (Gordon et al. 2005; Harari et al. 2007; Ioriatti et al. 2011). Endosymbionts are intercellular bacteria infecting arthropods and nematodes with the ability to modify their reproductive potential. Hence, some of them can be utilized as environmentally friendly tools in pest management either to reduce fitness of pests or to enhance fitness of natural enemies (Werren 1997).

Pérez-Hedo and Urbaneja (Chap. 7) demonstrate the use of mirid species as predators for controlling tomato pests in which *Nesidiocoris tenuis* is the most predominant. This predator is able to feed on different pest species (Urbaneja et al. 2012) such as thrips, whiteflies, leaf miners, leaf hoppers, aphids, spider mites, and lepidopteran pests (Urbaneja and Jacas 2008). Such an approach reduces the use of pesticides in tomato crops (Stansly et al. 2004). On the other hand, *N. tenuis* is considered as a pest of tomato due to its feeding behavior (Arno et al. 2010). The impact of fruit abortion on the yield in tomato crops could be compensated by an increase in the weight of the remaining fruit (Sanchez and Lacasa 2008). Bed bug (*Cimex lectularius*) infestations have increased dramatically in various parts of the world (Doggett et al. 2004; Potter et al. 2006; Harlan 2006).

Akhtar and Isman (Chap. 8) demonstrate a novel approach for controlling this pest, using IPM program which includes prevention, vacuuming, traps, heat and steam, fumigation, and the use of reduced risk pesticides such as silica gel, diatomaceous earth, neem, essential oil, and microbial-based products.

Polajnar et al. (Chap. 9) present an overview of an ongoing effort to develop a vibration-based method for mating disruption of the grapevine pest *Scaphoideus titanus*. Males are the active partners, usually searching for the females for initiating courtship. Reproductive interference by signal jamming between males and females suppresses efficiently development of a new generation of the pest (Polajnar et al. 2015). Such an approach could be used for controlling insect pests in other crops as well.

Swever and Smagghe (Chap. 10) report on an in vitro assay based on the use of EcR reporter system for acting as ecdysone receptor agonist or antagonist for developing novel insecticides. Such an assay could serve as a base for developing novel selective insecticides for controlling diversity of insect pests. There is a global need for developing new insecticides with novel modes of action along with nonchemical approaches, such as those based on genetically modified crops (Vontas et al. 2014).

The sweet potato whitefly *Bemisia tabaci,* the western flower thrips *Frankliniella occidentalis* and the onion (tobacco) thrips *Thrips tabaci* are major agricultural pests. Kliot et al. (Chap. 11) report on a resistance monitoring program for those pests that were found to be resistant to both spinosad and neonicotinoids. Management of *B. tabaci* relies heavily on chemical insecticides (Horowitz et al. 2005). The Q biotype was first recorded in Israel about 15 years ago (Horowitz et al. 2003) and since then has caused heavy losses. The western flower thrips was identified in Israel in 1987, and it considered a serious pest in major agricultural crops (Chyzik and Ucko 2002). The authors suggest that integration of novel biorational insecticides and biopesticides as a part of insecticide resistance management (IRM) strategy, along with long-term resistance monitoring programs for these pests, will contribute to the sustainability of these strategies.

Diamides, a new class of insecticides (Chap. 12), are derivatives of phthalic acid (flubendiamide) and anthranilic acid (chlorantraniliprole and cyantraniliprole). They activate the ryanodine receptor causing calcium depletion. Both insecticide types have been used for controlling a diversity of lepidopteran pests such as *Helicoverpa zea*, *Spodoptera exigua*, *Manduca sexta*, *Trichoplusia ni*, *and Chrysodeixis includens*. In addition to the activity against lepidopteran pests, chlorantraniliprole is very effective on some coleopteran, dipteran, isopteran, and hemipteran pests, exhibiting systemic and translaminar activity (Sattelle et al. 2008; Lahm et al. 2009). Reports of diamide resistance in the diamondback moth *Plutella xylostella* resulted in field control failures. In addition, recent reports have shown moderate to high resistance in other lepidopteran pest species, such as *Tuta absoluta* and *Spodoptera exigua*. This chapter discussed in detail resistance mechanisms, relating to metabolic and target sites, employed by the insect pests.

Diversity of resistance mechanisms in *Helicoverpa armigera* are described by Joussen and Heckel (Chap. 13) ranging from behavioral to physiological adaptation. A reduced penetration through the cuticle minimizes mutation of the target site which could occur from the use of pyrethroids, organochlorines, and oxadiazines. Both carboxylesterases (Wheelock et al. 2005) and cytochrome P_{450} monooxygenases (Feyereisen 2006) play an important role in insecticide resistance.

The chimeric P450 *CYP337B3* is an important gene that is present in the resistant and absent in the susceptible individuals. The corresponding enzyme metabolizes different pyrethroid insecticides and consequently confers resistance in *H. armigera* larvae. The authors deal with a new resistance mechanism by recombination that probably plays an important role in *H. armigera* populations.

Carrière et al. (Chap. 14) recommend integrating Bt crops with other management tactics to delay resistance to Bt toxins. Across the years and environments, yields were less stable from non-Bt corn than from Bt corn producing one or more toxins (Edgerton et al. 2012; Shi et al. 2013). Yield of Bt cotton and Bt corn is relatively higher in developed countries as compared to the developing ones because of less effective pest management in the developing countries (Carpenter 2010; Klümper and Quaim 2014). In a recent analysis, 12 of the 27 cases examined showed no significant increase in insect resistance after 2–15 years of exposure to Bt crops (Tabashnik and Carriere 2015). Of the remaining 15 cases, different levels of resistance were detected. The authors suggest diversity of procedures to delay resistance of various insect pests to Bt crops, e.g., effective refuge of at least 20 % even when pyramids, seed mixtures, or both are used along with other management tactics.

Paine and Brooke (Chap. 15) discuss the impact of insecticide resistance on vector control, mostly malaria vectors, and they report the recent developments in vector resistance research. Mosquito control is based mainly on indoor residual spraying and on treatments of bednets, especially with pyrethroids. As there are just a few effective insecticides for controlling the malaria mosquitoes and the necessity of pest and insecticide resistance managements against these pests, methods for identifying and characterizing insecticide resistance have been developed and applied. In addition, alternative methods have been examined to reduce incidence of malaria along with other vector-borne diseases.

Among the alternative methods is the SIT based on the use of laboratory mass-reared sterile males which are released into the natural environment of a target population. Sterile males disrupt progeny formation of the target population (Alphey et al. 2008; Dame et al. 2009). This technique is used successfully to eradicate the screwworm fly *Cochliomyia hominivorax* in the USA and Central America and the malaria control vector *Anopheles arabiensis* in northern Sudan and in South Africa (Klassen 2009; Munhenga et al. 2011).

Bielza (Chap. 16) reports on the importance of natural enemies' tolerance to pesticides which can improve the design of robust IPM strategies. In general, natural enemies possess inferior detoxification mechanism, suffer from food shortage, and are hence less likely to develop similar resistance as the insect pests (Tabashnik and Johnson 1999). Insecticide resistance among arthropod natural enemies received much attention in the 1970s and the 1980s (Croft and Morse 1979; Tabashnik and Johnson 1999) due to the effort made for the wide adoption of the IPM concept. High resistance to pyrethroids and organophosphates were detected in population of *Typhlodromus pyri* and *Amblyseius andersoni* in grape crops in France (Bonafos et al. 2007). These resistant populations played a crucial role in controlling tetranychid mites in commercial grape production (Bonafos et al. 2007). Lady

beetles tend to be more tolerant to insecticides than other aphidophagous insects (Hodek 2014). Resistance of natural enemies can be increased through artificial selection. Their use can prevent outbreak of pest resurgence (Mansoor et al. 2013).

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Chapter 2
Principles of IPM in Cultivated Crops and Implementation of Innovative Strategies for Sustainable Plant Protection

Jürgen Gross and Gerhard Gündermann

Abstract In this chapter, the concepts of integrated pest management (IPM) and integrated production (IP) are explained, and the most important definitions are given. The legal framework for regulation of IPM in the European Union is specified, and the general principles are explained. The EU Framework Directive requires that all EU member states develop a national action plan (NAP), which ensures that a set of eight general principles of IPM are implemented by all professional pesticide users. Along these principles, the authors present an overview on important examples for new and innovative developments and attempts in plant protection to enhance sustainable agriculture. They give short introductions in selective and biorational pesticides, anti-resistance strategies, and new methods for monitoring pest insects by semiochemicals. Furthermore, they give an overview on the diversity of nonchemical methods in pest control. These methods include mating disruption techniques mediated by semiochemicals and substrate vibrations, mass trapping, attract-and-kill techniques, the use of repellents, antifeedants and deterrents, as well as more complex push-and-pull strategies.

2.1 Introduction

The concept of integrated pest management (IPM) is an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides. Pests in cultivated crops include pest animals (including arthropods, mollusks, nematodes,

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and vertebrates), plant pathogens (viruses, bacteria including phytoplasmas and *Liberibacter*, fungi), and weeds. IPM aims to suppress pest populations below a specific economic injury level (EIL). The EIL is defined as "The lowest population density of a pest that will cause economic damage; or the amount of pest injury which will justify the cost of control" (Stern et al. 1959). Regarding IPM, currently 67 different definitions are available in the worldwide literature. The Food and Agriculture Organization of the United Nations (FAO), e.g., defines IPM as follows: "Integrated Pest Management means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO 2015). The International Organization for Biological and Integrated Control-West Palearctic Regional Section (IOBC-WPRS) defines further the concept of integrated production (IP). It is a concept of sustainable agriculture developed in 1976 which has gained international recognition and application. The concept is based on the use of natural resources and regulating mechanisms to replace potentially polluting inputs. The agronomic preventive measures and biological/ physical/chemical methods are carefully selected and balanced taking into account the protection of the environment and the health of farmers and consumers (Boller et al. 2004). The 2004 IOBC Standard for Integrated Production covers ecological, ethical, and social aspects of agricultural production as well as aspects of food quality and safety. The current set of IP guidelines and related tools has proven helpful and inspirational for farmers' organizations looking for a feasible way to work with integrated production in the premium food segment (Boller et al. 2004).

2.2 Regulation of IPM in Europe

IPM in Europe is regulated in Directive 2009/128/EC. A definition similar to the definition published by the FAO is provided by the European Union Framework Directive on the sustainable use of pesticides in Art. 3 No. 6: "Integrated pest management" means careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment. "Integrated pest management" emphasizes the growth of a healthy crop with the least possible disruption to agroecosystems and encourages natural pest control mechanisms" (Directive 2009/128/EC). The general principles are published in Annex III of Directive 2009/128/EC. Establishing incentives for professionals to implement guidelines for IPM can be found in Art. 14 No. 5 and the obligation of

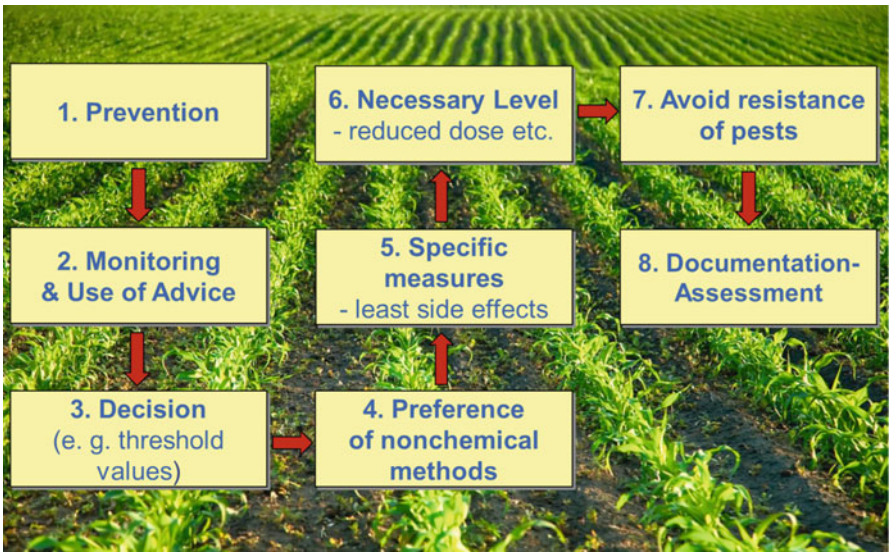

Fig. 2.1 The set of eight general principles of IPM

EU-ME to report how to ensure the general principles of IPM in Art. 14 No. 4: Member states shall describe in their national action plans (NAP) how they ensure that the general principles of integrated pest management as set out in Annex III are implemented by all professional users by 1 January 2014. Thus, all 27 member states of the European Union are to transpose this directive into national legislation.

The EU Framework Directive requires that all EU member states develop an NAP, which ensures that a set of eight general principles (Fig. 2.1) of IPM (Annex III) are implemented by all professional pesticide users compulsory from 1 January 2014 (European Union 2009):

1. Prevention and suppression of harmful organisms
2. Monitoring, warning, forecasting, and the use of advice
3. Decision-making (e.g., threshold values)
4. Preference of nonchemical methods
5. Pesticide selection (least side effects)
6. Necessary level (reduced pesticide use)
7. Anti-resistance strategy
8. Documentation and assessment

Member states shall establish appropriate incentives to encourage professional users to implement crop- or sector-specific guidelines for integrated pest management on a voluntary basis. Public authorities and/or organizations representing particular professional users may draw up such guidelines. Member states shall refer to those guidelines that they consider relevant and appropriate in their NAPs (Directive 2009/128/EC, Art 14 No. 5).

In Germany, guidelines (cultural or sector-related production) are developed through associations of farmers, gardeners, and associations with scientific support under a strong consideration of practical relevance. The Integrated Production Commission of the IOBC-WPRS, e.g., elaborates crop-specific guidelines for integrated production of agricultural crops that have formed the basis for IPM programs in Switzerland, the Czech Republic, and Italy. Further, it develops and standardizes methods of testing effects of pesticides on beneficial organisms. In the fulfillment of its objectives, it collaborates with other international organizations, notably FAO, WHO, the Commission of the European Union (CEU), and the European Plant Protection Organization (EPPO). The IOBC-WPRS has already published crop-specific IP guidelines for a large number of crops (pome fruits, stone fruits, arable crops, grapes, soft fruits (berries), olives, citrus, and field grown vegetables). Further guidelines have to be developed for sugar beet (Gummert et al. 2012), wine growing, urban greening, etc. Up to 2018, guidelines should be available in every plant production system including minor crops (mostly vegetables, fruits, nursery stock, and ornamentals). At present, around 30 % of farmers observe guidelines 3 years after their establishment.

The guidelines must include the following topics:

1. Prevention: crop rotation, cultivation (non-plow tillage), resistance, etc.
2. Observation: the use of learned lessons
3. Decision: check of pest development and thresholds
4. Preference of nonchemical methods: biotechnical, physical, and technical methods, biologicals, etc.
5. Pesticide: specific, environmental, reduced dose, and necessary level
6. Documentation and control of results

For minor crops, sustainability can only be realized by the continued availability of crop protection solutions for pest control. Since the last decade, European farmers have far less new technology to drive agricultural production than their competitors in other regions of the world. In Europe, particularly the number of minor crops without viable solutions for plant protection has increased (Lamichhane et al. 2015). This is mainly because many effective compounds once registered on an EU level have not been reauthorized due to stricter regulation processes. The limited range of available pesticides has increased the risk of pesticide resistance development since, in the absence of several pesticides with various modes of action, farmers must apply only a narrow spectrum of active ingredients (Lamichhane et al. 2015). Research and development (R&D) for new crop protection products needed by European farmers is in decline, according to an analysis of market trends in the EU and around the world. A recent study reveals that the number of active ingredients being developed and introduced in the EU is steadily decreasing – even as global expenditure on agricultural R&D is on the rise (Anonymous 2013). The share of global crop protection R&D focused on European markets has decreased from 33 % in the 1980s to only 16 % today. Moreover, the European market's share of total worldwide R&D expenditure for new product development in agricultural life sciences is just 7.7 %

today compared to 33 % in the 1980s (Anonymous 2013). The most important reasons behind the reduction in R&D investment in crop protection products for the European market are the nonacceptance of GMOs and the harsh regulatory environment. Thus, the future development and implementation of innovative IPM solutions are very important.

2.3 Demonstration Farms for IPM in Germany

In the year 2007, the network of reference farms, called "Demonstration Farms for Integrated Plant Protection" (DF-IPP), an important component of the NAP in Germany, has been started. It is a joint project of the Federal Ministry of Food and Agriculture of Germany (BMEL), the plant protection services in the federal states and the Julius Kühn-Institut, Federal Research Centre for Cultivated Plants (JKI). The network focuses on surveying representative farms to demonstrate the feasibility of IPM in representative regions and crops and to obtain annual data on plant protection product (PPP) uses in major crops and to generate additional information relevant to crop protection. All PPP treatments are evaluated to determine their actual use intensities based on the so-called treatment frequency index (TFI) and the necessary minimum of PPP use, as determined by experts from the plant protection services (Hommel et al. 2014). Various checklists were developed to evaluate the implementation of IPM on demonstration farms (Peters et al. 2015). The following parameters are used to explain differences in TFI scores between farms, within or between regions: field and farm size, soil quality, previous crop, tillage, sowing date, cultivar resistance, and the use of decision support systems (Hommel et al. 2014). The data are pooled for four selected regions (north, east, south, and west) and for Germany in total. In arable farming, e.g., about 75 farms are surveyed annually. Integrated plant protection (IPP) guidelines are to be applied in the model and demonstration project DF-IPP (Hommel et al. 2014). Thus, new developments and innovative strategies for sustainable plant protection can easily be implemented in practice and according to a circle of measurements and assessments (Fig. 2.1) under practical conditions evaluated (Gündermann 2014). The outcome of the IPM measures and assessments will be communicated to farmers and public (Fig. 2.2).

2.4 Certification of Plant Protection Knowledge

Professional users of PPPs must be certified (plant protection certification) to use plant protection products. Undergoing training in handling and using plant protection products, related to the certification, is voluntary in all member states. The state certificate includes a written and oral exam or an approved associated

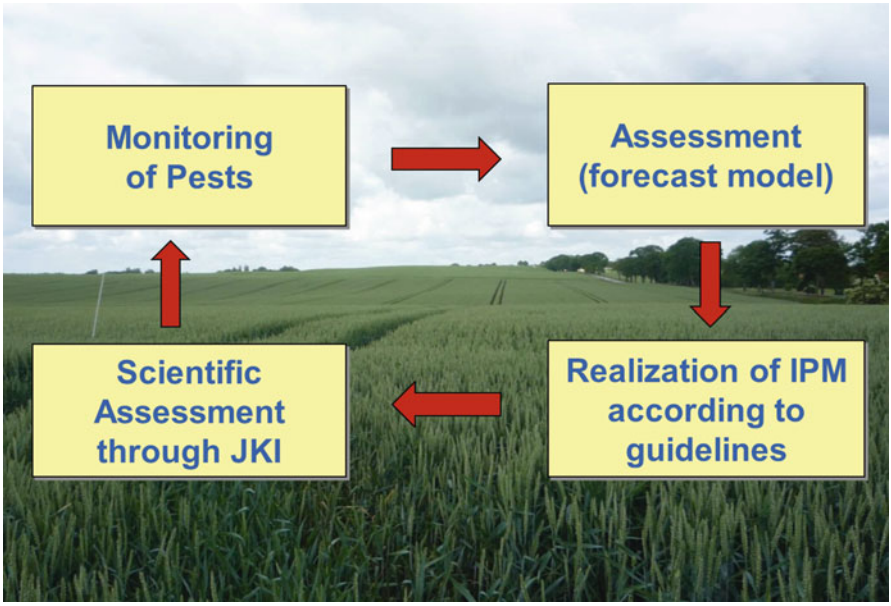

Fig. 2.2 The circle of measurements and assessments on DF-IPP in Germany (Gündermann 2014)

degree. Independent training and advice for farmers are regulated by the national plant protection acts and accompanied regulations, such as the regulation on professional knowledge. In Germany, e.g., each of the 16 federal states is responsible for advice, awareness raising, and training, in particular on good plant protection practice including IPP and the implementation of NAP measures (Hommel et al. 2014). The federal states are also required to diagnose and monitor pests, carry out field experiments and PPPs trials, and maintain databases and forecasting platforms in the Internet. The German Internet platform ISIP (Information System for Integrated Plant Production: www.isip.de) integrates weather data in disease models and provides regional decision support in major crops (Racca et al. 2011). In Denmark, an extensive monitoring system is operating linked to the farm advisory system, which has a high impact on reduced pesticide uses (Kudsk and Jensen 2014). In Germany, the organization of public extension differs from state to state (Hommel et al. 2014). Very common schemes to distribute knowledge are training courses in winter time and open field days during the growing season. During the latter, information is mainly transmitted via online portals, monitoring systems, prognosis models, and specific decision support systems (DSS) (Hommel et al. 2014). Professional users, advisors in plant protection, and distributors of PPPs have to renew their certification every 3 years. In Germany, training details and education topics have to be specified by region and crop through the responsible authorities (Freier and Zornbach 2008).

2.5 Development of Innovative Plant Protection Strategies

The future of agricultural plant production is threatened by the emergence of pest resistance and a declining availability of active substances in plant protection products (Lamichhane et al. 2015). Additionally, invasive species like the brown marmorated stink bug (*Halyomorpha halys*) (Leskey et al. 2012) and the spotted wing drosophila (*Drosophila suzukii*) are expanding their native range to other continents (Cini et al. 2014; Vogt et al. 2012). Thus, the numbers of severe agricultural pests registered in pest information databases like the "Pest Information Wiki" (PestinfoWiki Contributors 2015) are increasing continuously. In conclusion, there is a need to design cropping systems less dependent on synthetic pesticides, which integrate innovative plant protection strategies (Barzman et al. 2014). According to the eight principles of IPM formulated in Annex III of Directive 2009/128/EC, we give in the following an overview on important examples for new developments and attempts in plant protection.

2.5.1 Selective and Biorational Pesticides

Pesticides applied on crops shall be as specific as possible for the target pest and shall have the least side effects on human health, nontarget organisms, and the environment (Principle 5 of IPM – pesticide selection). Thus, those insecticides that are efficacious against target pests but less detrimental to beneficials, the so-called biorational pesticides, should be the first choice in IPM programs. This term describes any type of insecticide including botanicals and microbials active against pest populations but relatively innocuous to nontarget organisms and therefore nondisruptive to biological control (Schuster and Stansly 2015). Applied separately, biorational pesticides may perform with less biocidal power and appear more costly than conventional synthetic pesticides. By integrating several alternative methods, they may generate synergies resulting in satisfactory pest control (Barzman et al. 2015). In Chaps. 5 and 8 of this book, examples will be presented dealing with this aspect (Wise 2016; Akhatar and Isman 2016).

2.5.2 Anti-resistance Strategies

Where the risk of resistance against a plant protection measure is known and where the level of harmful organisms requires repeated application of pesticides to the crops, available anti-resistance strategies should be applied to maintain the effectiveness of the products. This should include the use of or rotation of multiple pesticides with different modes of action (Barzman et al. 2015) (Priciple 7 of

IPM – anti-resistance strategies). Some studies on this important principle are given in Chaps. 11, 12, 13, 14, 15, and 16 of this book.

2.5.3 Monitoring of Pest Insects by Semiochemicals

Harmful organisms must be monitored by adequate methods and tools. Such adequate tools should include trappings and observations in the field as well as scientifically sound warning, forecasting, and early diagnosis systems, where feasible, as well as the use of advice from professionally qualified advisors (Barzman et al. 2015) (Principle 2 of IPM – monitoring). IPM strategies require a full understanding of the biology and life history of a pest organism and its natural enemies within any ecosystem (Dent 1991). While the adult life stage will be the target for monitoring of pest insects by semiochemicals regularly, in some cases, other ontogenetic stages could be the target of monitoring depending of their biology and ecology. For monitoring of, e.g., mosquito populations, so-called ovitraps mimicking their preferred breeding sites were developed acting as an early warning signal by counting the eggs to preempt any impending dengue outbreaks (Jacob and Bevier 1969). Also in moth control, the monitoring of caterpillars or eggs could be easier than very mobile adult stages (see below). For innovative monitoring methods, which use semiochemicals for luring different life stages of target insects, only adult and egg monitoring are appropriate because no methods exist for luring relative immobile larval stages by chemical components in traps, while the mating and egg laying behavior of adult insects could be much better exploited for the development of chemically lured monitoring traps.

2.5.3.1 Adult Monitoring

Traps consisting of sticky foils or filled with toxic fluids are often equipped with artificial dispensers emitting synthetic sexual pheromones. They are widely used for monitoring the population dynamics of insect pests, which is the basis for decisions regarding chemical control and for calculating the optimal timing for spraying insecticides. Many insect pheromones have been identified in the past and are commercially available today for trapping moths, beetles, flies, and many other pest insects. The Internet database “Pherobase” lists hundreds of sexual pheromones for monitoring purposes (El Sayed 2014), and many of them are today commercially available. Traps equipped with these species-specific lures have allowed for significant advancement of IPM decision-making. However, they also have some weaknesses: the amount of caught specimen is not only influenced by population densities but also by weather conditions, dispenser specifications,

and the actual reach of attractive plumes from traps (Miller et al. 2015). Finally, they have to compete against the natural sources of pheromones, mainly female insects themselves, as male-produced pheromones are very rare (Gross 2013). Key parameters influencing the number of trapped organisms from any specified distance of origin from the trap are the probabilities that the trap is found (findability) and that the organism is captured after arriving at the trap (efficiency) and retained (retention) until the trap is emptied by the farmer (Miller et al. 2015).

Besides pheromones, also allelochemicals can be used as lures in traps. Recently, new findings have been reported for psyllids, which could be used for the development of new chemically lured traps for monitoring and also mass trapping (Mayer et al. 2008a, b, 2009, 2011; Rid et al. 2016). In this case, also compounds with a high specificity for the target pest should be used to reduce bycatches of beneficial insects (Weintraub and Gross 2013). The emission rates of the attractive compounds, often plant-produced kairomones, have to be multiple times higher than by using pheromones.

2.5.3.2 Egg Monitoring

Even though in most cases eggs are more difficult to monitor in the field than adults, the advantage of monitoring the egg stage is established in systems, in which robust degree-day models exist for the period of time for egg development, before the damaging larval stage begins. The use of egg traps, e.g., equipped with kairomones from almond, is common and recommended in IPM for monitoring the navel orangeworm (*Amyelois transitella*) in the USA and Mexico (Anonymous 2002). This method is applied to determine when navel orangeworm eggs will hatch in relation to hull split of infested almonds so a chemical treatment can be timed precisely (Anonymous 2002). The European grapevine moth *Lobesia botrana* and European grape-berry moth *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) are the most damaging insect pests in European viticulture. Larvae injure fructiferous organs by feeding and thus promote infestation with bacteria and fungi, such as gray mold *Botrytis cinerea*. To achieve effective chemical control, insecticide treatments have to be conducted before hatching of the larvae. For this purpose, an egg monitoring is necessary but not practicable. To prevent immoderate insecticide application, which would not be in compliance to IPM, a decision support system for growers is needed, which enables the timing and necessity of pest control. Such a tool, called "moth oviposition card" (M-OVICARD) consisting of volatile and nonvolatile compounds, supported by visual and tactile cues, is currently under development (Greif et al. 2015). The number of eggs, deposited on such a monitoring card, should correlate with actual pest infestation in grape vines and may help to determine the perfect spraying time, resulting in a reduced amount of applied insecticides (Greif et al. 2015).

2.5.4 Nonchemical Methods

Sustainable biological, biotechnical, physical, and other nonchemical methods must be preferred to chemical methods if they provide satisfactory pest control (Principle 4 of IPM – nonchemical methods) (Barzman et al. 2015). Plant protection strategies based on olfactory, gustatory, or acoustic signals are very divers and have a high potential to improve existing IPM strategies. For instance, there are many possibilities for the integration of semiochemical use in IPM strategies. Infochemicals convey information in interactions between individuals, leading in the receiver to a behavioral or physiological response. We distinguish between pheromones, which mediate interactions between organisms of the same species, and allelochemicals, which mediate interactions between two individuals that belong to different species (Dicke and Sabelis 1988). In most cases of pest control, pheromones are used due to their high species specificity. Sex pheromones are both used for monitoring of pest insects and for disturbing mating behavior (Gross 2013; Harari 2016, Chap. 6). Aggregation pheromones can be used for mass trapping, but this type of pheromone occurs only in a few known species (Gross 2013). For the so-called attract-and-kill or lure-and-kill systems, an attractive compound (a pheromone or a kairomone) is combined with a toxic component, like an insecticide or pathogenic microorganism. Ultimately, the potential of infochemicals for plant protection can be used in complex push-and-pull strategies.

2.5.4.1 Mating Disruption by Semiochemicals

The most prominent and environmentally friendly application method for pheromones in plant protection is the mating disruption technique. Females emit an airborne trail of sex pheromones, the so-called pheromone plume, which may be used by males to locate them. This technique exploits the male insects' natural response to follow the corresponding plume by introducing artificial dispensers emitting synthetic pheromones into their habitat. The synthetic pheromone is a volatile organic chemical designed to mimic the species-specific sex pheromone produced by the female insect. It is important to divide between competitive and noncompetitive mating disruption (Miller et al. 2015). Under competitive attraction operating in a crop treated with multiple point dispensers of synthetic pheromone, the frequency with which males find calling females is reduced because males are diverted from orienting to females or traps due to preoccupation with more numerous nearby dispensers that first attract responders and then arrest and possibly deactivate them (Miller et al. 2010). In contrast, noncompetitive disruption includes masking of females by pheromone dispensers and desensitization of responder sensory systems without first requiring attraction (Miller et al. 2010). Consequently, the male population experiences a reduced probability of successfully locating and mating with females, which can lead to termination of breeding followed by the collapse of insect infestation (Witzgall et al. 2010). The Internet database

"Pherobase" currently lists 149 species, for which mating disruption techniques have been proven, and 133 of these are Lepidoptera (El Sayed 2014). To date, important successes include codling moth *Cydia pomonella* in pome fruit, oriental fruit moth *Grapholita molesta* in peaches and nectarines, tomato pinworm *Keiferia lycopersicella* in vegetables, pink bollworm *Pectinophora gossypiella* in cotton (Welter et al. 2005), and omnivorous leafroller *Platynota stultana*, European grapevine moth *L. botrana*, and grape-berry moth *E. ambiguella* in vineyards (Gross 2013; Welter et al. 2005). The use of the mating disruption technique has been explored against stored-product pests such as *Plodia interpunctella* and *Sitotroga cerealella* (Fadamiro and Baker 2002). While most successes have been with Lepidoptera, research on stink bugs and beetles showed promising results (McBrien et al. 2002; Millar et al. 2002).

2.5.4.2 Mating Disruption by Substrate Vibrations

Mating disruption is generally based on sex pheromones and allows sustainable pest control resulting in strong reduction of pesticides sprayed (Witzgall et al. 2010). A very original and innovative approach in mating disruption using substrate vibrations was recently published by Eriksson et al. (2012). They applied disruptive acoustic signals (calls from a rival male) to grapevine plants through a supporting wire which decreased the mating frequency of the leafhopper *Scaphoideus titanus*, significantly. Read more on this new strategy in Chap. 9 of this book (Polajnar et al. 2016). In case that both acoustic and chemical signals are involved in courtship behavior like known from *Drosophila* (Fabre et al. 2012) or pear psyllids (Eben et al. 2014; Guedot et al. 2009; Lubanga et al. 2014), the acoustic mating disruption system could be supplemented by specific pheromone dispensers.

2.5.4.3 Mass Trapping

For the biological control of species that produce aggregation pheromones, mass trapping systems can be developed. Mass trapping is a direct control strategy, in which large numbers of pests are captured and removed from the system. Excellent results of the mass trapping technique were obtained in Central and South America by using sophisticated pheromone traps emitting male-produced aggregation pheromones of different weevil species, e.g., the West Indian sugarcane weevil, the banana weevil, and the American palm weevil (Giblin-Davis et al. 1996). In oil palm plantations in Central and South America, the palm weevil (*Rhynchophorus palmarum*) is a vector of the lethal red ring nematode. Today, the principal control method is a pheromone-based mass trapping, using one trap per acre (Oehlschlager et al. 2002). The key biological factors appear to be the relatively long life and slow reproductive rate of the tropical weevils and the fact that the aggregation pheromones attract both sexes. Success is critically dependent

on efficient mass trapping to remove weevils faster than they can reproduce (Welter et al. 2005). Although there are only a few examples of this technique with a satisfactory effectiveness in temperate regions, two prominent examples are known from the control of the bark beetle *Ips typographus* in Europe and the Mountain pine beetle *Dendroctonus ponderosae* in North America (Witzgall et al. 2010). Allelochemicals like kairomones are less used in mass trapping as pheromones but are sometimes used in combination. A well-known example for an effective kairomone is the pear ester (ethyl (*E*,*Z*)-2,4-decadienoate), a characteristic volatile component of ripe pear. This kairomone is an attractant for adult and larval stages of codling moth *C. pomonella*. Its identification has allowed the development of several new approaches to successful monitoring and mass trapping of this pest (Light et al. 2001; Knight and Light 2001; Knight et al. 2002). In total, 111 compounds are listed in "Pherobase," which have the potential for mass trapping applications (El Sayed 2014), but only a few are used in today's pest control strategies.

2.5.4.4 Attract and Kill (Lure and Kill)

Another approach using sex pheromones or other attractive compounds is the attract-and-kill or lure-and-kill method. A viscous paste, gel, or a spray containing an attractant mixed with an insecticide, sterilant, or insect pathogen (e.g., entomopathogenic fungus or granulosis virus) can be distributed as small droplets, dollops, or a film on twigs or leaves of cultivated plants, eliminating individuals that contact the lure. When a female sex pheromone was used as attractant and a contact insecticide as toxin, males are lured to the droplet, try to mate with it and finally get killed (Charmillot et al. 2000). Huang et al. (2013) suggest that an effective attract-and-kill device should keep target insects from contacting the pheromone source directly to avoid desensitization of the olfactory apparatus, while providing a surface that allows them sufficient time to contact the toxicant and acquire a lethal dosage. Conventional attract-and-kill formulations in which sex pheromones are mixed with an insecticide would discourage moths contacting the insecticide surface for prolonged times. Thus, the better approach would be to spatially separate the sex pheromone or another desensitizing attractant from the toxicant (Huang et al. 2013). A newly developed prototype device for the control of the oriental fruit moth *G. molesta* was reported recently: It consisted of a fabric pouch that was impregnated with a contact insecticide and baited with a separate female sex pheromone lure, which was placed inside the pouch (Huang et al. 2015). This construction provided a large insecticide-treated surface for males to interact with but also prevented them from directly contacting the attractant for minimizing the risk of moths overloading their sensory system with sex pheromones (Huang et al. 2015). In other cases, an insect could be lured by a plant kairomone like pear ester and killed by an insecticide or granulosis virus after feeding on the droplet (Light 2007). Many limitations of mass trapping by aggregation pheromones also apply to attract-and-kill strategies that target males only. Depending on how the system is implemented, it may also interfere with the male's location of females through false-trail following,

as well as the primary effect of the male's attraction to insecticide-laced baits (Welter et al. 2005). In total, 35 allelochemicals are listed in "Pherobase," which have the potential as new lures in attract-and-kill applications (El Sayed 2014). In conclusion, the attract-and-kill technique is a promising approach for IPM, but all knowledge on the biology and behavior of the target pest organisms must be included in the development of more efficient devices (Huang et al. 2013).

2.5.4.5 Repellents, Antifeedants, and Deterrents

There are actually rare examples for the use of repellent components in pest control. Some products containing repellent components, which produce a displeasing smell for mammals like deers, rodents, or wild boar, are commercially available. Throughout the sub-Saharan African countries, in which populations of the African elephant (*Loxodonta africana*) exist, farmers come into conflict with these pachyderms. Attracted by nutritious crops on the fields, they destroy substantial amounts of harvest by crossing through the plantations and feeding on the crops. As this species is protected and listed as a threatened species by the IUCN Red List and therefore must not be killed (Blanc 2008), new ways need to be found to repel or at least not attract the pachyderms to fields. It was shown recently that garlic, ginger, and lemon grass, plants which contain higher amounts of secondary plant products, were less attractive to elephants than maize (Gross et al. 2016). However, they may not be completely unpalatable or even repellent to them, but their activity could cause an avoidance behavior, which terms the active compound an antifeedant. The selection of appropriate, less attractive, or even unpalatable crops might be a solution for the agricultural sector in or close to elephant dwelled habitats to tackle these conflicts (Gross et al. 2016).

For the control of insect pests, both in greenhouse or field, no repellents have been approved so far worldwide. But there are some research activities trying to develop oviposition deterrents, e.g., for the invasive pest *D. suzukii* in the field (Stensmyr et al. 2012; Wallingford et al. 2015). The alcohols geosmin and 1-octen-3-ol were found to be deterrent to females of *D. suzukii* in laboratory choice tests (Wallingford et al. 2015). Furthermore, field experiments revealed that fewer eggs were observed in fruits at harvest and fewer adult *D. suzukii* were reared from fruits associated with 1-octen-3-ol odors than control fruit of cultivated red raspberry (Wallingford et al. 2015). In other cases, repellents are combined with attractants to so-called push-and-pull strategies (see below).

2.5.4.6 Push-and-Pull Strategies

More complex approaches for using the potential of allelochemicals in plant protection are the so-called push-and-pull strategies (Khan et al. 2010). They consist of cropping systems, in which specifically chosen companion plants are grown in between and around the main crop. Some of these companion plants

(intercrop) release infochemicals that repel insect pests from the main crop ("push" component). Furthermore, crops which attract insect pests more strongly than the main crop are planted in its surroundings ("pull" component) (Cook et al. 2007). Future directions for improving existing push-and-pull strategies or the development of new techniques may also include biotechnical applications consisting of artificial dispensers emitting synthetic repellent compounds and traps supplied with synthetic attractants.

2.6 Conclusion

As the R&D for new chemical pesticides needed by European farmers is in decline, the resulting limited range of available pesticides has increased the risk of pesticide resistance development (Lamichhane et al. 2015). Thus, new attempts in the development of nonchemical methods for plant protection in IPM will help to enhance sustainable control of pest organisms and to reduce the amount of applied pesticides. The likeliness of successful pest control will be improved by a combination of different techniques using volatile chemicals for monitoring and semiochemicals as well as vibrational signals for direct control of pest organisms together with a controlled application of biorational or synthetic pesticides. Last but not least, by integrating also functional ecology aspects in IPM strategies like the impact of "ecosystem services" (Wise and Whalon 2009) delivered by native pollinators and pest organisms' natural enemies (pathogens, predators, parasitoids) will be the way leading to a sustainable agricultural crop production in the twenty-first century.

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Chapter 3
Biological Control and Pollination Services on Organic Farms

Elias H. Bloom and David W. Crowder

Abstract Organic farming is an alternative agricultural system that encompasses holistic production tactics that promote and enhance ecosystem health. Organic farms rely on diverse communities of beneficial insects to provide critical ecosystem functions such as decomposition, biological control, and pollination. However, the conservation of ecosystem services in agricultural ecosystems including organic farms is a complex challenge, in part due to factors such as climate change and habitat loss. Organic farmers have begun to meet this challenge by adopting on-farm and landscape-level measures to preserve and restore ecosystem services, although more work is needed to stem the loss of global biodiversity. Here, we review the impacts of organic farming on communities of natural enemies and pollinators, and the services they provide. We also describe strategies currently used, and future research opportunities, that could further promote the conservation of these beneficial groups and their services in organic systems. Our review suggests that the conservation of natural enemies and pollinators on organic farms will require a multi-scale approach in which on-farm and landscape-level conservations are of equal importance. However, more research is needed to identify the particular practices that promote both of these beneficial groups simultaneously.

3.1 Introduction

Organic farming is an ecologically responsible method of food production encompassing "holistic production systems that promote and enhance agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity" (Dabbert et al. 2004) (Fig. 3.1). In contrast, many conventional farming systems sacrifice ecosystem services including biological control, pollination, and soil conservation for short-term increases in yield (Fig. 3.1). By conserving ecosystem services, organic farms generally produce yields only slightly lower than conventional farms with significantly less inputs (Crowder and Reganold 2015)

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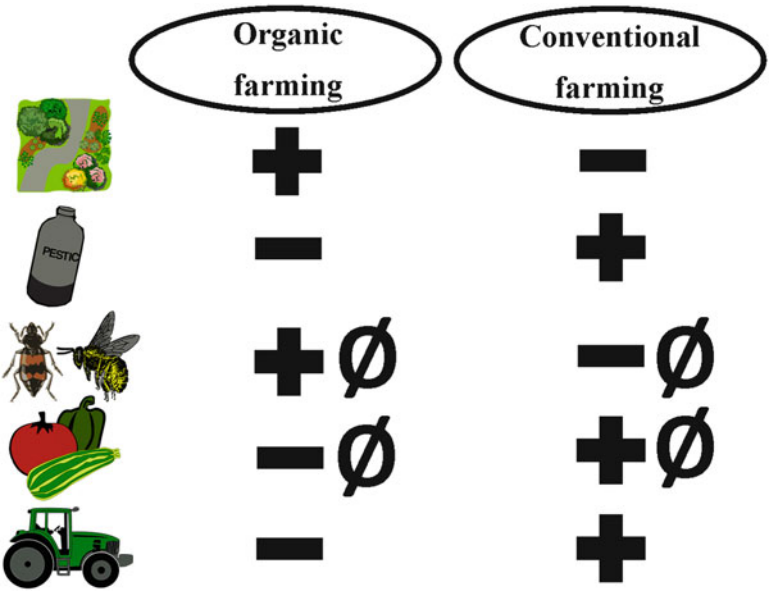

Fig. 3.1 Relative effects of organic and conventional farming on (symbols from *top* to *bottom*): floral diversity, pesticide use, biodiversity and ecosystem services, yields, and negative externalities. The farming practice that promotes these factors relative to the other is shown with a + sign; the practice that reduces these factors is shown with a −. If it is unknown whether organic farming affects these factors, a neutral symbol (Ø) is shown

(Fig. 3.1). The combination of high yields and organic premiums, which are paid by consumers in part because organic farming is considered more environmentally friendly, allows organic farms to often exceed the profits of conventional farms (Crowder and Reganold 2015).

Conventional farming systems often make extensive use of agrochemicals to manage pests. While organic farms can also use pesticides, they typically rely heavily on natural communities of beneficial insects to provide biological control services (Sandhu et al. 2010); indeed, chemical inputs on organic farms are significantly lower than conventional farms (Crowder and Reganold 2015) (Fig. 3.1). Insects perform many functions on organic farms such as decomposition, biological control, and pollination (Losey and Vaughan 2006). Organic farms use these services to produce crops without costly and toxic synthetic inputs. Thus, these services not only impact the value of crops produced but also reduce negative externalities associated with conventional farming such as erosion and pollution (Sandhu et al. 2010; Crowder and Reganold 2015) (Fig. 3.1).

Conventional farming practices also threaten global biodiversity (Butchart et al. 2010) and may thus indirectly harm sustainable farming systems that rely on diverse communities to provide ecological services (Fig. 3.1). Organic farmers and regulatory groups have begun to address this challenge by incentivizing farmers

to conserve biodiversity and ecosystem services (Gonthier et al. 2014; Batáry et al. 2015). However, the conservation of diverse communities in agricultural ecosystems is a complex challenge, in part due to factors such as climate change and large-scale habitat loss. Organic farmers have begun to meet this challenge by adopting on-farm and landscape-level measures to preserve and restore ecosystem services, although more work is needed to stem the loss of global biodiversity (Gabriel et al. 2010).

The methods organic farmers use to conserve beneficial species and their services will be the emphasis of this chapter. We focus on two ecosystem services, biological control and pollination. Biological control is a central tenet of organic farming, allowing farmers to control pests while reducing harmful pesticide inputs. Many organic practices that affect natural pest control also impact pollinators. Thus, pollinators should be considered whenever strategies for insect control are being implemented. Both biological control agents and pollinators are also indicators of ecosystem health (Paoletti 1999). Thus, they are model organisms to evaluate the virtues of organic methods and how these methods impact insects and their services.

In Sects. 3.2 and 3.3, we detail the impacts of organic farming on communities of natural enemies and pollinators and the services they provide. In Sects. 3.4 and 3.5, we describe strategies currently used and future research opportunities that could further promote the conservation of these beneficial groups and their services in organic systems. Our goal is not only to highlight the critical role these arthropods play in natural pest control and food production but also to spur research into global conservation of these groups and the services they provide to farms.

3.2 Promoting Natural Enemies and Biological Control on Organic Farms

Biological control is a key ecosystem service on farms provided by diverse communities of predators, parasitoids, and pathogens. The value of biological control of insect pests has been conservatively estimated at $4.5 billion annually in the USA (Losey and Vaughan 2006). If we assume that organic farms receive equal biological control as conventional farms, then the value provided to organic farms in the USA would be $45 million, as approximately 1 % of cropland is organic (Crowder and Reganold 2015). If weeds and noninsect crop pests were included in these valuations, these numbers would certainly be much higher. However, evidence suggests organic farms receive particularly effective biological control by promoting diverse and abundant natural enemy communities (Crowder et al. 2010; Crowder and Jabbour 2014). Here we highlight the impacts of organic farming systems on natural enemies and biological control services.

3.2.1 *Global Trends of Natural Enemy Communities in Organic Farming Systems*

Several global meta-analyses have shown that organic farming promotes abundant and diverse natural enemy communities (Fig. 3.2). Bengtsson et al. (2005) showed that both overall predator communities and the family Carabidae, which contains many predator species, were significantly more abundant and species rich on organic compared to conventional farms. Tuck et al. (2014) showed that predators were 12.5 % more species rich on organic farms compared to conventional farms. The benefits of such diversity increases have also been studied. Letourneau et al. (2009) showed that in 71 % of studies examined, increasing natural enemy species richness strengthened biological control on crop pests (Fig. 3.2). Moreover, these results were stronger in agricultural compared to natural ecosystems. Similarly, Griffin et al. (2013) used meta-analyses to show that more diverse predator communities typically improve natural pest suppression.

Crowder et al. (2010, 2012) also showed that organic farming systems provided significant benefits for the promotion of species evenness compared with conventional farming systems. Across natural enemy groups, evenness increased on average by 7 % in organic farming systems (Crowder et al. 2010). While this may

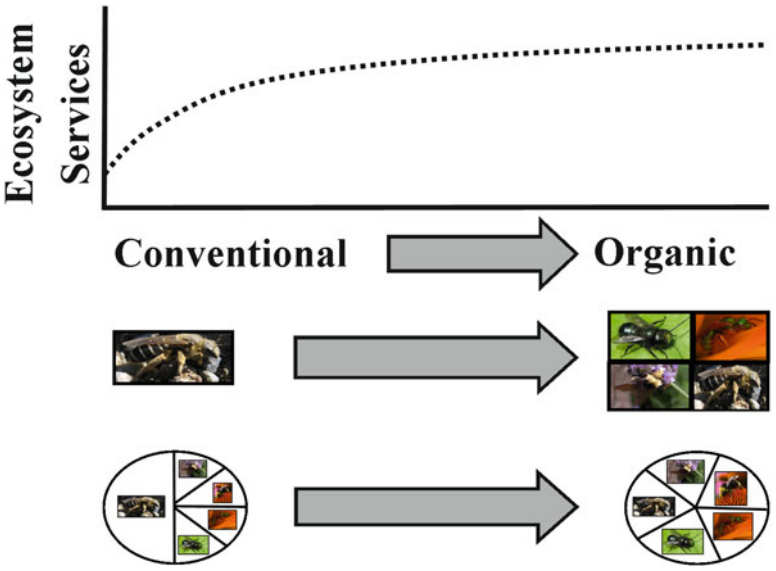

Fig. 3.2 General effects of organic farming on ecosystem services and biodiversity. The X-axis shows that as farms transition from conventional to organic farming, they typically see gains in species richness (shown is a comparison of a community with 1 vs 4 species) and species evenness (shown is a comparison of an unbalanced and balanced community). The Y-axis shows that as these gains in diversity are realized, ecosystem services generally increase (shown as a *dashed line*)

seem small, this increase led to up to 30 % higher aboveground biomass because more even natural enemy communities increased pest control (Crowder et al. 2010) (Fig. 3.2). Organic farming has also been shown to increase evenness by providing the greatest benefits to species that are rare in conventional systems (Crowder et al. 2012). This implies that switching from conventional to organic production may disproportionally benefit species that are threatened with local extinction, providing a beneficial conservation outcome.

Food webs have also been shown to be more diverse on organic compared to conventional farms. MacFadyen et al. (2009) examined the food web structure of ten pairs of conventional and organic farms and found that herbivores were attacked by more species of parasitoids in organic farms. While this study did not show a concurrent reduction in pest densities on organic farms, this was attributed to intensive management practices on conventional farms rather than a lack of natural pest control on organic farms (MacFadyen et al. 2009). Similar studies in the tropics have shown that more diverse agricultural habitats support more complex food webs and have shown increased parasitism rates in sustainable farming systems (Tylianakis et al. 2007).

By supporting diverse and abundant natural enemy communities, organic farmers likely receive stronger natural pest control than conventional farmers (Fig. 3.2). For example, a meta-analysis found that pest densities were similar on organic and conventional farms (Bengtsson et al. 2005). This suggests that the increased effectiveness of natural enemies in organic farming systems might enable farmers to achieve equivalent, or nearly equivalent, pest control to that achieved with pesticides. While biological control is unlikely to eliminate the need for pesticides, organic farming and other sustainable practices that promote diverse and abundant natural enemy communities will see substantial economic benefits and reduced negative externalities.

3.2.2 Factors Influencing Predator Communities and Biological Control on Organic Farms

i. *Agrochemical use*. Natural enemies are often highly susceptible to synthetic pesticides, which can weaken biological control (Geiger et al. 2010; Roubos et al. 2014). Intensive agrochemical use to control pests can also indirectly impact natural enemies by removing critical plant food resources used for shelter or nutrients (Roubos et al. 2014). Pesticide use can lead to secondary pest outbreaks, where early-season insecticide applications kill natural enemies and cause late-season outbreaks of pests. In California cotton crops, early-season pesticides used for *Lygus* bug control kill many natural enemies, resulting in late-season outbreaks of other pests and significant costs for growers (Gross and Rosenheim 2011). Organic farming systems limit such outbreaks by reducing the amount of pesticide inputs and their effects (Hole et al. 2005).

Modern decision support systems can help organic farmers decide which pesticides to use to manage pests while limiting harmful impacts on natural enemies. One of the greatest examples of this is the decision aid system for tree fruit growers in Washington State, USA (Jones et al. 2010). Within the decision aid system growers and pest control advisors can get recommendations of organically approved insecticides that have a narrow spectrum, thus killing pests while limiting harmful effects on natural enemy populations. Such decision support aids could be a major boon to organic farmers, allowing them to minimize harmful impacts on natural enemies while still achieving adequate pest control.

ii. *Habitat diversity on farms*. Meta-analyses have shown that organic farms support more diverse plant communities than conventional farms (Bengtsson et al. 2005; Crowder et al. 2012; Tuck et al. 2014). The increase in plant diversity on organic farms is particularly great at field edges (Gabriel et al. 2006). Natural enemies require diverse floral resources for shelter and nutrition (Letourneau et al. 2011; Chaplin-Kramer et al. 2011; Chisholm et al. 2014), such that increased plant diversity on organic farms provides many benefits. Diversity at field edges can promote populations of mobile predators that can disperse into crop centers and control pests (Gabriel et al. 2006). For example, planting rose and strawberries adjacent to apple orchards has been shown to increase densities of parasitoids, leading to improved biological control of leafrollers (Unruh et al. 2012).

Within crop fields, intercropping and undersowing are two strategies used by organic farmers to increase the diversity and effectiveness of natural enemy populations. Undersowing, or planting a secondary crop under a primary cash crop, increases vegetative heterogeneity on organic farms. In turn, undersowing enhances populations of natural enemies such as carabids and spiders (Sunderland and Samu 2000). Similarly, intercropping has been shown to increase the abundance and diversity of generalist predator species on organic farms (Sunderland and Samu 2000). While these studies did not evaluate impacts of these practices on biological control, the more diverse and abundant natural enemy communities produced by undersowing and intercropping are likely to strengthen natural pest control and reduce the need for pesticides.

iii. *Availability of foraging and nesting habitat across landscapes*. Organic farming systems embedded in heterogeneous landscapes generally have more diverse and abundant natural enemy assemblages than those in homogeneous landscapes of crops (Chisholm et al. 2014). In turn, damage from pests has been shown to decrease on organic farms embedded in complex landscapes (Letourneau et al. 2011), a result likely attributed at least in part to increased biological control. At landscape-level scales, organic farms have shown increased beta diversity in plant communities compared to their conventional counterparts (Gabriel et al. 2006, 2010).

Studies from conventional systems suggest that landscape diversification provides value for pest control. Landis et al. (2008) examined the economics of soybean aphid biological control by a suite of natural enemies across a mixture of crop and non-crop landscapes in the Midwestern USA. Biological control services were valued at $239 million annually in these landscapes, but conversion of native prairie habitat to maize farming systems reduced this value by $58 million. Similarly, Werling et al. (2011) showed that perennial grasslands had significantly greater pest suppression and diversity of natural enemy communities compared to maize crops. These studies, and results from meta-analyses (Letourneau et al. 2011; Chaplin-Kramer et al. 2011), suggest that biological control will be strongest when landscapes are managed for complexity.

However, the impacts of landscape complexity on biological control can be mediated by the floral diversity present on organic farms. In simplified landscapes, organic farmers might be able to achieve significant gains in biological control services by planting floral strips to promote habitat for natural enemies (Jonsson et al. 2015). In contrast, diversifying floral resources on farms embedded in complex landscapes might have little impact on biological control services, as these landscapes already provide an abundant source of natural enemies (Jonsson et al. 2015). Thus, organic farmers need to consider their surrounding landscape when deciding whether to implement strategies to diversify their farming systems.

iv. *Climate change*. Climate change might have complex effects on biological control in organic farming systems. For example, two parasitoid species exerted stronger biological control of the green peach aphid during extended heat waves because variation in the physiology of parasitoid species caused them to act more complementarily as temperatures warmed (Gillespie et al. 2012). In contrast, Barton and Schmitz (2009) found that a spider, *Pisaurina mira*, moved lower in the plant canopy as temperatures warmed, which caused it to overlap with an actively foraging spider, *Phidippus rimator*. This led to intraguild predation and local extinction of one spider species, which weakened control of a grasshopper pest (Barton and Schmitz 2009).

While these studies were not conducted in organic systems, they underscore the complex interactions that influence biological control. If herbivores adapt more readily to climate change than natural enemies, we would expect increased pest outbreaks (de Sassi and Tylianakis 2012). Since many studies suggest that higher trophic levels are most sensitive to climate change (Gilman et al. 2010), we might predict that negative impacts of climate change on diversity in natural enemy communities will magnify over time, which could weaken biological control.

3.3 Promoting Bee Communities and Pollination Services on Organic Farms

The global value of pollination services in agroecosystems is hundreds of billions of dollars annually (Winfree et al. 2011; Hanley et al. 2015). Farmers rely on bee communities that are abundant and diverse (Bommarco et al. 2012) to achieve effective pollination services due to spatial and temporal floral partitioning. While organic farms have abundant and diverse pollination needs, the value of bee pollination specifically for organic farms is unknown.

Bees can be broadly grouped into (1) honey bees, which are managed for pollination services, and (2) wild species, which are unmanaged. Honey bees became the poster child for the global pollination crisis in 2006 due to colony collapse disorder, a condition caused by a suite of stressors including disease, parasites, and insecticide use (Goulson et al. 2015). Little data exist for the health of honey bees on organic farms, although honey bee health may improve on organic farms due to increased floral resources compared to conventional farms where those resources may be lacking (Decourtye et al. 2010; Crowder et al. 2012). Wild pollinators also contribute to pollination in most crops. A global meta-analysis showed that fruit set increased significantly when wild pollinators were present for 41 crop species, regardless of honey bee abundance (Garibaldi et al. 2014). However, like honey bees, wild bees are declining worldwide.

3.3.1 Global Trends of Bee Communities in Organic Farming Systems

A global meta-analysis showed that organic farms have on average 74 % greater wild bee abundance, and 50 % more species, than conventional farms (Kennedy et al. 2013). By promoting diverse and abundant wild bee communities, organic farms have increased pollination services compared to other systems (Holzschuh et al. 2008; Gabriel et al. 2013) (Fig. 3.2). Reflecting these trends, European countries have made substantial efforts to conserve wild bees through the de-intensification of farming practices. Conservation farming methods have helped limit declines of European pollinators (Carvalheiro et al. 2013; Goulson et al. 2015). However, global trends indicate that wild bees continue to be threatened by intensified farming (Goulson et al. 2015).

3.3.2 Factors Influencing Bee Communities and Pollination Services on Organic Farms

i. *Agrochemical use*. Exposure to agrochemicals is a major contributor to global pollinator declines (Goulson et al. 2015). Bee communities are threatened in regions with extensive and intensive agrochemical use (Brittain et al. 2010),

especially on farms that use conventional practices or calendar spray schedules. To date, there is a clear lack of information on the effects of pesticides for many wild bee species, although honey bees and several bumblebee species have been well studied. One review suggests that overall levels of exposure to agrochemicals, rather than any single chemical, are the greatest detriment to bee health (Goulson et al. 2015).

Surprisingly, honey bees have been shown to prefer resources that contain neonicotinoids, even though these food resources decrease foraging efficiency and learning (Kessler et al. 2015). Therefore, organic farms near conventional fields that use neonicotinoids may find their bees in jeopardy. Conversely, when given a choice, bumblebees forage in areas that have not been sprayed with herbicides and fungicides, indicating that not all agrochemicals act as attractants for bees (Sprayberry et al. 2013). This supports the value of organic farms for bee conservation in agricultural landscapes, especially when those farms do not use any synthetic chemicals.

Approved agrochemicals used on organic farms can still be lethal or induce sublethal behavioral and physiological changes in honey bees and wild bees (Biondi et al. 2012; Kessler et al. 2015). For example, significant decreases in foraging efficiency were documented when *Bombus impatiens* was treated with realistic field levels of spinosad (Biondi et al. 2012). In contrast, some research indicates that certain pesticides may indirectly benefit pollinators. For example, the use of organic fungicides and snail bait was shown to increase butterfly and bumblebee abundance by reducing pest pressure on flower production by plants (Muratet and Fontaine 2015). However, pollinator health is widely believed to be negatively affected by pesticides. To prevent the deleterious effects of agrochemical use, organic farmers should be particularly aware of wild bee activity and only spray when bees are not present. The best time to apply chemicals is likely in the early morning and late evening when bees are the least active.

ii. *Ground-nesting habitat on farms*. Approximately 70 % of all bees live in the ground, thus maintaining this habitat for bee nests is critical for pollinator conservation (Goulson et al. 2015). Organic farms appear to provide belowground habitat for many bees and in some cases may be superior to natural areas and conventional farms. For example, organic farms hosted *Andrena* species that were not found in conventional systems, and irrigation and bare soil increased belowground nesting of some species compared to natural habitat (Forrest 2015). Yet, the value of natural lands proximal to organic farms should not be undervalued, since some wild bees may have specific habitat requirements that are not provided on farms (Greenleaf and Kremen 2006). This may be particularly true for rare species or aboveground cavity-nesting bees that do not adapt well to landscape management (Carré et al. 2009; Le Féon et al. 2013).

Tillage is a common pest control strategy on organic farms and is likely a contributor to the decline of many ground-dwelling bee species (Shuler et al. 2005). Thus, no-till organic systems may be one means of belowground bee conservation (Shuler et al. 2005). Crop rotation and fallow periods may also

influence bee communities, particularly rare species that require multiple floral resources or low disturbance (Le Féon et al. 2013). No-till, crop rotation, and fallow periods are widely known by organic farmers as a means to improve soil quality and health, but may also improve the richness and diversity of wild bee species on farms (Le Féon et al. 2013). In addition, farmers may also consider the introduction of less intensive cover crops such as temporary grasslands or flowering cover crops as part of crop rotations. Both of these techniques have been shown to increase wild bee community health (Le Féon et al. 2013). Moreover, pastureland either in-rotation or near crop systems may dramatically improve in-field wild bee abundance and diversity, particularly for bumblebees (Morandin et al. 2007).

iii. *Crop diversity on farms*. Access to multiple floral resources increases pollinator health, particularly for wild bees that need many resources to fulfill dietary requirements. For example, monocultures negatively affect the health of wild bees and their ability to provide pollination services to a crop (Girard et al. 2012). Studies that address the value of pollen diversity on wild bee health are lacking, but honey bees show improved colony health when foraging on multiple pollen sources (Girard et al. 2012). Management of floral diversity in large agronomic systems may be one of the best means to conserve global wild bee communities. Bees are widely viewed as bio-indicators of floral resources (Kevan 1999); therefore, it is likely that organic farms with diverse assemblages of wild bees have diverse and abundant plant communities.

iv. *Availability of foraging and nesting habitat across landscapes*. Natural habitat aids in conserving many bee species (Kremen et al. 2002; Kennedy et al. 2013; Martins et al. 2015). Monoculture production systems in particular isolate bees from natural lands containing nesting and food resources (Tscharntke et al. 2005; Carvalheiro et al. 2010). Organic farms, which are typically more diverse than their conventional counterparts, can buffer the detrimental attributes of homogenous landscapes, particularly for bumblebees (Rundlöf et al. 2008). However, organic farms in homogenous landscapes have less diverse bee communities than those closer to natural areas (Kennedy et al. 2013). Moreover, wild bee communities are more abundant and provide more effective pollination services on farms in heterogeneous landscapes (Carvalheiro et al. 2013). Organic farms may be similar to natural areas in forage and nesting value for wild bees, although future development of all farming systems must include a thoughtful approach to spatial connectivity with natural lands to optimize pollination services from wild bees.

v. *Urbanization*. Urbanization jeopardizes bees and pollination services by causing habitat fragmentation (McFrederick and LeBuhn 2006; Baldock et al. 2015). Spatial isolation makes wild bee community restoration particularly difficult in urban systems, and restoration efforts must preserve habitat and increase connectivity of urban organic farms with natural land. However, particular bee groups have been shown to prosper in urban farms (Matteson and Langellotto 2010). Urban gardens may be one habitat type that conserves bees and pollination services (Matteson and Langellotto 2010). These systems often use organic

methods and serve as a source of abundant nest and floral resources. In turn, the conservation of urban organic farms may serve as a source of abundant and diverse bee communities that spillover into the landscape.

vi. *Climate change*. Insects forage in specific climatic niches, and wild bees are active only at certain points within a given day. Similarly, flowering responses of plants are dependent on temperature. Serious concerns therefore exist with regard to the health of wild bees and their subsequent function due to climate change. Diverse assemblages of wild bees will likely buffer against effects of climate change, because increased number of species can provide insurance against the loss of services from any one species (Christmann and Aw-Hassan 2012; Brittain et al. 2013). Yet, asynchrony between flower blooms and pollinator activity is a major concern, especially in farming systems with low levels of pollinator diversity (Willmer 2012; Kudo 2013). As some wild bees undergo range contraction, farms outside of the range of those bees may no longer receive optimal pollination services for their crops, a trend observed in some nonfood production systems (Miller-Struttmann et al. 2015). Alternately, some bees may expand their range as global temperatures warm, competing with native bees for floral resources.

3.4 Strategies to Conserve Natural Enemies and Bees on Organic Farms

3.4.1 Increasing Connectivity Between Agricultural and Natural Landscapes

Conservation of natural lands that increase spatial connectivity between organic farms and suitable foraging and nesting habitat is the most important factor for on-farm conservation of natural enemy and bee community health (Krewenka et al. 2011; Burkman and Gardiner 2014). To promote healthy natural enemy and bee communities, organic farmers depend on valuable landscape features such as prairie and woodlands and overall spatial heterogeneity. The types of habitats that provide the greatest benefits are often unknown, however. Organic farmers might contribute to conservation of native bees and natural enemies by working with researchers to identify the specific landscape features that provide the greatest benefits to bees on farms. This might involve conducting on-farm research to identify the combinations of native or alien plants that provide the greatest benefit for beneficial arthropod communities.

Augmenting habitat for natural enemies and native bees by diversifying floral resources along roadsides may be one of the most effective strategies for conservation and for connecting organic farms with natural land. Governments could enact programs to plant natural enemy and pollinator-friendly plantings on the majority of these roadsides, which would provide wide-ranging benefits to these beneficial

groups and increase ecosystem services provided to farms (Hopwood 2010). In the USA alone, there are over four million hectares of publically owned land along the side of roads, including urban streets and rural highways (Forman et al. 2003). A small body of research indicates that spatial connectivity through powerline easements may also improve spatial connectivity of wild bees and resources (Russell et al. 2005). This could be particularly true for organic farmers in urban areas, where such habitats have been shown to increase the abundance and services of natural enemies (Burkman and Gardiner 2014). Policy makers in government should work to diversify these publically held resources to increase spatial connectivity for natural enemies and wild bees. Such efforts are underway in the USA and other countries as part of initiatives to conserve pollinators.

Fig. 3.3 Example of an aboveground structure that provides nesting habitat for cavity-nesting bees. The structure consists of large wooden blocks with holes drilled into them of various size; rolled cardboard tubes are inserted into these holes to provide nesting habitat

3.4.2 *Artificial Habitat, Floral Resources, and On-Farm Management*

Artificial above- and belowground bee habitat may also serve to improve natural enemy and bee community health (Williams et al. 2010; Burkman and Gardiner 2014). For example, it is likely that organic farmers who create and maintain nest substrate for bees (Fig. 3.3) will in turn derive greater pollination services. Similarly, farmers that create habitat patches suitable for natural enemies are likely to see increased abundance and diversity of these communities and a strengthening of biological control (Chisholm et al. 2014; Crowder and Jabbour 2014).

Extrafloral resources also improve the health of natural enemies and wild bees and their services on organic farms (Cole et al. 2015). All organic farmers should introduce resources for natural enemies and bees on their farm margins if feasible. Care must be taken to carefully select plants that are not invasive and provide resources for both generalists and specialists (Morandin and Kremen 2013; Burkman and Gardiner 2014; Pardee and Philpott 2014). Native plants, which have a long establishment period compared to some exotics, are most effective for natural enemy and wild bee management (Fiedler and Landis 2007; Morandin and Kremen 2013).

Organic farmers should also be cognizant of their surrounding landscape as part of their on-farm management. Natural enemies and wild bees often benefit most from diversification of floral resources on farms when the surrounding landscape is homogeneous (Tuck et al. 2014). This suggests that farmers embedded in a matrix of intensified areas have the greatest incentive to plant hedgerows or deploy nesting resources (Tuck et al. 2014). While these strategies will also likely benefit organic farms near natural habitat, returns will be diminished. More research should identify the economic benefits of floral diversification on organic farms embedded in landscapes of varying complexity to determine when and where organic farmers would benefit.

3.5 Opportunities for Future Study

3.5.1 *Improving Methods or Studying Ecosystem Services*

While there are standard procedures for measuring the biodiversity of natural enemy and bee communities, measuring ecosystem services remains difficult. Yet, when studying biological control, some researchers have found success using sentinel deployments of larvae or eggs to measure predation (Gardiner et al. 2014). Molecular gut-content analyses may also provide powerful tools to determine food web structure and which predators feed on which pests (Chisholm et al. 2014). When measuring pollination services, researchers typically use fruit set or pollen tube development. However, all of these methods are difficult and may not

precisely capture ecosystem services on farms. Chisholm et al. (2014) described a myriad of techniques that can be used to standardize the measurement of biological control services in variable landscapes through a combination of sentinel prey deployment and molecular gut-content analyses, but we know of no such literature for pollination services. Pollination researchers should work together to identify the techniques for capturing ecosystem services that are most effective and work to standardize studies based on the "best practices."

3.5.2 Developing Citizen Science Programs for Organic Farmers

Citizen science, the involvement of volunteers in research, may be one means to gather high-quality data on biodiversity and ecosystem services on organic farms over broad regions and long temporal scales (Turner 2003; Vance et al. 2003). Both pollinators and natural enemies have been studied using citizen science, including lady beetles, wild bees, and butterflies (Howard and Davis 2009; Kremen et al. 2011; Gardiner et al. 2012). However, to our understanding, no research programs have used citizen science to specifically study organic farms (Devictor et al. 2010; Kaartinen et al. 2013). As costs associated with research continue to escalate (Gardiner et al. 2012), organic farmers that double as citizen scientists could greatly benefit researchers that would like to gather large ecological datasets. Citizen science performed by organic farmers may also serve as a critical link between education, extension, and research.

3.5.3 Consideration of Natural Enemies and Bees in On-Farm Planning

Many farming practices that benefit bees also benefit predatory and parasitoid insects that provide biological control. Because both pollinators and natural enemies benefit from nectar-providing flowers, it has been suggested that management for pollination services will benefit predatory insects (Stallman 2011). However, interactions between pollinators and predators can also be negative. Ants that eat pests on plants can also harass pollinators, reducing seed set (Ness 2006). A recent meta-analysis of studies that measured either pollinator or predator responses to landscape complexity found mixed results (Shackelford et al. 2013). Thus, there is a clear need for research on whether these two groups of beneficial insects interact in a positive, negative, or neutral manner. Ideally, strategies implemented by organic farmers would provide simultaneous benefits to both groups while also helping reduce pest densities.

3.5.4 *Valuing Ecosystem Services*

Organic farmers would be more likely to implement conservation strategies promoting natural enemies and bees if they knew the value such practices might provide (Crowder and Reganold 2015). It is possible through changes in policy that systems could be developed whereby farmers that promote ecosystem services might reap greater financial benefits in terms of higher prices (Crowder and Reganold 2015). However, few studies have calculated the monetary value of biodiversity conservation or ecosystem services on organic farms (Wratten et al. 2012). In one of the few examples, Sandhu et al. (2010) showed that the economic value of biological pest control, soil formation, and mineralization of plant nutrients on organic farms was $86 per hectare per year more than on conventional farms. Our review strongly suggests that other farming systems are likely to see similar increases in value from organic farming practices. Determining the economic value of biological control and pollination in more farming systems would provide a strong financial incentive for farmers to endure the difficult 3-year transition to organic farming. This is

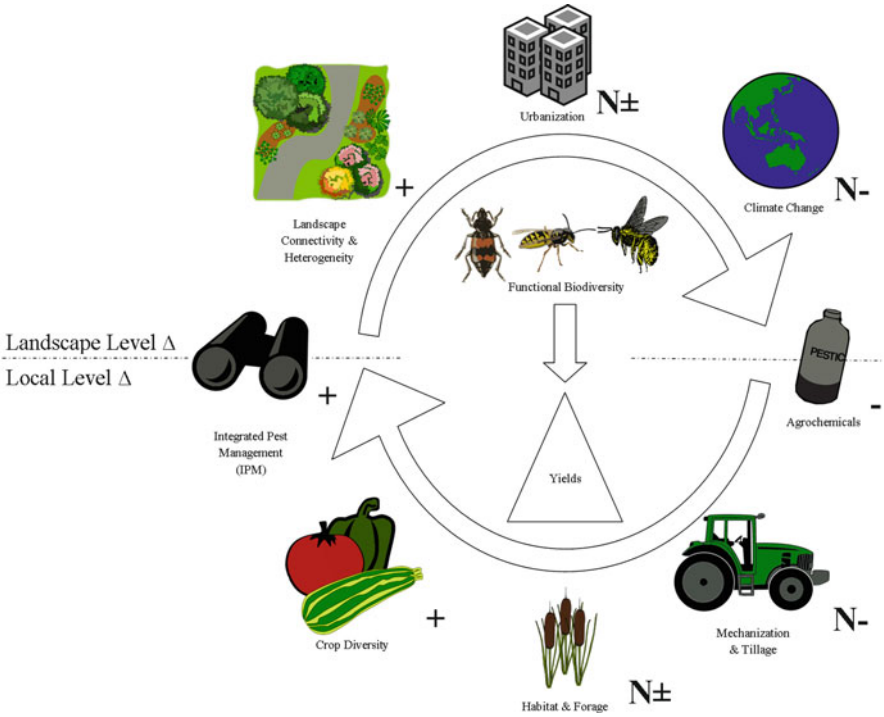

Fig. 3.4 Local and landscape factors that influence change (Δ) in biodiversity and ecosystem services on organic farms. Factors with positive (+) and negative (−) symbols have strong evidence to support effects on diversity and ecosystem services. Neutral (*N*) indicates factors where evidence is lacking. Some neutral factors may have conflicting evidence indicated by both a positive (+) and a negative (−) symbol or have a (+) or (−) based on the trends in the literature

particularly true considering that organic farming systems have been shown to be significantly more profitable than their conventional counterparts, even when ecosystem services are not considered (Crowder and Reganold 2015).

3.6 Conclusion

Conservation of natural enemies and pollinators on organic farms requires a multi-scale approach in which on-farm and landscape-level conservations are of equal importance (Fig. 3.4). There will be no substitute for both levels of conservation, since both tactics are essential to the longevity of natural enemies and wild bees on farms (Fig. 3.4). Conservation of natural enemies and pollinators is tightly linked with pest management practices on farms. Reduction in synthetic chemical use and promotion of biological control are likely to benefit pollinators. However, more research is needed to identify the particular practices that promote both of these beneficial groups. Organic growers and regulators must work together to reduce the intensity of their practices both on farms and across landscapes (Fig. 3.4). This would create temporal and spatial stability of nest and floral resources for the holistic conservation of natural enemies and wild bee species and the critical services these species provide for pest management and sustainability.

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Chapter 4
The Evolution of Alternative Control Strategies in a Traditional Crop: Economy and Policy as Drivers of Olive Fly Control

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Abstract The present essay links historical socioeconomic processes with pest control activities and research and development (R&D) trends in plant protection. We selected the olive orchard agroecosystem, especially in Southern Europe, as a model system. We specifically followed the evolution of olive fly (*Bactrocera oleae*) control strategies and research activities and linked them with economic processes in the producing countries and with European policy directives. Our analysis includes the period following the Second World War and until recent times. Our main aim was to understand the socioeconomic forces that shape agroecosystem management, especially pest control. Although we only developed the case for the olive fly in Southern Europe, we believe that most human agricultural environments are subjected to similar economic, social, and environmental processes and forces. This historical account shows the complexity involved in the management of the agroecosystem and the effect of global and local factors on plant protection activities

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and developments, highlighting the need for a holistic approach when agricultural and research policies are formulated.

4.1 Introduction

Plant protection does not occur in a vacuum. Pest control is an important activity of plant production, and as such it is completely influenced by the economic, and political, milieu in which it is practiced. Control methods or strategies are adopted and applied by individual growers and/or local cooperatives as a function of the household economic situation, the economy of the region, the economic prospects of the crop, the farmer perception of risk, as well as economic incentives and regulatory policies (Nestel 1995; Zadoks 2002). This relationship was recently emphasized in the study of Bebber et al. (2014) that showed the association between economic development and the global geographic distribution of crop pests and pathogens. Adoption of pest control strategies is also highly linked to the marketing strategies and interests of private corporations and industries and on government policies promoted through credit, extension, and research lines (Dent 1991; Norgaard 1976). As an example, a large portion of the research in plant protection has been driven by private interest and national policies that promote the utilization of inputs into agricultural production (Levins and Lewontin 1985). Research in plant protection has also been fueled and implemented by international organizations as part of regional agreements and the need to solve problems that transcend national boundaries. The program and partial implementation of the sterile insect technique to control the Mediterranean fruit fly in the Near East region, as an example, were strongly supported by the International Atomic Energy Agency (IAEA), and the program developed thanks to the positive political environment that reigned in that region at the end of the last century and due to the common interests between neighboring states to solve plant protection problems (IAEA 1997).

Olive cultivation and utilization is as old as Mediterranean civilizations. Olive oil has been produced and utilized since ancient times, and it is mentioned in many tales and old scriptures. Although olive cultivation has been practiced for more than 6000 years in the Mediterranean Basin (Besnard et al. 2013), the origin of the lineage is the wild oleaster shrub, possibly originating in Central Africa (Levinson and Levinson 1984). The way the plant winded up into the Mediterranean is not known, but the result of its domestication is the present *Olea europaea* from which all of the olive oil in the world derives (Tzanakakis 2003). Production of olive oil throughout history has been driven by the market. Production in the past was directed, in general, to the local market and to the farmer's household. In historic time, some of the production was also "internationally" traded through the Mediterranean Sea to Africa and Europe. During more recent times, especially during the second half of the last century, trade of Mediterranean olive oil globalized. The sharp increase in olive production during the second half of the last century is mainly the response

of growers and central administrations to the increasing global demand for olive oil and marketing campaigns that highlighted the benefits of this product (Katsoyannos 1992). Although certain areas of the Mediterranean largely modified their landscape to accommodate land for the increasing demand of olive oil in the world, becoming super-intensive modern plantations, in many places of the basin, production is still being practiced following traditional cultivation procedures, and in many cases olive production is still kept for domestic consumption (low-input traditional plantations) (Beaufoy 2001).

The key pest of olives in the Mediterranean and other areas of the world, like California, USA (Rice 2000), is the olive fruit fly, *Bactrocera oleae*. The olive fly originated from Central Africa together with its main hosts (Metcalf 1990; Nardi et al. 2005; Tzanakakis 2003, 2006). When the olive fly arrived to the Mediterranean is not known, but it is probably as ancient as the domestication of the crop (Nardi et al. 2005). Reproductively mature and mated female olive flies search for appropriate olive fruit to lay, usually, a single egg per fruit. Eggs are laid in the mesocarp of the fruit, and larvae feeding in the flesh (leaving a tunnel) lead into severe damage that may be intensified by secondary infestation of bacteria and fungi. Yield loss and economic damage is associated to both early fruit drop and microbial transformation of the mesocarp and acidification of the olive oil that diminish its qualitative properties and market price (Neuenschwander and Michelakis 1978). If remain unmanaged, infestation rates inflicted by the olive fly can reach up to 100 % (Tzanakakis 2003, 2006).

The olive fly is known and mentioned since ancient times (Daane and Johnson 2010). However, there is hardly any quote in these old scripts of human interventions against olive flies. Control activities exerted directly against the olive fly are probably a more modern aspect of the crop and can probably be dated back to the early twentieth century (Silvestri 1913). Intensification in the application of control tactics against the olive fly and the development of research agendas for this pest occurred mainly during the second half of the twentieth century, after the Second World War (WWII). This intensification in research and development (R&D), and application of control methods, is mainly linked to the economic resurgence in Europe after the WWII and the commercialization and expansion of the crop and the socioeconomic processes occurring in the Mediterranean during this period. Furthermore, pest control in general, and olive fly control in particular, has been also dependent of the availability of different technologies in a given moment. For example, the current use of precision agriculture applied to pest control has been possible only recently due to the development of highly sophisticated methods for data process and telecommunication. The present essay intends to narrate key aspects in the application of control methods and research strategies against the olive fly in some major producing areas of the Mediterranean. Our main aim is to show the link between R&D and control strategies against the olive fly with the economic and social tendencies of the Mediterranean during the second half of the twentieth century.

4.2 Economy and Olive Cultivation in the Mediterranean Basin After WWII

Most of the commercial olive production in the world is mainly concentrated in the Mediterranean basin, especially in Southern Europe. In the 1980s more than 98 % of the world olive oil was produced in the Mediterranean, with more than half of the production occurring in three main European countries: Greece (14.7 %), Italy (22.6 %), and Spain (23.5 %) (Katsoyannos 1992). In the Eastern and Southern Mediterranean countries, production of commercial olive oil during the 1980s was of importance in Tunisia and Turkey (7 % and 4 % of world total, respectively) (Katsoyannos 1992). At that time, some other emerging olive oil-producing countries included the Arab Republic of Syria and Morocco (Katsoyannos 1992). Present production volumes still indicate that Southern Europe is the main world area of olive oil production (Fig. 4.1). After the 1990s Spain was steadily increasing its olive oil production, quadruplicating the produced amount by 2010 (Fig. 4.1), and becoming the main olive oil producer in the world. Italy and Greece kept production at around the same level (Fig. 4.1). During the last 5 years, 95 % of the world olive oil has been produced in Spain (61.6 %), Italy (21.1 %), and Greece (13.5 %) (www.internationaloliveoil.org), highlighting their hegemony, especially that of Spain.

Increases in olive oil production can be obtained by incrementing land devoted to the crop or by intensifying the cultivation, mainly with agronomic and technical modifications. Spain is a good example of the intensification of the crop, which included changes in agronomic practices, irrigation, and fertilization and an increment in plant density. In approximately 60 years following WWII, olive oil production tripled in Spain, while land cultivated with olives expanded only by 10 % (Fig. 4.2a). Yield increments during this period raised from an average of 180 kg/ha in 1951–1960 to 500 kg/ha in 2001–2010 (Fig. 4.2a). Irrigation and plant density

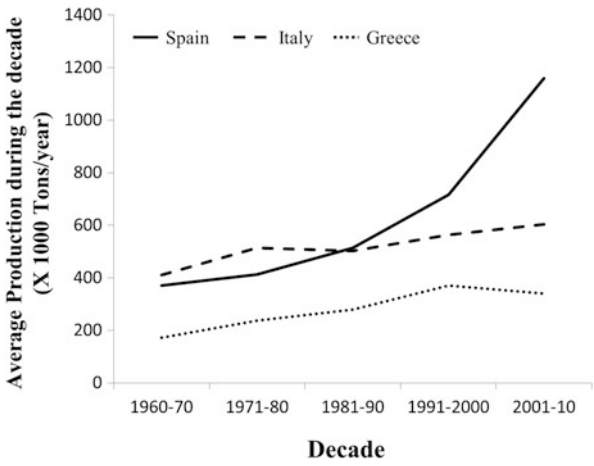

Fig. 4.1 Production trend of virgin olive oil in the Mediterranean Basin (Italy, Spain, Greece) from 1960 to 2013, (FAOSTAT 2015)

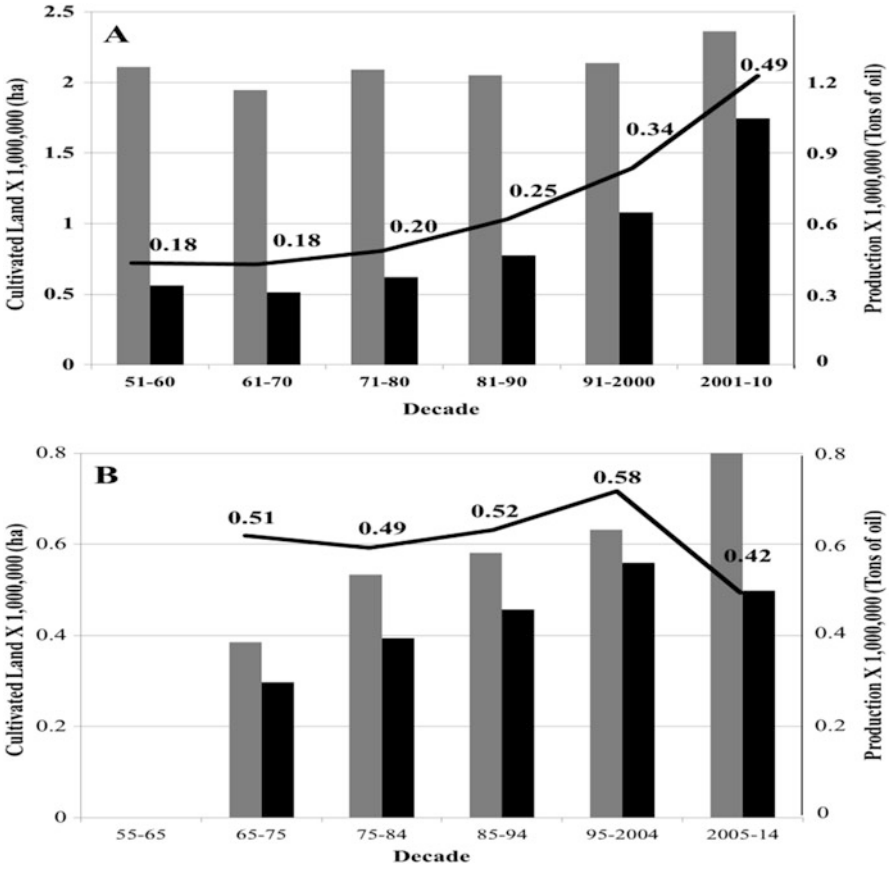

Fig. 4.2 Changes in cultivated land (*black bars*) and olive production (*gray bars*) in Spain since the WWII (**a**) and in Greece (**b**). *Line* shows the increments in yield since the1950s (*numbers on the line*, yield in tons/ha) (Data source from EUROSTAT (2015) for both Spain and Greece; Gomez et al. 2014 for Spain; Giannopoulou 1990 for Greece). Calculations of land under olive cultivation for Greece were derived from several sources and estimated as follows: for 1965–1975, the estimation came from Greek statistics on number of olive trees and the assumption of a density of 200 trees per hectare. The assumption was checked with data on number of trees for later years, when land area statistics existed for Greece (i.e., in Giannopoulou 1990 and EUROSTAT 2015). Estimations for the years after 1995 were obtained from EUROSTAT (2015). The estimated amount of land for the period 1985–1994 was assumed as being the midpoint between the previous statistics in 1985–1995 (Giannopoulou 1990) and the posterior available statistics for 1995 found in EUROSTAT (2015)

constitute two structural changes that highly modified the ability of Spain to increase olive oil production: by the end of 2010, almost 20 % of olive production in Spain was under irrigation and more than 50 % of the olive plots have tree densities that surpass 100 trees/ha (28 % of the olive cultivated land has densities of >200 trees/ha) (Gomez et al. 2014). This partially resulted from the establishment of corporate

agriculture in Spain at the beginning of the twenty-first century. This type of olive land exploitation already constitutes around 40 % of the total production in Spain and is characterized by super-intensive management, hedgerow cultivation system, mechanized pruning and harvesting, and short life of the agroecosystems. At the same time, an increase in the demand of organic olive products created a niche for artisan producers and the preservation of small holds and high-quality brands. This later tendency is not characterizing only Spain but appears as a trend in all major olive oil-producing countries.

In contrast to Spain, Greece partially increased production by expanding olive cultivation from approximately half a million hectares in the 1970s to more than 800,000 ha in the last decade (Fig. 4.2b). Olive oil production increased at a comparable pace to land expansion, resulting in a similar yield throughout the last 50 years (Fig. 4.2b). It is important to note that yields per area in Greece were substantially higher to those of Spain during most of the studied period (around 500 kg of oil per hectare) and that during the last decade, Greek yield declined by 20 % (Fig. 4.2b). The differences between Greece and Spain are probably related to olive varieties and environmental conditions. On the other hand, the decline in olive production in Greece during the last 10 years might be related to a series of low-production years and the time needed for new plantations to achieve maximal production (most of the new plantations in Greece were established after 2003).

The increments in olive oil production and intensification, especially in Spain, were encouraged by global demand for olive oil, market prices, and the incorporation of Spain into the EU (Gomez et al. 2014). In addition, structural changes in olive oil production were stimulated from the adoption of the European Common Agricultural Policy (CAP) by Greece in 1981 and Spain in 1986 (Lefebvre et al. 2012). Commodity price was an important stimulus for the transformation of the olive oil sector in all Mediterranean countries. International market prices doubled between 1973 and 1979 and tripled by 1991 (Fig. 4.3). In addition, the adoption of the CAP by Greece and Spain provided the institutional framework to transform the sector. The CAP was established in 1962 as a postwar instrument to ensure a streamline food production for Europe. Its aims were to establish a unified market for the free movement of agricultural products in Europe, promote financial solidarity between member states, foster preference for EU products, and provide the agricultural sector with parity to other sectors of the society (Delayen 2007). Of the three main olive oil-producing countries, Italy was part of the CAP since its conception (1962). Spain and Greece joined the CAP after their admission to the EU in the 1980s. The main direction of the CAP regarding olive production was toward supporting large-scale producers by subsidizing part of their production volume and small-scale producers by subsidizing them per number of trees (Lefebvre et al. 2012). The “imposed” minimal price for olive oil had no effect in the 1980s and 1990s since market prices were well above the established ones. The effect of CAP on the olive sector was an intensification of the production through mechanization, irrigation, increments in planting densities, and a reduction in number of varieties. All these changes had an important effect upon the landscape and the agronomic practices of the crop, including crop protection.

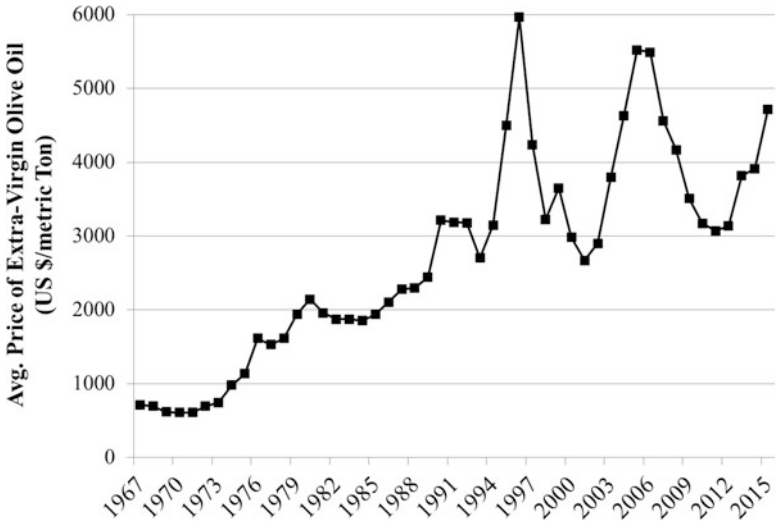

Fig. 4.3 International prices of extra virgin olive oil in the international market from 1967 to 2014 (Index Mundi 2015)

As shown previously, CAP importantly affected the olive sector both in Spain and Greece, where there was an intensification and/or expansion of the crop. In Italy, the effect of the CAP on the landscape and intensification was less pronounced (Lefebvre et al. 2012). After 1998, the original CAP arrangement was reformed by decoupling the payments from the produced volumes (Delayen 2007). Spain, however, decided to keep the arrangement as previously set, while Greece decided to support the conservation of traditional production systems and the production of high-quality olive. The new rules supported environmental protection practices, organic production, and biodiversity conservation. This policy was also applied to R&D funding through the LIFE Programme of the EC (Lefebvre et al. 2012). It seems that the continuation of high market values (Fig. 4.3) at the beginning of the new century still drives tendencies for the expansion and intensification of olive cultivation in both Greece and Spain (Fig. 4.2). However, big fluctuations in prices after 1990 might have had mixed effects on management and intensification trends.

Expansion tendencies of olive cultivation and commercialization in Southern Europe followed the economic processes in the old world. Europe's economic recovery after WWII was slow and dependent on the allies' plans (Marshall Plan) to restructure and support destroyed economies in Western and South Europe (Jackson 1979). Per capita gross domestic product (per capita GDP) started to increase in important olive oil-producing Mediterranean countries only after the 1970s (Fig. 4.4). In Greece, Italy, and Spain, per capita GDP doubled from 1970 to 1980 and increased to approximately 15,000 US dollars per capita by the end of the last century (Fig. 4.4). This tendency continued until 2008, when the eurozone entered a recession period that strongly hit these three main olive-producing countries;

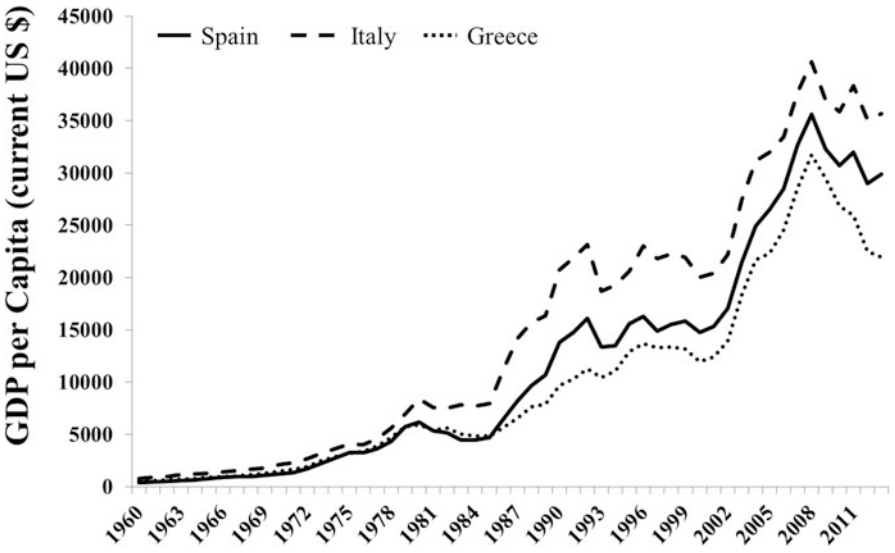

Fig. 4.4 Per capita gross domestic product (in current US $) in Spain, Italy, and Greece since 1960 (World Bank 2015)

between 2008 and 2012, the three economies suffered a loss of more than 10 % in GDP. Throughout the post WWII period, Spain and Italy showed a stronger economic performance than Greece (Fig. 4.4). The severe deterioration of the Greek economy between 2008 and 2012 reduced the per capita GDP by more than 20 %, a reduction larger than those experienced by Italy and Spain (Fig. 4.4).

To facilitate further discussion related to olive fly control, we decided to divide the postwar era into four major periods (Fig. 4.5): (1) Postwar economic stabilization (1945–1980); (2) effects of the Common Agricultural Policy (1980–1995); (3) stricter EU legislation on the use of pesticides, environmental protection, and control of toxic residues on agricultural products (1995–2008); and (4) Euro-crisis (from 2008 to the submission of this paper). The four periods were divided based on both economic trends and policy landmarks. Thus, the economic stabilization period is accompanied by the effects of the Marshall Plan throughout Western Europe which contributed to an economic and political stabilization of the region (Jackson 1979) and which resulted in the substantial growth of Southern European economies (Fig. 4.4). During this period, Spain enters the EU (1986). In the 1980s, the CAP is implemented by Greece and Spain, having an important impact upon olive production and retransformation of the sector, and in the economies of olive-producing countries. During the mid-1990s, R&D (through LIFE Programme) is redirected to finding alternative production systems and pest control methods that are less damaging to the environment, especially to water resources and human health. This, together with the ban on the use of certain pesticides (such as DDT in the 1980s, malathion in the early 2000s, and other organophosphates),

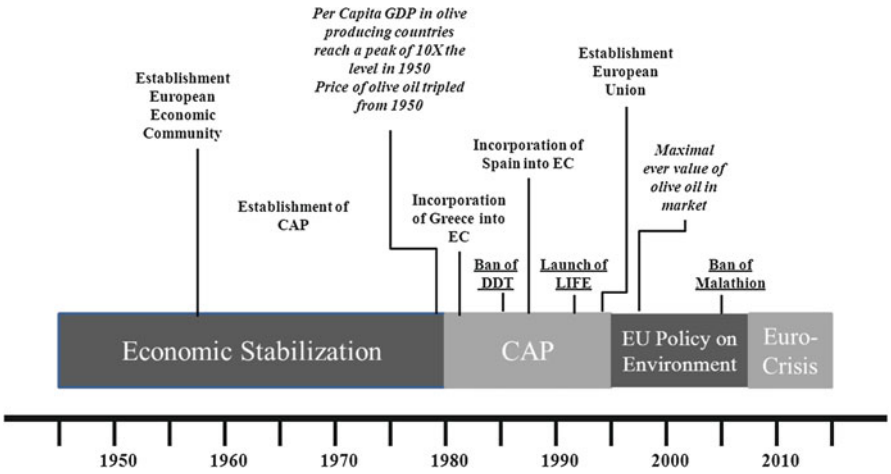

Fig. 4.5 Diagram showing the post WWII period and the division of this period in subperiods based on economic and political trends. The diagram shows several economic and policy landmarks useful for the discussion of the essay

and the imposition of stricter legislations in Europe since the 1980s, leads to agricultural producing countries to look for substitutes that will provide solutions to pest problems (Hislop 1993; Valerio 1994; http://www.pan-europe.info/Resources/Links/Banned_in_the_EU.pdf). Finally, in 2008 the Euro-crisis hit all olive oil-producing countries, changing the ability of these countries to invest in R&D, produce olive oil, and control insect pests.

4.3 Historical Overview of Olive Fly Control as Related to Economic Trends in the Mediterranean Basin

4.3.1 Available Pest Control Tools During the Economic Stabilization of Southern Europe

First organized attempts to control the olive fruit fly in the Mediterranean appeared at around WWI. Those attempts were based on the Berlese method, which consisted of spraying the olive trees with a mixture of lead arsenate and molasses (Haniotakis et al. 1986). This methodology provided limited control of the pest and was soon substituted by more potent insect-killing agents, such as nicotine and copper (Casida and Quistad 1998). The synthetic organophosphates and organochlorinated toxic chemicals, which derived from WWII, were introduced into agricultural practices during the 1940s and 1950s, becoming widespread insecticides by the end of WWII (Casida and Quistad 1998; Yu 2008). Notably DDT, parathion, and malathion

became widely used in olive orchards from the early 1950s to control the damage inflicted by the olive fly. Simultaneously, molasses were substituted as baits by protein hydrolyzate or ammonia-releasing salt solutions (Haniotakis et al. 1986).

Throughout most of this early period of olive fruit fly control, pesticides were applied either in combination with baits ("bait spraying"), mainly molasses, or without bait in the form of cover spraying. In the case of bait sprays, the adults are the main target, while cover sprays target all the stages of the insect, including the larvae feeding inside the fruit (Tzanakakis 2003). Application methods followed the technological innovations available at the time. Pesticide applications in the early times involved ground treatment of individual trees by people walking on the field with knapsack sprayers. Later on, tractors equipped with spraying appliances (compressors, tanks and multiple nozzles) substituted the manual applications. Tractor-driven applications were only possible with farmers and organizations having the adequate means and where the landscape allowed so (Hislop 1993). The use of aerial spraying with small airplanes and helicopters was adopted during the 1960–1970s and derived from other extensive crops (e.g., cotton and maize) outside of the Mediterranean (Lavers 1993). This practice was soon discontinued due to public environmental concern.

4.3.2 Olive Fly Control During the Intensification and Expansion of the Crop and the Incorporation of Greece and Spain to the CAP

Since the adoption of synthetic insecticides with and without attractants, spraying against the olive fly was conducted year-round following a calendar date approach. This application approach followed the philosophy of "no insect goal" (Arundel 1948). This concept, which was adopted around the globe during that time, intended to completely reduce insect pests and insect-borne diseases by indiscriminately applying potent insecticides like DDT. In olive orchards, this trend continued for approximately three decades. During this time, however, knowledge on the biology and ecology of the olive fly dramatically increased, fueling spraying from a calendar and regular basis to applications based on monitoring and the ecology of the fly (i.e., IPM). As an example, the discovery of olive fly reproductive dormancy during the early fruiting stages proved that spraying early in the season was redundant (Economopoulos et al. 1982; Kapatos and Fletcher 1986). Similarly, the identification of better attractants and trapping systems (Economopoulos et al. 1986; Haniotakis et al. 1986) provided the means for more efficient monitoring that could be used by the centralized management system to make spraying and decision-making more efficient and effective.

Management of the olive fly in Greece is centrally coordinated since the 1950s. The Greek national program against the olive fly was established in 1953 with a specific legal framework that, with some small modifications, is still in place today.

In brief the program is coordinated by the Ministry of Agricultural Development and Food, which: (a) provides financial support and manages the budget for the project, (b) hires the essential seasonal personnel (trapping personnel, sprayings contractors), and (c) provides the essential consumables that are purchased following open procurements. The implementation of the project is assigned to regional authorities (Directorates of Rural Economy and Veterinary), which establish the protocol for population monitoring and bait spray application following standard procedures. Since the start of the project, the olive fly population monitoring is based on adult trapping in glass McPhail traps (McPhail 1937) baited with an aqueous solution of 2 % ammonium sulfate or ammonium bicarbonate and fruit sampling to determine fertile (accompanied by the presence of immature) or total infestation that in addition includes oviposition stings that are not accompanied by eggs, larvae, or pupae in the fruit. The trapping protocol includes deployment of one McPhail trap at every 2000 trees that is checked every 5 days to count the captured adults and replace the ammonia solution. The trapping grid can be denser with one trap per 1000 trees in areas that historically suffer of high infestation rates. The program is financially supported by the Greek government and indirectly by the olive growers who contribute approximately 2 % of the total value of the produced olive oil as a tax fee. From 1950s to 2009, the application of bait sprays included organophosphates, such as fenthion and dimethoate, which is still registered for bait and cover sprays on olives. Since 2007 spinosad has been included in the list of alternative insecticides, and since 2008 and 2009, the pyrethroids alpha-cypermethrin and beta-cyfluthrin. Molasses syrup was the main bait since the beginning of the project, but in recent years it has been replaced by protein-based attractants such as Dacus bait and Entomella (commercial names of plant protein-based lures). Bait sprays are since 1997 applied from the ground only because areal insecticide applications were banned that year following environmental and health concerns (Tzanakakis and Katsoyannos 2003). Figure 4.6 gives an example of the importance of the centralized management and the expansion of its activities during the 1980s and until today. Data is for the prefecture of Larisa (central Greece): The area covered by the national program against the olive fly was rather stable before the 1980s, at approximately 4650 ha, increasing in the 1990s to 5714 ha and in 2014 to 6300 ha (ca. 1 % of total olive-producing land). The same tendencies can be found in all of the other producing areas of Greece (data not presented).

In 1962 the Spanish central government established by law areas of olive fly compulsory control (S.G.S.H.V.F 2012), initiating the Spanish centralized management mechanism. This mechanism was also the basis for the development of a coordinated olive fly research plan in Spain, which was launched in 1979. Similar to the Greek experience, the centralized management of the olive fly was already functioning when the EC through the CAP directed the need of coordinated control of the pest in olive-producing countries in 1989/1990. As in Greece, the financial resource for the centralized management of the fly came from a 2 % production tax. The 1989/1990 EU directive was used as a baseline for launching the Spanish national program for the improvement in the quality of olive oil production. Further financial aid for the program came from European funds. Since 2005, the plan

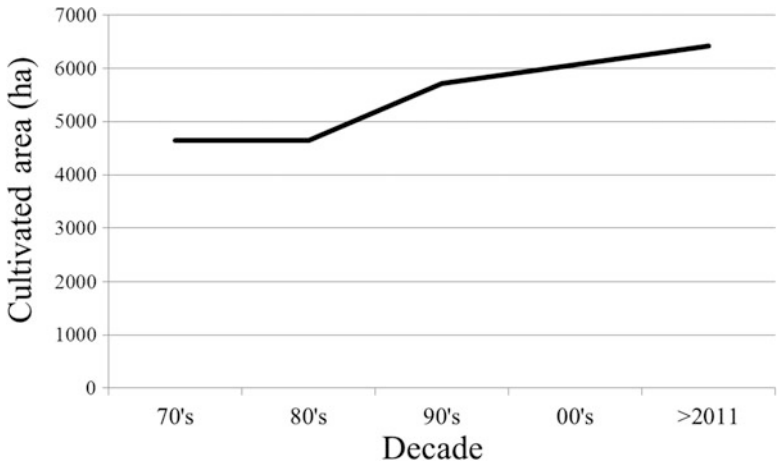

Fig. 4.6 The area of olive orchards included in the national projects against the olive fly in the prefecture of Larisa (central Greece) from 1970 to 2014 (Data provided by the Directorate of Rural Economy and Veterinary of Larisa)

became the "Red Dacus" which aim is to centrally detect and assess the risk and coordinate the control of the olive fly using the most efficient methods (S.G.S.H.V.F. 2012). The centralized management in Spain operates similar to the one described for Greece.

4.3.3 European Concern over the Environment and Euro-Crisis

As a result of the rising pressure of environmental organization during the 1980s, and changes in environmental policies and public awareness in Europe, the aerial spraying was completely abandoned in the olive sector (Tzanakakis and Katsoyannos 2003). Consumers' concern in Europe over olive oil-production methods, and specially pest control, also affected other aspects of olive fly pest control and directed research into the study of alternatives methods. During that period, corporative R&D started to introduce biorational killing agents (such as pyrethroids and spinosad) into the market. In addition, the accumulated knowledge on the biology of the olive fly prompted the Greek Ministry of Agricultural, Development and Food to invest on the development and commercialization of lure and kill devices, which started to appear in the farmers' fields at the end of the previous century (Broumas et al. 2002). Field trials supported by the Ministry of Agriculture were substantially reduced following the economic crisis that prevailed since 2009. Finally, the demand for organic olive products stimulated farmers to implement alternative control methodologies, such as mass trapping and lure and kill and,

more recently, kaoline film of the tree. This shift in production and pest control, which in Greece currently comprises around 10 % of the cultivated area, required the abandonment of the centralized management system toward individual-based monitoring and control schemes.

4.4 Research on Alternative Control Strategies: Economy and Policy

General government spending on all types of (i.e., science and technology) R&D activities since WWII in olive-producing countries, especially Spain and Greece, was below the world average (OECD 2015). Greek investment on R&D has been below 1 % of the GDP for all this period (an average of 0.5 %), while Spain's investment steadily increased after 1995, reaching a level of 1.35 % of the GDP in 2010, which is close to half of the world average (OECD 2015). Information regarding internal spending on Agricultural Sciences R&D, and specifically on research on the olive fly, is harder to obtain. Data for Spain shows that government spending on Agricultural Sciences R&D has been in general below 10 % (around 7 %) of the total investment in R&D, except for a few years in the 1970s and 1980s when investment in Agricultural Sciences R&D exceeded 10 % (National Statistics Institute of Spain 2015).

The assignment of research resources by the European Commission to research on the control and biology of the olive fly has been slim. Between 1985 and 2014, we were able to find only six projects financed by the general frameworks of the Research Council that contemplate olive fly control or has some relation to the study of its biology (CORDIS 2015). Earlier EU-funded projects (from 1986 to 1993) focused on the investigation of alternative (to chemical control) methods of olive fly control, while more recent ones (after 2005) included the development of wireless monitoring systems (CORDIS 2015) as part of an integrated pest management strategy. Besides EU, the International Atomic Energy Agency (through the Department of Technical Cooperation and coordinated research programs), FAO, and local governments have also provided resources and supported research on olive fly control, especially in non-EU Mediterranean countries (IAEA 2015).

An exploration of scientific publications on the olive fly control and biology since the 1950s resulted in 551 papers (Scopus® Data Base 2015). Of these, 331 papers were clearly related to pest control (the other 220 papers were more basic studies dealing with physiology, molecular biology, and ecology of the olive fly). Most of the reported studies belong to the post-1995 period (Fig. 4.7). Considering key words and not entering into the details of the content of the studies per se, lure and kill and mass trapping included the largest number of publications (105). It was followed by biological control (81 papers), chemical control (57), SIT (45), and precision pest management (42). The figure also shows the dominance of research topics during the analyzed period. As an example, while chemical control

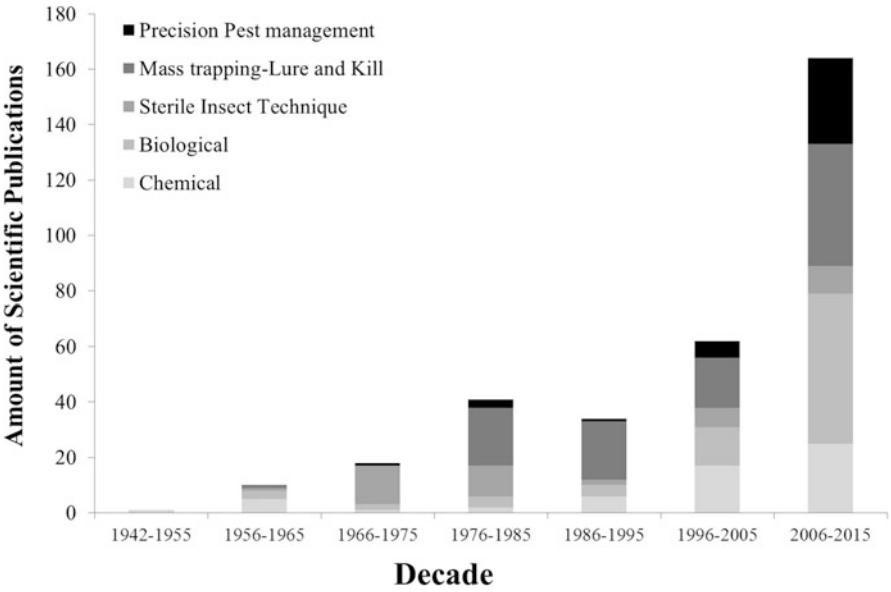

Fig. 4.7 Number of publications linking olive fly and control since 1950 per control type category and period of publication (Scopus 2015)

studies predominate during the first decades, lure and kill and mass trapping became important research subjects after the 1980s. Research on SIT became an important subject of research during the 1960s–1980s and again at the beginning of the new century (Fig. 4.7). Precision pest management of the olive fly starts to become an important research paradigm during the new century (Fig. 4.7). Biological control has also been thoroughly investigated, and a comprehensive review has recently appeared (Daane and Johnson 2010). In the following sections, we will summarize research and developments concerning the control of the olive fly using biological control, the sterile insect technique (SIT), mass trapping, and lure and kill and the recent research direction toward precision management of the olive fly.

4.4.1 Biological Control

First surveys for olive fly parasitoids were conducted by Silvestri (1914) in South Africa, where the natural enemies of the olive fly keep the populations low. More recent foreign exploration for natural enemies of the olive fly include that of Neuenschwander (1982), who described the parasitoid fauna of the olive fly more extensively, and the one conducted by American scientists in Africa, India, Pakistan, and China after the establishment of the olive fly in California (Daane and Johnson 2010).

The largest-scale releases of mass-reared parasitoids were conducted in a Sicilian island by Monastero and Delanoue (1966a, b). This field trial in Lipari Islands included the release of more than eight million *Psyttalia concolor* (Hymenoptera: Braconidae) to protect 250,000 olive trees. The study showed an impact on olive fly and recovery of parasitoids after each inundative release. However, the impact only lasted for the release season, and the parasitoid was in general unable to keep in check the olive fly populations (Daane and Johnson 2010). After that large-scale field trial, different species of parasitoids have been released in Greece and California. Their impact on olive fly populations, however, has been low.

Although natural control seems to be effective in South Africa, the impact of biological control of the olive fly in the Mediterranean and California has been limited. Daane and Johnson (2010) suggest that the hypothesis of Latiere (1917) may in fact provide an explanation to the reasons precluding the effectiveness of biological control in commercial orchards of the Mediterranean and California. Latiere's hypothesis suggests that parasitoids originating from Africa, where the olive fly infests wild olive verities with small fruits, are unable to effectively attack the larvae of the olive fly in the more fleshly European cultivars. That is, larvae are able to escape the short ovipositor of most parasitoid species.

The last intensive project in California was not successful (Daane and Johnson 2010) and probably reduced the hopes of using classical biological control as a method to control the olive fly. Currently, we are not aware of any other important project on biological control of the olive fly in the Mediterranean and California.

4.4.2 The Rise and Decline of Olive Fly SIT

The sterile insect technique is an environmentally friendly pest and vector control method that employs regular releases of big numbers of mass-reared and sterilized individuals of the target species (when technically feasible, preferably males) into a geographically delineated area (Knipling 1955, 1959). The released sterile males compete with their wild counterparts for inseminating reproductive mature feral females, transferring sperm carrying dominant lethal mutations that result in the production of unviable zygotes and the oviposition of infertile eggs (Robinson 2005). Knipling's model suggests a quick decline and complete elimination of the wild population if the sterile released to wild male ratio is high. However, in the practice of SIT, suppression and decline of the population of feral flies are a much longer process that hardly leads to eradication. The first (and most successful to date) application of this methodology was the eradication of the parasitic screwworm fly *Cochliomyia hominivorax* (Coquerel) from the North Americas in a massive campaign that started at the end of the 1950s, and it is still ongoing (Wyss 2000). Currently the screwworm distribution area is confined to South America, with the maintenance of a buffer zone of regular sterile releases in Panama. The particular application has proved to be very cost effective: a cost of 13 million

US$/year in facilities and personnel vs. the estimated savings of up to 1 billion US$/year in damages to animal capital (Vargas-Teran et al. 2005).

Although the induction of sterility with the use of radiation was already proposed early in the twentieth century (Muller and Altenberg 1930), R&D started at the end of WWII. The emerging use of nuclear power for both, military and peaceful, applications provided a strong incentive for the development of nuclear-related technologies, with SIT being a promising example of agricultural and peaceful application of nuclear technologies (Krafsur 1998). SIT features some unique characteristics that differentiate it from other pest control methods: it is logistically complex and cannot be applied individually by single farmers but only in the framework of area-wide control programs, often under the supportive active participation of national and international authorities (Suckling et al. 2014). It is also very demanding in R&D. Every time a new species is targeted with this method, a considerable amount of research is required to define radiation sterility doses, mass-rearing methodology, effective release techniques, maintenance of quality in the field, etc. (Dyck et al. 2005). These perquisites usually mandate years of preparation before any actual release is conducted. Thus, SIT use is not only dictated by sheer economic parameters (e.g., agricultural product values that might fluctuate yearly) but also by long-term decision-making and policies at the national and, more often, multinational level.

Following the successful case of screwworm eradication (Vargas-Teran et al. 2005), and partially due to the general high costs of chemical control and labor, and the considerable high value of olive products, olive fly was one of the first insects to be considered for the expansion of SIT application as a control or eradication method. In a meeting with the Greek Atomic Energy Commission authorities in Athens on February 1959, Dr. H.J. Gomberg, of the Michigan State University, suggested that the olive fly problem was of similar nature with the screwworm problem, and it could be considered for SIT development and application (Economopoulos 2001). This was one of the first attempts of the USDA to expand the use of the newly applied SIT to other pest species. It should be noted that by that time (early 1960s), the olive fly was not posing a direct threat to the US olive cultivation industry since it was not present in California, the major olive-producing US state. The olive fly was introduced into California much later, toward the end of the twentieth century (Rice 2000). Since this early contact on behalf of USDA, and with the support of UN organizations (FAO and IAEA), extensive research has been devoted in the Mediterranean Basin in the development of SIT against the olive fly. This research has been conducted in many laboratories, with the most prominent one among them being the NCSR Demokritos entomology laboratory in Athens, Greece. Overall, a wide range of research topics has been investigated during a period of about 20 years, resulting in the establishment of basic knowledge that was related, but not limited, to the following important aspects of olive fly biology:

(a) Mass rearing: this included the development of larval and adult diets (Moore 1962) and caging and egging system for the flies (Tsitsipis 1982). Due to the

monophagous nature of olive fly larva, a very specific, and special, diet needed to be developed for mass rearing. In addition, R&D concentrated on adjusting all the details of the cages, especially egg collection methods (Tzanakakis 1989).

(b) Olive fly radiation biology: the R&D on olive fly sterilization initially concentrated on studying the use of chemosterilants (Haniotakis and Galachtiou 1972) and later gamma irradiation as standard method for successful sterilization of the olive fly. The research resulted in standardizing irradiation doses (under nitrogen or carbon dioxide atmospheres) that effectively sterilized males without causing major negative somatic damage (Economopoulos 1977a).

(c) Behavior: the mating rhythm of the olive fly and the factors affecting it were a very important aspect of the SIT R&D package (Loher and Zervas 1979). It has been proven in the past that slight alterations in the complex mating behavior of the fly could be responsible for reduced efficacy of the release of sterile males (Zervas and Economopoulos 1982).

(d) Colonization effects and genetics: genetic studies using olive fruit fly were among the first ones reported for fruit flies (Krimbas 1963; Zouros and Krimbas 1970). Additionally, the olive fly case was one of the first demonstrations of the adverse effects of colonization upon the quality of the strains (Economopoulos 1977a), with the notable example of the rapid changes in allelic frequencies of basic metabolic genes (e.g., alcohol dehydrogenase) during the first few generations under laboratory rearing (Zouros et al. 1982, 1986; Konstantopoulou et al. 1996, 1999).

(e) Chemical ecology: the study of the mating system and the mass rearing of olive fruit fly in large cages led eventually into the research on the reproductive system of males and females, with the detailed description of the rectal glands (Economopoulos et al. 1971). Later, research led to the identification and characterization of the sexual pheromone blend emitted by mature females (Baker et al. 1980). The major pheromone component (1,7-dioxaspiro (5,5) undecane) was produced synthetically, and tested in the field (Mazomenos and Haniotakis 1985), and today is widely used for monitoring and in mass-trapping control systems of the olive fly in organic and conventional farms (see next section).

(f) Trapping systems: the research showing the dramatic changes during the colonization also provided detailed knowledge on the spectral sensitivity of the olive fly (Remund et al. 1981) and led into the identification of the most attractive colors (i.e., green-yellow) to be widely used later in monitoring systems. Important research has been conducted toward this aim, with the use of traps of various colors and shapes (Prokopy and Economopoulos 1975; Economopoulos 1989; Katsoyannos 1989).

The efforts to implement SIT on olive fly lasted approximately 20 years, before being abandoned due to unsolved issues in mass rearing, high cost, and low quality of the mass-produced flies. Low sterile fly quality was associated with the rather poor results from pilot field applications in olive groves of mainland Greece and in island situations (Economopoulos 1977a; Zervas and Economopoulos 1982).

Nevertheless, work on the olive fly nutrition, selection under artificial rearing, alterations in the olive fly symbiotic microflora, and other subjects, directly or indirectly related to SIT, continued in different laboratories (Konstantopoulou 1997). In the years of olive fly SIT hiatus, important research efforts also concentrated on the chemistry of the sexual pheromone(s) and its application for managing olive fly populations (Haniotakis et al. 1991; Broumas et al. 2002).

The beginning of the twenty-first century marked a resurgence of interest for olive fly SIT (Economopoulos 2001). There were several factors contributed to this renewed efforts: (a) olive fly populations were detected by 1998 in California, USA (following an accidental introduction), establishing rapidly and spreading to almost all olive-producing areas (Rice 2000); (b) market prices of olive oil (Fig. 4.3) and the intensification of olive cultivation provided new ground and opportunities for the use of novel methodologies for pest control; (c) the ban of organophosphate insecticides (among the major olive fly control chemicals for decades) from the EU zone (Fig. 4.5) created a vacuum in control methods and opened up needs for new developments; and (d) the advancement of molecular techniques, and especially genetic transformation, provided the tools for the establishment of new, improved, olive fly strains (Koukidou et al. 2006). With the use of specific transposable elements (Franz and Savakis 1991) and genetic markers, it has become possible to develop constructs that allow for easy identification of the mass-reared insects in the field, and most importantly, for easy genetic sexing at the early embryonic level, by eliminating females from mass production and releases (Ant et al. 2012).

The involvement of international organizations, such as FAO/IAEA that promotes SIT (Suckling et al. 2014), was crucial for the new initiatives regarding the olive fly. A good demonstration of this involvement is the initiation of many IAEA technical cooperation (TC) projects that helped to expand knowledge in new olive-producing countries and agroecosystems (e.g., arid environments in Israel, TC ISR/5/012) (Rempoulakis and Nestel 2012; Estes et al. 2012). This renewal in olive fly SIT was eventually accompanied with the interest of the private sector for future commercialization of the method (e.g., Bio-Fly company, in Israel).

This second period of interest for olive fly SIT lasted for about a decade and produced a wealth of knowledge (Dimou et al. 2010; Estes et al. 2012; Rempoulakis and Nestel 2012; Rempoulakis et al. 2014) but unfortunately did not lead to a large-scale implementation of this methodology. One of the reasons was the general financial declining situation of the south European countries following the financial crisis of 2008 (Fig. 4.4), which greatly reduced their power to invest in national research schemes and applications in large scale. That in turn discouraged the private sector for further investment in R&D and commercialization of the method. Also, despite the new findings, no major breakthrough in olive fly mass rearing and preservation of insect quality was established. As a result, the rearing of great numbers of insects continued to be costly and labor intensive. Finally, a pilot application in Israel during 2010–2011 did not provide spectacular results in infestation reduction (Nestel et al. 2012). Although olive fly research is currently ongoing with the use of new molecular methods, the main aim has focused into the discovery of mechanisms responsible for insecticide resistance (e.g., detoxification

enzymes) (Kakani and Mathiopoulos 2008; Pavlidi et al. 2013), genetic studies (Zygouridis et al. 2014), sexual communication (Mavraganis et al. 2010; Benelli et al. 2012; Canale et al. 2012; Levi-Zada et al. 2012), or the use of the insect as a subject for population dynamics and climatic and ecological modeling (Gutierrez et al. 2009; Ordano et al. 2015; Blum et al. 2013, 2015).

4.4.3 Olive Fly Lure and Kill and Mass-Trapping Methodologies Under Different Economic Settings

Since the beginning of the last century, various types of traps have been used for either fruit fly population monitoring or control purposes. For the olive fly, one of the most widely used trap types for monitoring has been the glass McPhail trap baited with various proteinaceous attracting substances (Economopoulos 1989; Mazomenos et al. 2002). In Greece, this trap is still being the primary mean of population estimation for the centralized olive fly monitoring and control system. Similarly, this type of trap was widely used during the 20 years of the national surveillance program for *B. oleae* in Spain (plan Dacus). The trap is relatively simple in operation, requires little specialization from the scouting personnel, and provides a somehow reliable estimation of the field population. However, plastic traps, especially handmade ones (such as the OLIPE trap type), are currently substituting glass McPhail traps (Tabic et al. 2011; Yokoyama 2014). The use of traps (sometimes the same monitoring traps) in population control is an idea that appeared several decades ago (Orphanidis et al. 1958), following the changes in the perception of pest control toward more environmentally sustainable methods and the development of more powerful, long-range, and more species-specific combinations of trap attractants. In addition, cost-effective industry technology was available for producing high number of plastic or cardboard traps with low price. Further, domestic materials (i.e., plastic bottles) were adapted to olive fly traps (i.e., OLIPE traps). The end of the DDT and organophosphates period and the substantial knowledge acquired from the studies of insect behavior (some of them related to SIT and other ecological studies as mentioned earlier) brought advancements toward the creation of new more-efficient trapping systems. In addition, the shift from the "zero insect" policy toward keeping the population below the economic damage threshold (i.e., IPM) affected significantly the management of fruit flies (Kapatos 1989), and the monitoring of the pest arose as a cornerstone of economic effective strategies.

Initial attempts to control the olive fruit fly by lure and kill methods can be traced back to the 1960s (Mazomenos et al. 2002). McPhail traps baited with a solution of protein hydrolyzate were used to attract olive flies (Orphanidis et al. 1958). Visual (yellow-color) sticky traps have also been used experimentally to control olive fly (Economopoulos 1977b), but as many authors have emphasized, these traps could be detrimental to beneficial insects (i.e., parasitic wasps) that also respond to the visual lures (Broumas et al. 1983; Kapatos and Fletcher 1983; Jones 1987). The

period starting from the late 1970s to early 1980s is when the knowledge of olive fly biology in food attractants, new trap designs, and synthetic pheromones started to show applied outcomes that could be capitalized in mass trapping. As a result of the identification and characterization of the olive fruit fly pheromones (Baker et al. 1980; Mazomenos and Haniotakis 1981, 1985), pheromone traps have been developed and tested as monitoring and control tools (Mazomenos et al. 2002 and references therein). Several studies have demonstrated the usefulness of the major compound of the mix (1,7-dioxaspiro (5,5) undecane) (common name "olean"), in baiting traps. Olean has been found to attract only the males of olive fly, making it difficult to target the egg-bearing, and exclusively damaging, females with the same trapping devise. As a result, in most of the cases, the pheromone is used in combination with some food lure (usually ammonium salts) that attracts females as well (Broumas and Haniotakis 1987). Traps baited with this combination have been used both for detection (Rice et al. 2003) and as mass-trapping devices aimed at controlling olive infestation (Navarro-Llopis and Vacas 2014 and references therein). The expansion of the European Union in the 1980s with the addition of Greece and Spain signaled a turning point in the olive fly control (see landmarks in Fig. 4.5). As mentioned earlier, together with Italy (that was part of the union since its conception), the three countries produced most of the olive oil of the world. Changes in olive orchard management, including plant protection especially against the olive fly, were significantly guided by the CAP through the incentive of alternative control strategies for the crop pests. Additionally, the European policy actions regarding the environment (a typical example of which is the LIFE Programme) promoted more environmentally friendly methods for olive fly control (Fig. 4.5). Overall, the abandonment of potent synthetic insecticides in combination with EU environmental considerations and policies (e.g., regulation on chemicals and residues and subsidies) created a vacuum where all the existing knowledge of olive fly biology could be utilized to provide alternative solutions. As a result, markets for new methods of pest control opened, and the private sectors invested in new products for lure and kill and/or mass-trapping applications. Some examples of lure and kill products specifically targeting the olive fly include the Eco-Trap® (Greece), OLIPE® (Spain), Dacus Trap® (Spain), Biofeed® (Israel), Magnet OL (UK/USA), etc.

The application and adoption of this alternative approach to control damage inflicted by the olive fly, however, depend on the economic conditions of the region and farmers, on the availability of specific markets for organic olive oil, and on the existence of subsidies (either from the state, international organizations, or local organization of producers) supporting alternative agricultural production. While in certain areas and economies an alternative control system is economic and feasible, in other areas the same system may become economically prohibitive. As mentioned earlier, the development and adoption of lure and kill systems against the olive fruit fly in Southern Europe have been supported by local governments and the EU commission through subsidies, high prices of olive oil in the market, and wealthier economies (at least until 2008). In contrast, low-income economies and farmers that do not have state subsidies, or subsidies from other organizations, find themselves

unable to adopt high-priced commercial devices. An example of this situation was experienced by some of the authors of this essay. During the last years, a project in Palestine favoring a local olive oil organization of producers (through the Japanese NGO, NICCOD) pursued the aim of producing "organic olive oil" for very lucrative markets. One of the aims of the project was to control the olive fly damage with lure and kill devices (Yasin et al. 2014). Field trials were initially performed using a well-known and broadly used commercial lure and kill device produced in Greece, the Vioryl S.A. Eco-Trap®, that combines the sex pheromone and a trophic lure in a bag with a contact insecticide (Broumas et al. 2002). The results of this trial were very good, and farmers were able to produce good-quality certified organic olive oil. The imported Eco-Traps were subsidized by the project, and based on a questionnaire applied to the beneficiaries of the project to measure satisfaction (Nestel unpublished survey), more than 90 % of the engaged farmers were willing to continue using the Eco-Trap but only if this was subsidized by the project or the state (the cost of alternative control using the imported Eco-Trap was prohibitive for the farmers). In order to find a solution, thus, an alternative lure and kill system, based on labor-intensive methods and not on capital-intensive systems, was tested. A yellow sticky trap system lured with ammonium salt and recyclable boards was tested as a cheaper and labor-intensive solution giving comparable results to the relatively expensive, and unaffordable to the Palestinian economy, Eco-Trap (Yasin et al. 2014). As demonstrated from this example, thus, adoptions of alternatives control systems are "economy dependent," and what is recommendable and possible for one economy may not be possible for other economy.

4.4.4 New Direction in Olive Fly Management: Precision Pest Targeting

Classic integrated pest management (IPM), or the control of agricultural pest populations using as a framework the temporal dimension and a combination of control tools and tactics, was the paradigm that prevailed during the second half of the previous century (Nestel et al. 2004). Classic IPM is based on forecasting models, monitoring of pests, and the application of control measures, which are mainly applied at the local farm level (Kapatos 1989). Since the beginning of the new millennia, and in conjunction with the development of digital geographic tools and models, IPM started to incorporate the spatial dimension, shifting the pest control paradigm to the newer evolving field of precision agriculture (Nestel et al. 2004). An earlier version of this new trend is the area-wide management approach, which incorporates both the temporal and the spatial dimensions beyond the local farm level (Klassen 2000; Lindquist 2000). The objective of area-wide control is "to reduce pest population within the target area to a non-economic level by attacking the entire insect pest population in the entire target area" (Lindquist 2000). The target "area" is usually a geographic continuum that includes agricultural

and nonagricultural elements in the landscape where the insect host can be found. The limits of the "area" are usually geographic or political boundaries. Area-wide, however, takes the geographic region as a whole and applies control techniques, such as the release of sterile flies, on all "areas" of the targeted region. In contrast, the new developing paradigm intends to more precisely target pests by following the development in space and time of the pest population and damage (Nestel et al. 2004).

Early attempts to manage the olive fly with the concept of precision targeting includes the work conducted in Italy by Petacchi et al. (2002) and Guidotti et al. (2005) and in Israel by Nestel et al. (2002, 2004). Petacchi et al. (2002) and Guidotti et al. (2005) developed a system that included geo-referenced data on farm damage that was provided weekly by agricultural extension people and a decision support system helping people involved to take decision on management at the local level. The study in Israel included an olive production farm in northwestern Israel (Nestel et al. 2004). Different olive orchards in this farm (with different varieties and agronomic practices) are scattered throughout the heterogeneous landscape and intercalated with other fruit crops, covering a relatively large area of several hectares. For the purpose of olive fly management, all the olive orchards were subdivided into management units of 0.2 ha. Management included monitoring of adult olive flies (with yellow sticky panels) and fruit damage. Olive fly management was based on the preventive use of mass-trapping devices (Eco-Trap®, Vioryl S.A., Greece) that were distributed throughout the farm from the beginning of the season. Intervention management through the season was based on locally incrementing the number of Eco-Traps in the management units that showed temporal hot spots of relatively high fruit damage. At the end of the fruit growing season, the precision targeting management of the olive fly resulted in a very low rate of damage (<7 % in highly susceptible varieties), allowing the production of extra virgin olive oil from the cultivated olives of the farm (Nestel et al. 2004).

Precision targeting of olive fly has also been applied in the monitoring of the fly. The knowledge of the spatial dispersion of the olive fly in Northern Greece (Kounatidis et al. 2008) provided the basis for a posterior analysis and development of monitoring zones, which are expected to have an economic impact on management (Castrignano et al. 2012). The proposal utilized the fact that olive fly trapping and spatial patterns have a clear seasonality which is related to elevation in the region (Castrignano et al. 2012). In a similar way, different attempts to predict olive fly phenology including spatial variables, such as altitude and distance from the sea, have been conducted (Petacchi et al. 2015). Finally, precision targeting of olive fly has matured up to a point that field data on damage and population trapping levels drives pesticide application services directly to the "hot-spot" area for "precise" spraying of pesticide against the olive fly (Pontikakos et al. 2010). The olive fly LAS (location-aware system) targeting may be such that only a few trees may be sprayed during an intervention event, which is remotely determined and driven by the expert from the office using Internet services (Pontikakos et al. 2010). This idea was further developed and fine-tuned through an EU project funded by the

ENPI-CBC Med Programme. The *FruiFlyNet* Project integrated the concept of LAS with the semi-automatic monitoring of the olive fly that used the developed Real-Time Insect Counting Traps, tailored for different species of fruit flies (Tsiligiridis et al. 2014). This project was a good example of implementation of technology (i.e., telecommunication) primarily not designed for pest control but rapidly adopted by the current IMP procedures due to the increasing pressure to reduce environmental impact of insecticide sprayings and the cheaper cost of wireless technology.

4.5 Concluding Remarks

As mentioned in the introduction, plant protection is a mirror of policy and economy, which can be viewed at different scales. As an example, national policies, like the establishment of a centralized monitoring and control system, may provide the background for the later formulation of policies that transcend the national boundaries. In the case of Greece and Spain, the already existing national policies served as the basis for the later application of the CAP guidelines to olive cultivation, which included the format for olive fly control. In addition to policy, olive cultivation and control were affected by economic incentives, such as national and EU subsidies to the crop and producers, and by international prices of the commodity. All these elements, together with achievements in R&D and with evolving environmental constraints, shaped the course of olive fly control during the last 60 years and continue molding the application of control methods and research directions.

Figure 4.8 shows the historic course of olive fly control in Southern Europe, highlighting the major policy, economic, and research landmarks since 1950. Although not exhaustive, they are helpful to narrate the complex setting in which olive fly control evolved during this period of time. As can be seen from the figure, three major periods can be distinguished. The shifts are relatively few, gradual, and prolonged, reflecting the nature of this perennial cultivation and contrasting with faster processes usually observed in annual cultivations, in which changes are more drastic and rapid. An additional factor explaining the observed gradual implementation of changes may be also related to the delayed transference of research innovations into the agricultural setting and to the long preparatory period required at the European level to formulate policy directives. Certain economical and policy tendencies and R&D developments have been more pivotal than other in shifting control paradigms (Fig. 4.8). The olive oil bonanza, which is characterized by high international demand of olive oil and sharp increases in prices after the 1970s (Fig. 4.3), is probably the main force behind the expansion of the cultivated land and the increment in the centralization of olive fly control. Similarly, the ban in the use of organophosphates and organochlorines (Fig. 4.8) stimulated the search and test of alternative pesticides and control methodologies, such as lure and kill, accelerating their field application and adoption. Finally, the resultant vector from two opposing forces (i.e., the intensification of the olive cultivation and the growing

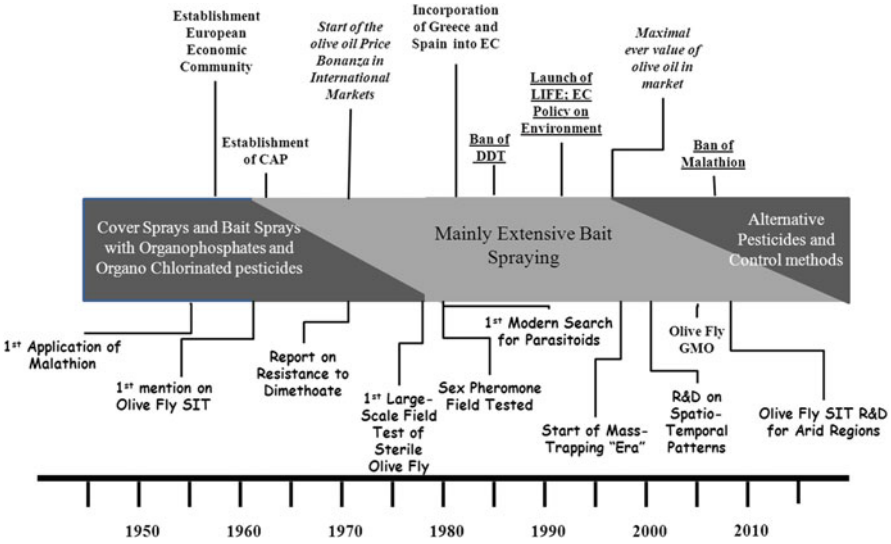

Fig. 4.8 Diagram summarizing olive fly control periods since WWII and until today. The diagram lists a series of economic (*italicized characters*), political (*bold and underlined characters*), and R&D (*stylized bold character*) landmarks that accompanied the suggested division of olive fly control in Southern Europe

demand for organic olive products) is the main driving force shaping olive fly control during the twenty-first century (Fig. 4.8).

Our intention with this essay was to understand the forces that shape the management of the olive agroecosystem, especially pest control. Although we only developed the case for the olive fly in Southern Europe, we believe that most human agricultural environments are subjected to similar processes and forces. While this is a historical account of a specific pest and its management, it shows the complexity involved in the management of the agroecosystem and the effect of global and local processes on plant protection activities and developments. Our analysis, thus, attempted to highlight the complexities of plant protection and demonstrate the need for a holistic approach when agricultural and research policies are formulated. We hope that this approach is a useful tool for future analysis and policy making in plant protection.

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Chapter 5
Enhancing Resistance Management and Performance of Biorational Insecticides with Novel Delivery Systems in Tree Fruit IPM

John C. Wise

Abstract Twentieth century delivery systems co-evolved with insecticide discovery to meet the demands of pest management in an era of increasingly mechanized agriculture production. The attributes of ground air-assisted sprayers met the practical demands of farmers to deliver broad spectrum pesticides into large fields of crops in an economical and timely basis. In an age of cheap contact poisons and limited understanding of environmental risks, the logical and optimal placement of insecticides was on the foliar canopy of crops, lending to maximum contact toxicity to the target pests. The twenty-first century has witnessed the development of an array of biorational insecticide chemistries, with performance attributes distinct from those introduced in the twentieth century. None-the-less, farmers have continued to rely upon twentieth century delivery systems to apply both new and old materials, with little consideration of what changes might improve their performance.

Trunk injection represents an alternative delivery system for biorational insecticides of trees, including tree fruit crops, which has the potential of maximizing the ingestive exposure of the compound to the target pest. Residue profile analysis of trunk injected insecticides shows that vascular delivery is predominantly to foliage, with fruit residues below USEPA maximum residue limits. Field and laboratory studies demonstrate seasonal effectiveness of trunk injected insecticides against key apple insect pests, suggesting that this is a promising delivering system for tree fruit IPM and resistance management.

5.1 Introduction

The market demand for specialty crop production has grown in recent years in response to public awareness of the health benefits of fruits and vegetables, as well as for their preventative attributes to various forms of cancer and heart disease

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(Wu et al. 2004). The economic contribution of specialty crop production has grown in importance as other traditional industries like manufacturing have declined. Public awareness of the importance of fruits as a source of cholesterol free, fat free, dietary fiber that lowers cholesterol is at an all-time high. In turn, the US Food and Drug Administration (FDA) have reordered fruits and vegetables as to their importance for human dietary intake, as well as their place in the Food Pyramid (Squires 2005). The rise in global demand for specialty crop production is well documented in the USA and European Union, but is also gaining attention in developing countries around the world (FAO 2001).

Profitability in domestic fruit markets requires meeting high food quality standards, often through the judicious use of pesticides (Wise and Whalon 2009). As tree fruit producers enter the twenty-first century, it is important to note that even though there has been significant evolution of the tools (i.e., reduced-risk pesticide chemistries) being used in pest management (USEPA 1997; Agnello et al. 2009), application equipment has remained relatively unchanged (McCartney and Obermiller 2008). Scientists, like Pimentel (1995), estimate that with conventional sprayers as little as 0.4 % of the pesticide contacts the target pest. Other studies show that airblast sprayers are a relatively inefficient means of delivering pesticides to their target, with only 29–56 % of the applied spray solution being deposited on the tree canopy, and the remaining product drifting to ground or other off-target end points (Steiner 1969; Reichard et al. 1979; Zhu et al. 2006; Perry et al. 1998) (Fig. 5.1). Some technical advancements have come to the conventional ground sprayer, such as adding towers or nozzle sensors (Landers and Farooq 2005; Landers 2002), but the fundamental elements for delivering materials to the tree canopy have remained the same.

Twentieth century delivery systems co-evolved with insecticide discovery to meet the demands of pest management in an era of increasingly mechanized agriculture production. The attributes of ground air-assisted sprays met the practical

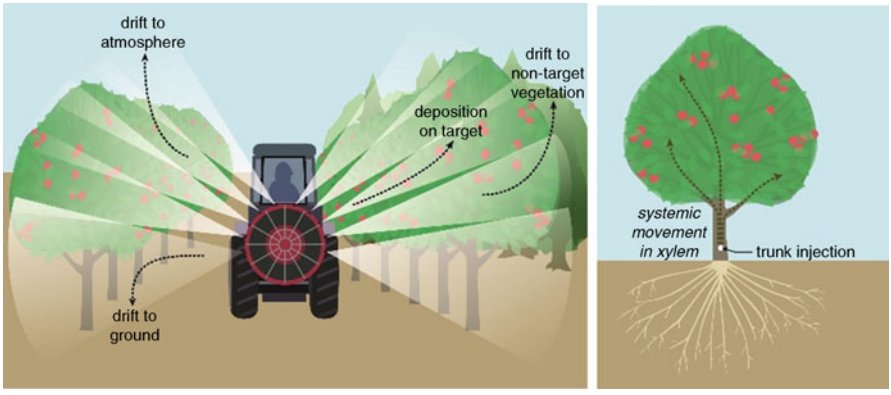

Fig. 5.1 Non-target drift from airblast sprayer foliar sprays versus trunk injection delivery (Images by Marlene Cameron)

demands of farmers to deliver broad spectrum pesticides into large fields of crops in an economical and timely basis. In an age of cheap contact poisons and limited understanding of environmental risks, the logical and optimal placement of insecticides was on the foliar canopy of crops, lending to maximum contact toxicity to the target pests. The twenty-first century has witnessed an array of biorational insecticide chemistries, with performance attributes distinct from those introduced in the twentieth century. Most biorational insecticides (spinosyns, insect growth regulators, avermectins, diamides, neonicotinoids) are ingestion-active and hold various degrees of plant-systemic capabilities. Nonetheless, farmers have continued to rely upon twentieth century delivery systems to apply both new and old materials, with a little consideration of what tactical changes might improve the performance of their crop protection materials.

As regulatory scrutiny on water quality, carbon abatement and non-target drift increases, the high cost of pesticides exacerbates the penalty of wasting active ingredient and the need for creative alternatives heightens (Brundtland 1987). Trunk injection represents an alternative delivery system for biorational insecticides of trees, including tree fruit crops, which has the potential of enhancing resistance management and maximizing the performance of compounds on the target pest. Arborists have developed a variety of techniques for injecting pesticides into sap systems of woody plants. To be effective, the injected compounds must be translocated from the injection site to the areas of insect feeding or disease infection (Fig. 5.1). Once in the xylem, chemicals are dependent upon the transpiration stream to move mainly upward and be distributed throughout the tree canopy (Mendel 1998; Harrell 2006; Aćimović et al. 2014). Trunk injection of systemic insecticides has become a preferred method for controlling emerald ash borer (EAB) in urban landscapes because of minimal risks for human exposure, or the negative impacts of pesticide drift on non-target organisms (McCullough et al. 2005; Tanis et al. 2006; Mota-Sanchez et al. 2008a). This chapter explores the potential advantages and disadvantages of trunk injection as a delivery system for "plant medicines" in tree fruit crop production.

5.2 Advantages of Trunk Injection for Tree Fruit IPM

5.2.1 Elimination of Spray Drift, Reduced Worker Exposure and Dietary Risks to Consumers

The elimination of spray drift is the first most obvious benefit of trunk injection over conventional foliar application methods. Off-target spray drift not only negatively affects the environment, but also is a pointless waste of active ingredient, since the material no longer contributes to the pest control it was intended for (Pimentel 1995). US and Canadian fruit growers in the Great Lakes regions face increasing scrutiny over pesticide use around the highly valuable and sensitive fresh water

resources (USGOA 2005). The US Endangered Species Act (1973) has also resulted in restrictions on commercial pesticide use when farms reside within several kilometers of lands holding protected species, such as the Karner Blue Butterfly (Lycaeides *melissa samuelis*) (http://ecos.fws.gov/speciesProfile/profile/speciesProfile?spcode=I00F). Restrictions on insecticides, like methoxyfenozide, are based on the perceived risks of spray drift from ground or aerial applications (http://www.epa.gov/oppfead1/cb/csb_page/updates/2009/blue-butterfly.html).

The proximity of commercial fruit production acreage to major population centers both provides economic opportunity from access to "local markets", but can also heighten tensions at the agriculture-urban interface. Fruit farmers in these areas are under continual pressure to grow high quality crops while managing the fears and concerns of adjacent residents about the hazards of spray drift. Even if growers use the safest crop protection materials available, neighbors have been known to complain about the noise associated with ground sprayers. Trunk injection has the potential to reduce the negative publicity associated with conventional ground application techniques.

Pesticide dietary tolerances set by the US Environmental Protection Agency (EPA), as well as CODEX global maximum residue limits (MRLs), must be considered with any new pesticide delivery system. Field research (Wise et al. 2014) on apples indicates that applying insecticides by trunk injection results in a *discriminatory distribution* in the tree that is favorable in terms of dietary tolerances, with the vast majority of residues ending up in foliage versus fruit. For the two compounds studied in 2009, emamectin benzoate and imidacloprid, residues in fruit at harvest were well below the current US EPA MRLs for pome fruit crops (Figs. 5.2 and 5.3).

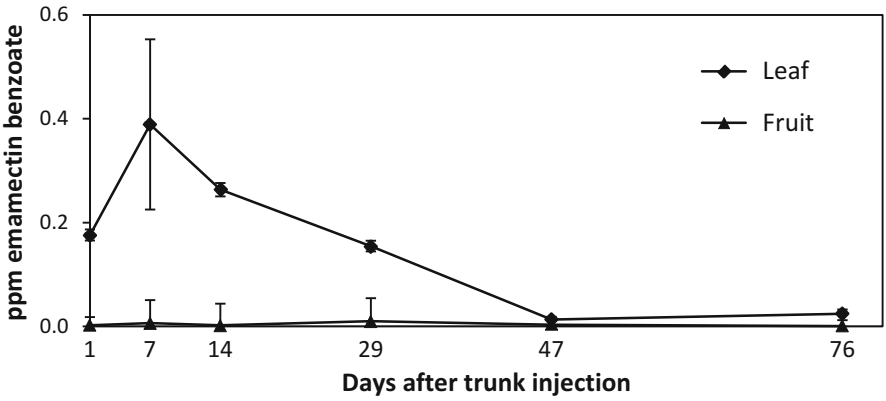

Fig. 5.2 Temporal distribution profile of trunk-injected emamectin benzoate in apple canopy during 2009 based on residue concentration in leaves and fruits (Wise et al. 2014). MRL for emamectin benzoate in apple fruits is 0.02 ppm, set by the USEPA

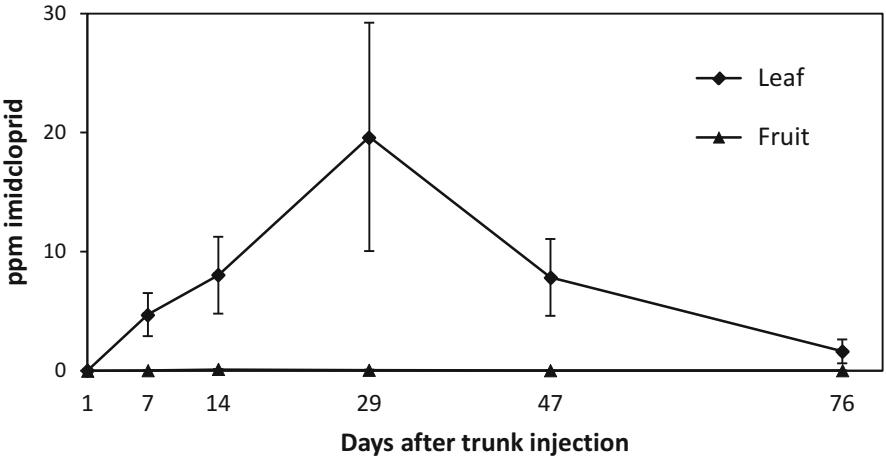

Fig. 5.3 Temporal distribution profile of trunk-injected imidacloprid in apple canopy during 2009 based on residue concentration in leaves and fruits (Wise et al. 2014). MRL for imidacloprid in apple fruits is 0.5 ppm, set by the USEPA

5.2.2 Prolonged Residual Activity of Materials on Target Pests, and Reduced UV Degradation and Wash-Off

Biorational insecticides are generally viewed favorably for IPM because of their safety profile for consumers, farm workers and the environment. Under a conventional foliar application model, however, the pesticide load on the plant is excessive at the time of application, in order to assure that sufficient active ingredient remains at the final days of the spray interval to protect the crop from pests (Wise et al. 2006; Wise and Whalon 2009). Pesticide residues on the surface of the plant are susceptible to degradation from ultraviolet (UV) light and wash-off from environmental elements like precipitation (Bostanian et al. 2012). Biopesticides in particular, like azadirachtin, to date have shown limited commercial presence in tree fruit production, in part because they tend to be short-lived when applied as foliar sprays. Because of their sensitivity to UV degradation, they require a multitude of foliar sprays over the "control period", thus generally costing more to the farmer than conventional alternatives. Delivering biopesticides and biorational materials by injection eliminates that economic deficiency from their performance portfolio when considering control options in an IPM program. While metabolic degradation processes will also ensue for compounds within the plant, our research shows that reduced UV exposure on the plant surface results in prolonged activity against target pests. Practical comparisons of foliar versus injection delivery of common insecticides used in tree fruit systems show dramatic improvements in the duration of residual control following trunk injection (Table 5.1). In all cases, the duration of pest control is lengthened substantially for materials that are injected compared to

Table 5.1 Comparison of insecticide residual activity following foliar application or trunk injection

Active ingredient	Insecticide class	Days of residual activity	
		Foliar spray	Trunk injection
Emamectin benzoate	Avermectin	7–10	90+
Imidacloprid	Neonicotinoid	14–21	90
Chlorantraniliprole	Diamide	14–21	90
Azadirachtin[a]	Botanical	5–7	60–90

[a]Unpublished data, J. Wise (2012)
– Data based on VanWoerkom et al. (2014), Wise et al. (2006), Mota-Sanchez et al. (2008b)

when sprayed on the tree canopy. In addition, insecticides applied as foliar sprays are often susceptible to wash-off from precipitation. While most modern insecticides are viewed to be more resistant to wash-off than conventional organophosphates, they are still expected to encounter 30–50 % loss of residues following 2.54 cm of rain (Wise et al. 2015, 2016; Hulbert et al. 2011, 2012). Beyond the challenge of maximizing the duration of active residues, the unpredictability of wind and rain often make it difficult for farmers to properly time applications using ground sprayers. Trunk injection eliminates much of the concern over the negative impacts of weather on pest management.

5.2.3 Vascular Delivery Overcomes Negative Impacts of Growth Dilution, and Enhances Ingestive Exposure

Since biopesticides and most biorational insecticides are predominantly ingestion-active materials, they often perform more poorly than conventional contact poisons when foliar-applied to the crop canopy. Whereas the surface of the crop canopy is the logical place to deliver a contact poison, foliar application of ingestion-active biopesticides puts these materials in a great disadvantage. Trunk injection, however, has the potential to enhance the ingestive exposure of biopesticides to the pest and improve in-plant distribution and longevity in the crop.

Growth dilution refers to the physical phenomenon whereby the concentration of insecticide residue on the plant is diluted as a direct result of growth of plant tissues (Willis and McDowell 1987; Steffan 2005). Measurements made on apple (*Malus domestica* Borkhausen) leaves and fruit (Wise et al. 2001 unpublished data) shortly after petal fall stage showed that the surface areas roughly tripled over a two week period (Table 5.2). Thus, not even accounting for environmental degradation forces, the "killing power" of an insecticide is reduced three-fold just from growth dilution alone.

This problem is further magnified when attempting to protect meristematic-active vegetative tips of growing terminals from foliar pests, like the obliquebanded leafroller (OBLR) (*Choristoneura rosaceana*) (Harris) or the potato leafhopper

Table 5.2 Measurements taken from open leaves (a) and fruit (b) at 1, 7 and 14 days post-petal fall

Time (days)	Diameter (mm)	Surface area (cm^2)	Weight (g)
(a)			
1	5.2	19.6	4.6
7	6.5	29.7	6.4
14	8.8	51.7	11.2
(b)			
1	10.6	3.64	6.1
7	14.8	6.93	16.2
14	18.0	10.22	26.3

Mean values based on 10 units from each composite sample; diameter values based on the average of the longitudinal and radial measures; surface area based on formula calculations of a sphere; weight measures in grams

(PLH) *(Empoasca fabae)* (Harris). Vantimmeren et al. (2011) showed dramatic losses of insecticide on young grape leaves from growth dilution as compared to when sprays were made to mature leaves, and vascular deliver (i.e., soil applications) showed the best duration of PLH control. VanWoerkom et al. (2014) showed 90+ days of OBLR activity following a single injection of emamectin benzoate, whereas a foliar spray of the same material would be expected to provide 7–10 days of residual control. Vascular deliver of insecticides eliminates the struggle to keep growing plant tissue covered, because compounds are readily transported to meristematic-active leaf terminals, thus providing toxic doses to young larvae or nymphs where they feed.

5.2.4 Enhances SAR Effects of Selected Compounds

Systemic acquired resistance (SAR) is a form of induced resistance, whereby plants develop systemic resistance to pathogen attack following infection (reviewed in Pieterse and Van Loon 2007). SAR, induced by pathogen infection, is mediated by salicylic acid (SA) and associated with the accumulation of pathogenesis-related (PR) proteins. SAR can also be induced by chemical inducers, such as acibenzolar-*S*-methyl, to control of a number of plant diseases (Walters and Fountaine 2009). Phosphorous acid is also thought to induce SAR to pathogen attack and has shown efficacy against bacterial pathogens (Wen et al. 2009).

SAR for insect control is commonly associated with the jasmonic acid signaling pathway. Ford et al. (2010) showed that the neonicotinoid compounds, imidacloprid, thiamethoxam and clothianidin, induce SA-associated plant responses through their 6-chloropyridinyl-3-carboxylic acid and 2-chlorothiazolyl-5-carboxylic acid metabolites. These neonicotinoids were shown to induce SA responses associated with reduced growth of the powdery mildew pathogen, *Arabidopsis thaliana*, and

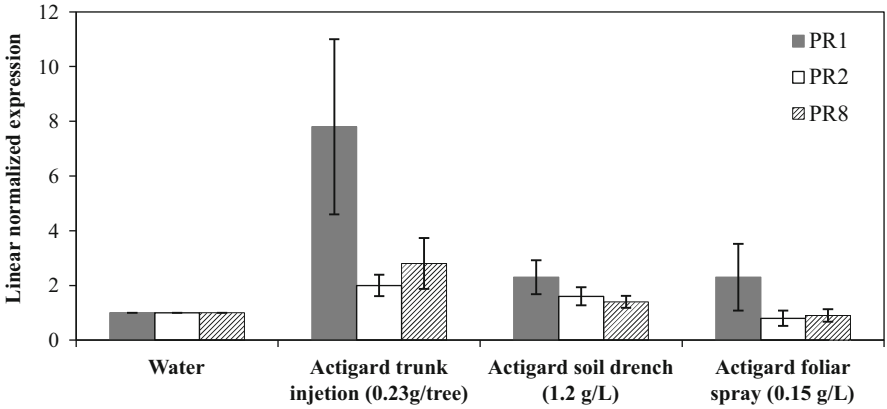

Fig. 5.4 Linear relative gene expression relative to water for proteins PR1, PR2 and PR8, after three ways of delivery of acibenzolar-S-methyl (Aćimović 2014)

enhanced stress tolerance (Ford et al. 2010). SAR inducer imidacloprid has been associated with enhanced resistance against microbial pathogens, and inducing (a)biotic stress tolerance. Preliminary research at Michigan State University utilizing headspace analysis showed heighten levels of farnesene from apple foliage following trunk injection of imidacloprid (Wise et al. unpublished data 2011). Investigation of SAR-inducing compounds, using vascular delivery systems like trunk injection, warrants further research in tree fruit production systems.

Preliminary results from acibenzolar-*S*-methyl trunk injection in Michigan apples (Aćimović 2014) showed higher levels of gene expression (PR1) when applied with trunk injection compared to the foliar spray (Fig. 5.4). Injections of potassium salts of phosphorous acid or acibenzolar-*S*-methyl similarly showed higher levels of gene expression (PR1, 2, 8) in apples (Aćimović et al. 2015). The injection of potassium salts of phosphorous acid also showed significant protection from of apple scab, *V. inaequalis* (Cooke), infections (VanWoerkom et al. 2014; Aćimović 2016). The apple scab control resulting from the injection of phosphorous acid is noteworthy because foliar application of this material in apples is not recognized as an effective alternative for disease control (Jamar 2011; Sundin et al. 2010).

5.2.5 Resistance Management

Pesticide resistance management is a key component of any IPM program (Wise and Whalon 2009). Successful insect resistance management depends on (1) optimal performance of a selected chemical tool when targeting a given pest; while assuring

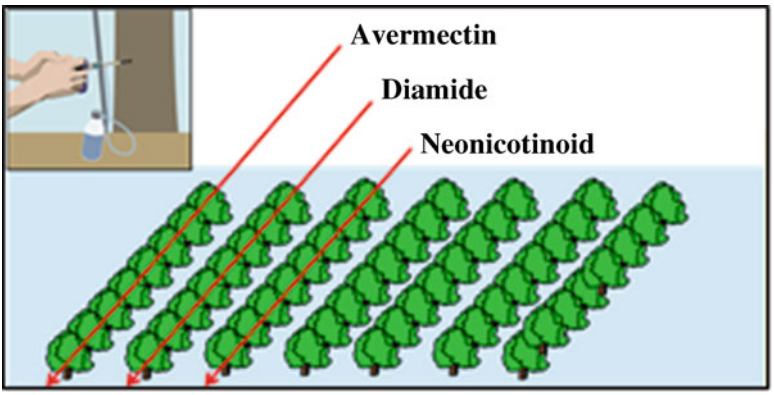

Fig. 5.5 Spatial strategy for resistance management via trunk injection, utilizing three classes of insecticides with distinct modes of action (Image by Marlene Cameron)

a limited duration of selection pressure through (2) rotation to uniquely different modes-of-action in the subsequent season or generation of the pest. Tree fruit pest management has historically relied upon a *temporal* strategy for resistance management, by rotating chemistries (modes of action) between generations of a target pest, thus changing the selection pressure on the population over time. For example, when managing the codling moth, *Cydia pomonella* (L.), in apples, generally two insecticide applications with a common mode-of-action would be used in the first generation, and rotation to materials with a different mode of action used in the second generation. This conventional tactic is effective to the extent that arthropod generations are distinct and not overlapping. Some insect pest species or certain seasonal weather patterns will result in overlapping generations, thus weakening the separation of selection pressures. Delivering insecticides by trunk injection provides an opportunity to employ a *spatial* strategy for resistance management, whereby individual trees or rows of trees can treated with different chemistries, thus exposing an arthropod population to a complex (or mosaic) of modes-of-action that may more effectively slow the development of resistance (Fig. 5.5). A spatial strategy is common in field crops utilizing insecticide-coated seed treatments and with the stacking of gene traits. In the realm of disease control, there is another important advantage. Research has shown that when non-target bacteria are exposed to aerial drift of pesticides in the agro-ecosystem following ground foliar sprays, they can acquire resistance genes and then transfer these genes to target organisms, such as the fire blight pathogen, *Erwinia amylovora*, thus hastening the development of resistance (Chiou and Jones 1993; Sundin et al. 1995). Trunk injection provides an opportunity for precision delivery of disease control materials, while eliminating exposure to non-target organisms in the environment.

5.3 Disadvantages and Risks Demanding Further Research

5.3.1 Wounding and Long-Term Tree Health

Injury to the tree following an injection procedure is often the first concern cited in discussions about this delivery system. Research in ornamental and forestry arenas have reported the range of acute forms of injury to tree trunks from various injection tools and long-term risks from secondary insect and disease infestations (Shortle et al. 2010; Smith and Lewis 2005). In general, the drill-based tools are the most invasive because of the large cavity left after drilling (reviewed in Perry et al. 1991; Wasniewski et al. 1993). Even though woody plants will compartmentalize the wound as new cambium and bark form with annual growth, the disruption of active xylem tissue and exposure to infections can be a concern for some plant species (Doccola et al. 2011, Neely 1988). Blade or needle-based systems do not remove woody tissue from the tree, but injury may include bark cracking in the season following injection (Aćimović et al. 2015; Montecchio 2013). In cases of pressurized injection systems, there have been reports of embolism and other inner-tissue damage resulting from the attempt to force transport of injection solutions beyond capacity transpiration rates of the tree (Sachs et al. 1977; Navarro et al. 1992). Most concerns about long-term risks to tree health appear to be in minimally-managed forest systems, where intervention is uncommon and the likelihood of monitoring individual trees is unrealistic. In these cases, where expectations of tree life can be in the hundreds of years, it is understandably a priority to minimize the risks from unnatural wounds for the long-term health of forest trees.

Tree fruit production systems are different in many respects. First, tree fruits are of the most intensively managed woody plants in the world. The management of apples, for example, includes the monocultural planting of several thousand dwarf trees per hectare and targeting full-bearing production within 3 or 4 years after establishment. Apple trees undergo aggressive horticultural pruning annually, for which as much as 20 % of canopy wood is removed to maintain the desired tree size and optimal bearing wood. Commercial apple growers commonly protect trees from a wide range of arthropods and diseases with as many as 14 applications of crop protection materials per season (Wise et al. 2015). My conversations with fruit growers about the risks associated with injection technology and tree wounding has brought little-to-no negative reaction. They point to the wounds resulting from annual pruning and the relative fast growth and healing of apple trees as the reason for their lack of concern. Our field research suggests that injection wounds are largely healed-over within 1–2 growing seasons (Aćimović 2014). This is supported by other research, showing that vigorously growing trees have the capacity to quickly heal, resulting in minimal disruption of function (Wasniewski et al. 1993). Continued observation is warranted to assure that there will not be long-term health impacts of economic consequence, but for the time being this does not appear to be a significant obstacle.

5.3.2 Limited Fruit Protection Demands Tactical Partnering with Mating Disruption or Other Selective Tools

The *discriminatory distribution* of trunk injection materials to foliage over fruit is favorable in light of food safety, but there is a downside in relation to controlling direct fruit pests. Injection can contribute significantly to the control of those direct pests that feed dually on apple fruit and foliage, such as the Oriental fruit moth, *Grapholita molesta* (Busck), obliquebanded leafroller, and rosy apple aphid, *Dysaphis plantaginea* (Passerini). Unfortunately, insect pests that solely infest fruit, like codling moth and apple maggot, *Rhagoletis pomonella* (Walsh), will require the integration of additional tactics into an apple IPM program. Codling moth mating disruption is an excellent IPM partner with trunk injection because it selectively targets key direct fruit pests, like codling moth, but needs the addition of other IPM tools to address other pests (Gut et al. 2004). For control of apple maggot, various "Attract and Kill" tactics, such as pesticide-treated spheres (Stelinsky et al. 2001) or insecticide-comprising baits (Pelz et al. 2005), complement the strengths of injection.

5.3.3 Impacts on Beneficials and Risk to Pollinators

Maintaining beneficial arthropods, including pollinators, in apple agro-ecosystems is an important priority for long-term sustainability of apple production (Theiling and Croft 1988; Altieri and Nicholls 2004). Eliminating season-long prophylactic surface residues in the tree canopy is likely to reduce toxic effects to a wide array of natural enemies and pollinators. In some cases insecticides, like imidacloprid, have been detected in nectar and pollen of treated crops, raising concerns over potential toxic affects to honey bees (Johansen 1977; Johansen and Mayer 1990). Initial trunk injection residue data (VanWoerkom et al. 2014) showed zero insecticide detections in apple flower parts during bloom-stage in the year following injections, but comprehensive analysis of nectar and pollen samples during bloom stage are needed to evaluate the relative risks of trunk injected insecticides to pollinators. Comparing outcomes of product rate ranges and injection timings, the associated risks to pollinators must be understood before proceeding to implementation.

5.3.4 Speed of Application (Economics)

A perceived weakness of trunk injection as a delivery system in tree fruit crops is the speed of application. If a typical tractor-driven ground sprayer runs at 3.7 km per hour (2.3 mph), then the time required to spray 1 ha of semi-dwarf apples

is approximately 30 min if row spacing is 5.4 m (12 min to cover 1 acre for 18 ft row spacing). A high density orchard with 3.6 m row spacing would take approximately 44 min (18 min to cover 1 acre with 12 ft row spacing). Fourteen individual foliar sprays in a growing season would equal 420 min per hectare (168 min per acre) for semi-dwarf orchard and 616 min per hectare (252 min per acre) for a dwarf apple orchard. With current commercially available injection tools, the same semi-dwarf orchard (approximately 617 trees per hectare or 250 trees per acre) will take 1542 min per hectare (6250 min her acre) to inject all trees with four ports each. A dwarf apple orchard (approximately 2470 trees per hectare or 1000 trees per acre) will take 3705 min per hectare (1500 min her acre) to inject all trees with two ports each (smaller diameter trees require fewer ports to provide uniform canopy distribution). So, using the current commercially available injection tools, the seasonal application time for trunk injection is 3.5–6 times more than using a ground sprayer. Of course other variables, such as equipment maintenance, fuel, capital expenditures contribute to the overall cost of production, but this analysis suggests that more efficient injection tools would be needed to make this delivery technique competitive in the commercial fruit system. It is no surprise that the current commercially available injection tools are not optimal for commercial fruit production, since they were developed primarily for non-crop ornamental and shade-tree uses. The forces driving equipment development have included minimized wounding, ease of transport and use and to a lesser degree injection-time. If trunk injection will find a place in commercial fruit production, tools will have to evolve to become more highly mechanized to match the capital-intensive technology currently used to meet economic efficiency demands of the competitive global food market.

5.3.5 Regulatory Hurdles

There appear to be a good selection of pesticide candidates for trunk injection, which are already labeled for pome and stone fruits, and for which our tested use-patterns fall within the established seasonally limits of active ingredients and MRLs. Even so, we anticipate that GLP (Good Laboratory Practices) Field Residue Trials in cooperation with the USDA IR-4 Project (Dorschner et al. 2009) will be necessary for USEPA to add trunk injection as a labeled use for the candidate compounds. For those compounds, discussions with USEPA officials will determine what bridging data are necessary for attaining sufficient GLP field residue data for USEPA evaluation, registration and eventual new labeled uses for tree fruit producers.

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Chapter 6
Manipulation of Insect Reproductive Systems as a Tool in Pest Control

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6.1 Introduction

Insect pests must reproduce in order to exist. In sexual organisms, sperm and ova need to synchronically meet in a specific environment under conditions that may include temperature, time, food, day length, and many others. Mating events can therefore be predicted and as such open for manipulation.

Many insects use airborne sex pheromones to locate their mates. Exploring the various means to interfere with or to exploit this communication channel to disrupt reproduction is the aim of this chapter (Table 6.1). Repeated use of toxic insecticides has led to the development of resistance in insect pests of various taxa (see, e.g., Denholm and Rowland 1992; Liu et al. 2010; Boyer et al. 2012). The necessity to combat the pests, together with increasing demands from consumers and farmers to refine the use of pesticides and reduce toxic residues on edible products, calls for alternative, environmentally friendly methods of managing insect pests. Developing methods to interfere with the pest mating behavior and sabotaging reproductive potential by transferring pathogens during mating or through manipulating endosymbiotic bacteria are effective answers to this demand.

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Table 6.1 Tactics for manipulating sexual reproduction for management of insect pests

Technique	Tactic	Details	Sample reference
Pheromones	Monitoring	All pheromone-producing insects	Reddy and Tangtrakulwanich (2014)
	Mass trapping males	♀-Produced sex pheromones retain ♂♂ in a trap	El-Sayed et al. (2006)
	Attract and kill	Sex or aggregation pheromones used with killing agent	El-Sayed et al. (2009)
	Mating disruption	Synthetic off blends that mimic natural ♀ pheromone prevent ♂♂ from finding ♀♀ and mating	Witzgall et al. (2010)
Irradiation	Sterile insect technique	Diptera; sterile ♂♂ are released	Vargas-Teran et al. (2005)
	Inherited sterility	Lepidoptera; partially sterilized ♂♂ are released to produce defective F1 offspring	Bloem et al. (2005)
Symbionts	Parthenogenesis inducing	Asexual production of offspring	Silva et al. (2000)
	Cytoplasmic incompatibility	Mating of individuals possessing or not symbionts causes embryo death	Vavre et al. (2000)
	Antagonistic effects	Reduces fitness parameters by mixed infections	Gottlieb et al. (2010)
Pathogens	Virus	Viruses are released and sexually transmitted to manage populations	Huger (2005)
	Fungi	Internally and externally growing fungi are transmitted by spores or mating attempts	Riddick and Schaefer (2005)
	Mites	Released to control *Harmonia*, now adversely affects coccinellid biological control agents	Rhule et al. (2010)

6.2 Sex Pheromones

Pheromones are chemical signals that are used to convey information among individuals of the same species (Karlson and Luscher 1959; Wyatt 2003). As such, sex pheromones are species-specific and contain information about the species and gender of the releaser (Svensson 1996; Johansson and Jones 2007; Wyatt 2003). There is accumulating evidence that the properties of the species-specific sex pheromones also provide information about the phenotypic conditions of the releaser (Harari et al. 2011; Steiger and Stökl 2014; see review by Harari and Steinitz 2013).

Typically, in most insects as in most sexual organisms, males invest less than females in reproduction per se; males may mate repeatedly and therefore put more energy into attracting or searching for females (Trivers 1972). In moths, for example, females produce the highly species-specific sex pheromone, and

males search for calling (signaling) females. These pheromones are released in minute amounts (nanograms) and evaporate quickly, thus selecting against specialist predators and parasitoids and for high-quality searching males (Greenfield 1981; Wyatt 2003; Harari and Steinitz 2013).

Male-produced sex pheromones occur most notably in beetles and flies. In both taxa, the male-produced pheromones are associated with local host plant availability, such that arriving females gain mate, food, and oviposition sites at the same place and time (Landolt and Philips 1997). Male sex pheromones are often produced in large amounts and are synergized by volatiles associated with food or oviposition sites (Landolt and Philips 1997; Wyatt 2003). Often, male-produced pheromone attracts both males and females and is termed "aggregation pheromone" (Wyatt 2003).

6.3 Pheromones as a Monitoring Tool

Using pheromone-baited traps for population monitoring has several advantages:

1. The pheromone is species specific and thus easy to use by nonprofessionals.
2. Small and large populations of a pest can be trapped.
3. Catches the pest in early stages of infestation or invasion.
4. Can be used to delimit areas of infestation.
5. Allows discrimination of pest phenology (generations).
6. Permits estimation of population size.
7. Saves field scouting labor by revealing times of treatment need.

All of the above make the pheromone-baited traps a useful tool in integrated pest management (IPM) decision making: timing control measures with susceptible developmental stages of the pest, before populations increase, and evaluation of the efficacy of chemical applications.

The trap design may also influence the number of individuals caught, as the behavior of the pest should be considered. In designing a trap for click beetles, for example, the male tendency to crawl toward the pheromone-releasing female and his ability to escape from the trap must be considered (Vernon and Toth 2007; Reddy and Tangtrakulwanich 2014). The proper trap placement may also affect trapping efficacy. For example, the European corn borer, *Ostrinia nubilalis*, feeds, rests, and mates in grassy vegetation adjacent to corn fields; therefore, pheromone traps should be placed on the grassy edges of sweet corn fields (Knodel et al. 1995). Traps developed to attract the leopard moth (*Zeuzera pyrina*) a serious pest of apple (Haniotakis et al. 1999) and olive (Hegazi et al. 2015) trees, should be placed above the canopy (Hegazi et al. 2015) as the nonflying females climb to the top branches and call for males, whereas traps for the codling moth (*Cydia pomonella*) or the European berry moth (*Lobesia botrana*) should be placed in the upper third of the canopy (Anshelevich et al. 1994; Witzgall et al. 2008).

The stored products pests may particularly illustrate the need of proper monitoring schemes. Historically, pest management of stored products has relied on toxic chemical pesticides. In the attempt to reduce chemical applications, accurate pest monitoring was needed to assess the occurrence and potential damage of the various insect pest species. In three department and two pet stores, monitoring schemes took place employing an array of traps baited with pheromones of suspected moth and beetle pests to monitor them over time (Arbogast et al. 2000). The analysis of the data allowed effective location of infestation foci of certain pest species, and to follow fluctuations in their population size. The most abundant pests in their study were *Plodia interpunctella*, *Lasioderma serricorne*, *Oryzaephilus mercator*, *Tribolium castaneum*, and *Cryptolestes pusillus*. The results obtained from pheromone-baited traps have established the effectiveness of regular monitoring regime in providing a strong management tool against stored products. This allows for defining hot spots of certain pests and targeting control measures against these pests only, at the precise time and space. Suppressing of pest population is therefore achieved with reduced pesticide cost and risk.

6.4 Using Sex Pheromones in Management Tactics

6.4.1 Mass Trapping

Understanding the role of volatile pheromones for mate location, coupled with the knowledge of pheromone synthesis, has led researchers to the development of mass trapping as an important part of the arsenal of pest management tools. In this method, either sex or aggregation pheromone is used to attract numbers of males or both males and females, respectively. If a female sex pheromone is used, then males are removed from the population before mating. The reduced male availability results in fewer matings with calling females (El-Sayed et al. 2006; Byers 2007). In this form of mass trapping, females may eventually mate, but due to the low numbers of males in the population, some females may mate at an old age, thus suffering from decreased oviposition potential (Weissling and Knight 1996). Furthermore, most moth females that eventually do mate do so with experienced males that have smaller spermatophores that contain reduced amounts of nutrition and sperm, which significantly harm their reproductive success (Simmons 2001). When aggregation pheromones are used in mass trapping, the efficacy is greatly enhanced when females are lured and killed as fewer females are able to oviposit, and the population decreases rapidly.

In mass trapping, pheromone-baited traps typically are uniformly distributed in the treatment area. The relative contribution of the sex pheromone release rate and its pattern of distribution are in debate (Byers 2007), i.e., small distance among many dispenser sources of low release rate of pheromone (Suckling and Angerilli 1996) or large distance among the few sources that release pheromone at relatively

high concentration rate (Alford and Silk 1983). Many successful trials have used small numbers of high release rate pheromone (see El-Sayed et al. 2006 for review).

A significant decrease in pest populations using a high density of pheromone dispensers, each with a low release rate, was also demonstrated. For example, a high density of pheromone points, similar to that used for mating disruption, but operating with lower release rates and equipped with sticky surface, significantly decreased codling moth, *C. pomonella*, and the obliquebanded leafroller, *Choristoneura rosaceana*, male captures in these traps (Miller et al. 2010; Reinke et al. 2012). These researchers suggested that control achieved by mass trapping was superior to mating disruption (see below) due to male removing, such that in each consecutive day, fewer males were available in the population for mating.

Mass trapping has been used mainly to control Lepidoptera, Coleoptera, Diptera, and Hemiptera. In cases of low infestation and isolated plots that preclude reinfestation of the crops, significant reduction in pest population and damage to yield have been achieved (codling moth: Madsen and Carty 1979; the pink bollworm: Mafra Neto and Habib 1996). In some cases, early mass trapping has led to delayed and reduced insecticide applications (Teich et al. 1979).

In most moth species, female-produced sex pheromone attracts males only. Male moths typically mate repeatedly; therefore, a high proportion of the males must be removed from a local population to significantly reduce the amount of mated females. In the case of male protandry (males have a shorter developmental time and eclose before females), males can be selectively removed at the beginning of the season to reduce the number of mating events. This may have a long-term effect on the population increase, preventing economic damage or at least delaying the first insecticide application (Liebhold and Tobin 2008). For example, the moth *Leucinodes orbonalis*, the eggplant fruit and shoot borer, is a key pest of eggplant in Southeast Asia. Insecticides are not effective in controlling the pest that dwells deep within the fruit, and intensive use of insecticides has led to larval resistance. Thorough research, optimizing the pheromone blend, trap design, and distribution pattern in the eggplant field, has led to a 50 % increase in marketable yield and excluded the need for insecticide implementations (Cork et al. 2005).

6.4.2 Attract and Kill with Sex Pheromones

Pheromone baits in mass trapping of males may contain insecticides or pathogens that not only attract the males but also kill them. In this case, a trap is only for temporary containment, as a touch point for the attracted male and the killing agent (Brockerhoff and Suckling 1999; see review in El-Sayed et al. 2009). This method is also termed "lure and kill," "attract and kill," "male annihilation," "bait sprays," and "attracticide." The efficacy of the method, regardless of the poison used, depends on the pest contact with the killing agent, the efficacy of the pesticide, killing the male before further inseminating females, the efficacy of the combined lure (pheromone

and a killing agent), and avoiding any repellent action. However, the success of the method depends on the species' sexual behavior (monandry, polyandry, or polygeny), population density, and flight ability of both males and females. Thus a comprehensive understanding of courting and mating behavior of the specific pest is required.

Byers (1993) demonstrated that the proportion of individuals removed using attract and kill methods is independent of the initial pest population size. However, the time to reach a population level that is below the economic threshold is positively correlated with the initial population size (population dependent). Thus, when a pest population is high, density-independent control methods, e.g., chemical insecticides, are needed to reduce the targeted pest population size before implementing mass trapping. In addition, measures should be taken to avoid repeated immigration into the treated area (El-Sayed et al. 2006). There is general agreement, though, that mass trapping is more effective with low populations of the pest, when finding a female is more costly for the remaining males (Sternlicht 1982; El-Sayed et al. 2006; Byers 2007).

In southern Europe, attempts were made to suppress the olive fruit fly (*Bactrocera* (*Dacus*) *oleae*), a major pest of olives in the Mediterranean region, using components of the male sex pheromone and food baits in traps; an average reduction of four insecticide applications each season was achieved (Petacchi et al. 2003). The house fly, *Musca domestica*, is commonly trapped using "feeding stations" enriched with insecticides but also with the female-produced pheromone, muscalure. Attract and kill traps are typically placed in yards and livestock stables (Butler et al. 2007; Geden et al. 2009).

6.4.3 Attract and Kill with Aggregation Pheromone

Some male cerambycid beetles produce aggregation pheromones that attract both males and females. The beetles typically cause much damage at low population densities, as one larva can kill a tree. This characteristic, together with the long life cycle but short adult stage, renders them good candidates for attract and kill control (Hall et al. 2006).

Bark beetles are key pests of forests worldwide and many release aggregation pheromones. The inaccessibility of insecticide treatments in forests and the costs involved in covering large areas has led to the increasing use of mass trapping efforts to control these devastating pests. Furthermore, whereas an egg-laying moth may cause substantial damage to a crop, large numbers of bark beetles are needed in order to overcome the host tree's resistance. Bark beetles positively respond to only one or two components of their species-specific pheromone, and the purity of the component is often not critical for their attraction to bait (Schlyter and Birgersson 1999). One disadvantage of some bark beetle pheromones for mass trapping is the involvement of host (tree) volatiles in the attractant of the beetle that may compete with pheromone volatiles in the traps.

A 3-year trial to suppress the population of the bark beetle *Ips duplicatus* was conducted in 2000 ha of isolated Mongolian spruce (*Picea mongolica*) forest in Inner Mongolia, China, using 80 traps baited with the beetle aggregation pheromone (Schlyter et al. 2001). In three successive years of this treatment, tree mortality was reduced significantly. The synthetic blend of the aggregation pheromone was highly competitive with the de novo-produced, male-released pheromone (Byers et al. 1990; Ivarsson et al. 1993).

The male boll weevil, *Anthonomus grandis*, is a major pest of cotton in North, Central, and South America and produces grandlure, an aggregation pheromone (Tumlinson et al. 1969). When the pheromone was incorporated into control tubes that were distributed in 14 stations per hectare, using an "attract and kill" tactic, the weevil reached "eradication level" in US cotton fields.

6.4.4 Mass Trapping with Pheromones and Food

Mass trapping with a species-specific pheromone in combination with a food-based kairomone is often used against beetle pests. The weevil *Rhynchophorus palmarum*, a key pest of oil (*Elaeis guineensis*) and coconut (*Cocos nucifera*) palms in tropical America and a vector of the red ring nematode (*Bursaphelenchus cocophilus*) disease, was treated by mass trapping baited with synthetic male-released aggregation pheromone and synergized with sugarcane (Oehlschlanger et al. 1995). During 1 year of treatment when trap densities were less than one trap per 5 ha, the red ring nematode disease was reduced by 80 % regardless of the initial infection level of the disease or the beetles' capture rate. This pest's management was based on integrated control that included the removal of infested trees in the orchard and the surrounding areas (Oehlschlager et al. 2002).

The red palm weevil, *Rhynchophorus ferrugineus*, is a devastating pest of coconut and date (*Phoenix dactylifera*) palms in Southeast Asia and the Middle East. Controlling the early invasion of the weevil to date plantations in Israel was achieved by applying mass trapping using 4500 traps (10/ha) baited with the synergetic combination of food (ethyl acetate and a fermenting mixture of dates and sugarcane molasses) and ferrugineol, the male release aggregation pheromone (Soroker et al. 2005). No further infestation was detected between 2002 and 2009 (Soroker et al. 2013). Unfortunately, subsequent multiple invasions of the pest have led to the spread of the pest in Israel attacking mostly *Phoenix canariensis* in the city's avenues and private gardens.

6.4.5 Mating Disruption

Mating disruption occurs when the immediate aerial environment around a pest is saturated with its species-specific (synthetic) pheromone (Wright 1965), causing

disorientation and interrupting communication between the sexes and thus delaying, reducing, or preventing fertilization of females. The use of mating disruption as a tool in IPM is highly recommended since, as opposed to conventional chemical insecticides, sex pheromones are species specific (and therefore incapable of developing resistance or tolerance), do not harm beneficial insects, and are not toxic to people.

Unlike mass trapping, synthetic pheromones used in mating disruption can be "off blends" to some extent, as the full plume-following behavior and landing on or near the pheromone source are not required. Moreover, the non-precise mimic of the natural pheromone may further add to male confusion when searching for females (McCormick et al. 2012).

Mating disruption has gained acceptance through years of successful control of various moth pests (Rice and Kirsch 1990; Harari et al. 2007; Witzgall et al. 2010). The success of the method depends on (1) the physical attributes of the pheromones and (2) the physical characteristics of the target area. The pheromone attributes include the pheromone blend components and their ratio, pheromone release rate, placement of pheromone source (height, canopy), time of release, pheromone movement and concentration in air, and the extent of the synthetics competition with wild female pheromone (population density). The geographical characteristics of the plot, including topography and the plot's size and shape – larger plots with reduced border lines decrease the possibility of gravid female invasion into the treated plot – and the pheromone concentration dilution at the borders (Cardé et al. 1998; Gut et al. 2004).

Other factors that affect the efficacy of mating disruption are male dependent:

1. Sensory fatigue – when males are continuously exposed to high level of pheromone. This may lead to desensitization of the receptors on the antennae, the habituation of the central nerve system, or both (Cardé et al. 1998; Judd et al. 2005; Stelinski et al. 2005)
2. Competitive attraction – pheromone release points compete with females for males. Therefore, pheromone dispensers should be evenly spaced, avoiding voids in pheromone concentration or "holes" containing diminished amounts of pheromone in the open air (Stelinski et al. 2004; Miller et al. 2006a, b)
3. Camouflage – where the female-released pheromone is masked by the synthetic pheromone in the background. This may happen if the homogenously released synthetic pheromone is highly similar to that of the female, refraining the males from detecting and following their preferred female-released pheromone blend (Schofield et al. 2003).

Excessive amounts of pheromone in the environment may also affect females and contribute to the success of the methods. Detecting their own pheromone (Harari et al. 2015), females may increase the rate and/or time of calling (Palaniswamy and Seabrook 1985) both of which may have an energetic cost that negatively affects their reproductive potential (Weissling and Knight 1996; Harari et al. 2011). The delay in mating, due to the pheromone-saturated environment, leads to mating in old age in which there is a reduction in reproductive potential of both males and females (Stelinski and Gut 2009).

Apparently natural mortality (predation, exhaustion/starvation) and senescence of both females and males may contribute to the success of mating disruption (Harari et al. 2011).

A potential shortcoming of mating disruption arises if dispensers are not distributed early in the season when the pest population is still low and maintained for the entire season to avoid population buildups. This requires costly, long-lasting pheromone dispensers or repeated application of the pheromone. Specifically timed pheromone release, using sprayers and puffers to suit that of the pest mating behavior, was developed to circumvent this problem.

Mating disruption has been effectively used as an area-wide (140,000 ha) control tool in vineyards in Germany, Italy, Spain, France, Switzerland, Austria, and Israel against the European grapevine moth, *L. botrana*, and the European grape-berry moth, *Eupoecilia ambiguella* (Ioriatti et al. 2008, 2011). Mating disruption strategy has led to significantly reduced insecticide applications without an increase in damage or control costs (Gordon et al. 2005; Harari et al. 2007; Ioriatti et al. 2011).

In orchards, mating disruption is used against the codling moth, *C. pomonella*; oriental fruit moth, *Grapholita molesta*; and the light-brown apple moth, *Epiphyas postvittana*. The codling moth is a cosmopolitan, oligophagous pest feeding mainly on apple, pear, and walnut. The larvae penetrate the fruit as neonates, where they are protected from insecticides. There are few effective insecticides; some have been banned, whereas resistance has evolved against others (Reyes et al. 2009). Area-wide mating disruption is applied against the pest in Europe and apple-growing regions in the USA (Witzgall et al. 2010).

The largest operation of the mating disruption tactic was against the gypsy moth, *Lymantria dispar*. The gypsy moth was introduced from Europe to North America in 1869 (Tobin et al. 2012) and has since became a key pest of northeastern US forests causing the defoliation of hundreds of thousands of hectares (Cameron et al. 1974). The polyphagous larvae attack more than 300 tree species which include forest and shade trees and edible fruits and nuts such as apples, apricot, blueberry pear, pistachio, and others (Miller et al. 1987). An area-wide mating disruption trial was operated by the USDA Forest Service with the goal to reduce the pest expansion to uninfested regions. The goal was to eliminate isolated, low-density colonies bordering infested areas (Tobin and Blackburn 2007) and to suppress occasional, sporadic outbreaks of the pest in already established zones (Haynes et al. 2009). Approximately 1200–1600 km^2 were treated each year in the control program using mating disruption tactics. Since 2000, this program has reduced *L. dispar* spread from historical rates of about 21 km per year (Liebhold et al. 1992) to less than 4 km per year (Roberts et al. 2011). The efficacy of the program is estimated in preventing new infestations on more than 400,000 km^2 between 2000 and 2010 (Tobin et al. 2012).

Although mating disruption is most often used against lepidopterans where males are searching for the female-released sex pheromone, attempts to use this method to control beetles and scale insects have also been promising. Recently, mating disruption was developed against mealybugs and, particularly, the vine mealybug *Planococcus ficus*. This mealybug is a key pest of vines in the Mediterranean Basin, South Africa, and California. During high infestation levels, this mealybug causes

direct damage rendering table grapes unmarketable and reduces the quality of wine. Furthermore, *P. ficus* is a vector of a few viral diseases, e.g., grapevine leafroll-associated virus and corky bark disease (Rosciglione and Gugerli 1989; Tanne et al. 1989); as such, it is a key pest even at low population densities. Insecticide applications are often not effective as most of the mealybug lifecycle is concealed under the bark or underground in the root system (Lentini et al. 2008). The short lifespan of the males and the restricted mobility of the females render this species a good candidate for mating disruption. Two-year mating disruption trials conducted in Italy and Israel demonstrated a significantly reduced number of males captured in pheromone traps (up to 95 %), a reduced density (Cocco et al. 2014), and a significant lower percentage of infested vines (Sharon R. personal observation).

Mating disruption was also developed to control beetles such as *Megaplatypus mutatus*, an ambrosia beetle native to South America that attacks living trees. The weakening of the tree may lead to stem breakage and tree mortality. In addition, the commercial value of the tree is reduced due to the dark staining caused by the associated fungi (Funes et al. 2016). The beetle was introduced to Italy in 1998, and the risk of its further spreading to other parts of Europe has led to the development of an area-wide program using mating disruption. Uniquely the method is targeted to disrupt females searching for mates. Several factors contributed to the success of the methods against the ambrosia beetle: the life cycle of most stages is inside the host tree and protected from insecticides. The sex pheromone released by the male is known and can be formulated in controlled release devices. The adults are relatively immobile, with females laying eggs inside one tree, and do not disperse further. This behavior reduces the risk of gravid females invading into the treated area and allows managers to focus on hot spots only. The mean number of new galleries, evidence for new attacks, was reduced dramatically after the deployment of the mating disruption dispensers (Funes et al. 2011, 2016).

6.5 Irradiation Techniques

An alternative, environmentally friendly method to manage insect pests is the release of irradiated males: sterile insect technique (SIT) is primarily for dipterans which are sensitive and can be fully sterilized without affecting behavior and courtship performance in the field. The mating of sterile males with wild females results in zero offspring. Another radiation technique is partial male sterility technique (IS) (also called inherited sterility or F1 sterility) and is primarily for lepidopterans which are somewhat resistant to radiation, as full sterilization affects field performance. The mating of partially sterilized males with wild females results in nonviable embryos or sterile, male-biased offspring. For both methods, there is no adverse effect on other taxa (Gamble et al. 2010). A surplus of sterile males is released in the infested area in order to compete numerically with the local existing wild males. The minimum number of sterilized males to be released is called the

critical flooding ratio and is dependent on the distribution of the pest; clumped distributions require higher release numbers. Population models suggest that the integration of mating disruption or attract and kill with SIT-IS should result in additive or synergistic effects. The few females that do mate may mate equally with sterile or wild males (Carpenter 1992).

An example of SIT is the attempt to eradicate the screwworm, *Cochliomyia hominivorax*, and release of millions of sterilized males which took part in the 1960s to control screwworm fly from southern USA. Wild female flies that mated with the sterile males did not produce viable offspring leading to the decline of the population and eventually to the pest eradication (Myers et al. 1998). Eradication of the pest is now maintained from North America to the Isthmus of Panama (Vargas-Terán et al. 2005).

The Mediterranean fruit fly (*Ceratitis capitata*) is one of the most destructive insect pests worldwide due to its extremely wide range of hosts in various environments. Because of the risk of establishment in Central and North America of the fly and its immense potential for economic damage, the US agricultural authorities together with those of Mexico and Guatemala joined efforts to eradicate the pest and establish a fly-free zone. The IPM approach included the sterile insect technique, for which a production of 500 million sterile insect were reared weekly (Schwarz et al. 1985, 1989). After 30 years of the program, the area was declared pest-free and is maintained by repeated eradication actions followed by extensive and intense monitoring schemes. The Mediterranean fruit fly pest status was defined for most of Mexico as "pest absent" (Enkerlin et al. 2005).

IS-established programs are generally against lepidopteran pests (Bloem et al. 2005), including the codling moth *C. pomonella* (Carpenter et al. 2005), the pink bollworm *Pectinophora gossypiella* (Bloem et al. 2005), and the painted apple moth *Teia anartoides* (Suckling et al. 2007).

6.6 Sexually Transmitted Pathogens for Insect Management

Sexually transmitted pathogens have been documented in insects since 1875 (Peyritsch cited in Whisler 1968). Peyritsch showed that male houseflies (*Musca domestica*) infected with the fungus, *Stigmatomyces baeri*, transmitted fungal thalli to females during mating. Although there was no indication that the fungus was pathogenic or shortened the fly lifespan, sexual transmission was clearly demonstrated. Since that time, a number of viruses, protozoans, fungi, nematodes, and even mites have been shown to be transmitted during copulation (see review of Knell and Webberley 2004). The most frequently documented sexually transmitted pathogens are viruses and fungi, followed closely by arthropods, and the fewest are nematodes and protozoans. Most often, there is a reduction in fecundity and fertility when infested by a virus or fungus, and sometimes there is a shortening of lifespan or an effect on the offspring. Usually there is little to no effect on mating behavior

which allows for the continued transmission of these pathogens. Only insects of economic interest in crops and trials with a field component will be discussed in this section.

6.6.1 Viruses

There are a few viruses (e.g., dengue fever virus, St. Louis encephalitis virus) that can be sexually transmitted but are normally maintained in vector populations by transmission and acquisition to and from mammalian hosts (Tesh 1981). There is also an example of a plant-pathogenic virus transmitted sexually in the whitefly, *Bemisia tabaci* (Ghanim and Czosnek 2000). These viruses do not seriously affect the insect life history and are therefore not suitable for biological control. In fact, sexual transmission of these viruses may serve to maintain them in the vector population when vertebrate or plant hosts are scarce.

One of the early successes in using sexually transmitted pathogens occurred in 1967 in Western Samoa with a virus originally isolated from *Oryctes rhinoceros*, the rhinoceros beetle. The virus reduced the beetle populations by 50 % (Marschall and Loane 1982). This is a large, horned, scarabid beetle that attacks the leaf axil of developing fronds of Raffia, coconut, and other varieties of palm, eventually killing the tree by boring down into the meristematic tissue. The virus was originally classified as a baculovirus but is now in its own category (*Oryctes* virus) (Evans and Shapiro 1997). This virus replicates in the midgut epithelial cells, fat bodies, and testicular and ovarian cells. Zelazny (1976) showed that *Oryctes* virus was transmitted during copulation between infected and uninfected partners. Additionally, the virus could be transmitted to adults when they visited breeding sites that contained virus-infected dead larvae. Zelazny et al. (1990) released five different strains, singly or in combination, of *Oryctes* virus on islands in the Maldives in 1984–1985. Over the course of 4 years, the beetle population was reduced to 10–20 % of the original level, and on some islands, where the virus was not released, it appeared and significantly reduced beetle populations. *Oryctes* virus has also been released for rhinoceros beetle control in other South Pacific islands and India (see review of Huger 2005).

6.6.2 Fungi

There are monographs describing and cataloging the ascomycetous *Laboulbeniales* fungi that infect insects (Thaxter 1896; Weir 1996). These fungi are primarily ectoparasites, often species and location specific on the host. Although easily transmitted during copulation, they cause few if any adverse effects on the host (Riddick and Schaefer 2005). However, another group of fungi, the *Entomophthorales*, as its

name suggests, does cause severe damage and can be lethal. Soper (1963, 1981) worked with *Massospora* spp. pathogenic to various species of cicada. The fungus penetrated the abdomen to the point where it fell off. However, even in the absence of the part of the abdomen, the cicadas still attempt to mate, thus transferring the entomopathogenic fungus. While these fungi are lethal, their hosts are a nuisance pest but not of economic importance.

A final order of entomopathogenic fungi is the *Hypocreales* in which the genera *Metarhizium* (known as green muscardine fungi because of the color of the conidia that appear outside the integument a few days after death) and *Beauveria* (known as the white muscardine) are found. Species of these fungi are being applied for direct biological control of pests under limited conditions, usually elevated humidity. However, evidence suggests that there is the potential for developing these fungi for direct transmission during copulation.

Ips spp. bark beetles are phloem-feeders, making extensive galleries just under the bark of coniferous trees. They are naturally attracted to stressed or dying trees and serve an important role in the decomposition process; however, in large numbers, they can kill a tree. Since the galleries are in sapwood, fungi would be a logical means of beetle management. In laboratory trials, Kreutz et al. (2004) found that the transmission of *B. bassiana* from infected *I. typographus* to uninfected beetles was highly dependent on the ratio of the former to the later: at a 1:1 ratio, there was 96 % mortality at 4.3 days post-infection down to 75 % mortality at 7 days when the ratio of infected to uninfected was 1:20. In field trials, they found that there was a significant reduction in the length of all galleries and the number of bore holes.

The use of *B. bassiana* is being explored with the red palm weevil, *R. ferrugineus*. A sterile insect release program has been developed, and based on the success with *Oryctes*, irradiated males are treated with fungus. Results (Llacer et al. 2013) show that there is transfer of the fungus during mating and females show postmortem hyphal growth. While this is still in the early stages, the combination of irradiation for sterile insect technique (SIT) and fungus will result in greater efficiency in beetle management.

Fruit flies attack fruit in the early stages causing them to be deformed or drop off the plant. Because the larvae feed within the fruit, they are not susceptible to insecticides. Fruit flies are often distributed in humid, tropical regions; therefore, entomopathogenic fungi are a management option. The Mediterranean fruit fly, *C. capitata*, has been distributed worldwide and is one of the most destructive pests of ripening fruit. As such, and because the public has a preference for "green" management measures, there have been several programs on this and other fruit flies. One of the best methods to manage the medfly is by SIT, where millions of irradiated, sterile, male flies are released. While no control measure is 100 % efficient, this technique can be improved by additionally treating males with *B. bassiana*. In a large, 7000 ha, coffee growing area in Guatemala, researchers (Flores et al. 2013) found that 44 % of the wild medflies caught in dry traps were infected with *B. bassiana*. Fungi could only have been transmitted during leks, mating or mating attempts. The Mexican fruit fly, *Anastrepha ludens*, is another fruit fly for

which there is an SIT program. Work with two different commercial varieties of *B. bassiana* demonstrated that mating success of inoculated males was not affected and transmission to females through copulation was more than 80 % through direct mating and more than 15 % through attempted mating (Toledo et al. 2007). However, these researchers cautioned that more tests are required before the technique should be introduced commercially.

Bactrocera cucurbitae, known as the melon fruit fly, is an economically important pest of more than 80 species of plants in the Cucurbitaceae. Initial trials with *B. bassiana* looked good; however, Thaochan and Ngampongsai (2015) found that the mating propensity and competitiveness was significantly lower in *M. guizhouense*-infected males and females. In spite of this, there is a potential for pest management by treating male fruit flies and work is continuing.

6.6.3 Pheromones and Fungi

As described elsewhere in this chapter, there are many uses for pheromones. Female pheromones are used to attract males in conjunction with autodissemination traps containing both *B. bassiana* and/or *M.* (*anisopliae*) *brunneum*. Maniania et al. (2011) worked with *Busseola fusca*, a noctuid stem borer that is an important pest of corn and sorghum in eastern and southern Africa. Larvae feed on young leaves from which they enter the stems, reducing grain production or killing the plant outright. Researchers found that 94 % of the females that mated with infected males died from *B. bassiana* and 100 % from *M. brunneum*. In field trial, Yasuda (1999) used only *B. bassiana* in the female pheromone trap for the sweet potato weevil, *Cylas formicarius*. This pest attacks sweet potato crops, but in the absence of this host, it survives on morning glory, bindweed, and other Convolvulaceae. By 21 days post-infection, 57.9 % and 31.6 % of captured males and females, respectively, were infected with the fungus. Since the trap attracted only males, it was assumed that the females became infected through copulation.

6.6.4 Nematodes

At present there are very few known exoparasitic, entomopathogenic nematodes transmitted sexually and only one that has the potential for development as a biological control agent (BCA). *Spodoptera frugiperda*, the fall armyworm, is a voracious pest that will decimate many crops but prefers grass and small grain crops including corn and sorghum. The nematode, *Noctuidonema guyanense*, is an external parasite of adult moths and feeds internally with a very long (>100 μm) stylet (Simmons and Rogers 1996). The nematode moves actively and passively to a new host during mating, and the level of the new infestation is dependent on

the parasite load (typically 30–40 nematodes) of the infected host and the duration of mating; 15 min is enough for nematodes to transfer hosts, although mating can take up to hours. Furthermore, unlike endoparasitic nematodes, *N. guyanense* can withstand temperature fluctuations and humidity as low as 20 % RH. Infested armyworms have decreased survival with males being more affected.

6.6.5 Mites

Adalia bipunctata is also known as the two-spot ladybird. It is reared commercially and released as a BCA against aphids and other small insects. *Coccipolipus hippodamiae* is an ectoparasitic mite known to infest at least 19 species of coccinellids and is native to Europe. It resides on the underside of the elytra, feeding on the hemolymph, and immatures are transmitted between beetles during copulation. Once the immature mite has moved to the new host, it commences feeding and molts to the adult. Because this mite infests a BCA, much work has been done to understand the system (Hurst et al. 1995; Webberley et al. 2004). The mite infestation has a strong negative effect on coccinellid species, reducing female fecundity and egg viability. It is because of these deleterious effects that the mite is being examined as a potential control agent for the ladybird beetle, *Harmonia axyridis* (Rhule et al. 2010). *Harmonia axyridis* was originally released as a BCA against aphids and coccids in Europe; however, when released from its native site (Asia) became a voracious, polyphagous predator and even preyed on other coccinellids. Since *H. axyridis* has not evolved with the mite, artificial releases of mite-infested individuals may lead to sexual spread of the mite and reduction in the *Harmonia* population.

6.6.6 Adhesives to Enhance Infection and Transmission

While most of this section has been devoted to sexually transmitted pathogens, there are other developments to enhance pest management through mating. Rogers et al. (2014) tested two different adhesive powders specifically with the aim of attaching the insecticide spinosad to the medfly for a long enough duration for mating to occur. Of the adhesives they tested, the commercially available Entostat powder gave the best performance. The lethal time for males was over 9 h which was sufficient for them to mate. When males were treated, 70–78 % of the mated females had a knockdown time of less than 40 h. If females do not lay eggs during this time, the population is expected to significantly decline. It should be noted that these authors intend to use more species-specific insecticides, like trimedlure, to improve efficacy and prevent contamination of the environment.

6.7 Effects of Bacterial Endosymbionts on Arthropods

Many intercellular bacteria are endosymbionts infecting arthropods and nematodes and are capable of altering the reproductive potential of their hosts. This ability renders some endosymbionts as potential environmentally friendly tools in pest control either to enhance fitness of natural enemies or reduce fitness of pests (Werren 1997; Floate et al. 2006; Zindel et al. 2011).

Endosymbiotic associations can be obligatory (primary symbionts) or facultative (secondary symbionts) and are commonly found in diverse arthropod groups (Kono et al. 2008; Zindel et al. 2011). Obligatory endosymbionts are responsible for nutrition uptake (Baumann 2005), thus indirectly affecting development, reproduction, and overall fitness (Clark et al. 2010). In the absence of obligatory endosymbiotic bacteria, the host cannot survive. Facultative endosymbionts, although not critical to the host survival, can dramatically affect its fitness (Oliver et al. 2003; Perotti et al. 2006; Jones et al. 2007). Facultative endosymbiotic bacteria may affect their host feeding range or efficacy (Gunduz and Douglas 2009; Hosokawa et al. 2010), its immune function (Cordaux et al. 2011), defense against natural enemies (Oliver et al. 2009), and resistance to insecticides (Kontsedalov et al. 2008; Xia et al. 2013). Interestingly, facultative endosymbionts can be beneficial to their host under certain situations but costly in others (Haine 2007).

Secondary endosymbionts are mostly transmitted vertically from mother to offspring, but horizontal transfer is also known (Lipsitch et al. 1995; Lively et al. 2005). Males do not transfer their endosymbionts due to the low volume (if any) of cytoplasm transferred by their sperm.

In recent years, ample evidence has accumulated demonstrating various effects of bacterial endosymbionts on the reproductive potential of many insect taxa (Table 6.2). Endosymbionts can induce a female-biased sex ratio in offspring, thereby increasing the number of females in the population (Cordaux et al. 2011). The induction of the host sex ratio distortion can be via feminization (conversion of genetic males to phenotypic females) (F) and parthenogenesis (conversion of haploid males into diploid females) (PI) and through male killing (MK) (male

Table 6.2 Endosymbiont-induced reproductive manipulations

Endosymbiont	Bacterial group	Infected hosts	Manipulation
Wolbachia	α-*Proteobacteria*	Insects, crustaceans, mites, spiders	F, PI, CI, MK
Cardinium	*Bacteroidetes*	Insects, mites, spiders	F, PI, MK
Rickettsia	α-*Proteobacteria*	Insects, spiders	PI, MK
Spiroplasma	*Mollicutes*	Insects	MK
Flavobacteriia	*Mollicutes*	Insects	MK
Arsenophonus	γ-*Proteobacteria*	Insects	MK

F feminization of genetic males, *PI* parthenogenesis induction, *CI* cytoplasmic incompatibility, *MK* male killing

offspring die during embryonic development). Another reproductive manipulation is via cytoplasmic incompatibility (CI), induced by endosymbiont-bearing males that mate with uninfected females.

Often, reproduction-manipulating bacteria induce higher fitness in their hosts. For example, when females mated multiple times, infected flour beetle males (*Tribolium confusum*) gain higher fecundity through outcompeting the sperm of noninfected males (Wade and Chang 1995).

Of the known host reproduction-manipulating endosymbionts, *Wolbachia* is the most studied bacteria; *Wolbachia* is involved in several strategies including feminization, parthenogenesis, male killing, and cytoplasmic incompatibility; all of these lead to an increase in frequency of *Wolbachia*-infected females in the host populations (Saridaki and Bourtzis 2010).

6.7.1 Parthenogenesis-Inducing Endosymbionts

Increased understanding of the endosymbiont-insect relationship effect on reproduction of the insect host can open new arrays of pest control methods. For example, endosymbionts that are linked to parthenogenesis have been hypothesized to allow higher sustainability of their arthropod host; parthenogenesis permits faster increase of the population as they do not produce males, and low host populations do not harm their mate finding, as males are not needed to reproduce. For example, *Trichogramma* wasps are egg parasitoids and commonly used as biocontrol agents against pest eggs. *Wolbachia*-infected *Trichogramma* are parthenogenic but often suffer from reduced fecundity. Stouthamer (1993) compared the efficacy of *Trichogramma* females in controlling the population of a pest moth and demonstrated that a high density of the pests' sexually reproducing females produced more offspring and attacked more hosts, but in a low host population density, the parthenogenic, asexual females parasitized more eggs. Occasional augmentation with laboratory-reared *Wolbachia*-infected wasps may increase the overall success of managing a pest population due to the increased number of female progeny of the parthenogenic wasps, compared to the noninfected wasps that produce both males and females (Silva et al. 2000). Experimentally, parthenogenesis-inducing endosymbionts were transferred to sexually reproductive parasitoid wasps to obtain benefits when wasps were released as natural enemy agent. However, this resulted in limited induced parthenogenesis (Grenier et al. 1998).

6.7.2 Cytoplasmic Incompatibility-Inducing Endosymbionts

Mating of endosymbiont-bearing males with uninfected females may result in inviable offspring in diploid species and male only in haplodiploid species, whereas when both males and females harbor the same endosymbionts, the offspring are

viable (Vavre et al. 2000). Interestingly, when two or more strains of endosymbionts co-occur and each of the strain independently induces CI, singly infected females produce viable offspring after mating only with singly infected males carrying an overlapping strand, whereas females infected with all strains produce viable eggs after mating with males infested with any of the shared strands (Mouton et al. 2005).

In a biocontrol approach, endosymbionts inducing cytoplasmic incompatibility were transferred to a novel host in the attempt to release infected males that will mate with local uninfected wild-type females. In this approach, similar to SIT, treated males compete for copulation with the local males. Mating with treated males should result with no offspring and population decrease. This approach was taken to control the Mediterranean fruit fly *C. capitata* by releasing males infected with CI strain isolated from the fruit fly *Rhagoletis cerasi* (Zabalou et al. 2004, 2009). Similarly, *Wolbachia* taken from *Aedes albopictus* was transplanted in *A. aegypti* and resulted in high level of cytoplasmic incompatibility (Xi et al. 2005).

6.7.3 Endosymbionts in Pathogen-Vectoring Hosts

A different approach has been developed to combat vector-borne pathogens such as malaria, dengue fever, Chagas diseases, and filariasis, which are transmitted through feeding. In an attempt to control the dengue virus transmission through its vector, the mosquitoes *A. albopictus*, a *Wolbachia* strain was taken from *Drosophila melanogaster* and introduced into *A. albopictus*. The presence of the foreign *Wolbachia* eliminated the transmission ability of dengue virus in the treated mosquitoes (Blagrove et al. 2012; see also Moreira et al. 2009; Frentiu et al. 2010). The *Wolbachia*-infested mosquitoes when released into wild population of *A. albopictus* are expected to reach local fixation due to cytoplasmic incompatibility. This is because infected males produce no offspring after mating with local females (CI), followed by a decrease of the local mosquito populations and a relative increase of *Wolbachia*-infected females that so not transmit the virus.

The combination of the two strategies offered by the same *Wolbachia* species, i.e., releasing *Wolbachia*-infected females that do not transmit the virus, together with *Wolbachia*-infected males that limit the abundance of dengue, leads to a rapid decrease in dengue-carrying females in the population. This is a promising tactic to block the spread of the pathogens (Iturbe-Ormaetxe et al. 2011; Hoffmann et al. 2011; Walker et al. 2011).

Newly acquired dengue virus requires a sufficient incubation time in the host before the titer is high enough for transmission. Thus, the spread of dengue virus is strongly dependent on the mosquito's lifespan. Infecting mosquitoes with a *Wolbachia* that leads to a shortened lifespan and early senescence, before the virus can complete its development, has the potential to further limit the spread of the pathogen (Sinkins and O'Neill 2000; Texeira et al. 2008; Tahir et al. 2015).

6.7.4 Mixed Endosymbiont Infections

Another potential route for biological control using endosymbionts evolved with an improved technique to transfer bacteria between insects. There is an intriguing relationship between two different symbiotic bacterial communities that evolved with surprising effect on the host. *Wolbachia* and *Cardinium*, both of which have been documented to cause CI in insects, are also hosted by the same hosts: the spider mite *Bryobia sarothamni* and the parasitoid wasp *Encarsia inaron*. The two endosymbionts cause diverse reproductive phenotypes in their host; *Cardinium*, but not *Wolbachia*, induces strong cytoplasmic incompatibility. Apparently, in the presence of *Wolbachia*, *Cardinium* cannot perform the reproductive manipulation (Saridaki and Bourtzis 2010). If sexual transmission between members of the same population becomes a common practice, the introduction of the reciprocal symbiont can neutralize the reproduction manipulation. That is, mating of *Wolbachia*-infected males with *Wolbachia*-uninfected females that carry *Cardinium* can neutralize the *Cardinium* affect.

This antagonistic effect might be applied to the whitefly, *Bemisia tabaci* biotypes B and Q. These biotypes differ in various fitness parameters and the ability to induce damage in plants. The Q biotype is known to develop higher resistance to insecticides and both differ in the secondary endosymbionts they carry. *Hamiltonella* has been detected only in the B biotype, while *Wolbachia* and *Arsenophonus* have been found only in the Q biotype. The transmission efficiency of tomato yellow leaf curl virus by *B. tabaci* differs between the two biotypes; the B biotype is able to transmit the virus while the Q biotype is a poor transmitter. It is suggested that these differences are correlated with the endosymbionts and the two host types (Gottlieb et al. 2010). Thus, transmission of *Wolbachia* and/or *Arsenophonus* from the Q biotype to the B biotype might create diverse reproductive phenotypes.

6.8 Conclusion

In order to avoid the spread and extent of insect resistance to insecticides and to meet the public demands for reducing insecticide residues on edible products, the use of alternative tools to manage arthropod pests needs to be continually examined and developed.

Pheromone-based control tactics have been established against a variety of insect pests and found to be especially effective in area-wide programs. In planning future IPM programs, the use of these methods should be considered on large geographical scales, taking under consideration the pests' ecological and behavioral patterns, the distribution pattern of its hosts, and the available natural enemies that may contribute to the pest maintenance in low densities. The adoption of these environmental

friendly tools leads to a decrease in pesticide use and therefore conservation and increase of natural enemies that lead to a decrease of secondary pests (Ioriatti et al. 2008).

Insect pathogens have most frequently been developed as biological insecticides, but there have been some significant successes when they have been transmitted during mating of their hosts. As was shown in this chapter, pathogens can be used in conjunction with various pheromones in lure and kill or attracticide tactics.

Despite the plethora of literature of the subject, endosymbionts have not yet reached their expectation as biocontrol agents since there are relatively few concrete applied examples. This may be due to the complexity of the endosymbionts' lifestyle, the lack of technology, or the still uncovered attributes of the agents to either improve their host fitness (Dedeine et al. 2001) or reduce it (Silva et al. 2000; Zabalou et al. 2004).

All organisms must reproduce, thus a thorough understanding of the target pest's reproductive biology and behavior should be allied in order to properly use the available tactics, as discussed in this chapter, and develop new ones in order to face and overcome the challenge of minimizing the costly effect of hostile insects.

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Chapter 7
The Zoophytophagous Predator *Nesidiocoris tenuis*: A Successful But Controversial Biocontrol Agent in Tomato Crops

Meritxell Pérez-Hedo and Alberto Urbaneja

Abstract Protected tomatoes and sweet peppers are perhaps the crops whose use of augmentative biological control is the most established in the Mediterranean basin. In both crops, most phytophagous pests can be managed with the release and/or conservation of natural enemies; thus, the use of pesticides is rare. The lack of pesticide use has strengthened export markets, as they exert strong restrictions on pesticide residues. In the case of tomato crops, this change resulted from the development of integrated pest management (IPM) programs based on the use of mirid predators (Hemiptera: Miridae). There are several mirid species found in tomatoes in southern Europe, but *Nesidiocoris tenuis* is by far the most predominant. Primarily as a result of the use of *N. tenuis* in south-eastern Spain, IPM in tomatoes has considerably reduced pesticide use and increased the resilience of tomato crops against invasive pests. In this chapter, we present all of the attributes of *N. tenuis* that made these successes possible as well as the limitations that its use may pose.

7.1 Introduction

During the most recent decade, pest management of protected tomatoes in the Mediterranean basin has been evolving progressively from pest management based on the use of purely chemical pesticides to strategies based almost exclusively on

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the biological control of pests, where pesticides are rarely used. This process began at the end of the 1970s of last century with the use of *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae) and *Phytoseiulus persimilis* Athias-Henriot (Acari Phytoseiidae) for whitefly and spider mite control (Gabarra et al. 2008). Later, releases of leafminer and aphid parasitoids and the release and/or conservation of the predatory mirid *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) started to be common in some specific areas of the Mediterranean basin, although their use was scarce compared to tomato crops of northern Europe (van der Blom 2002). During the 1990s, the use of bumblebees for pollination prompted Mediterranean farmers to choose those pesticides that were selective with these pollinators. Consequently, the use of broad-spectrum pesticides significantly decreased in most tomato crops in the Mediterranean basin and opened the door to the release of natural enemies in this crop (van der Blom 2002). Nevertheless, chemical control still was the primary control measure to combat pests (Stansly et al. 2004). The appearance of the invasive pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in 2006, in Spain (Desneux et al. 2010), prompted the application of strategies to control this threatening tomato pest based on the use of mirid predators (Hemiptera: Miridae) (Urbaneja et al. 2012). Among the options placed into practice [e.g., releases of *Trichogramma achaeae* (Nagaraja & Nagarkatti) (Hymenoptera: Trichogrammatidae) (Cabello et al. 2012), treated with *Bacillus thuringiensis* (González-Cabrera et al. 2011)], the use of mirid predators was the most effective (Calvo et al. 2012; Urbaneja et al. 2009, 2012). There are several species of mirids that are reported to prey upon *T. absoluta*, such as *Nesidiocoris tenuis* (Reuter), *M. pygmaeus*, *Dicyphus errans* (Wolff), *D. maroccanus* (Wagner), *Campyloneuropsis infumatus* (Carvallo), *Engytatus varians* (Distant) and *Macrolophus basicornis* (Stal) (Ingegno et al. 2013; Abbas et al. 2014; Urbaneja et al. 2009; Bueno et al. 2013), but thus far only two of them, *M. pygmaeus* and *N. tenuis*, have been effectively employed within IPM programs (Urbaneja et al. 2012; De Backer et al. 2014; Calvo et al. 2012).

In southern Spain, where the largest protected tomato area in the Mediterranean basin is concentrated, the predatory mirid bug *N. tenuis* has successfully been incorporated into IPM programs (Fig. 7.1). Most tomato pests are controlled by the inoculation and conservation of *N. tenuis*, with a few exceptions, such as the eriophyid mite *Aculops lycopersici* (Masse) (Acari: Eriophydae). In only two crop seasons (2011–2012 and 2012–2013), the use of *N. tenuis* was widespread in a majority of the protected tomatoes in south-eastern Spain. In Spain, tomato plant protection using the natural enemies of pests is cheaper than not using a biological control (Velden van der et al. 2012). Nevertheless, as observed in Fig. 7.1, the total surface of the protected tomatoes using *N. tenuis* has not yet been reached. This might be attributed to some tomato growers being afraid of the plant damage released *N. tenuis* may inflict on their crops when high populations are reached, and they decide thus to resort to using selective insecticides instead. Indeed, until recently, *N. tenuis* had been considered a pest to many crops due to its plant feeding

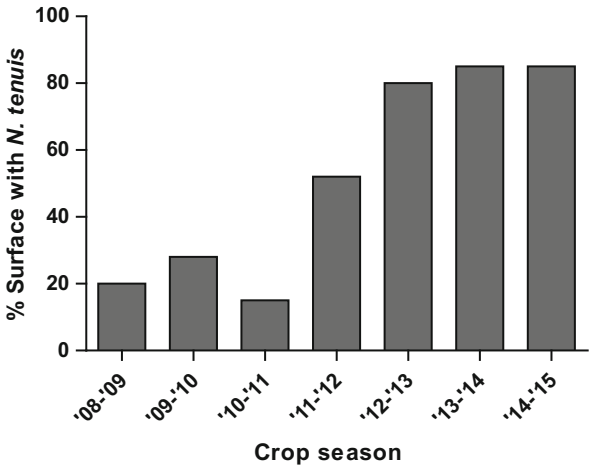

Fig. 7.1 Development of Integrated Pest Management based on the use of *Nesidiocoris tenuis* as the main component in the region of Almería (south-eastern Spain), where approximately 10,200 ha of protected tomatoes are cultivated. Source: Jan van der Blom (Coexphal, Almeria, Spain)

behaviour and, thus, its mere presence was countered with chemical treatments [see more details in Castañé et al. (2011)]. However, due to its wide success in tomato pest control, its use has been widespread and is currently considered a key natural enemy used for release and conservation purposes.

The recent success of *N. tenuis* has encouraged the scientific community to research this predatory mirid. Consequently, the number of scientific publications with a Science Citation Index (SCI) that have appeared in recent years related to *N. tenuis*, as well as the number of citations these articles have received, have considerably increased in comparison to previous years (Fig. 7.2). In this respect, it is curious that for such a well-known and global mirid as *N. tenuis* (formerly *Cyrtopeltis tenuis*), only two SCI research articles exist before the year 2000.

The success of this predator in controlling the invasive pest *T. absoluta* has prompted researchers from other geographical areas where *N. tenuis* is also present to use this mirid against *T. absoluta* or other tomato pests. It is very important that those in other regions know what was previously been achieved with this predator and to know what the present situation is to avoid repeating past mistakes. This book chapter was undertaken to bring together, in one single document, the existing information ["the good" and "the bad"] on *N. tenuis* as a biological control agent. Hence, firstly we provide basic information about the characteristics of *N. tenuis*, its distribution and biology and especially on its thermal requirements. Second, we document its zoophytophagous behaviour and its implication on the practical use of *N. tenuis*. Third, we review its potential as a natural enemy in different crops and the added benefits that *N. tenuis* can induce due to its plant feeding behaviour, which trigger indirect plant defences. Finally, we discuss possibilities to improve the use of *N. tenuis* and, especially, its ability to reduce plant damage.

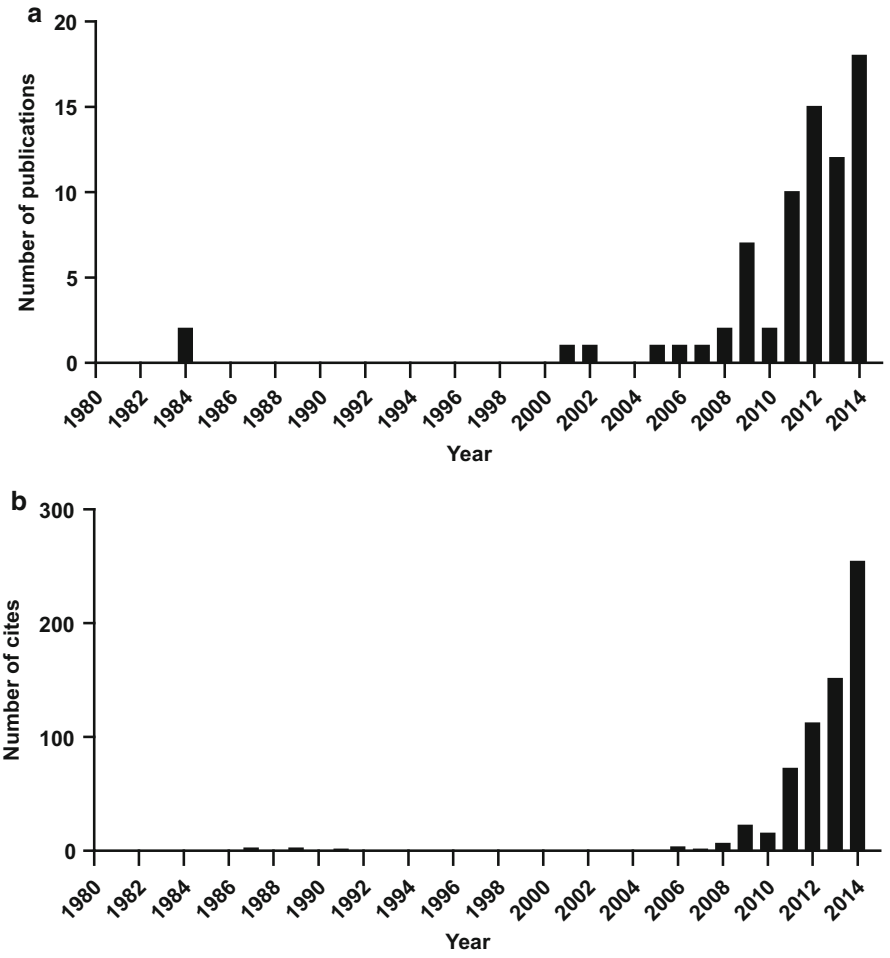

Fig. 7.2 Number of scientific publications devoted to *Nesidiocoris tenuis* (**a**) and number of citations (**b**) these articles have received from 1900 until 2014, according to the Science Citation Index (SCI). Values of articles and citations were obtained using the Web of Knowledge search engine with the search terms "Nesidiocoris tenuis" and "Cyrtopeltis tenuis"

7.2 Description and Distribution of *N. tenuis*

N. tenuis was described as *Cyrtopeltis tenuis* by Reuter from individuals collected in China in 1895 (Reuter 1894), although it had been previously described as *Dicyphus tamaricis* by Puton, in 1886, from individuals collected in Tunisia (Puton 1886). According to Wheeler and Henry (1992), this species is of palaeotropical origin, but has been transported widely with goods; today it is almost cosmopolitan in its distribution (Fig. 7.3). *N. tenuis* has a worldwide distribution and has been found in

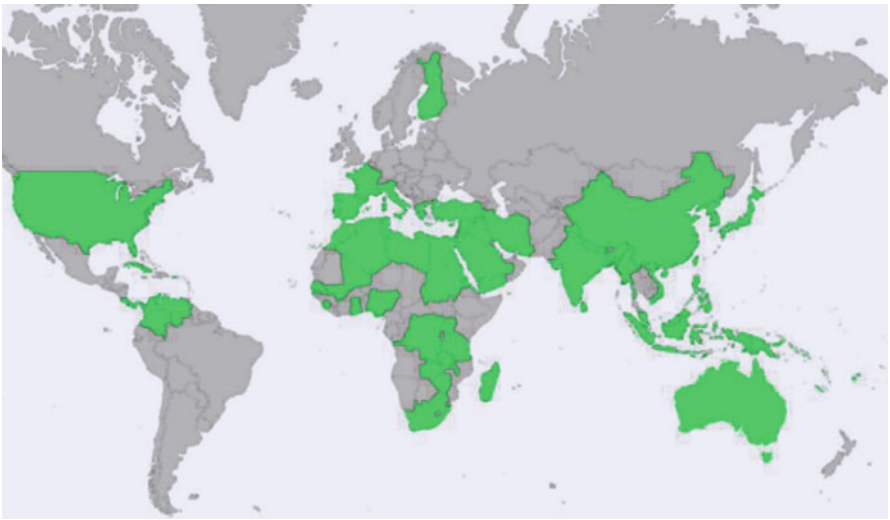

Fig. 7.3 World map distribution of *Nesidiocoris tenuis* (Source: CABI (2015) and the authors' personal observations)

all regions except some countries of South America, northern Europe and Russia. *N. tenuis* has been commercially or accidentally released or studied under laboratory conditions in cold areas such as the Netherlands, Belgium, Russia and the United Kingdom (De Puysseleyr et al. 2013; Hughes et al. 2009; Messelink et al. 2015; Pazyuk et al. 2014), where its permanent establishment has not been confirmed. Hughes et al. (2009) demonstrated that the permanent establishment of *N. tenuis* in northern Europe is unlikely to occur because this species cannot endure winter conditions in this geographical area for more than 4 weeks.

7.3 Biology of *N. tenuis*

N. tenuis, as a hemimetabolous insect, develops through the egg, nymphal and adult stages. The nymphal stage is further comprised of five instars, extending from the emergence of the nymph until it reaches the adult state. After emergence, nymphs have an average length of 1 mm with red eyes and a white colour immediately after emergence, then becoming yellow (Fig. 7.4a).

Nymphs grow to reach the fifth stage with a length of 2.5 mm and a green colouration. On this last instar, the primordial wings reach the fourth abdominal segment and the genitalia begin to be externally visible. The duration of the life cycle decreases as temperature increases, with an average development time of 86.7 days at 15 °C, 38.2 days at 20 °C, 21.8 days at 25 °C, 17.2 days at 30 °C and 14.9 days at 35 °C, when reared on tomato and using *Ephestia kuehniella* Zeller (Lepidoptera:

Fig. 7.4 (**a**) First nymphal instar of *Nesidiocoris tenuis*: (**b**) Female and male *N. tenuis* on a tomato plant

Pyralidae) as prey (Fig. 7.5) (Sánchez et al. 2009). Sánchez et al. (2009) found that *N. tenuis* is unable to develop at 40 °C, and lower development thresholds for eggs and nymphs were estimated to occur at 10.3 °C and 11.7 °C, respectively. The same authors established the thermal constant to be 148.6 and 182.3° days for eggs and nymphs, respectively. Hughes et al. (2009) estimated the developmental threshold (from egg to adult) to be 12.9 °C, with a thermal constant of 278 DD when *N. tenuis* was reared preying ad libitum on *E. kuehniella* on tobacco. On tomato, *N. tenuis* reaches maximum survival at approximately 25 °C, with an increased mortality above and below this temperature (Fig. 7.3). However, on tobacco leaf discs, Hughes et al. (2009) found the maximum survival (100 %) to be at temperatures of 30 and 32 °C.

Adults have brown eyes and the wings have dark brown spots. Males differ from females in that they have a slimmer abdomen and their genitalia are a black dot at the end of the abdomen, whereas females have a domed abdomen and inverted T-shaped genitalia (Fig. 7.4.b). Males and females have similar developmental times (Hughes et al. 2009). Females insert eggs into the epidermis of stems and leaf nerves, excluding the ligule (appendix for respiratory protruding from plant tissue). The egg is opalescent white, elongated and slightly curved.

When reared on tomato and fed *E. kuehniella* on tomato plants at 25 °C, *N. tenuis* females are able to produce more than 80 nymphs, with a sex ratio that

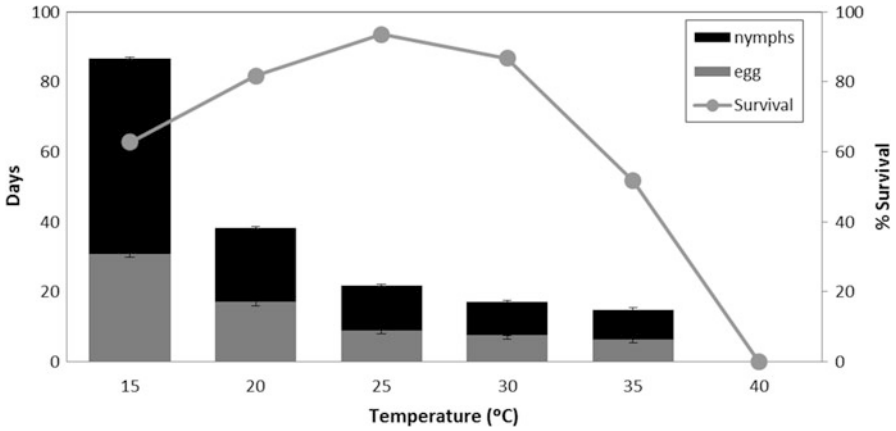

Fig. 7.5 Hatching time (*grey bar*; X ± SE), nymphal development time (*black bar*; X ± SE) and survival (%) for *Nesidiocoris tenuis* at different temperatures (Source: Sánchez et al. 2009)

is approximately 1:1 (Mollá et al. 2014). At this temperature, females survive approximately 18 days. This mirid is able to generate between 62 and 82 offspring nymphs per female in the temperature range 20–35 °C (Sánchez et al. 2009). To date, it has not been shown that this species enters diapause (Hughes et al. 2009). In addition to the aforementioned biological values, this clearly indicates that *N. tenuis* is a species adapted to warm environments (Sánchez et al. 2009) and will have difficulty surviving in cold climates (Hughes et al. 2010).

7.4 Zoophytophagy of *N. tenuis*

Zoophytophagous mirids are a special type of generalist predators that can also feed on the plants upon which they live. This group of predators may utilize different food resources, having the ability to feed at more than one trophic level, such as eating alternative prey and/or plant material, which further facilitates its establishment prior to pest infestation and its survival during periods of prey scarcity, resulting in a system of crops that are more resilient to pest attacks.

7.4.1 Plant Hosts

N. tenuis has been recorded on wild and cultivated plant hosts but it seems to prefer sticky plants with glandular trichomes that produce adhesive and viscous exudates on which *N. tenuis* is perfectly adapted to move and actively hunt for prey

Table 7.1 List of plant hosts on which *Nesidiocoris tenuis* has been recorded. Based on the references included in the text and the authors' personal observations

Family	Species	Common name
Solanaceae	*Solanum lycopersicum* L.	Tomato
	Capsicum annuum L	Pepper
	Nicotiana tabacum L.	Tobacco
	Solanum melongena L	Eggplant
	Solanum tuberosum L.	Potato
	Solanum nigrum L.	Black nightshade
Cucurbitaceae	*Cucurbita pepo* L.	Zucchini
	Cucumis melo L.	Melon
	Lagenaria siceraria (Molina) Standl.	Calabash
Asteraceae	*Gerbera* spp.	Gerbera
	Dittrichia viscosa (L.)	False Yellowhead
Pedaliaceae	*Sesamum indicum* L.	Sesame

(Hameed et al. 1976; Goula and Alomar 1994; Nath and Pal 1975; Prasad et al. 1979; Cano et al. 2009; Pérez-Hedo and Urbaneja 2015; Nucifora and Calabretta 1986) (Table 7.1).

The amount of plant feeding by *N. tenuis* decreases with increased prey feeding (Arnó et al. 2010; Sánchez 2009). *N. tenuis* is able to develop without having to feed on the plant (De Puysseleyr et al. 2013) but not without the availability of prey (Urbaneja-Bernat et al. 2013; Urbaneja et al. 2005). Urbaneja et al. (2005) showed that *N. tenuis* was unable to completely develop on sweet pepper, eggplant and tomato without supplemental food. However, tomato proved to be the most suitable plant food, enabling up to one-third of the nymphs to survive through the third instar. Eggplant was an intermediate plant host, with one-third surviving through the second instar but none further. Sweet pepper was the least suitable plant host, allowing only 10 % of the nymphs to survive through the first instar. Furthermore, when prey was also available, the biological parameters of *N. tenuis* varied depending on the host plant from which it fed. This mirid was able to completely develop on sweet pepper, eggplant and tomato host plants when supplemented with *E. kuehniella* eggs. However, the lowest survivorship was observed on pepper (64.3 %), compared to 73.7 and 72.7 % on eggplant and tomato, respectively. In addition, nymphal developmental time was longer on sweet pepper (14.3 days) than either eggplant (12.6) or tomato (12.9). From the above results, it might be concluded that animal prey is a required dietary component for *N. tenuis.*

El-Dessouki et al. (1976) observed that *N. tenuis* is frequently attracted to fresh wounds and returns to previous feeding sites where nymphs and adults aggregate. When comparing the tissues injured by *N. tenuis* to healthy tissues, a reduction of 34 % in the total protein content in the former was found (Raman and Sanjayan 1984).

7.4.2 Prey

This predator, similar to most mirid predators, displays a high degree of polyphagous behaviour and is able to feed on several different pest species (Urbaneja et al. 2003, 2005). As mentioned above, the availability of prey is essential for this species because *N. tenuis* cannot complete its life cycle without feeding on prey (Urbaneja et al. 2005). The range of prey includes different species of thrips, leaf miners, leafhoppers, aphids, spider mites and lepidopteran pests (Urbaneja and Jacas 2008). Depending on the prey upon which *N. tenuis* feeds, its life history traits can vary. Urbaneja et al. (2003) determined that the time needed to reach adulthood varied according to the prey species. Accordingly, when *N. tenuis* fed ad libitum on the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae), the developmental time from the first nymphal instar until adulthood was longer than when it fed on the thrips *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), and longer than when it fed on the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae) and on the eggs of *E. kuehniella* (Fig. 7.6a). Conversely, the survival was greatest when *N. tenuis* was fed *E. kuehniella* eggs (Fig. 7.6b). Therefore, it might be thought that when prey are motionless, the biological parameters of *N. tenuis* are better than when prey are mobile and, consequently, can escape causing *N. tenuis* to increase its energy expenditure to catch its prey. However, apart from prey mobility, the key determinant is the nutritional quality of the prey and how it meets the nutritional requirements of *N. tenuis* because adequate nutrition is essential for organisms to attain optimal fitness and to realise their maximal reproductive potential. Mollá et al. (2014) confirmed that *N. tenuis* could successfully develop and reproduce when it fed separately ad libitum on eggs of *T. absoluta* and *E. kuehniella*, under laboratory conditions. However, biological parameters were much better when *N. tenuis* fed on *E. kuehniella* eggs. The same authors speculated that *N. tenuis* is possibly able to compensate for the suboptimal nutrition offered by a particular prey in the field by also feeding on tomato plant tissue. However, due to the high polyphagy of *N. tenuis*, the presence of two or more prey under field conditions could be complementary and, therefore, increase its population levels. Further study of the nutritional requirements of *N. tenuis* could facilitate biological pest control by optimising the *N. tenuis* diet.

7.5 Plant Damage

N. tenuis has traditionally been classified as a pest of tomatoes due to its feeding behaviour (Arnó et al. 2010; El-Dessouki et al. 1976; CABI 2015; Raman and Sanjayan 1984; Kajita 1978; Malausa 1989; Malausa and Henao 1988; Trottin-Caudal and Millot 1997; Vacante and Grazia 1994). The phytophagous habits of

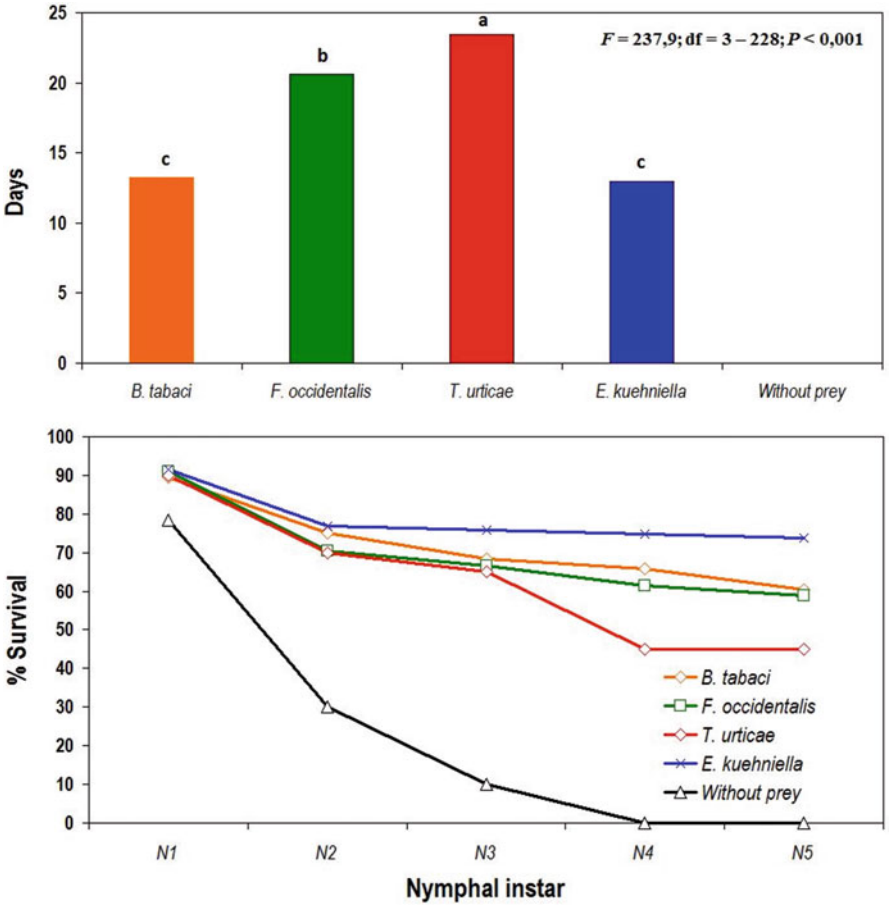

Fig. 7.6 (**a**) Development time (first instar nymph to adult) and (**b**) nymphal survival (%) for *Nesidiocoris tenuis* when reared on mature nymphs of *Bemisia tabaci*, mature nymphs and adults of *Frankliniella occidentalis*, all stages of *Tetranychus urticae* and eggs of *Ephestia kuehniella* at 25 °C (Source: Urbaneja et al. (2003))

N. tenuis as a phloem feeder could cause lesions to plant tissues because *N. tenuis* feeds on the vascular tissues by frequent stylet insertion, which produces a brown discoloration around tender stems and petioles known as necrotic rings (Fig. 7.7a), followed by the drying of flower stalks (Fig. 7.7b), flower abortion and whitish halos in fruits (Raman and Sanjayan 1984; Wheeler 2001; Castañé et al. 2011) and, ultimately, to yield loss under certain conditions (Sánchez 2009; Arnó et al. 2010).

The intensity of injury to tomato crops has been observed to decrease with the availability of prey (Arnó et al. 2010; Calvo et al. 2009). *N. tenuis* phytophagy is inversely proportional to the availability of prey (Sánchez 2009). The impact of fruit abortion on yield in tomato crops could be compensated by an increase

Fig. 7.7 (**a**) A young apical shoot with substantial *Nesidiocoris tenuis* damage. The *black arrows* indicate the presence of necrotic rings, whereas the red arrows show how new leaves wither due to the blockage of the phloem. (**b**) A new tomato shoot is broken at the point where the necrotic ring is

in the weight of the remaining fruits (Sánchez and Lacasa 2008). Particular care should be taken in cherry cultivars and those cultivars with short trusses, as they are particularly susceptible to *N. tenuis* damage. There are many references to *N. tenuis* causing damage to tomato crops. However, *N. tenuis* is also able to induce damage on other cultivated crops such as melon, sesame, tobacco, sweet pepper, calabash, some cucurbits and some ornamental crops (Prasad et al. 1979; Nath and Pal 1975; Hameed et al. 1976). Provided these conditions, before using *N. tenuis*, the potential risk of crop damage from this mirid should be considered (Albajes and Alomar 2008).

7.6 *N. tenuis* as a Biocontrol Agent

Due to its entomophagous behaviour *N. tenuis* has been observed to contribute to the control of whiteflies, thrips, leafhoppers, leaf miners, spider mites and Lepidoptera species in greenhouses (Arzone et al. 1990; Calvo and Urbaneja 2003; Carnero-Hernández et al. 2000; Marcos and Rejesus 1992; Solsoloy et al. 1994; Trottin-Caudal and Millot 1997; Urbaneja et al. 2009; Vacante and Grazia 1994; Nucifora and Calabretta 1986; Atherton 1933).

N. tenuis has effectively suppressed populations of Lepidopteran pests such as the tomato fruit borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Devi et al. 2002), and it can prey on eggs and young instar larvae from other related species such as *Spodoptera litura* Frabicius (Lepidoptera: Noctuidae) (Wei et al. 1998).

As previously mentioned, *N. tenuis* may use different food resources, being able to feed on more than one trophic level, such as alternative prey and/or plant material, which further facilitates its establishment before pest infestation and its survival in the crops for periods of prey scarcity, resulting in system crops that are more resilient to pest attacks. These characteristics have been especially exploited in tomato crops. The cornerstone of the strategies currently in use with *N. tenuis* entails pest control with either biological or selective insecticides until the population of predators is high enough to control the pest by themselves. To this end, and depending on the type of tomato crop and the area of cultivation, predators are released and/or conserved.

In tomato greenhouses, inoculative releases of *N. tenuis* (1–2 individuals/m^2) are usually conducted several weeks after transplanting [i.e., in greenhouse tomatoes of Murcia (southeast Spain), where approximately 3.000 ha are managed under IPM that is based in the inoculation and/or conservation of this predatory mirid bug] (Urbaneja et al. 2012). This strategy has been used successfully to control whitefly populations once a certain number of *N. tenuis* is present in the crop. Sánchez et al. (2014) determined that densities of 0.2 individuals per leaf during the linear population growth phase of *T. absoluta* (0.5–3 larvae per leaf) did not prevent outbreaks, which confirms that with this pest *N. tenuis* has to be well established in the crop before pest arrival. However, reaching this number of natural enemies requires five to eight weeks after the release in spring-summer crops (Mollá et al. 2011). Furthermore, it should be noted that this strategy does not work for all crop conditions. For example, in late crop cycles without heating, the mirid reproduction rate is not high enough to reach the desired population levels (Urbaneja et al. 2012). To shorten the establishment period and improve the distribution of *N. tenuis* in the crop, especially when weather conditions are less favourable, releases of predators have been made in the seedling nurseries (Calvo et al. 2012). In Almería (Spain), during the tomato growing season of 2010–2011, inoculation of *N. tenuis* in the nursery proved to be a good strategy for controlling *T. absoluta* and has been successfully implemented in approximately all tomato growing areas. This strategy entails transplanting tomato plants upon which *N. tenuis* individuals have already laid eggs in the nursery. A dose of approximately ½–1 *N. tenuis* per plant is released in the nursery in addition to *E. kuehniella* eggs as an alternative prey. Following this strategy, and if the environmental conditions are favourable, the population of *N. tenuis* can rapidly increase to enough individuals necessary to control tomato pests. Unfortunately, if the number of *N. tenuis* is too high, they might cause damages to the crop (necrotic rings and flower abortion), suggesting the implementation of regular monitoring to properly manage this approach.

7.7 Plant Defence Induction by *N. tenuis*

Plants respond to herbivore attacks through several signalling pathways, resulting in the production of herbivore-induced plant volatiles (HIPVs) (Dicke et al. 2009). Mirid predators can activate on the plant the same defence mechanisms that repel strict herbivores (Halitschke et al. 2011; Kessler and Baldwin 2004; De Puysseleyr et al. 2011; Pappas et al. 2015; Pérez-Hedo et al. 2015b; Pérez-Hedo et al. 2015a). These HIPVs can modify the behaviour of both the phytophagous pests and their natural enemies (Pare and Tumlinson 1997). The phytophagous activity of *N. tenuis* on tomato plants activates metabolic pathways, which causes a physiological response in the plant. This response results in a double-defence response: Abscise acid synthesis increases to activate volatiles resulting in an effect of the non-preference of the whitefly *B. tabaci*, and jasmonate acid synthesis increases to activate volatiles that attract the parasitoid whitefly *E. formosa*. In addition, the volatiles of the feeding punctured plant by *N. tenuis* can induce defences in neighbouring plants that are in good condition through the JA route, also resulting in the attraction of parasitoids by these intact plants (Pérez-Hedo et al. 2015b). However, not all zoophytophagous predators have the same capacity to induce responses in tomato plants. Tomato plants may have different degrees of attraction to natural enemies and pests depending on whether the phytophagy is produced, for example, by *N. tenuis*, *M. pygmaeus* or *D. maroccanus* (Pérez-Hedo et al. 2015a). Thus, while the plants damaged by *N. tenuis* repel *B. tabaci* and *T. absoluta*, the phytophagy of *M. pygmaeus* and *D. maroccanus* has no effect on the repellency on *B. tabaci* and also attracts *T. absoluta*. The activity of the three phytophagous mirid predators also results in the attraction of *E. formosa* (Fig. 7.8).

7.8 Conclusion

Undoubtedly, the incorporation of *N. tenuis* as a part of IPM programs in tomato in the Mediterranean basin has contributed to the success of pest control in this crop, in the reduction of pesticide use and in an increase in the resilience of the crop against invasive pests. Nevertheless, it is still possible to improve the potential of this mirid. Indeed, the most important issue to address is reducing the negative impact on the plant by *N. tenuis*. As mentioned previously, *N. tenuis* can damage plants as a result of their behaviour when the level of phytophagous prey is scarce. Because of the damage caused by *N. tenuis*, its use is limited in some geographical areas where it is considered exceptionally dangerous; even in the south of Spain, where its beneficial effect is amply demonstrated, it is normal to frequently treat crops with pesticides against *N. tenuis* populations to reduce and limit damages to the crop. An extreme case is Finland, where *N. tenuis* is listed as an invasive

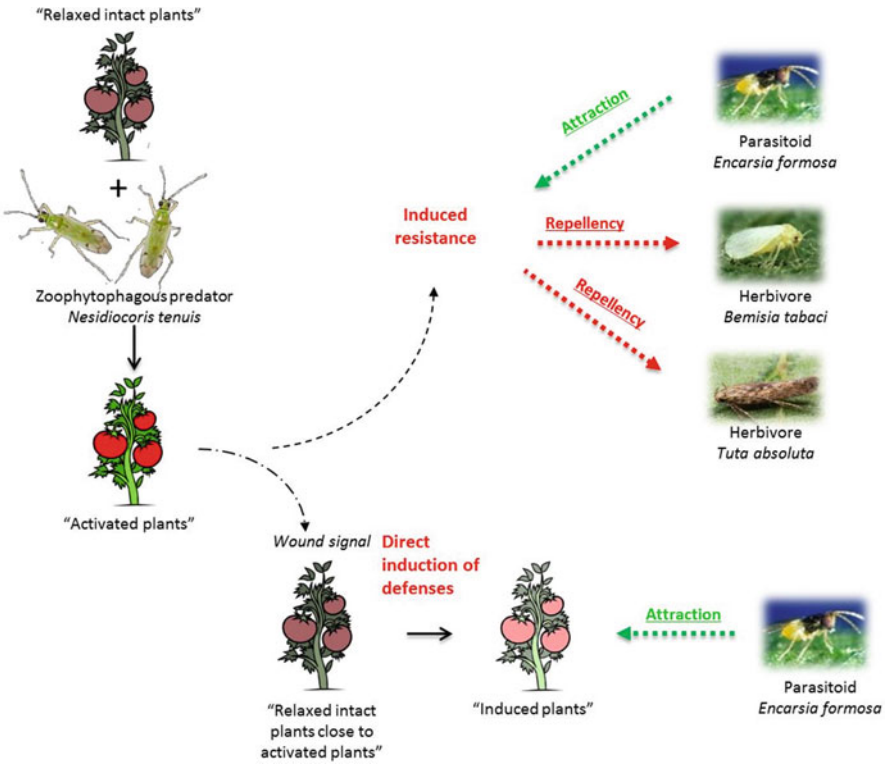

Fig. 7.8 A conceptual model of plant benefits induced by the zoophytophagous predator *Nesidiocoris tenuis* (Adapted from Pérez-Hedo et al. (2015a, b)). At the *top left* of the flow chart, a relaxed tomato plant is induced by *N. tenuis* feeding. Activated tomato plants resulted in a repellence effect on the whitefly *Bemisia tabaci* and the lepidopteran *Tuta absoluta*, and in an attraction of the whitefly parasitoid *Encarsia formosa*. Some of the chemical changes in the punctured plant may act as wound signals to undamaged, adjacent tomato plants. The JA pathway is activated in induced tomato plants, which results in the attraction of the parasitoid *E. formosa*

species and classified as a considerable nuisance, as it damages tomato plants in protected crops, especially in conditions with artificial lighting (MMM.FI 2012). To reduce the amount of plant damage inflicted by *N. tenuis*, new research has recently been launched within the European consortium BINGO (Breeding Invertebrates for Next Generation BioControl; http://www.bingo-itn.eu/en/bingo.htm), which aims to determine the metabolites causing damage to plants and, through artificial selection, produce strains of *N. tenuis* with less negative plant impacts.

Another limitation is to ensure the establishment and subsequent permanence of *N. tenuis* in periods of food scarcity. It would therefore be interesting to find alternatives to the eggs of *E. kuehniella*, which currently are conservatively used due to their high price (approximately $400 per kg of fresh eggs) (Urbaneja-Bernat et al. 2015). In recent years, several options have been evaluated, of which some

have shown promising results. For example, the decapsulated cyst of *Artemia* sp. or the complementary use of sugars may reduce the use of eggs of *E. kuehniella* (Urbaneja-Bernat et al. 2013, 2015; Mollá et al. 2015).

The defensive induction caused by *N. tenuis* on tomato crops could partly explain its great success as a key biocontrol agent. As mentioned previously, its action is not only beneficial for the direct effect of its entomophagy but also indirectly by its phytophagy, which causes a physiological response in the tomato plant. Further research is needed to better understand this interesting phenomenon and for the possibilities to be exploited in crop protection practices.

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Chapter 8
Development of Semiochemicals and Diatomaceous Earth Formulations for Bed Bug Pest Management

Yasmin Akhtar and Murray B. Isman

Abstract Bed bugs are obligate blood feeders on humans. In recent years, bed bug, *Cimex lectularius* L., (Hemiptera: Cimicidae) infestations have increased dramatically in many parts of the world including Canada and the USA, leading to a renewed interest in the chemical ecology of these pests to design better control options. According to Health Canada, bed bugs can now be found everywhere from homeless shelters to five-star hotels and from single-family dwellings to public transportation. Given that bed bugs are among the most difficult pests to eradicate, along with their demonstrated resistance to conventional insecticides and ease of transport, the key objective of our research is to facilitate the development of products for management of bed bugs, based on semiochemicals – nontoxic behavior-modifying substances or natural products such as diatomaceous earth. A more thorough understanding of how such chemicals influence bed bugs will inform the most effective uses of the formulated products as part of a bed bug pest management system. Although the consumer market is currently flooded with products of dubious composition and efficacy, these products are rarely adopted by pest management practitioners due to the lack of scientific data supporting claims of control. Our research involves helping our industry partners advance to the forefront in the development of safe and effective products for management of these public health pests. We have identified lead compounds as repellents as well as attractants and have developed specific diatomaceous earth (DE) dust formulations as part of a bed bug management strategy.

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8.1 Introduction

Bed bugs (*Cimex lectularius* L.; Hemiptera: Cimicidae) have reemerged as important public health pests in the past 15 years, with increasing intensity of urban infestations in Canada, the USA, western Europe, and Australia (Doggett et al. 2004; Potter et al. 2006; Harlan 2006). Low-income communities are more likely to suffer chronic and increased bed bug infestations due to limited financial resources available to provide effective community-wide management of infestations (Wang et al. 2011). This pest has a negative impact on the hospitality industry due to adverse publicity and litigation by persons who are bitten while staying in hotel rooms (Doggett et al. 2004; Potter et al. 2006). Since bed bugs can arrive on clothing or in suitcases of guests from infested homes or other hotels harboring the pests, hotels may become heavily infested with bed bugs (MedicineNet 2013). In addition to hotels, bed bugs have been found in movie theaters, office buildings, apartments, single-family dwellings, college dormitories, health-care facilities, laundries, shelters, transportation vehicles, and other locations where people congregate (MedicineNet 2013; Hwang et al. 2005). Bed bugs prompted the closure of the New Westminster, Canada, Public Library for 48 h in 2011 following the discovery of bed bugs in books (newwestcity 2011). At the Vancouver (Canada) Public Library, bed bugs were discovered across 12 of the Library's 22 locations in 2012 (Woo 2012). More than a third of pest management companies in the USA have treated bed bug infestations in hospitals in 2012, 6 % more than the year before and more than twice as many as in 2010, according to a survey released by the National Pest Management Association (Wjeczner 2013). The percentage of exterminators dealing with bed bugs in nursing homes also almost has doubled since 2010, to 46 % (Wjeczner 2013).

The exact cause of this resurgence is unclear, but may be a consequence of (i) the development of resistance in bed bugs to commonly used domestic insecticides; (ii) increased human movement – both travel and migration – (iii) more frequent exchange of secondhand furnishings among homes; (iv) decreased public awareness; and (v) global warming (Harlan 2006; Romero et al. 2007; Reinhardt and Silva-Jothy 2000). "Bed bugs are making a comeback. People now travel more than ever before, and bed bugs are hitching rides on clothing and luggage. Anyone can get an infestation of bed bugs and this does not mean a lack of cleanliness" (Health Canada 2013). The US Centers for Disease Control and Prevention (CDC) and the US Environmental Protection Agency (EPA) consider bed bugs a pest of "significant public health importance" and an emerging public health problem across the USA (CDC and EPA 2010).

Bed bugs are obligate blood feeders that require blood meals for growth and development throughout their life cycle (Fig. 8.1). Bed bug nymphs typically take 4–5 weeks to complete development and reach sexual maturity (Omori 1941). Blood feeding can result in allergic reactions in human beings due to the presence of vasodilatory substances (nitric oxide) in bed bug saliva Weeks et al. 2010;

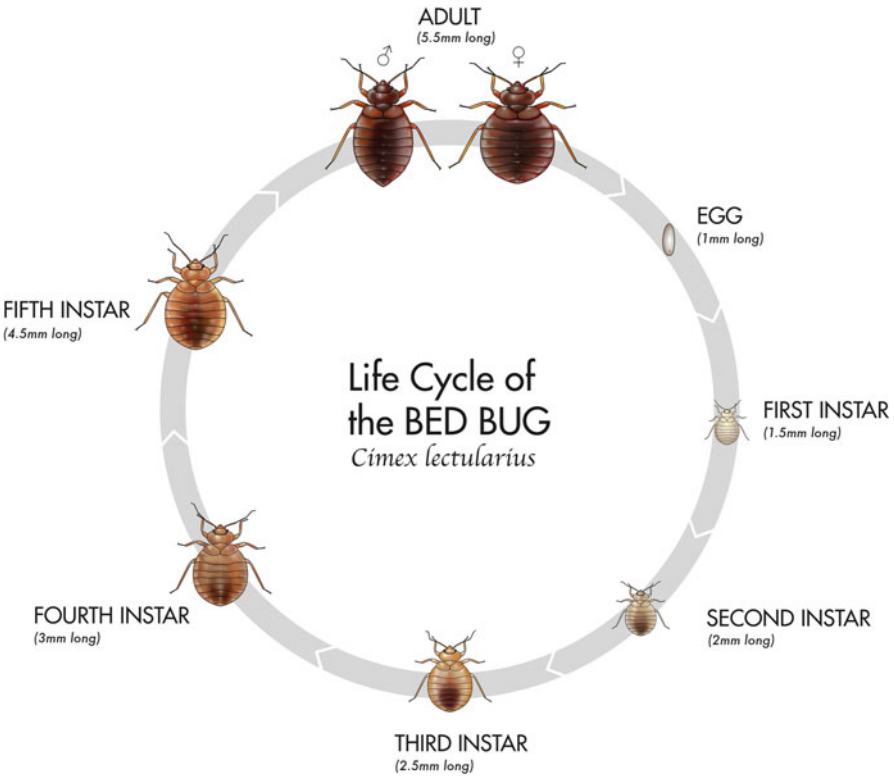

Fig. 8.1 Life cycle of *Cimex lectularius*

Doggett et al. 2012). Although there is no direct evidence that they can transmit disease between human hosts, they cause a range of emotional problems, discomfort, anxiety, and sleeplessness (Reinhardt and Silva-Jothy 2000; Doggett et al. 2012). Bed bug infestations often require expensive ongoing inspections and treatments, disposal and replacement of infested beds and other furnishings, and quarantine of infested areas (Romero et al. 2007). Treatment of bed bug infestations cost consumers in North America over $2 billion in 2010 alone. There is an urgent need to develop pest management tools that are not only effective in suppressing bed bug populations, but that do not themselves have undue negative impacts on human health (Haynes et al. 2010; Berg 2010). The latter is imperative given that bed bugs most often occur in and around bedding (up to 85 % of the population in infested rooms) (Wang et al. 2007) where humans spend up to a third of their lives. We discuss the major control strategies commonly used for the management of bed bugs with special emphasis on the use of natural products including diatomaceous earth and behavior-modifying substances or semiochemicals.

8.2 Bed Bug Control Strategies

8.2.1 Synthetic Insecticides

According to the EPA (2015), registered active ingredients for bed bug control include 29 chemicals: 16 pyrethrins and pyrethroids, 4 neonicotinoids, 3 inorganic compounds, chlorfenapyr, dichlorvos (DDVP), propoxur, S-hydroprene, alcohol, and neem oil. The majority are pyrethroids, which have limited field efficacy due to widespread resistance in urban bed bug populations (Romero et al. 2007; Zhu et al. 2010; Wang et al. 2014). Pyrethroid resistance (Table 8.1) in bed bugs has been reported in North America, Australia, Asia, and Europe and is widespread throughout the USA and presumably elsewhere (Romero et al. 2007; Zhu et al. 2010; Wang et al. 2014; Adelman et al. 2011; Kilpinen et al. 2011; Tawatsin et al. 2011; Dang et al. 2015; Doggett et al. 2011a; Seong et al. 2010). Several attempts have been made to characterize the mechanisms of resistance in these resurgent bed bug populations (Adelman et al. 2011). While a target site mutation, or *kdr* resistance, was identified as the primary mechanism of resistance in most resistant populations (Romero et al. 2007; Zhu et al. 2010; Yoon et al. 2008), other mechanisms of resistance such as enhanced detoxification enzyme activity also have been reported in a few cases (Romero et al. 2009a) or a combination of both (Adelman et al. 2011). Zhu et al. (2013) reported a unique resistant strategy in which resistant genes on the cuticle served to slow down the toxins from reaching target sites.

The development of pyrethroid resistance in bed bugs and the withdrawal of several effective insecticides, registered for bed bugs from the UK and US markets, have further reduced the options for control (Hwang et al. 2005). Chlorfenapyr, a prospective alternative to pyrethroids, is registered for bed bug control and is increasingly being used commercially (Moore and Miller 2006; Potter et al. 2008; Wang et al. 2009a; Romero 2011). Chlorfenapyr may not always control bed bugs in a timely manner (Moore and Miller 2006; Romero et al. 2010). Chlorfenapyr, used as a dry residue, produced >50 % mortality after 3 days of continuous exposure to bed bugs (Romero et al. 2010). Bed bugs exposed to chlorfenapyr EC-treated headboards took a longer exposure period to achieve 50 % mortality compared with synthetic pyrethroids (Moore and Miller 2006). The slower action of chlorfenapyr can be explained on the basis of its different mode of action (electron transport chain inhibitor) compared with synthetic pyrethroids (neurotoxin).

Pyrethroid resistance in bed bugs has prompted a shift to commercial insecticide products based on mixtures of a pyrethroid and a neonicotinoid by urban pest management professionals in the USA (Romero et al. 2010; Potter et al. 2012). These two classes of insecticides exhibit different modes of action (Gordon et al. 2014). Currently, the combination (pyrethroid/neonicotinoid) products are some of the most effective choices for control in the field (Romero et al. 2010; Potter et al. 2012). At least, four such combination products are being marketed for bed bugs including Temprid® (Bayer Environmental Science), Transport® (FMC Professional Solutions), Tandem® (Syngenta Professional Pest Management), and Bedlam Plus®

Table 8.1 Response of bed bugs to synthetic insecticides

Insecticide	Effect	Additional note	References
Deltamethrin	Widespread distribution of knockdown resistance mutation	L925I or V419L mutations responsible for knockdown resistance to deltamethrin	Zhu et al. (2010) and Yoon et al. (2008)
Deltamethrin	Based on the LD_{50} values, resistant ratios were ~5200-fold to deltamethrin	Bed bugs exhibit both kdr-type (L925I) and increased metabolic resistance to pyrethroid insecticides	Adelman et al. (2011)
Dust band (1 % cyfluthrin)	Mortality	Both dust band and IPM resulted in higher bed bug reduction than the control	Wang et al. (2013b)
β-Cyfluthrin	Unique resistance strategy	Resistant genes on cuticle slow down the toxins from reaching target sites	Zhu et al. (2013)
Deltamethrin and lambda-cyhalothrin	Mortality	Resistance in field populations in Kentucky and Ohio	Romero et al. (2007) and Zhu et al. (2010)
Pyrethroid/ neonicotinoid	Mortality	Combination products – more lethal to bed bugs than active ingredient alone	Potter et al. (2012, 2013a)
Deltamethrin	Mortality	Field strain less susceptible than lab strain	Seong et al. (2010) and Moore and Miller (2006)
Deltamethrin	Mortality	Efficacy varies with feeding status	Potter et al. (2013a)
Deltamethrin + piperonyl butoxide	Synergistic ratio varied in different populations	P450 and other resistance mechanisms (enhanced metabolic activity) may be involved	Yoon et al. (2008)

(MGK), with the expectation that there will be more in the future. Laboratory and field reports indicate the products are more efficacious than pyrethroids alone and are now the most utilized category for bed bug treatment (Romero et al. 2010; Potter et al. 2013a).

To minimize the risk of insecticide exposure and the amount of insecticide used, Wang et al. (2013a, b) designed and evaluated a dust-treated band technique. Both laboratory and field data suggested that 1 % cyfluthrin dust-treated bands were highly effective in killing bed bugs. There was no significant difference in the final counts of bed bugs between dust-treated band and integrated pest management (IPM) treatments. A recent study (Devries et al. 2015) demonstrated the role of

feeding status of bed bugs in the toxicity of deltamethrin; 21-day-starved bugs had a significantly lower LD_{50} [0.221 ng·bug^{-1}] compared with 2- and 9-day-starved bugs.

8.2.2 Behavior-Modifying Substances

Semiochemicals (behavior- and physiology-modifying chemicals) could be exploited for management of bed bugs (Logan and Birkett 2007) especially in multi-dwelling buildings including hotels and school dormitories (Weeks et al. 2010). Behavior-modifying substances can be based on natural pheromones eliciting a repellent or an attractant response by the bed bug. Alarm pheromones of the bed bug could be used as a repellent to deter bed bugs from human hosts, and aggregation pheromones could be used in traps to monitor or control bed bug populations in an infested area. Although some semiochemicals have been identified previously, our knowledge of how they mediate bed bug behavior and consequently how they could be utilized for bed bug management remains incomplete.

8.2.2.1 Use of Repellents to Deter Bed Bugs from Human Hosts

A potential strategy is the use of repellents to drive bed bugs away from places where human beings are sleeping in an effort to reduce bed bug bites. Insect repellents can be of three different types including semiochemicals (alarm pheromones), botanicals (based on plant essential oils), or synthetics (e.g., DEET).

Semiochemicals (e.g., pheromones) are chemical substances emitted by an organism that produce behavioral and physiological changes in receivers. Both adult and nymphal stages of bed bugs emit secretions that are repulsive to conspecifics. Of the ten compounds constituting *C. lectularius* nest odors, Levinson and Bar Ilan (1971) identified (*E*)-2-hexenal and (*E*)-2-octenal, secreted from dorsal abdominal glands in nymphs and metathoracic glands in adults, acting primarily as an alarm pheromone and responsible for eliciting dispersal behavior in conspecifics (Levinson et al. 1974).

Alarm pheromones are released by all stages of bed bugs under stress and have a dual function (Ryne 2009). In addition to causing conspecifics to disperse, it can also act as a mating deterrent. Homosexual mating is a common behavior in bed bugs. In order to avoid homosexual mating and abdominal injuries, newly fed nymphs secrete (*E*)-2-hexenal:(*E*)-2-octenal in a nymph-specific ratio [2:5], 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal (Table 8.2), although 4-oxo-(*E*)-2-hexenal may also exert repellent effect on its own against males. Newly fed males also release alarm pheromones to signal their sex and avoid/reduce the risk of homosexual mating (Ryne 2009; Harraca et al. 2010; Liedtke et al. 2011; Feldlaufer et al. 2010). A comprehensive review of bed bug chemical ecology has been provided by Weeks et al. (2013) and Benoit (2011), among others.

Table 8.2 Response of bed bugs to semiochemicals

Semiochemicals	Effect	Additional note	References
4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal	Mating deterrent against *C. lectularius* and *C. hemipterus* adults	Absent in headspace collections of adults	Liedtke et al. (2011)
E)-2-hexenal, (*E*)-2-octenal, 4-oxo-(*E*)-2-hexenal, and 4-oxo-(*E*)-2-octenal	Mating deterrent	Nymphs of both species but were stage specific	Harraca et al. (2010)
1-octen-3-ol, lactic acid, heat, and CO_2 (dry ice)	Attractant (pitfall trap)	Caught 80 % of bed bugs in small arenas and 57 % of bed bugs overnight in large arenas. Also effective in infested apartments	Siljander et al. (2011)
Proprionic, butyric, valeric, octenol, and L-lactic acid + heat (37.2–42.2 °C) and CO_2	Attractant	Captured more bed bugs than other traps	Pfiester et al. (2008)
CO_2, heat, and natural lure (1-octen-3-ol, spearmint oil, and coriander oil)	Attractant (pitfall trap and experimental arena)	Chemical lure and CO_2 are essential for designing effective bed bug monitors – heat less important	Wang et al. (2011)

Commercially available insect repellents can be divided into two categories – synthetic chemicals and plant-derived essential oils. *N*, *N*-diethyl-3-methylbenzamide (DEET) has remained the most widely used insect repellent since 1957 and has demonstrated activity against a number of arthropods including mosquitoes, biting flies, chiggers, fleas, and ticks (Syed and Leal 2008; Pickett et al. 2008). However, DEET has a strong smell and dissolves certain plastic materials. Since many consumers are reluctant to apply DEET to their skin, there remains the need to develop new and safe repellent products (Fradin and Day 2002).

Most plant-based insect repellents currently available in the market are based on essential oils including citronella, peppermint, eucalyptus, lemongrass, geranium, and soybean among others. Most of the essential oil-based insect repellents available in the market provide a short protection time compared with DEET (Fradin and Day 2002). The repellent containing oil of eucalyptus marketed as Repel Lemon Eucalyptus Insect Repellent (WPC Brands) and Fite Bite Plant-Based Insect Repellent (Travel Medicine) provided a mean protection time of 120.1 ± 44.8 min against mosquitoes; the insect repellent, Herbal Armor® (All Terrain), consisting of 25 % essential oils (citronella, geranium, cedar, peppermint, lemongrass, and soybean) as active ingredients provided a protection time of 20 min or less (Fradin and Day 2002). The synthetic IR3535-based repellent and a formulation

containing 23.8 % DEET provided an average protection time of 22.9 and 301.5 min, respectively, against mosquito bites in arm-in-cage studies (Fradin and Day 2002). Three repellent products based on plant essential oils including EcoSMART® insect repellent have been commercially registered against bed bugs in the USA (EcoSMART 2014) but not in Canada.

Wang et al. (2013a, b) evaluated the repellency of three commercially available insect repellents including DEET (97 % purity, Spectrum Laboratory Products Inc., Gardena, Ca), Cutter® Advanced Insect Repellent (7 % picaridin, United Industries Corporation, St. Louis, MO), and Rest Easy™ Bed Bug and Insect Control (0.5 % permethrin, Eaton, Twinsburg, OH) along with five nonregistered materials (two recently reported natural repellents, isolongifolenone and isolongifolanone [derived from *Humiria balsamifera*, a plant commonly found in South America] and three novel potential insect repellents developed by Bedoukian Research Inc., Danbury, CT, including 3-methyl-5-hexyl-2-cyclohexenone, propyl dihydrojasmonate, and γ-methyl tridecalactone) against *C. lectularius*. Cutter® Advanced Insect Repellent and Rest Easy™ Bed Bug and Insect Control were not active repellents against bed bugs; DEET provided a high level of repellency against bed bugs. A 10 % DEET, in the presence of carbon dioxide as a host cue, provided ≥94 % repellence for a period of 9 h. Although both isolongifolenone and isolongifolanone exhibited strong repellent effects against bed bugs, they were significantly less active than DEET. The three novel compounds (i.e., 3-methyl-5-hexyl-2-cyclohexenone, propyl dihydrojasmonate, and γ-methyl tridecalactone) exhibited similar levels of repellency and residual action as DEET in repelling bed bugs (Wang et al. 2013a).

8.2.2.2 Screening of Putative Bed Bug Repellents in the Laboratory

We have screened several naturally occurring semiochemicals or their structural and functional analogs (Gilbert 2014) for repellent (Table 8.3) and attractant effects (Table 8.4). Approximately 20 of 120 compounds, including both natural and synthetic semiochemical analogs, demonstrated sufficient bioactivity against *C. lectularius* at 24 h and a screening concentration of 1 % to be considered for continued repellent formulation development (Table 8.3). Methyl trans-4-oxo-2-pentenoate and 1-furan-2-yl-2-methylbutan-1-one provided 100 % repellence (Table 8.3) followed by (E)-1-hydroxyoct-2-en-4-one, 6,10-dimethyl-5,9-undecadien-2-one, furfuryl propionate, 2-butyrylfuran, 1-(furan-2-yl)-pentan-1-ol, and (E)-3-methylhept-3-ene-2,5-dione. Furfuryl propionate has been formulated as a fast-acting aerosol (Table 8.5) and as slow-release beads (Table 8.6). These products are currently under review by the joint US Environmental Protection Agency (US EPA) and Health Canada's Pest Management Regulatory Agency (PMRA). Potential target markets for these products include commercial pest control operators, first responders who are often required to enter infested locations, the hospitality industry (e.g., hotels, cruise lines), and travelers.

Table 8.3 Bed bug repellents screened in the laboratory at 1 % $N = 30$ (three replicates of ten insects)

Compounds	Repellence (%)	Compounds	Repellence (%)
Methyl trans-4-oxo-2-pentenoate	100	1-(Furan-2-yl)-propan-1-ol	79
1-Furan-2-yl-2-methylbutan-1-one	100	6,6-Diethoxyhex-4-yn-3-one	78
(*E*)-1-Hydroxyoct-2-en-4-one	98	(*E*)-3-Methylhept-3-ene-2,5-dione	76
6,10-Dimethyl-5,9-undecadien-2-one	98	(*E*)-Oct-4-ene-3,6-dione	74
Furfuryl propionate	94	(*E*)-1-(Furan-2-yl)pent-1-en-3-one	73
2-Butyrylfuran	92	Ethyl 2-furoate	73
1-(Furan-2-yl)-pentan-1-ol	92	1-(Furan-2-yl)-ethanol	71
(*E*)-3-Methylhept-3-ene-2,5-dione	90	Ethyl 3-methyl-4-oxocrotonate	72

Table 8.4 Bed bug attractants screened in the laboratory at 1 %

Compounds	Attractance (%)
(*E*)-6-Hydroxyhex-4-en-3-one	100
Allyl propionate	96
3-Hexanone	85
3-Nonanone	100
Vinyl propionate	92
N-Methylpropionamide	100
Methyl-4-oxobutanoate	100
3-Pentanone	90

$N = 30$ (three replicates of ten insects)

One lead compound, furfuryl propionate, demonstrated consistent repellence for over 24 h (Fig. 8.2) and has been developed into a rapid-release aerosol formulation and slow-release beads. Repellent effects of the formulation and beads are shown in Tables 8.5 and 8.6, respectively.

8.2.2.3 Repellent Effects of Compounds and an Aerosol Formulation in Glass Arenas

Glass box arenas (Fig. 8.3) consist of rectangular containers ($23.5 \times 18.5 \times 7.0$ cm) with lids modified to fit a mesh screen allowing air movement between the box interior and exterior. Test solutions (control versus treated) were applied onto pieces of cloth (10×10 cm) placed at opposite ends of the box arena. The position of test subjects was monitored at specific time intervals (1, 2, 4, 6, 8, 12, and 24 h) after initial introduction and repellence were determined for each treatment.

Table 8.5 Repellent effect of furfuryl propionate aerosol formulation in a glass box arena

Time (h)	Number of bed bugs		Repellence (%)
	Control	Treated	
1	26	0	100
2	27	0	100
4	24	0	100
6	28	0	100
8	29	0	100
12	29	0	100
24	23	5	82.1

$N = 30$ (three replicates of ten insects); control = isopropyl alcohol (IPA); treated = furfuryl propionate (5 %)
Only individuals that are in close contact with the treatment or control substrates were recorded. Bed bugs elsewhere in the arena were not included in repellence calculations

Table 8.6 Repellent effect of furfuryl propionate-treated beads in cardboard box arena

Time (h)	Number of bed bugs		Repellence (%)
	Control box	Treated box	
24	20	6	76.9
48	21	7	75.0
72	19	9	67.8
96	21	6	77.8
120	21	6	77.8
Average	20.4	6.8	75.0

$N = 30$ (three replicates of ten insects); control box = untreated beads; treated box = beads treated with furfuryl propionate (25 %)

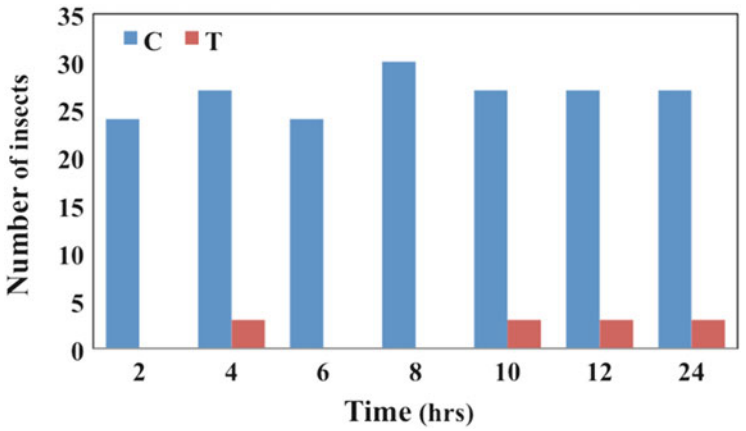

Fig. 8.2 Response of bed bugs to furfuryl propionate (2.5 %) at different time intervals. *Asterisks* indicate significant differences between the control and treated groups for that time period (Tukey's test; $p < 0.05$). Control was isopropyl alcohol (100 %); $N = 30$ (three replicates of ten insects)

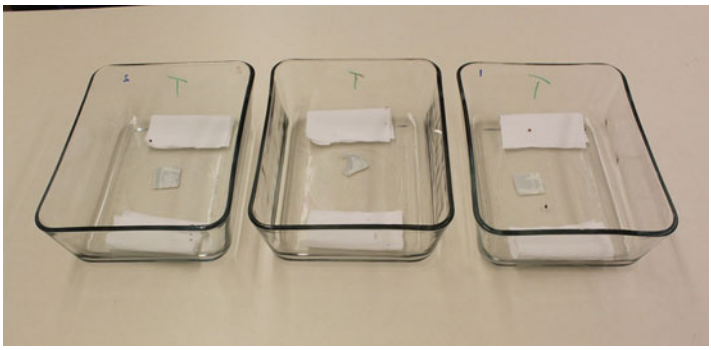

Fig. 8.3 Glass box arenas

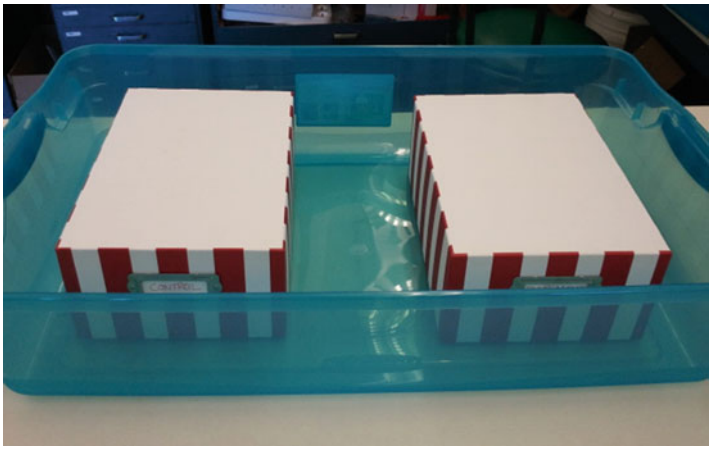

Fig. 8.4 Cardboard box arena

8.2.2.4 Repellent Effects of Beads in Cardboard Boxes

Beads were tested in a cardboard box (suitcase mimic) (Fig. 8.4) arena ($28.2 \times 19.3 \times 11.0$ cm). Control/treated beads were introduced into glass Petri dishes (5 cm diameter), placed on each side of a plastic container. Cardboard boxes were inverted over the Petri dishes containing the beads, one for the control and one for the treated beads. Following 24 h of saturation, ten bed bugs were introduced into the center of the plastic container and were allowed to crawl freely under the cardboard boxes. The position of the insects was monitored after 24, 48, 72, 96, and 120 h. Readings were taken by flipping the control box only using the formula: number of bed bugs on the treated beads = number of bed bugs introduced – number of bed bugs on the control and outside the boxes.

Overall, almost 50 % of the compounds demonstrated good activity at a concentration of 1 % against nymphs and adult bed bugs as repellents; 15 compounds

consistently produced over 80 % repellence, generally meeting regulatory performance standards for pest control. The most potent compounds produced an average of 100 % repellence.

8.2.2.5 Traps Based on Aggregation Pheromone

Aggregation pheromones are responsible for the formation of conspecific groups of mixed age and sex. The study of aggregation pheromones is the area of bed bug chemical ecology currently attracting the most interest from the scientific community (Weeks et al. 2010; Siljander et al. 2007, 2008; Olson et al. 2009; Gries et al. 2015). The importance of using an aggregation pheromone for monitoring or control purposes is based on the premise that it will be effective against all members of the colony regardless of sex and developmental stage. Recent studies have demonstrated that aggregation of bed bugs is mediated by a combination of airborne (Siljander et al. 2008) and contact pheromones (Siljander et al. 2007; Olson et al. 2009). An airborne aggregation pheromone (Table 8.2) composed of several short-chain aldehydes and monoterpenes occurring in the exoskeleton of immature bed bugs has recently been shown to stimulate aggregation of adult and immature bed bug in harborages (Siljander et al. 2008). Ten compounds (nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, benzaldehyde, (+)- and (−)-limonene, sulcatone, benzyl alcohol) proved to be essential components of the *C. lectularius* airborne aggregation pheromone (Siljander et al. 2008). According to Gries et al., (60) essential attractive volatile pheromone components present in bed bug feces consist of a blend of sulfide compounds (dimethyl disulfide and dimethyl trisulfide), (*E*)-2-hexenal, (*E*)-2-octenal, and 2-hexanone. Although (*E*)-2-hexenal and (*E*)-2-octenal (Levinson and Bar Ilan 1971, 1974; Siljander et al. 2008) have been previously reported as alarm and aggregation pheromone components, the two sulfides and 2-hexanone represent new volatile pheromone components.

Some of the natural constituents of the aggregation pheromone also function as an alarm pheromone at higher concentrations that could be useful as a repellent (Siljander et al. 2008). (*E*)-2-Hexenal and (*E*)-2-octenal were the most abundant compounds associated with aggregation in the headspace collections from bed bug colonies (Siljander et al. 2008). Both of these compounds serve as alarm pheromones at higher concentrations elicited by mechanical disturbance or agitation (Siljander et al. 2008).

8.2.2.6 Traps Based on Heat, Carbon Dioxide, or Chemical Lures

Traps that claim to attract bed bugs currently on the market use either heat (Kells and Goblirsch 2011; Puckett et al. 2013), chemical lures, or both (Anderson et al. 2009). Heated traps mimic a human host to attract hungry bed bugs by producing heat a few degrees higher than the ambient temperature (Moore and Miller 2009). Chemical-baited devices often rely on the use of attractant semiochemicals to lure bed bugs

from their refuges and into a trap. Carbon dioxide and heat have been proven to be the two most effective attractants used for bed bug monitoring (Puckett et al. 2013; Singh et al. 2012). Another study also suggests a combination of chemical lure (i.e., 1-octen-3-ol, spearmint oil, and coriander Egyptian oil) and CO_2 to design effective bed bug monitors (Wang et al. 2009b).

Several traps also use a combination of chemical lures, CO_2, and heat to attract bed bugs. A pitfall trap using a combination of heat (37.2–42.2 °C) and kairomones, including a gel lure, impregnated with propionic acid, butyric acid, valeric acid, (RS)-1-octen-3-ol, and L-lactic acid, and CO_2 (Anderson et al. 2009) captured more bed bugs than other traps in a vacant apartment (Table 8.2).

Two commercial monitors, CDC3000 (Cimex Science LLC, Portland, OR) and NightWatch (BioSensory Inc., Putnam, CT), that became available in early 2009 also use a combination of CO_2, heat, and a chemical lure to attract bed bugs. These traps were set up in occupied apartments along with a dry ice trap (Wang et al. 2011). The dry ice trap captured more bed bugs than CDC3000, which in turn was more active than NightWatch. In lightly infested apartments, the ClimbUp Insect Interceptor, a passive monitor without any attractant (operated for 7 days), trapped a similar number of bed bugs as the dry ice trap (operated for 1 day) and trapped more bed bugs than CDC3000 and NightWatch (operated for 1 day). The Interceptor also was more effective than visual inspections in detecting the presence of small numbers of bed bugs (Wang et al. 2011). The chemicals that make up the seven-component blend of CDC3000 have yet to be disclosed.

First Response Bed Bug Monitor (SpringStar Inc., Woodinville, WA, USA), which uses a combination of CO_2, heat, and a synthetic kairomone lure to attract bed bugs, was used to sample bed bugs from two established bed bug populations (Schaafsma et al. 2012). The number of first-instar nymphs caught in the trap was significantly higher than reported in previous studies employing different sampling methods. Another device patented by Siljander et al. (2008) is based on the release of a cocktail of bed bug aggregation pheromones and infrared radiation (Table 8.2).

These devices have several limitations including their high cost and lack of accessibility to the general public. Some of them are no longer available. Moreover, the use of CO_2 in traps is impractical for routine surveillance. Although a number of traps are commercially available, there are few scientific studies that have tested the efficacy of these devices.

Some studies (Anderson et al. 2009; Wang et al. 2011) have demonstrated the potential for trapping as a viable alternative to visual inspections in confirming the presence and size of a bed bug infestation. Wang et al. (2011) showed that certain traps could detect up to 100 % of infestations that have been previously identified by visual inspection.

The potential use of bed bug semiochemicals in monitoring and control of bed bugs has been reviewed (Weeks et al. 2010). Early detection of a bed bug infestation is very important because the larger the infestation, the more difficult eradication will be. Current routine monitoring is limited to visual inspections. Visual inspections are not only labor and time consuming but often seem to miss a large number of bed bugs (Wang et al. 2010).

Trained dogs have been used to detect bed bugs and identify active and inactive refuges (Weeks et al. 2010; Pfiester et al. 2008). This technique may be quicker and more effective than visual inspection, but requires proper training of both the dog and the handler (Fong et al. 2013). Canine inspections are costly (require ~ USD 900–1500 for inspecting a nursing home, apartment building, or a hotel) (Weeks et al. 2010; Miller 2007), and their performance is often unsatisfactory (Wang et al. 2011). More research is needed to determine factors responsible for canine detection and establishing standard procedures to evaluate the reliability of canine detection services (Wang et al. 2011).

Bed bug monitors are valuable tools in bed bug management. Although ClimbUp Insect Interceptor, dry ice trap, and NightWatch are the most effective monitors known at the present time, none of them provides 100 % (Wang et al. 2011) detection. Moreover, there are no effective commercial products available that are suitable for wide-scale routine surveillance (Weeks et al. 2010).

Therefore, a trap baited with attractive semiochemicals could be effectively used for monitoring bed bug infestations, followed by timely insecticide applications. Additionally, numbers of insects caught in the traps could be helpful in providing information about the geographic distribution of bed bugs (Weeks et al. 2010).

8.2.2.7 Screening Bed Bug Attractants in the Laboratory

We have screened close to 50 semiochemical analogs (Gilbert 2014) for attractant effects with bed bugs in the laboratory. Of this number, at least eight compounds (3-nonanone, 3-hexanone, 3-pentanone, *N*-methylpropionamide, methyl-4-oxobutanoate, (*E*)-6-hydroxyhex-4-en-3-one, vinyl propionate, and allyl propionate) showed sufficient performance (85–100 % attractance) to be considered for continued attractant formulation development (Table 8.4) and could offer the potential for a "push-pull" type of pest management system for bed bugs based on semiochemicals.

Attractant effects were determined in a custom glass Y-olfactometer (Fig. 8.5; length of each arm = 15.3 cm, length of the ally from point of introduction to the choice point = 13.5 cm, diameter of the tubes = 2 cm) at the University of British Columbia, Vancouver, BC, Canada. Each bioassay consisted of at least five test concentrations, each with 30 insects. Bioassays were replicated three times over subsequent days. Glass containers and the olfactometer were washed thoroughly with soap and warm water, baked, and cleaned with acetone before each use.

Attractant compounds offer the potential to produce baited traps for bed bug monitoring. Such monitoring traps could be used in hotels, libraries, hospitals, and other locations that are sensitive to infestation. Traps could be used in residences both to verify the presence of a suspected infestation and to assess the degree of bed bug eradication success. One great advantage of using attractants in monitoring traps is that they are not considered pest control products, so they do not require pesticide registrations in the USA and Canada.

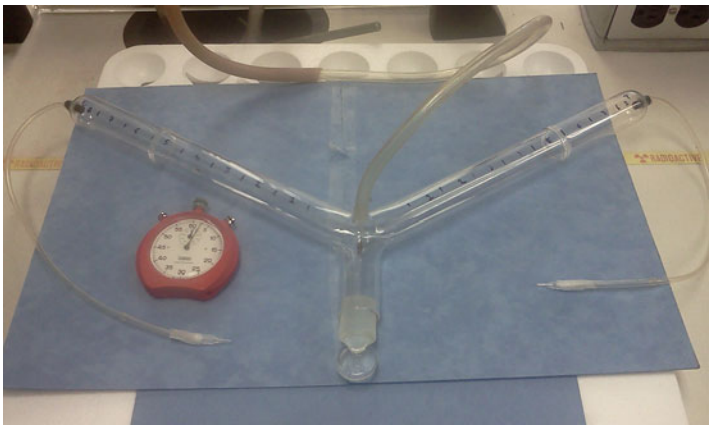

Fig. 8.5 Glass olfactometer

8.2.3 Role of Biopesticides in Bed Bug Management

Although a number of products are available in the market, they are rarely adopted by pest management practitioners, due to the lack of scientific data supporting claims of control to date. Nine commonly available biopesticides along with two synthetic insecticides were tested against bed bugs by researchers at Rutgers University. Most of the biopesticides tested failed to satisfactorily control bed bugs through direct spraying. Of the nine products tested, Bed Bug Patrol® (Nature's Innovation Inc., Buford, GA) and EcoRaider® (Reneotech Inc., North Bergen, NJ) showed some promise, but at a much slower speed than the synthetic insecticides tested for comparison (Singh et al. 2013).

Dusts have been used to ward off insects from grain storage for centuries, including "plant ash, lime, dolomite, certain types of soil, and diatomaceous earth (DE) or Kieselguhr" (Hill 1986). Of these, diatomaceous earth in particular has seen a revival as a nontoxic (when in amorphous form) residual pesticide for bed bug abatement. Desiccant dusts are among the oldest forms of insect control agents and are considered effective as long as the insects walk on them (Benoit et al. 2009). Diatomaceous earth chafes and abrades the waxy outer layer of the insect epicuticle resulting in death of the insect due to desiccation (Potter et al. 2013b).

Convincing reports of DE's effectiveness against bed bugs as a nontoxic, eco-friendly alternative include both laboratory (Benoit et al. 2009; Potter et al. 2013b; Akhtar and Isman 2013, 2016; Doggett et al. 2008; Romero et al. 2009b; Anderson and Cowles 2012) and field studies (Wang et al. 2009a). Insects exposed to diatomaceous earth may take several days to die (Benoit et al. 2009); therefore, there is a need to search for new methods to increase the efficacy of diatomaceous earth and decrease killing time. One study aiming to increase the efficacy of desiccant dusts and silica gels for bed bug control involved their application in

combination with alarm pheromone components. When (*E*)-2-hexenal and (*E*)-2-octenal were applied either singly or as a blend, in combination with Dri-die (silica aerogel, Fairfield American Corp., Frenchtown, NJ), water loss increased twofold and threefold, respectively, resulting in decreased survival time of first-instar nymphs from 4 days to 1 day (Benoit et al. 2009). A mixture of DE and the pheromone blend demonstrated a 50 % increase in water loss over controls and a decreased survival time from 4 to 2 days in first-instar nymphs. Mixture of the pheromone blend and desiccant dust was more effective than either component alone. Presumably, the addition of alarm pheromone enhanced crawling activity, thereby promoting cuticular damage that increases water loss. While these results are promising, field trials are necessary to determine whether the additive effect of the pheromone is maintained in a natural situation.

Romero et al. (2009a, b) reported high levels of mortality with some commercially available dusts against pyrethroid-resistant bed bug strains. They evaluated five different dusts, two pyrethroid-based dusts, DeltaDust® (0.05 % deltamethrin, Bayer Environmental Science, Montvale, NJ)) and Tempo 1 % Dust® (1 % cyfluthrin, Bayer Environmental Science, Montvale, NJ), and three desiccant dusts, Drione® (1 % pyrethrins, 10 % piperonyl butoxide, 40 % amorphous silica gel, Bayer Environmental Science, Montvale, NJ), MotherEarth D® (100 % diatomaceous earth, BASF), and NIC 325® (99.5 % limestone, ACM – Texas, Loveland, Colorado) against four different populations of bed bugs. Tempo® caused 100 % mortality of bed bugs in all four populations (two highly resistant from Cincinnati and New York, one moderately resistant from Los Angeles, and one susceptible population from New Jersey) within 24 h. Drione also killed 100 % of all the populations, but it took 72 h of continuous exposure, for the two resistant populations from Cincinnati and New York. DeltaDust® caused significant mortality (>90 %) only after 1 week of exposure (Romero 2011).

We have demonstrated strong residual effects of a specific diatomaceous earth (DX13™, DE Laboratories Inc., Vancouver, Canada) dust and an aerosol formulation thereof in the laboratory (Akhtar and Isman 2016) as a reduced-risk bed bug management strategy. The residual effect of DX13™-aerosol persisted for 21 days. The LT_{50} value (Fig. 8.6) increased from 9.1 h for freshly applied DE aerosol to 46.1 h for aerosol aged for 21 days in Petri dishes at an average dosage of 45.8 g m-2. Mortality of the bed bugs in the Petri dishes was >97 % at 72 h for DX13™-aerosol residue aged for 0 h and 14 days. Mortality was 81 % at 72 h for DX13™ aerosol residue aged for 21 days (Akhtar and Isman 2016).

Desiccant dusts, with their physical mode of action and long residual activity, appear to be superior to sprayable pyrethroid products for killing bed bugs (Anderson and Cowles 2012). Comparison of Mother Earth® DE-treated apartments with chlorfenapyr-treated apartments after 10 weeks showed an average 97.6 % bed bug reduction in DE-treated apartments versus 89.7 % reduction in chlorfenapyr-treated ones (Quarles 2015). Moreover, the dust IPM program was less expensive ($463/apartment) than the spray IPM program ($482/apartment) (Quarles 2015).

We also have demonstrated secondary and tertiary mortality of bed bugs through horizontal transfer of DX13™ from exposed to unexposed bed bugs (Akhtar and

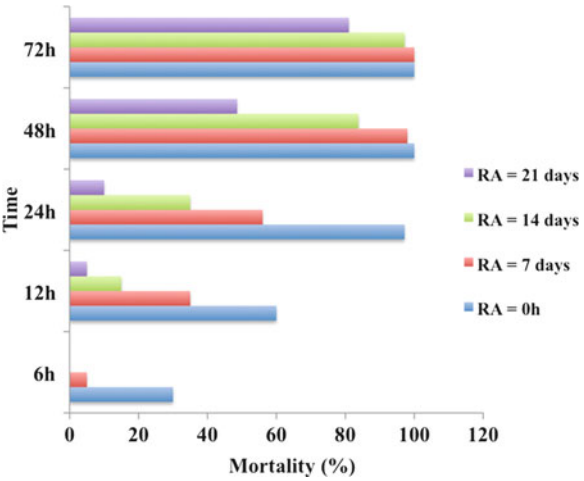

Fig. 8.6 Residual effects of DX13™-aerosol at an average dosage of 45.8 g m-2 (Akhtar and Isman 2016). $N = 5$ replicates of seven to eight insects. Bed bugs were introduced into Petri dishes with residual aging time (RA) of 0 h, 7, 14, and 21 days; mortality was assessed at different time intervals after introduction of insects to treated/control Petri dishes. There was no mortality in the control group

Isman 2013, 2016). Lethal concentrations causing 50 % mortality (LC_{50}) values varied from 24.4 mg of DX13™ dust at 48 h to 5.1 mg of dust at 216 h when a single exposed bed bug was placed with five unexposed bed bugs. Time to kill 50 % of bed bug (LT_{50}) values varied from 1.8 days to 8.4 days when a "donor" bed bug exposed to 20 and 5 mg of DX13™ dust, respectively, was placed with five "recipient" bed bugs (Akhtar and Isman 2016). This result is important because bed bugs live in hard-to-reach places (e.g., cracks, crevices, picture frames, books, furniture), and as such the close interactions between the members of the colony can be exploited for delivery and dissemination for designing effective control strategies.

Pest control operators have been marketing diatomaceous earth as a nontoxic, eco-friendly alternative for years. It is also recommended by government and academic institutions as part of a "Comprehensive integrated bed-bug management program" (Potter et al. 2013b).

8.2.4 Role of Microbials in the Management of Bed Bugs

Fungal species including *Beauveria bassiana* and *Metarhizium anisopliae* also have been used (Table 8.7) to control blood-feeding arthropods (Darbro et al. 2011; Fernandez et al. 2011; Pedrini et al. 2009). Barbarin et al. (2012) evaluated the efficacy of *B. bassiana* as a residual biopesticide against the common bed bug in laboratory conditions. *Beauveria bassiana* (I93-825) was highly virulent to bed bugs, causing rapid mortality (3–5 days) following short-term exposure to spray residues regardless of feeding status, sex, strain, or developmental stage of bed bugs. Barbarin et al. (2012) also evaluated autodissemination of conidia as a means to spread infection among bed bug populations in untreated, inaccessible areas. With respect to test substrates, jersey knit cotton was a better substrate for conidial

transfer than paper, probably due to the relatively contoured surface resulting in more conidia coming into contact with the insect cuticle. These results demonstrate that choice of substrate is important in both bioassay design and end product development.

Ulrich et al. (2014) exposed bed bugs to conidia of the entomopathogenic fungus, *Metarhizium anisopliae*, through feeding, aerosol spray, or contact with a treated surface. Mortality was high through feeding, but humidity dependent in other methods of application in laboratory bioassays. Based on the results, they concluded that *M. anisopliae* was a poor pathogen for use in control of bed bugs, particularly at the relative humidity that would likely be encountered under field conditions.

8.2.5 Role of Juvenile Hormone in the Management of Bed Bugs

Naylor et al. (2008) evaluated the effect of the juvenile hormone analog (S)-methoprene (Table 8.7) on adult and nymphal stages of *C. lectularius*. Exposure of nymphs to technical grade (S)-methoprene at a range of doses resulted in an incomplete eclosion, uneven cuticle formation, prolapses of the gut through the dorsal abdominal wall, and formation of supernumerary nymphs. The immature stages could not develop to fertile adults. Response was dose dependent and no normal adults were produced at the highest dose (30 mg/m^2). (S)-Methoprene was as effective against the pyrethroid- and carbamate-resistant strain as it was against the susceptible strain, suggesting that there is currently little or no field resistance or cross-resistance to this compound.

8.2.6 Nonchemical Tools

Although nonchemical tools, such as temperature treatments (e.g., steam and dry ice), mattress encasement, sanitation, and vacuuming, are available, only the spraying of insecticides provides long-term control and prevents against reinfestation of bed bugs (Doggett et al. 2004). Several studies have demonstrated the use of temperature to control bed bugs (Table 8.7). The lethal temperatures required to kill 99 % of adult bed bugs (LT_{99}) and their eggs were 48.3 °C and 54.8 °C, respectively; time to kill 99 % of adult bed bugs exposed to 45 °C was 94.8 min; eggs survived for 7 h at 45 °C but only 71.5 min at 48 °C (Kells and Goblirsch 2011). Puckett et al. (2013) exposed all stages of bed bugs to three steam treatment exposure periods and demonstrated that mortality of bed bug eggs was 100 % (regardless of duration of exposure) and that of nymphs and adults ranged from 88 % to 94 %. Rukke et al. (2015) exposed adult bed bugs to sublethal temperatures 34.0 °C, 35.5 °C, 37.0 °C, 38.5 °C, or 40.0 °C for 3, 6, or 9 days. The two uppermost

Table 8.7 Response of bed bugs to natural products ± insecticide/physical control

Source	Effect	Additional note	References
(S)-Methoprene (juvenile hormone analog)	Failure of immature stages to develop to fertile adults	Active against both resistant and susceptible strains	Naylor et al. (2008)
Metarhizium anisopliae	Dose-dependent mortality through feeding	Mortality was 100 % through feeding and humidity related through spraying and contact with the treated surface	Ulrich et al. (2014)
Temperature lethal/sublethal	Mortality sterilization	Strong correlation between mortality and temperature as well as different stages of bed bugs	Kells and Goblirsch (2011); Puckett et al. (2013)
Three steam treatment exposure periods using a portable device	Controlling localized infestations	100 % mortality – eggs Steam could be used as a practical component of IPM to manage bed bugs	Rukke et al. (2015)
Beauveria bassiana	Mortality, horizontal transfer of fungal spores	Bed bugs were exposed to paper and cotton jerseys treated with spore formulation of *B. bassiana* for an hour died within 5 days	Barbarin et al. (2012)
Desiccant dust + (*E*)-2-hexenal or (*E*)-2-octenal or their blend	Enhanced efficacy of dust	Increased movement of bugs enhanced exposure of bugs to desiccant dust leading to mortality and water loss	Benoit et al. (2009)
Desiccant dust and aerosol formulation	Mortality, horizontal transfer	Contact and residual effects; DE dust was transferred from infested bed bugs to uninfested bed bugs and caused mortality	Akhtar and Isman (2013, 2016)
Insecticidal dusts	Enhanced efficacy of dust	Dust products containing an insecticide had long residual activity and were more superior to sprayable pyrethroid products for killing bed bugs	Anderson and Cowles (2012)
Various laundering methods	Mortality	Washing clothes at 60 °C, drying at >41 °C, and freezing at −17 °C killed all stages of bed bugs	Naylor and Boase (2010)
Combination of chemical and nonchemical methods	Mortality/controlling infestation	Washing and cleaning/throwing away infested belongings combined with several insecticide applications	Fuentes et al. (2010)

temperatures induced 100 % mortality within 9 and 2 days, respectively, whereas 34.0 °C had no observable effect. The intermediate temperatures interacted with time to induce a limited level of mortality but had distinct effects on fecundity in terms of decreased number of eggs produced and hatching success (Rukke et al. 2015).

Comparison of various laundering methods (Table 8.7) to disinfect clothing infested with bed bugs demonstrated that washing at 60 °C, tumble drying for at least 30 min on the hot cycle (>40 °C), dry cleaning with perchloroethylene, or freezing for at >2 h at −17 °C killed all stages of bed bugs (Naylor et al. 2008). However, soaking items in detergent-free water for 24 h was sufficient to kill bed bug adults and nymphs but not the eggs (Naylor et al. 2008).

The concern over the itchy bites of bed bugs followed by development of secondary infections has led to the development of a new sterilization system (AsepticSure®, Medizone International Inc., Sausalito, CA) in hospitals that can kill the highly drug-resistant bacteria as well as the bed bugs (Wjeczner 2013). Medizone International Inc. has already started distributing its new disinfecting technology to hospitals in Canada and is seeking its approval to market it in the USA. Although this system took less than an hour to eradicate 100 % of bacteria, it took 24 h to kill bed bugs and 36 h to kill their eggs (Wjeczner 2013).

A combination of chemical and nonchemical means (washing and cleaning all affected belongings, throwing away infested belongings, and several insecticide applications) were required to control bed bug infestation in three homes (Table 8.7) in Valencia (Spain) (Fuentes et al. 2010), occupied by people who have acquired bed bugs during their travel to the UK, Spain, and Sweden prior to the study.

8.3 Future Directions

Evaluations of populations from across the USA and other parts of the world indicate that resistance to pyrethroid insecticides is widespread. This inability to control bed bugs with pyrethroids necessitates development of products with new modes of action, relabeling of existing efficacious products, and greater reliance on alternative tactics such as heat treatment, vacuuming, mattress encasements, or barriers (Romero et al. 2007). Development of delivery systems based on barrier treatments, such as a "bed skirt," positioned between the harborages and the human host demonstrates potential for effective control (Barbarin et al. 2012).

There will be an increased demand for the development of novel behavior-modifying substances such as effective repellents and attractants based on semiochemicals or other natural products. A trap (or traps) containing a lure based on a natural or synthetic blend of semiochemicals may be one strategy for diverting bugs from human hosts or to partially "trap out" a resident population. Targeting control to homes, rooms, and areas that are infested with bed bugs will reduce insecticide use (Reinhardt and Silva-Jothy 2000).

A further strategy is the stimulo-deterrent diversionary strategy, or "push-pull" strategy, that combines an attractant and a repellent. Yet another strategy is "attract and kill" that combines an attractant with a toxic product. For the development of such pest management systems, it is essential to establish a full understanding of bed bug chemical ecology and behavior.

In conclusion, insecticide-only treatments for bed bugs will likely fail due to resistance and cross-resistance development. The best hope is an IPM program using components such as prevention, monitoring, vacuuming, traps, repellents, heat and steam, fumigation, and use of reduced-risk pesticides such as silica gel, diatomaceous earth, neem, essential oils, and microbials. As diatomaceous earth (DE) has an extremely long residual action and as its mode of action limits the possibility of resistance developing, there is a strong potential for DE dust to be employed as a preventative insecticide, which further enhances the prospect for strong financial returns. A multidisciplinary strategy with several key components including a code of practice for the control of bed bug infestations that defines and promotes best practice for bed bug eradication, development of a policy and procedural guide for accommodation providers, and education of stakeholders should be adopted similar to Australia and other countries (Doggett et al. 2011b). Even with all these options, complete elimination of bed bugs from a structure is very difficult (Quarles 2015). Without the development of new tactics or approaches for bed bug management, further escalation of this pest should be expected.

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Chapter 9
Developing a Bioacoustic Method for Mating Disruption of a Leafhopper Pest in Grapevine

Jernej Polajnar, Anna Eriksson, Meta Virant-Doberlet, Andrea Lucchi, and Valerio Mazzoni

Abstract Widespread use of substrate-borne vibrational signals by insects presents a unique opportunity to develop alternative methods of pest control, enabled by better understanding insect behaviour and advances in technology. One such method is currently under development for use against the invasive leafhopper *Scaphoideus titanus*, a vector of Flavescence dorée in European vineyards. Basic understanding of the vector's sexual behaviour and observations of naturally occurring antagonistic interactions between males enabled development of vibrational broadcasts that obscured signal characteristics important for mate recognition and localization in small-scale field tests. The naturally occurring antagonistic interactions constitute acoustic noise that can be characterized, adjusted and broadcasted using modified acoustic technology. Steps in development of this technology to maximize reliability and energy efficiency are outlined, as well as plans for large-scale field testing

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and future perspectives. While several specific factors work in favour of using vibrational disruption in the system *S. titanus* (pest) and grapevine (host) and possibilities of direct transfer to other systems are limited, success of this approach is nevertheless hoped to stimulate the development of vibrational playback in general for control of other insect pests.

9.1 Introduction

The growing public health awareness and regulatory issues dealing with harmful side effects of pesticides in the recent decades are providing a strong impetus for developing innovative approaches in pest control that could reduce our reliance on toxic substances for achieving consistent and economically viable food supply (Epstein 2014). This goal is becoming more and more complex, however, because selective pressure exerted by commonly used pesticides leads to emergence of pesticide resistance in pest populations, and exotic pests are being introduced to new agricultural areas through international trade routes (Meyerson and Mooney 2007).

Insects comprise numerous economically important pests, and IPM practices have historically been focused on controlling harmful insects in agricultural environments (Kogan 1998; Ehler 2006). Harmful effects of pesticides can be reduced by either developing more targeted compounds that exhibit fewer side effects, or avoiding them altogether and developing non-toxic methods of pest management. Ironically, the shift from broad-spectrum pesticides that accompanied adoption of integrated control programs may have been a crucial factor responsible for the sudden and unexpected establishment of the invasive leafhopper *Scaphoideus titanus* Ball, 1932 in Europe, leading to widespread Flavescence dorée outbreaks (Belli et al. 2000). Thus, a targeted approach to combat this problem is needed.

In commercial farming, the most important issues influencing the decision which pest control method to use are cost/benefit ratio, reliability and convenience, which explains the prevalence of pesticide use, but also occasional breakdowns of pesticide treatments and manifestations of their adverse effects due to ecologically unsound decisions (Metcalf 1994). Despite problems inherent to pesticide treatments, such as adverse effect of toxic compounds on environment and human health, it is unrealistic to expect that a costlier, less reliable and/or less convenient alternative will be adopted just because it avoids those problems (unless accompanied by state legislation and regulations strongly favouring the alternative). On the other hand, one important constraint in the use of agrochemicals is difficulty in predicting negative consequences in the environment, which often makes the risk assessment unreliable (Sánchez-Bayo and Tennekes 2015). From this follows that alternatives are warranted, but should still be designed from the outset to match the results of available technology as best as possible. It is an uphill battle because the historical

and current prevalence of pesticides means that the industry is highly optimized by now, ensuring fulfilment of basic requirements (Metcalf 1994), but new discoveries in pest biology and modern technological advances are opening possibilities to replace harmful substances at least in niche applications.

One of the possibilities is behavioural manipulation by exploiting insect sensory biases to protect valuable resources (such as crops or human health). It has been used for centuries, more or less methodically, but often quite incidentally, since the mechanisms that guide animal behaviour are complex, diverse and not fully understood. Consequently, the tactic often involves a trial-and-error approach for management of a given pest (Foster and Harris 1997). Numerous successful cases have been reported nevertheless (e.g. Witzgall et al. 2008; Ioriatti et al. 2011), proving that exploitation of sensory processes can work even with an imperfect knowledge of underlying mechanisms (Cardé 1990), but there is a little recourse to constructive modification in case of failure if the artificial stimuli are poorly defined (Foster and Harris 1997). The two main tactics for implementing behavioural manipulation are "push-and-pull" and "attract-and-kill", working, as the name suggests, by either concentrating the individuals in an area where they can be conveniently eliminated, repelling them from the protected resource or disrupting key behaviours such as host finding, feeding, mating and oviposition (Foster and Harris 1997). Manipulation of chemical communication for mating inhibition and mass trapping by synthetic pheromones that have been under development for 50 years are currently two of the most widely recognized alternatives to pesticides (Gaston et al. 1967; Witzgall et al. 2010), integrated in pest management strategies in several important crops worldwide, particularly against moths (Cardé 1990; Cardé and Minks 1995). The drawback of this approach is that in some other major pests from various insect groups, most notably Auchenorrhyncha, long-range chemical communication appears to be largely absent, so the approach is ineffective in defence against some of the world's most destructive agricultural pests and plant disease vectors.

Vibrational communication is an important alternative, although historically overlooked modality used by numerous insect groups. In fact, it is widespread throughout the insect class and the most prevalent form of communication with mechanical stimuli, with a conservative estimate putting the number of users at 92 % or 195,000 described species in 80 % of all insect families, of which the majority uses vibrational signalling exclusively (Cocroft and Rodríguez 2005). To this, we must add a diverse range of spiders (Barth 1998), crustaceans (Popper et al. 2001) and other arthropods. There are several practical reasons for such ubiquity. Mechanical vibrations propagate rapidly and emission can be precisely controlled (unlike chemical signals), which makes them suitable for achieving specificity that is important for recognition. In the simplest form, production requires no anatomical or physiological adaptations, although different specialized structures do exist for this purpose in various groups. At the receiver's side, insects are pre-adapted for detecting substrate vibrations with surface hair sensillae and receptors located in legs and antennal joints, although, again, specialized receptors have evolved in

some groups that are tuned to a particular frequency range used in communication (Čokl and Virant-Doberlet 2003). Sound production, while far more widely known, is in fact an exception in insects, widespread only in Orthoptera and Cicadoidea (Cocroft and Rodríguez 2005), since in air-borne sound communication insects face severe physical constraints due to their small size (Bennet-Clark 1998). Nevertheless, it is sound production, in many cases audible to an unaided human ear that has received much attention of researchers, while vibrational communication was postulated, but completely unknown until the pioneering work of Frej Ossiannilsson 65 years ago. Even after that, the technological and conceptual issues in detecting and interpreting insect vibrational signals prevented rapid progress. As one of the consequences, exploiting vibrational communication for human benefit is still in its infancy (Mankin 2012; Polajnar et al. 2015).

In this chapter, we present an overview of an ongoing effort to develop a vibration-based method for mating disruption of the grapevine pest *S. titanus*, following a bottom-up approach – starting with a comprehensive study of the species' ecology and mating behaviour, then demonstrating that the stimulus is effective in laboratory conditions, and finally testing the method in semi-field and small-scale field conditions. This is the current level the research has been pursued so far, in collaboration between research groups at the Fondazione Edmund Mach (San Michele all'Adige, Italy), Pisa University (Pisa, Italy) and the National Institute of Biology (Ljubljana, Slovenia). The key papers published so far are summarized, along with theoretical background to explain the rationale behind each step. Lastly, the future direction for the next step – large scale field trials – is outlined in the hope of stimulating development of similar solutions against other pests.

9.2 Background

The subject of research effort outlined in this chapter is the American grapevine leafhopper *S. titanus*. This species originates in eastern and central parts of North America where it feeds on wild and cultivated Vitaceae, and is not regarded economically important (Chuche and Thiéry 2014). However, the population introduced to Europe more than 150 years ago serves as a vector for a dangerous grapevine disease Flavescence dorée, and for this reason, *S. titanus* is widely regarded an important pest in European vineyards throughout this part of the range. The causative agent of Flavescence dorée, phytoplasmas from groups 16SrV-C and -D (Angelini et al. 2003), is hypothesized to have also spread from North America (Maixner et al. 1993), and the disease has reached epidemic proportions in the affected areas of Europe since the vector's introduction (Laimer et al. 2009).

As a relatively recently introduced invasive species, *S. titanus* is unfortunately subject to a little predation or parasitism in Europe. Consequently, climatic conditions and the presence of hosts are the main factors determining the species' distribution in this part of the range. It is thought to be able to colonize most or all European wine growing regions, although it is still absent from the warmest

Mediterranean areas, in line with its North American origin (Chuche and Thiéry 2014). The species is univoltine, with cold winter temperatures important for regulating egg hatching in the period May–July (Vidano 1964; Chuche and Thiéry 2009).

Geographic distribution of *S. titanus* in Europe has been thoroughly studied. From the first records in 1958 from vineyards in South-Western France, the newcomer has spread throughout Central and South Europe, as far as Switzerland in the North and Serbia in the West (Papura et al. 2012; Chuche and Thiéry 2014). Molecular markers show that the European population originated from a single introduction event, supposedly as an unwanted side effect of importing large amounts of grapevine stock for use in breeding programs at that time (Papura et al. 2012). *Scaphoideus titanus* has since spread throughout the present range, despite Flavescence dorée being declared a quarantine organism on an international scale as early as 1983 (OEPP/EPPO 1983) and control measures involving mandatory large-scale pesticide treatments against the established populations of the vector along with other measures, such as hot water treatments of seedlings. The latter are used to destroy the eggs laid under the bark, but also the phytoplasmas themselves (Caudwell et al. 1997), while neurotoxic pyrethroids are predominantly used against nymphs and adults (Chuche and Thiéry 2014). In contrast with the North American populations, the host range appears to be largely limited to the genus *Vitis* in Europe (Vidano 1964, 1966; Lessio and Alma 2004a), which is a fortunate situation that simplifies control measures. Still, no alternative control methods are being widely used against this pest so far; although a "push–pull" strategy has been proposed, employing trap crops for attraction and kaolinite clay coating of grapevines for repelling (Chuche and Thiéry 2014).

9.3 Sexual Behaviour of *Scaphoideus titanus*

The research program on *S. titanus* presented here first focused on describing sexual behaviour of this species, which was thoroughly studied, starting with a brief report by Mazzoni et al. (2008) and later expanded by additional publications (Mazzoni et al. 2009a; Eriksson et al. 2011; Polajnar et al. 2014). The main features of the mating system were described by Lucchi et al. (2004) and Mazzoni et al. (2009a), and are presented here (also mentioned briefly by Chuche and Thiéry 2014 in their summary of *S. titanus* biology) with special reference to features important for utilizing this basic knowledge in practice.

In general, sexual behaviour of this species is fairly stereotyped, consisting of distinct phases with predictable and sequential transitions between phases (Fig. 9.1). Courtship activity and associated risks are almost exclusively assumed by the male who searches for the stationary female and initiates the mating sequence (Mazzoni et al. 2009a). Of note is the fact that *S. titanus*, like other leafhoppers, relies on vibrational modality alone and does not use chemical signalling (Claridge 1985; Mazzoni et al. 2009a), which greatly simplifies our understanding and facilitates

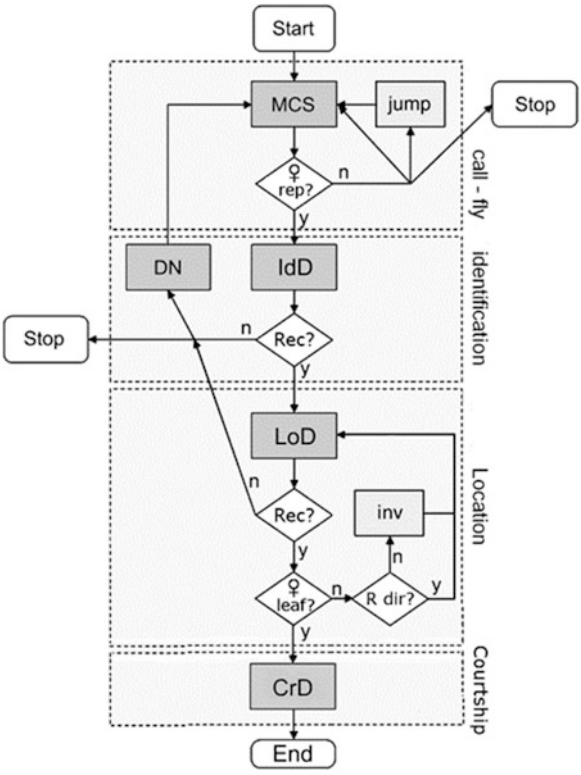

Fig. 9.1 Flow chart with the behavioural steps of a male *Scaphoideus titanus* searching for a female on a grapevine plant. *MCS* male calling signal, *DN* disturbance noise, *IdD* identification duet, *LoD* localization duet, *CrD* courtship duet, ♀ *rep* female reply, *Rec* recognition, ♀ *leaf* arrival at the female leaf, *R dir* right decision, *Inv* inversion of direction [From Polajnar et al. 2014, Fig. 3, p. 72, with permission from Elsevier)

manipulation of this system. Antennae of this species in particular exhibit strong reduction of the olfactory sensilla, both in terms of number of sensory structures and sensory neurons per sensillum (Mazzoni et al. 2009c; Rossi Stacconi and Romani 2012). Likewise, vision is of secondary importance and cannot play a prominent role in long-distance courtship because the individuals are active during the night (Mazzoni et al. 2009a), but even in daylight, the line of sight is usually blocked by foliage in such an environment as a tangle of grapevine shoots (Endler 1993). Its role is likely comparable to the role of the tactile sense, i.e. limited to the final phase of the courtship sequence when the partners are already in physical contact, or even completely negligible if the courtship takes place at night.

Males are the more active partner in a pair, searching for the female and initiating courtship, while the females are stationary and do not signal spontaneously. Their role is limited to replying to male calls, thereby guiding the male to immediate vicinity. During the mating season, sexually mature males search around the foliage by jumping between leaves and emitting a series of short vibrational pulses, repeated with a regular rhythm and increasing in amplitude throughout the signal – termed Male Calling Song or MCS. If a receptive female is present within the signal's active space (*sensu* Mazzoni et al. 2014), she responds by inserting own short pulses in some or all gaps between male pulses. The resulting duet – Identification Duet or

IdD – is crucial for mate recognition: female reply must arrive within a narrow time window after a pulse and before the following pulse in order to be recognized by a male. The possibility of false positives is further reduced by the male increasing the inter-pulse interval after each reply (Polajnar et al. 2014). Movement in this phase is random, but after identification is complete, the male starts to search for the female on the host plant by walking. Walking bouts are interspersed by short localization duets (LoD) characterized by reduced numbers of pulses with no inter-pulse interval increases. This movement is highly oriented, with males correcting their directional decisions after moving away from the female by chance (such as taking the wrong turn at the branching point). When a male arrives in close vicinity to the female, usually the same leaf lamina or the stalk of the leaf harbouring the female, he switches to the most complex part of his vibrational repertoire, the courtship song that includes a high-frequency buzz between pulses and double pulses in the second part of the song. Thus, a courtship duet (CrD) is established, with bouts of signalling again interspersed by walking – the final stage of approach until the animals are in direct contact. After that, the male uses the pauses between signalling bouts to position himself next to the female, and copulation directly follows the last call (Mazzoni et al. 2009a; Polajnar et al. 2014).

Vibrational signals in Auchenorrhyncha (with the notable exception of cicadas) are induced in the substrate with specialized muscle activity, usually without directly striking the substrate, but some signal components may involve percussion (Čokl and Virant-Doberlet 2003). Even if the first description of putative vibration-producing structures in Auchenorrhyncha by Ossiannilsson (1949) predates the proof that insects communicate with substrate vibrations, the exact mechanism of production is still unclear, owing to small size of the animals and large variability (Čokl and Virant-Doberlet 2003; Wessel et al. 2014). What is clear is that the amplitude of these vibrations is low by human standard and characterized by low to medium frequencies, which is important for efficient transmission. Frequencies above 500 Hz are strongly attenuated in herbaceous plant tissues due to elastic and resonant properties of those tissues (Michelsen et al. 1982), so their usefulness for vibrational communication on distances exceeding the range of centimetres is limited, except in strong signallers such as the large New Zealand tree wetas (Orthoptera: Stenopelmatidae) (McVean and Field 1996). In *S. titanus*, most of the energy of vibrational pulses is concentrated in a broad band between 40 and 250 Hz (Mazzoni et al. 2009a; Polajnar et al. 2014), while the high-frequency buzz is narrow-band with fundamental frequency around 280 Hz and clear harmonic structure (Mazzoni et al. 2009a).

The key feature of communication system in *S. titanus* is that males use the information present in the female signals' perceived amplitude and temporal synchrony with own signals, therefore utilizing not only female reply *per se* but also transmission properties of the substrate to guide their behaviour. The exchange is crucial for both identification and localization of the sexual partner, and the information flow must be constant: if signalling is interrupted for any reason, the male will revert to the calling phase and will stop signalling and, eventually, abandon the location if he does not detect an appropriate reply (Polajnar et

al. 2014). Frequency range is not species-specific, except perhaps the frequency of the harmonic buzz. It is understood as reflecting physical constraints of the acoustic environment instead, where high frequencies are rapidly attenuated (Michelsen et al. 1982) and the lowest frequency range is occupied by sources of ambient noise, such as wind, rain, movement of other animals in the same acoustic environment and even human activity (Barth et al. 1988; Virant-Doberlet et al. 2014). The relatively narrow frequency range that remains must be shared with all other users of the vibrational modality occupying the same acoustic environment at the same time. Reciprocal signal masking is a common problem experienced by animals whenever multiple signallers are present within signal range, especially in acoustic and bioluminescent communication (Greenfield 1994). Individuals of the same species are the most likely to be present in the same microhabitat and the most likely to emit signals at the same time due to shared ecological and behavioural features. Those signals will also, by definition, have common properties and their overlapping may mask features important for recognizing and/or locating the emitter, which fits the definition of noise (Brumm and Slabbekoorn 2005).

With this knowledge, it is possible to understand the next important feature of the vibrational communication system in *S. titanus*: emission of disruptive noise in antagonistic interactions between males. *S. titanus* is the only leafhopper species in which alternative courtship tactics have been so far studied in detail: in a setting where two males and a female are present in the same acoustic environment at the same time, vigorous rivalry develops between the males. The second male may disrupt the courtship duet between the first male and the female, by producing another kind of vibratory emission, termed »male disturbance signals«. Those are composed either of discrete pulses (male disturbance pulses, MDP), or continuous trains of those pulses (male disturbance noise, MDN), emitted without pauses (Fig. 9.2). When the rival male detects an ongoing duet, he may intersperse it with his own disturbance signals, either continuously (with MDN) or in response to each first male's pulse (with MDP), alternating with or overlapping those pulses. According to the current understanding of the information exchange between sexual partners, the disturbance signals cause masking of the crucial spectral and temporal features of communication signals, thus preventing the caller's identification and localization. Emission of disturbance signals is short but effective, usually causing the courting male to restart the CrD. The rival may then use the tactic of silent approach (i.e. without courtship signals) towards the female in an attempt to displace the courting male, using direct aggression if necessary, although rivalry often causes the female to stop responding altogether and move away (Mazzoni et al. 2009a). Specialized signals with similar function have been observed also in the leafhopper *Aphrodes makarovi* (Kuhelj et al. 2015), and the membracids (Hemiptera: Membracidae) *Ennya chrysura* (Miranda 2006) and *Tylopelta gibbera* (Legendre et al. 2012).

Nymphs seem to be silent and do not exhibit any behavioural response to vibrational stimuli (Chuche et al. 2011).

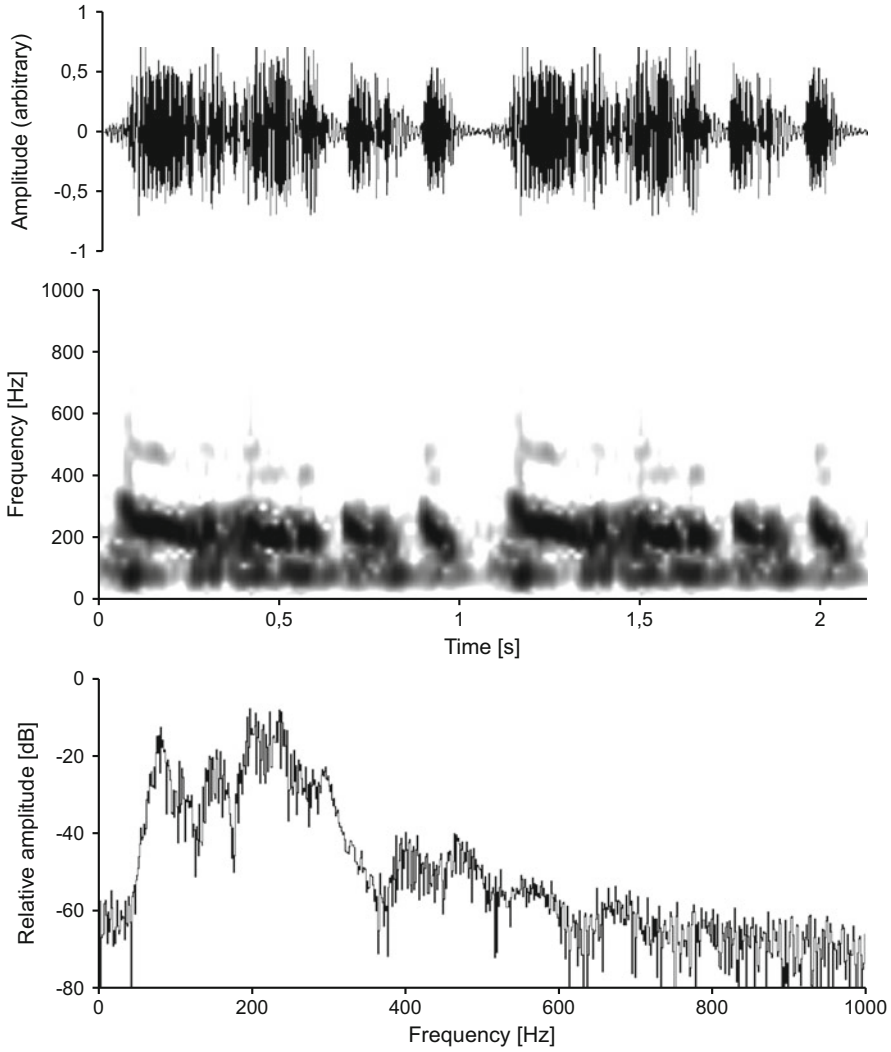

Fig. 9.2 Properties of the disturbance noise (DN) signal that was used for stimulation (Polajnar et al. 2016). *Above*: oscillogram, *middle*: spectrogram, *below*: frequency spectrum. Note that the oscillogram and the spectrogram share the time axis

9.4 From Proof of Concept to First Field Trials

The observation that the male–female duet is easily interrupted by emission of disruptive signals (Mazzoni et al. 2009a) led to the idea that the mating behaviour of *S. titanus* can be artificially disrupted by vibrational playback. The main conceptual

issue is that in natural circumstances, disruption is short-term, used for momentary gain of opportunity by rival males, so playback must be modified for long-term mating disruption. Therefore, it is crucial to understand the theoretical background in order to achieve that goal.

Reproductive interference by signal jamming between species is a common phenomenon in species communicating with airborne acoustic signals (Gröning and Hochkirch 2008). In signal theory, noise is any factor that reduces the ability of a receiver to detect a signal or discriminate between signals (Brumm and Slabbekoorn 2005), therefore other animals that interfere with communication between potential mates are a source of noise. Several strategies have evolved to counter the negative effect of such interference in animals, both at the emitter's and the receiver's side (Brumm and Slabbekoorn 2005). They include simple immediate responses, such as amplitude increase (the so-called "Lombard effect") that has been until now mostly described in vertebrates (Potash 1972; Sinnot et al. 1975), but also in a bushcricket (Hammond and Bailey 2003). More widespread is a noise-dependent adjustment of serial redundancy (repeating signal components) (Ronacher et al. 2000; Aubin and Jouventin 2002). In cases where noise inhibits signalling, the presence of noise may cause temporal shifts in signalling, which has been described on various time scales and in various animal taxa (Greenfield 1994). In the context of competitive signalling, such inhibition may also result in alternation between individuals and formation of choruses (Greenfield 1994). Finally, there is evidence of short-term frequency altering in the presence of noise within a narrow frequency band, most notably in frogs (Howard and Young 1998) and birds (Manabe 1997; Slabbekoorn and Peet 2003), but also in stink bugs (Polajnar and Čokl 2008). Disruptive playback should therefore be (a) strong enough to obscure key signal characteristics, (b) continuous, and (c) broadband, covering at least the whole natural frequency range of the leafhoppers' signals. The disturbance signals described above have evolved to fulfil all these prerequisites and were observed to be efficient in disrupting communication, which makes them an ideal starting point for developing a method for artificial disruption (Fig. 9.3). Continuity can be achieved by simply looping the signal.

9.4.1 Laboratory Trials

The first trials were conducted in the laboratories of the National Institute of Biology (Ljubljana, Slovenia), using a small experimental arena consisting of a grapevine cutting with one intact leaf where sexually mature *S. titanus* males and females were placed and left to start courting (Mazzoni et al. 2009b). Laser-Doppler vibrometry was used for the characterization of vibrations, both for setting up the playback and registering the animals' response. This non-contact recording method is widely used in studies of vibrational communication, because it avoids mass loading the substrate (as opposed to the alternative – piezoelectric detectors), thus preserving the delicate signal structure (Cocroft and Rodríguez 2005; Cocroft et al. 2014).

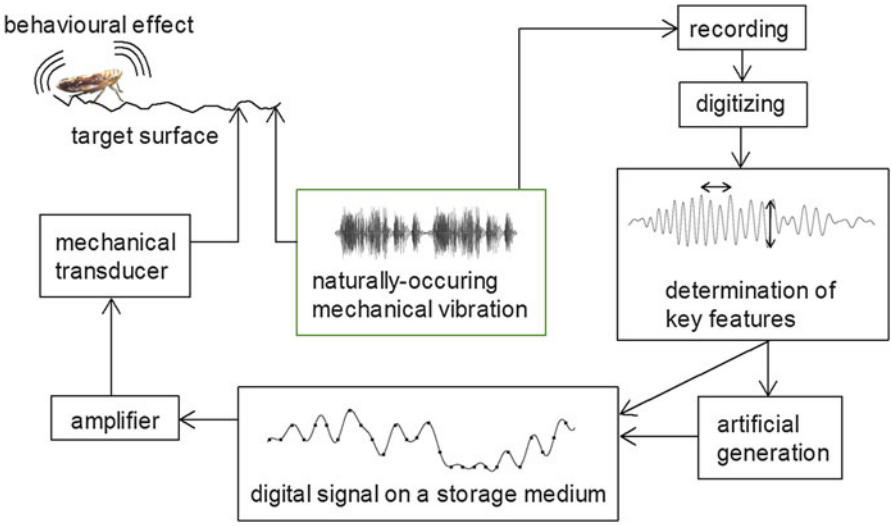

Fig. 9.3 Generalized approach for developing a novel method for exploiting the behavioural effect of mechanical vibrations. This process starts with identification of naturally occurring effects of vibrational stimuli. The stimulus is recorded using suitable acoustic equipment and analysed to determine its key features (amplitude, frequency, modulation, etc.). Complex stimuli can be directly used, or more simple vibration patterns retaining only the key features can be generated artificially. Playback to the target surface is done with electromechanical transducers that vibrate the target surface; this vibration is then transmitted to target organisms in which it evokes a behavioural effect (From Polajnar et al. 2015, Fig. 1, p. 17, with permission from John Wiley & Sons)

A specialized vibration transducer, or "minishaker" (Type 4810, Brüel and Kjær, Nærum, Denmark), controlled by a computer was used for laboratory playback in order to enable fine tuning of the stimulus, which was done by recording the playback with a laser vibrometer and modifying the sound file until it had desired properties. The transducer consists of an electromagnetic mechanism coupled to a metallic rod which is then attached to a point on the target surface, usually slightly away from the position where animals are placed, relying on efficient transmission of vibrations through the plant. Laser beam, on the other hand, is focused on a point near the animals so that the computer record closely corresponds to what is perceived by the animals. Such setup is standard in laboratory studies of insect vibrational communication (Elias and Mason 2014).

Several playback variants were initially tested, including pure tones at 60 and 200 Hz, white noise and pre-recorded MDN, all adjusted so that the peak amplitude matched the amplitude of signals normally produced by the animals. Sexually mature animals in the period of highest activity were used and playback was started after the pair started duetting, but even with such motivated individuals, all treatments were effective in reducing the mating success by at least 50–66 % on a relatively short time scale of 20 min. Two treatments stood out: continuously

played white noise and MDN, achieving perfect (100 %) disruption (Mazzoni et al. 2009b). This was the key result which confirmed the hypothesis that interfering with the vibrational modality alone is efficient in disrupting the mating behaviour. The trial duration, while short, was substantially longer than the normal duration of rival interactions or even than the complete mating sequence including mate recognition, searching and courtship in *S. titanus* (Mazzoni et al. 2009a; Polajnar et al. 2014). It is important to note that the flat frequency spectrum characteristic of white noise is only preserved in the immediate vicinity of the playback apparatus, but gets selectively filtered by the plant during transmission (Michelsen et al. 1982), so the spectral properties become more similar to those of MDN which contains vibrational energy in the range below 300 Hz, while retaining efficiency. With white noise, energy required to generate high frequencies is wasted, which has implications for efficient transducer design. Another significant finding of the study by Mazzoni et al. (2009b) is that the animals are able to exploit 5-s pauses in playback to complete the mating sequence, even at 1:1 duty cycle, which limits the possibilities for energy conservation.

9.4.2 Initial Field Trials and Transmission Tests

The second phase of this research took place at the agricultural research institute of Fondazione Edmund Mach in San Michele all'Adige and at Pisa University (Italy). The trials were first designed to simulate natural conditions, using potted plants placed in cages. The setup was a scaled-down replica of a vineyard row, with potted grapevine plants positioned in a row and attached to a metal wire running between them. Plants were enclosed within cages so a male–female pair could be released inside and recaptured after a set period – in this case, the trials were conducted overnight, between 5 pm and 10 am the following day. Parallel trials were also conducted in an experimental vineyard, using sleeves wrapped around individual shoots (Eriksson et al. 2012).

Since mating behaviour could not be monitored directly in this case, a crucial preliminary step was to develop a test for mated status of females. The approach was based on the observation that gravid females accumulate eggs if they do not have access to suitable oviposition substrate (grapevine bark). The females were therefore reared individually for 10 days in pots with cut grapevine leaves for feeding, but no bark. With a large enough sample, it became clear that gravid females indeed accumulated eggs, while virgin ones only produced a handful of eggs, so if 0–6 eggs were found inside the abdomen after 10 days, the female was considered virgin, while if 10 or more eggs were found, it was considered mated. Those with 7–9 eggs were discarded as unclear, to reduce the probability of error (Eriksson et al. 2012). Accumulation of eggs in a situation where suitable oviposition substrate is absent was also reported in the closely related species *Homalodisca vitripennis* (Sisterson 2012), and was a fortunate discovery, because it meant that the mated status of females could easily be determined post-hoc with simple dissection under

Fig. 9.4 A prototype shaker hung on the trellising wire in the experimental vineyard in San Michele all'Adige. A cage for constraining the animals during field trials is visible in the neighbouring row behind (Photo: J. Polajnar)

a stereo microscope. The eggs present in abdomens of virgin females are thought to be unfertilized and without potential for development, again analogous to *H. vitripennis* (Al-Wahaibi and Morse 2009).

A new field shaker device was used for the disruption trials, a prototype that was built by CBC Europe Ltd. (Milano, Italy). It operates on the same principle as a minishaker or a loudspeaker, but is simpler than the specialized minishaker used initially and its mechanism is coupled with a metal hook designed for hanging on the wire used for trellising in vineyards (Fig. 9.4). This device rated for 1 W of power was connected to an amplified audio output of an off-the-shelf MP3 player playing the looped MDN signal. Signal amplitude, expressed as peak velocity, i.e. velocity of the strongest component, was in the range of millimetres per second at the source, which is two orders of magnitude larger than naturally emitted signals (Eriksson et al. 2011; Polajnar et al. 2014). Plants attached to the wire at successive distance from the source, up to 940 cm away, were thus vibrated simultaneously, which provided disruption for the animals released onto them. The results were clear in both semi-field and field conditions: disruption prevented 77–100 % of pairs from mating, regardless of the distance from the source. In control conditions without disruption, only around 20 % females remained virgin after being left with a male overnight (Eriksson et al. 2012). Still, measuring the playback amplitude showed that attenuation did occur, although not enough to bring the disruption within the range of amplitudes perceived by the male if the female is signalling from the same leaf. It was reduced to the level of a female signalling from another leaf on the

same plant only at the furthest distance in the field setting (Eriksson et al. 2012). However, some females did manage to mate even in the presence of noise. It should be noted that the experimental procedure put the animals in a slightly unnatural situation where the male is constrained in close vicinity (same section of the stem with a handful of leaves) to the female for a prolonged duration, and cannot leave entirely when exhibiting "call & fly" behaviour (as described by Mazzoni et al. 2009a). Thus, the probability of landing directly next to the female is higher than in open space, which would facilitate courtship. Indeed, it is still unknown what is the role of both direct body contact and vision between potential mates and if they can override the absence of vibrational signals. Our observations (unpublished data) indicate that when partners are in close proximity, mating can occur even at the same MDN amplitude that in other experiments resulted in 100 % disruption. At the same time, plants are a complex substrate where bending waves travel and are reflected from end points, creating a pattern of nodes and antinodes (Michelsen et al. 1982; Čokl et al. 2007; Polajnar et al. 2012) so the amplitude of disruptive playback may drop below the threshold for efficiency at some locations, depending on the exact geometry of the individual plant. A perfect efficiency in natural or semi-natural situation therefore cannot be expected.

Aside from attenuation by transmission through the wire, transmission of vibrations through the plant itself is an important issue that was examined in several supporting studies. Transmission in plants is a complex physical question (Michelsen et al. 1982) that is usually generalized in studies on vibrational communication of insects, with exception of plants with the simplest structure where modelling was attempted (Polajnar et al. 2012). It is commonly accepted that insect-produced vibrations travel in the form of bending waves, where the substrate motion is perpendicular to the direction of propagation (Michelsen et al. 1982; Hill 2001; Čokl 2008). This motion is normally expressed as velocity, either as peak velocity of the entire signal in question or as peak velocity of one of its components, although it is not precisely known which feature the animals are sensitive to. The question is further complicated by directional nature of the excitation resulting in eccentric movement of the stem (McNett et al. 2006), by leaf structure (Magal et al. 2000) and of course by selective filtering of spectral components (Michelsen et al. 1982; Polajnar et al. 2012).

On a grapevine cutting, MCS playback adjusted to the normal level of signal emission induced vibrations in the same range of amplitude (>0.01 mm/s) throughout the same leaf and on the petiole of the neighbouring leaf. Most locations had amplitude one range lower (>0.001 mm/s), except the furthermost pair of leaves over 0.5 m away where the amplitude dropped below 0.001 mm/s, under the threshold of signal detection by *S. titanus* (Eriksson et al. 2012) (Fig. 9.5). Such a branched structure with a signaller at its root and delimited by furthest points where the active signal is still detectable by the receiver has been termed the Active Space Network (Mazzoni et al. 2014). Interestingly, propagation is also possible through the air between parallel leaves of neighbouring plants separated by a few centimetre wide gaps. In this case, the vibrating flat surface of the leaf induces air movement the same way as a loudspeaker membrane, which is then picked up by the other leaf, but progressively attenuated with increasing distance between them. *Scaphoideus*

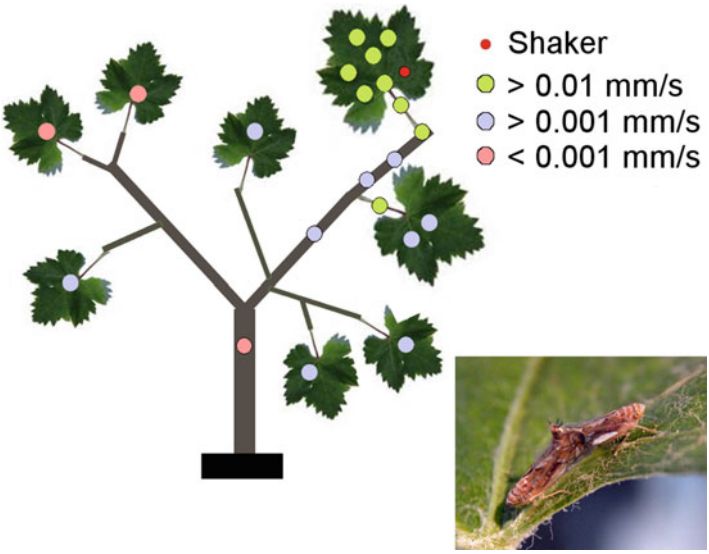

Fig. 9.5 Transmission of MCS through a grapevine plant. The uppermost leaf of potted grapevine plants was vibrated with male calling signal (*red dot*, shaker). The intensity of vibrational signals was measured at several points along the grapevine plants as substrate velocity at the dominant frequency (mm/s) and accordingly, three probability levels of successful mating communication were assigned to each point: "high", velocity of mating signals 0.01 mm/s, *green circles*; "median", velocity of mating signals between 0.001 and 0.01 mm/s, *blue circles*; "low", velocity of disruptive signals under 0.001 mm/s, *pink circles*. The latter is below the threshold level of signal detection of *S. titanus* (Eriksson et al. 2011) (Photo: A. Lucchi, Modified from Eriksson et al. 2012, Fig. 1, p. 2, doi:10.1371/journal.pone.0032954.g001; with permission from PLoS)

titanus leafhoppers are able to communicate through up to 6 cm wide gap (Eriksson et al. 2011). In a later study, animal-produced vibrations were carefully measured to determine whether the male could use the amplitude gradient for orientation, and were found to reflect distance from the source reliably, at least on short range (Polajnar et al. 2014). The absence of prominent amplitude fluctuations that characterize transmission of pure-tone signals (Polajnar et al. 2012) was probably caused by the broadband nature of the signal. We can therefore simplify the model and assume, with appropriate safety margin (yet to be determined), monotonous attenuation of vibrations with distance from the source.

9.4.3 Improving Efficiency

With the proof of concept complete, the attention shifted to determining limitations of the prototype technology and improving efficiency. Two issues were explored: time of activation and amplitude threshold, both factors affecting energy use and equipment wear.

9.4.3.1 Threshold vs. Range

Amplitude of mechanical vibrations is a continuous parameter. Assuming monotonous attenuation with distance from the source, the amplitude of disruptive signal will eventually drop to the level of normal background noise, ceasing to affect communication. There will be a certain amplitude threshold on this continuous scale where playback prevents mating of 50 % pairs (or some other desirable percentage). Therefore, intersection of the attenuation curve and this threshold value equals the effective range of disruption.

Experiments were designed to determine the amplitude threshold in the laboratory and test it with the prototype shaker in the field. The males were given the same task as before – locating the female on a different leaf of the same grapevine cutting and mating with her while DN was being broadcast. To avoid mass loading and preserve signal characteristics, a flat loudspeaker was placed parallel to one of the leaf laminas, so its sounds were picked up by the plant and propagated elsewhere as vibrations. Since the propagation of vibrations along plants is unpredictable (Michelsen et al. 1982; Čokl et al. 2007), male- and female-perceived amplitudes were measured post-hoc from exact locations of both and analyzed separately. With sufficient number of trials with different amplitudes, a clear pattern emerged: no effect on mating behaviour at very low amplitudes was observed, but there was a sharp drop in ratio of male searching success when the amplitude of DN was increased to above 0.0025 mm/s, and no male was able to locate the female above 0.015 mm/s. Almost all males who found the female were also able to mate successfully, meaning that recognition and searching are key components of mating behaviour on which disruption works. This was confirmed by the fact that the proportion of females replying to male signals formed the same pattern. Signals and male movement were not affected by the noise level in those males that successfully located the female, suggesting that the process is all-or-nothing – i.e. either working as normal or breaking down completely (Polajnar et al. 2016).

The threshold of zero success obtained in laboratory experiments – 0.015 mm/s was then used to assess the range. Measurements throughout the growing season revealed constant changing of the attenuation curve with time, related to the growth stage of grapevine plants. The attenuation gradually increased throughout this time, presumably because the plants grew heavier, but also gripped the conducting wire more firmly with vines and "tied" it to poles and to other wires. Furthermore, measuring the amplitude along one of the shoots revealed that there is a sudden 20 dB drop of amplitude at the wire-stem interface, but then the attenuation is greatly diminished and the amplitude remains largely constant down to the rootstock. Leaves vibrate more freely and slightly higher amplitude was measured there, so, considering that leafhoppers mostly perch on leaves, the relevant amplitude drop is approximately 15 dB (Polajnar et al. 2016). Summing the attenuation caused by transmission along the wire and attenuation caused by transmission to the plant, the worst-case scenario at the end of the season is a range 10 m to either side of the prototype shaker. Further away, the disruption amplitude falls below 0.015 μm/s, which should facilitate mating. This prediction was tested with field trials, using

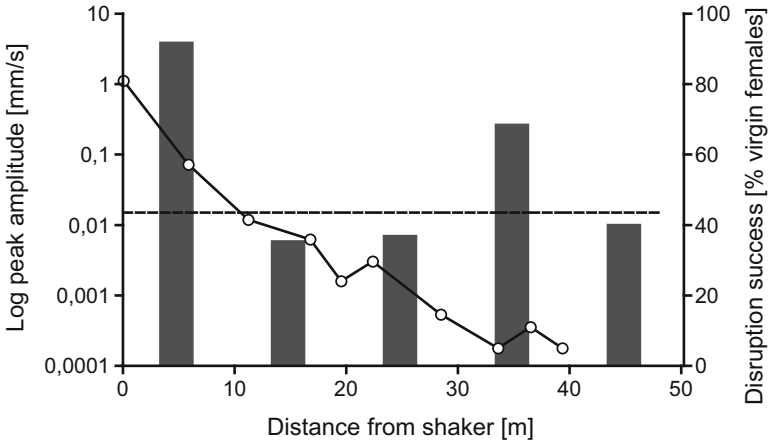

Fig. 9.6 Proportion of virgin females (*bars*) in test of distance from the shaker with disturbance noise (DN) emission. Amplitude values (*empty circles*) are taken from end-season measurements and reduced by 15 dB to account for extra attenuation during transmission along the shoot, an approximation of the actual amplitude perceived by the animals. Those values are compared with the threshold obtained by laboratory trials (0.015 mm/s, *dashed line*) (Modified from Polajnar et al. 2016 in press, with permission from Springer)

cages placed along the row at 5, 15, 25, 35 and 45 m, and 1 day of continuous disruption. Results confirmed the prediction, with only the closest cage reliably preventing mating (Polajnar et al. 2016) (Fig. 9.6), although this outcome is likely trellis system-specific.

9.4.3.2 Time of Activation

Systematic observations by Lessio and Alma (2004b) and Mazzoni et al. (2009a) revealed a diel pattern of *S. titanus* activity. Both reported that the animals are significantly less active during daytime, roughly from 0800 to 1600 h, which opens a possibility to save energy by switching off the minishaker in this period without giving the animals the opportunity to mate. Field trials were therefore designed to test whether disruption would retain efficiency if the minishaker was switched off during certain periods of the day, with focus on the period around noon. The method of Eriksson et al. (2012) was again employed in the experimental vineyard of the Fondazione Edmund Mach, but with pre-programmed silent periods that lasted from 3 to 20 h, depending on the treatment (Polajnar et al. 2016).

The results provided two major insights: as predicted from the data on daily activity, switching the disruption off between 1200 and 1500 h did not affect the efficiency at all, leaving approximately 90 % of females virgin after one day, same as control with continuous disruption (i.e. positive control). Extending the silent period to 8 h (between 0900 and 1700 h) caused a corresponding drop in the proportion of virgin females, although still not significantly different from

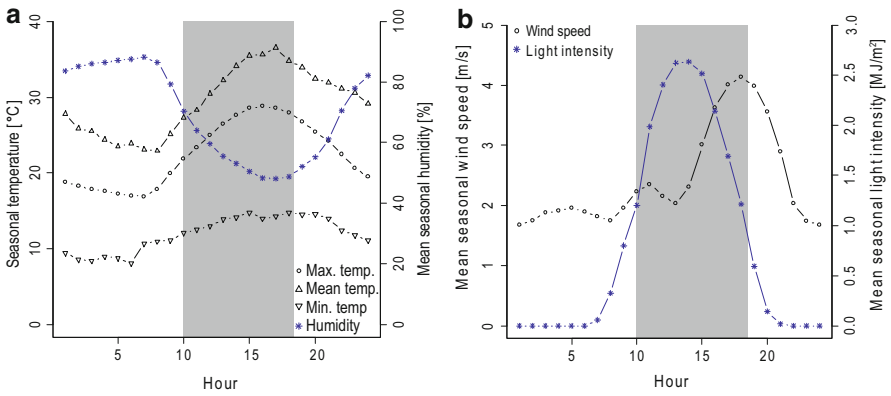

Fig. 9.7 Climatic conditions in the seasons 2012 and 2013 as recorded by the weather station in San Michele all'Adige. Combined times of silent windows that did not cause decrease of disruption efficiency are *grayed* (From Polajnar et al. 2016, in press, with permission from Springer)

positive control. Any other silent period resulted in efficiency below 50 %, which is not significantly different from control without disruption (i.e. negative control). Interestingly, the inefficient treatments included the one with silent period from 1000 to 1600 h and an additional short silent period between 2200 and 2400 h – the latter was apparently enough to enable most pairs to mate. Leaving the animals in a cage for 3 days with continuous disruption likewise decreased the effect, allowing almost half of the pairs to mate (Polajnar et al. 2016). Secondly, the results provided an indirect proof that *S. titanus* daily activity during the mating season is entrained by light intensity, as already observed by Lessio and Alma (2004b), and not any associated environmental factor such as temperature. The combined silent windows of treatments that did not result in decreased efficiency match the peak of average hourly light intensity as measured by a nearby weather station, whereas the temperature, humidity and wind speed are offset (Fig. 9.7). The period of activity can therefore be clearly characterized as crepuscular, but spanning the entire range of other environmental factors (Polajnar et al. 2016).

According to this result, it is possible to optimize disruption, using a simple timer to switch off the stimulation and conserve energy. It may also be possible to implement a light measuring device for precise matching to day/night cycle in a certain area, although the cost might not be worth the effort.

9.5 Perspectives

To summarize, the idea of control effort using mechanical vibrations as means of mating disruption against *S. titanus* in Europe is based on the observation that rival vibrational signals, emitted naturally in antagonistic interactions, are effective

in disrupting the communication between a male and a female. This approach is facilitated by the following facts:

- The species is monophagous in the introduced range, with the host range limited to the genus *Vitis*
- Courtship is almost exclusively unimodal, relying on substrate vibrations
- Animals exhibit low level of dispersal from host vineyard
- Standard in viticulture provides the means of delivery of vibrational energy to target surfaces

The idea is to use a series of stimulating devices attached to vineyard wires at suitable intervals in all rows to achieve sufficient coverage of all the plants, vibrating them with continuously played recording of a male disturbance noise (MDN) signal. Playback should be constant throughout the time that adults are present in the field, i.e. late June to September, depending on location (Chuche and Thiéry 2014), except for the period between 0800 and 1600 h each day when it can be switched off. Technology to achieve this includes electromagnetic shakers, audio playback devices, amplifiers and power sources, all based on or being mass-produced electronic components that would require relatively little effort to modify for this purpose and create a practically useful solution. According to the manufacturer, equipment costs could be brought to within 300€/ha at the outset, which is comparable to the cost of pesticide treatments if a minimum of 5-year lifetime of a unit is assumed – for comparison, the cost of basic products alone for pyrethrinoid treatments is up to 25 € per hectare per year in France (Chuche and Thiéry 2014).

As presented in this chapter, the solution is in the middle stage of development, as proof of concept successfully transferred to small-scale field conditions. Keeping in mind the main prerequisites for pest control methods as mentioned above (Metcalf 1994), two main tasks must be accomplished next: large-scale field trials, and developing the product as a package for convenient installation. Large-scale field trials, conducted in several successive years will be needed to establish whether the method is in fact feasible and efficient in practice. Technology is also still in the prototype stage, with a lot of potential for improvement. The shakers must be made robust enough to survive successive seasons exposed to the elements. Such technical details as the best way to attach to the wire or some other part of the trellis to achieve optimal propagation must be worked out. There are also different options on how to provide power supply. It will in particular be crucial to develop a tool with rechargeable batteries (i.e. solar lights), and entirely cable-free for convenient installation. Therefore it will be crucial to generate the required power, store it and to minimize energy waste (by improving the mechanical properties of the system and the materials of the vineyard construction). A further development stage would be the addition of useful sensors integrated in the device. Such sensors could for example detect light, leaf wetness, temperature and optionally relative humidity. Such sensors are necessary for further rationalization of functioning, depending on the type of mating activity of the insects, once the role of environmental conditions such as temperature and humidity is clarified.

It is not yet possible to predict the cost/benefit ratio of the solution, before performance on a large scale is known. Assessing efficiency of pest control methods is difficult in itself, relying on indirect methods such as counting trap catches or even taking inventory of the actual damage to the crop at the end of the growing season (Cardé and Minks 1995). Therefore, the planned approach is to conduct field trials in a suitably isolated large area that will provide data on effect over consecutive years. The efficiency needs to compare favourably to mandatory pesticide treatments that may achieve over 90 % reduction of the number of *S. titanus* nymphs, as determined by manual counts (Žežlina et al. 2013). Although not directly comparable and not taking the population density into account, the observed efficiency in semi-field conditions – 90 % reduction of mating success – at least gives hope that this level is achievable at equipment cost projected above. We believe the Mezzocorona viticultural area of Italy, where the pilot experiments described in this chapter were performed, is an optimal place for such activity: interaction between researchers and users is facilitated by the presence of a strong research institute on one hand and a regional agricultural cooperative on the other. Their cooperation recently enabled the success of pheromone mating disruption initiative against two other grapevine pests, the moths *Lobesia botrana* and *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) that eliminated the need for pesticide treatments against these pests in the area (Ioriatti et al. 2008).

9.5.1 Possible Concerns

The main constraint of the technique is migration of mated females from untreated areas, which is shared with conventional mating disruption techniques (Cardé and Minks 1995). The problem is exacerbated by the fact that infection dynamics are still poorly understood. As a worst case scenario, a single infected female may theoretically be enough to infect a plant (Caudwell et al. 1970), and then the subsequent generations would spread the phytoplasms to nearby plants. Comprehensive area-wide application of the technique will therefore be necessary to achieve reliability. A similar concern is the possibility of spreading away from host plants to vineyard surroundings where the animals could mate undisturbed. No reports about behaviour on other plants exist, but studies suggest that *S. titanus* is largely incapable of active dispersal away from its host plant, with females less likely to fly than males. The main dispersive behaviour is the male “call & fly” which, however, is limited to distances of few meters. After 1–2 months of adult life, which is the common species life-span, a few meters can nevertheless translate to movement of some hundreds of meters or even kilometres, but being non-directional, it is probably not enough to cause important dispersal. Actual dispersal will more critically depend on local environmental factors, such as wind and natural barriers (Lessio and Alma 2004a; Beanland et al. 2006; Riolo et al. 2014).

Untreated vineyards, however, would represent pools from which the animals could keep re-colonizing treated areas, as is the case with chemical control. Like-

wise, the method is unsuitable for head trained vines, pruned without wire trellises, unless it is modified to work on individual plants. For these reasons, it may be necessary to combine mechanical mating disruption with more conventional pest control techniques in some scenarios, which should be determined on a case-by-case basis.

Potential adverse effects on non-target organisms have been reviewed in Polajnar et al. (2015). Briefly, they fall into two classes: those affecting plants themselves and those affecting other beneficial organisms. Plants respond to mechanical perturbation by invoking a not yet completely understood network of metabolites, which causes slow changes in growth patterns and metabolite allocation (Chehab et al. 2009). The net effect might be deleterious or beneficial, depending on the stimulation and plant species, so this issue should be studied thoroughly before implementation. For example, experimentally induced intense sinusoidal vibrations have caused increased biomass allocation to the root system, reduction in dry weight of reproductive structures at maturity, delay in flowering and fruit formation and promoted senescence in *Capsella bursa-pastoris* (L.) Medik. (Niklas 1998). Conversely, stimulation by pure-tone airborne sound reportedly increased yield and various physiological parameters in several species of crop plants (Tianzhen et al. 2009; Lirong et al. 2010), although this phenomenon is still controversial. Other beneficial organisms include natural enemies of pests, such as spiders, with IPM methods actively promoting their abundance (Sunderland and Samu 2000; Landis et al. 2005). Low-frequency noise with amplitudes above 0.1 mm/s may decrease spider sensitivity to prey cues (Wu and Elias 2014), which could be a problem close to the shakers, so further research is needed. A similar problem may be expected in insect parasitoids, but experimental evidence is even scarcer in that group (Meyhöfer and Casas 1999; Laumann et al. 2007).

Large-scale field trials will be a good opportunity to explore those concerns and develop an ecologically sound strategy for application, again based on the existing knowledge about basic *S. titanus* biology.

As one of the minor issues, selective pressure by continuous playback of disruptive noise might eventually result in a temporal shift of sexual activity to exploit any predictable silent windows. Such behavioural plasticity has already been demonstrated in insect populations, in response to factors such as eavesdropping parasitoids (Vélez and Brockmann 2006) and predictable periods of intense wind (Tishechkin 2007, 2013; McNett et al. 2010). In this event, a reduction of the method's efficiency would be observed by long-term field sampling. The solution would be straightforward – narrowing or removal of the silent window – but would forfeit energy saving.

9.6 Conclusion

There is a strong market demand for alternatives to chemical pesticides in agriculture for several reasons. Consumers are more and more wary about potential risks from chemicals and chemical residues in fruit and vegetables, so large food retailers

are imposing more stringent limits than those in current legislation on residues. The continuous use of the same old active ingredients may increase the risk of emergence of pest resistance to insecticides. In the case of grapes, in those areas where pheromone mating disruption is already implemented, the adoption of the mechanical mating disruption will significantly reduce the need for insecticides (treatments against leafhoppers are almost the sole chemical treatments left). In particular, there is strong demand in several regions where the final aim is also to brand the territory in question with the label 'insecticide free area' for marketing purposes. The European Union has responded to concerns about harmful effects of pesticides as well, by adopting a policy on sustainable use of pesticides (c.f. directive 2009/128/EC). At the same time, regulation 1107/2009 imposed re-registration of pesticides, meaning that many old active ingredients are no longer available on the market. Together with the public perception that in the recent years favours "organic"/"eco"/etc. produce, these changes provide a strong impetus for farmers to adopt alternative technologies for pest management.

Therefore, the method of mating disruption presented here has tangible potential to provide a new perspective for controlling *S. titanus* in European vineyards, although it will still require substantial research effort before actual application is possible. Considering the widespread use of vibrational communication among insects, we hope that it will also stimulate development of innovative methods against other pests in different scenarios, along with other existing research initiatives in the field of behavioural manipulation with mechanical vibrations (Polajnar et al. 2016), most notably those targeting bark beetles (Hofstetter et al. 2014) and psyllids (Mankin et al. 2013; Rohde et al. 2013).

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Chapter 10
Cell-Based Screening Systems for Developing Novel Insecticides: Insights from the EcR-Reporter Paradigm

Luc Swevers and Guy Smagghe

Abstract For the discovery of new insecticides, there is an increasing interest in the development of in vitro methods to replace conventional insect toxicity tests. The ultimate goal is to achieve an alternative system that allows for rapid ("high-throughput") testing of candidate compounds enabling prediction of their efficacy at the whole animal level. Besides a dramatic increase in the number of compounds that can be screened, requirements for successful in vitro screening systems include reproducibility, predictive power for toxicity in vivo, and low cost/benefit ratio.

Here we present some relevant examples in the development of screening systems for discovery of biorational insecticides. The significant advances in ecdysone receptor (EcR)-based reporter systems are used as a paradigm to illustrate advantages and pitfalls of cell-based screening systems. While the EcR-based reporter assay can predict the hormonal activity of compounds accurately, unexpectedly, rather low predictive power exists whether such compounds with high activity in vitro can be developed and used as effective insecticides. Furthermore, the principles that guide effective insecticide activity for EcR agonists in larvae may differ among different insect groups. Thus, while in vitro systems can narrow down the number of compounds considerably, larvicidal assays at considerable scale remain necessary to assess efficacy.

The chapter concludes with a short presentation of two other applications of the EcR-reporter system: the identification of ecdysone antagonists and the testing of environmental compounds with endocrine disruptor activity.

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10.1 Introduction

Humans are exposed to insect threats both at the level of transmission of insect-borne diseases and at the level of agricultural output, which is at risk by insects that devastate crop production. In both areas, the use of insecticides is considered the most effective measure to control the insect threats. There is a global need for the development of new insecticides, preferably with new modes of action, while the importance of nonchemical approaches, such as those based on genetically modified crops, is also widely acknowledged (Vontas et al. 2014).

Insecticide management has become increasingly more critical over time since fewer new insecticides are being discovered, while older commercial chemistries are being reduced by increased regulation. This review addresses the question to what extent in vitro screening systems can be used to accelerate the discovery of new insecticides. In vitro systems are based on a simple assay that examines the inhibition or inappropriate activation of an essential target in insects and that can be used in high-throughput screening format for identification of new active substances. While in vitro assays are typically characterized by their rapidity and low cost, other essential properties of successful in vitro screening systems are of a high sensitivity (ability to identify relevant compounds accurately in compound libraries or collections of biological extracts) and a high specificity (inability to identify non-accurately nonrelevant compounds). Statistical parameters have been proposed to predict the "suitability" of an in vitro assay to be developed as a reliable high-throughput screening assay (Zhang et al. 1999). In this article, we report on a robust and specific in vitro screening system, for identification of ecdysone analogs (a class of biorational insecticides; Ishaaya et al. 2005), and its ability to predict the larvicidal efficiency of the identified ecdysone analogs.

10.2 Sensitive and Specific In Vitro Screening Systems for Identification of Ecdysone Analogs

Robust specific screening systems for identification of ecdysteroid mimics have been developed that are based on an ecdysone reporter assay in insect cell lines (Swevers et al. 2004). The principle of the assay is the induction of the expression of green fluorescent protein (GFP) or luciferase by the activated ecdysone receptor/ultraspiracle protein (EcR/USP) complex. The reporter plasmid contains a basal promoter preceded by seven repeats of the ecdysone-response element (EcRE) from the *Drosophila hsp27* gene (Koelle et al. 1991; Swevers et al. 2004) that confers robust and specific binding of the EcR/USP complex (Swevers et al. 1996). Because the ability of compounds to activate EcR/USP is based on stringent interactions, the assay is considered very specific for identification of ecdysone analogs. In

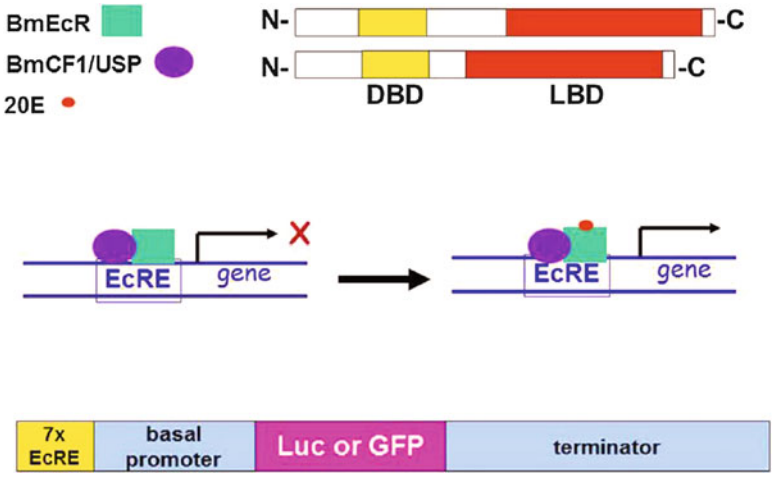

Fig. 10.1 Overview of the EcR-reporter assay. BmEcR and BmCF1/USP refer to the two components of the ecdysone receptor heterodimer (EcR/USP) in the silkworm (*Bombyx mori*). The structure of the nuclear receptors EcR and USP is also shown schematically. Abbreviations: *DBD* DNA-binding domain, *LBD* ligand-binding domain, *20E* 20-hydroxy-ecdysone

addition, the presence of seven EcRE repeats in the promoter region results in high inducibility of the reporter and a high sensitivity of the assay (Fig. 10.1).

Because the EcR/USP complex is expressed constitutively in insect cell lines, development of an in vitro assay only requires the introduction of the EcR-reporter plasmid through transfection. After transfection in the insect cell, the EcR-reporter plasmid recruits the EcR/USP heterodimer complex that is endogenously present. The EcR/USP heterodimer can be activated by the natural hormone 20-hydroxy-ecdysone (20E), the related ecdysteroid ponasterone A (PonA), or a synthetic ecdysone agonist. Experiments have shown that the EcR-reporter plasmid can be robustly activated by endogenously expressed EcR/USP heterodimers in insect cell lines, e.g., Lepidoptera (Bm5, *Bombyx mori*; Hi5, *Trichoplusia ni*; Sl2, *Spodoptera littoralis*; SE4, *Spodoptera exigua*; Swevers et al. 2004, 2008; Mosallanejad et al. 2008; Soin et al. 2010a), Diptera (S2, *Drosophila melanogaster*; Soin et al. 2010b), and Coleoptera (Ag3C, *Anthonomus grandis*; CPB, *Leptinotarsa decemlineata*; Soin et al. 2009; Ogura et al. 2012). While in most cases, the EcR-reporter plasmid could be introduced by transfection, it is noted that also transformed clonal Bm5 cell lines exist that have permanently incorporated the EcR-GFP reporter in their genomes (Swevers et al. 2004). In the case of the non-transfectable Se4 cell line (derived from *Spodoptera exigua*, Lepidoptera), transduction could be achieved using recombinant baculovirus expressing EcR-reporter cassette (Swevers et al. 2008).

10.2.1 Lepidopteran Cell Lines

Clonal Bm5/EcRE-GFP cell lines (permanently transformed cell lines with incorporated EcRE-GFP reporter cassettes) have a robust fluorescence response following addition of ecdysone agonists and were used to screen a library of more than 150 diacylhydrazine (DAH) compounds, a class of chemicals with strong ecdysone agonist activity in lepidopterans (Swevers et al. 2004; Wheelock et al. 2006). Effective mean concentration (EC_{50}) values could be calculated for most compounds which allowed quantitative structure-activity relationship (QSAR) analysis. The 3D-QSAR model was subsequently shown to fit well with the X-ray structure model of the ligand-binding pocket of EcR of *Heliothis virescens*, an important lepidopteran pest (Billas et al. 2003), thus validating the use of the cell-based screening system as a tool for identification of ligands of the EcR/USP complex (Wheelock et al. 2006). In addition, the cell-based screening system could be adapted to a droplet-based microfluidic system that allows detection of EcR activation in nanoliter droplets, thus further reducing significantly the cost of screening (Baret et al. 2010).

In the Bm5/EcRE-GFP-based screening system, the commercial DAH tebufenozide and methoxyfenozide (RH-5992 and RH-2485, respectively, from Rohm and Haas Co.) and chromafenozide (ANS-118 from Sankyo Agro Co. Ltd) were highly active, while halofenozide (RH-0345; Rohm and Haas Co), which is targeted against coleopterans (Dhadialla et al. 1998; Retnakaran et al. 2003; Smagghe et al. 2013), showed lower activity. Of interest was the identification of two compounds, KU-106 and KU-121, which showed a potency and efficacy in the EcR-reporter assay that was comparable to the highly active commercial compounds tebufenozide, methoxyfenozide, and chromafenozide (Swevers et al. 2004; Soin et al. 2010a).

When a collection of candidate ecdysone agonists, including DAHs, acylaminoketones (AAKs), and tetrahydroquinolines (THQs), were tested in two lepidopteran cell lines, *B. mori*-derived Bm5 and *S. littoralis*-derived Sl2, for EcR-reporter activation, very similar data were obtained with respect to potency and efficacy of the compounds ($R^2 = 0.977$; Fig. 10.2), indicating similar activation properties of EcR/USP in different lepidopteran species (Soin et al. 2010a). In these assays, DAH and AAK compounds showed much higher activity than THQs.

10.2.2 Dipteran Cell Lines

A similar collection of DAHs, AAKs, and THQs was also tested in a dipteran cell line, S2 derived from *D. melanogaster* (Soin et al. 2010b). In this case, 100–1000-fold lower potency was observed for DAHs and AAKs, as compared with lepidopteran cell lines, while also the efficacy (inducibility of the EcR reporter) was significantly reduced. While, in lepidopteran cell lines, some DAHs, such as tebufenozide, methoxyfenozide, and the experimental compounds KU-106 and

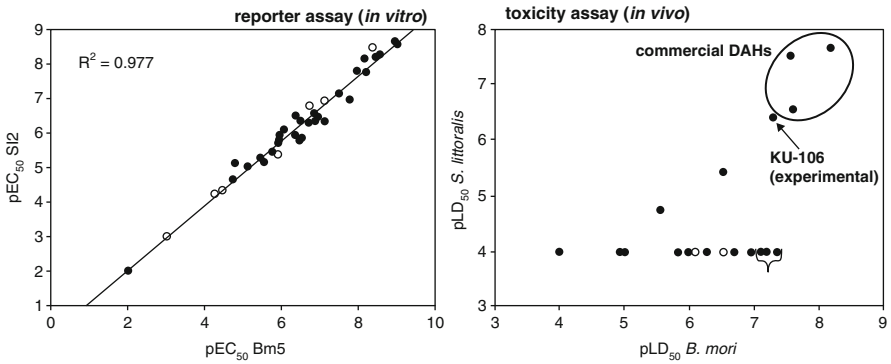

Fig. 10.2 Activity of diacylhydrazine (*DAH*) (*black dots*) and acylaminoketone (*AAK*) (*white dots*) ecdysone agonists in cell-based EcR-reporter assay (*left*) and insecticidal (toxicity) assay on larvae (*right*). Effective mean concentrations (shown as –log or pEC_{50} values) in reporter assays are compared between *B. mori*-derived Bm5 and *S. littoralis*-derived Sl2 cell lines. Lethal mean doses (shown as –log or pLD_{50} values) in larvicidal assays are compared between *B. mori* and *S. littoralis* larvae. For the toxicity assays, activities of the commercial DAHs tebufenozide, methoxyfenozide, and chromafenozide are indicated by a diagram, while the experimental compound KU-106 is indicated by an *arrow*. The activity of three DAHs with >1000 higher toxicity against *B. mori* larvae than against *S. littoralis* larvae is also indicated by a clamp. R^2 = coefficient of correlation (Reprinted by permission from John Wiley & Sons Ltd (Soin et al. 2010a))

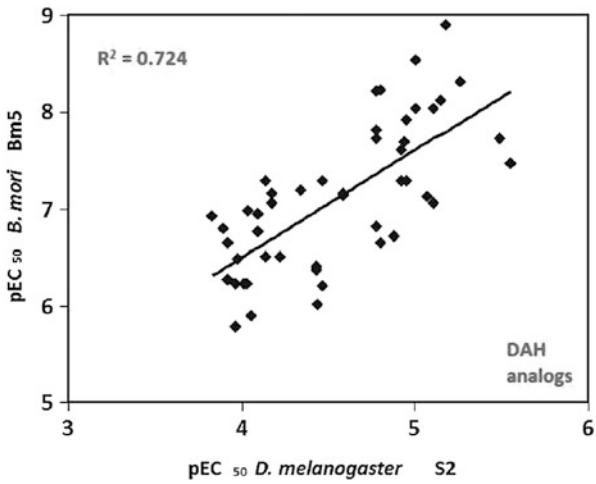

Fig. 10.3 Comparison of potency (pEC_{50}) of DAH compounds between dipteran S2 and lepidopteran Bm5 cells. DAH compounds are 100–1000-fold more active in lepidopteran cells. R^2 = coefficient of correlation (Reprinted by permission from John Wiley & Sons Ltd (Soin et al. 2010b))

KU-121, were active at nanomolar concentrations, in dipteran cell lines, not one DAH could be identified with higher potency as the natural hormone 20E (EC_{50} = 75–150 nM) (Fig. 10.3). Moreover, not one DAH or AAK compound was identified with higher potency in dipteran than in lepidopteran cells, reflecting the high specificity of these classes of compounds for EcR/USP of Lepidoptera (Soin et al. 2010b). For THQs, on the other hand, two compounds could be identified that

were active in S2 cells but not in Bm5 cells, indicating the potential for development of Diptera-specific THQs for pest control. However, considerable optimization is necessary since their EC_{50} in the EcR-reporter assay is high (~10 μM) and THQs also exhibit general cell toxicity and thus need to be modified to increase their safety (Soin et al. 2010b).

10.2.3 Coleopteran Cell Lines

The Ag3C cell line derived from the boll weevil, *Anthonomus grandis*, was found to be easily transfectable with the EcR-reporter plasmid and therefore could be used for the testing of ecdysone agonists (Soin et al. 2009). As was the case for the dipteran S2 cell line, DAHs showed much lower activity in the Ag3C cell line than in the lepidopteran cell lines Bm5 and Sl2. For the commercial DAHs tebufenozide, methoxyfenozide, and halofenozide, potencies of 1, 6, and 87 nM were observed in Bm5 cells, respectively, while the corresponding potencies in Ag3C cells were 7, 5, and 15 μM, a difference ranging from 200-fold (halofenozide) to 1000-fold (tebufenozide and methoxyfenozide). This observation contrasts with the very similar potencies of ecdysteroids in both types of cell lines (100–200 nM for 20E and 5–10 nM for PonA) (Fig. 10.4; Soin et al. 2009).

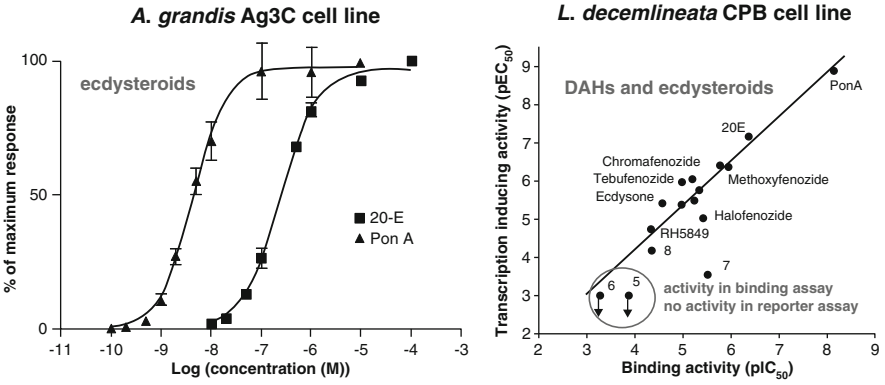

Fig. 10.4 Activity of ecdysteroids and ecdysone analogs (*DAHs*) in coleopteran cell lines. *Left* Dose response of the ecdysteroids 20E and PonA in Ag3C cells derived from the boll weevil (*Anthonomus grandis*). *Right* Comparison of the activity of ecdysteroids (20E, PonA, Ecdysone) and DAHs (unsubstituted RH-5849, tebufenozide, methoxyfenozide, halofenozide, chromafenozide, and the experimental compounds 1–8) in biochemical binding assays and EcR-reporter assays (transcription-inducing activity) using CPB cells derived from the Colorado potato beetle (*Leptinotarsa decemlineata*). Compounds without activity in the reporter assay and with significant binding activity are indicated by a diagram. Abbreviations: pIC_{50}, mean inhibitory concentration (–log value); pEC_{50}, mean effective concentration (–log value) (Reprinted by permission from Elsevier and John Wiley & Sons Ltd (Soin et al. 2009; Ogura et al. 2012))

The CPB cell line, derived from the Colorado potato beetle *Leptinotarsa decemlineata*, on the other hand, required extensive optimization in transfection protocols to establish effective delivery of the EcR-reporter plasmid (Ogura et al. 2012). Although five- to tenfold higher potencies could be reported for commercial DAHs in CPB cells compared with Ag3C cells, EC_{50}s remained 50–500-fold higher than in lepidopteran cells. When experimental DAHs were tested in CPB cells, EcR-reporter activity could be correlated with binding activity to the EcR/USP complex in biochemical assays, although exceptions were noted (Fig. 10.4; Ogura et al. 2012). Significant binding to EcR/USP without measurable activity in the EcR-reporter assays could be indicative of ecdysone antagonist activity (Fig. 10.4).

10.3 Validation of Insecticidal Activity of Ecdysone Agonists in Toxicity Assays with Insect Larvae

It is well established that the commercial DAHs (tebufenozide, methoxyfenozide, halofenozide, chromafenozide) exert their action by the induction of a premature molt that ultimately is lethal (Retnakaran et al. 1995; Dhadialla et al. 1998; Nakagawa 2005). Within hours after the application of ecdysone agonist, larvae stop feeding and the process of apolysis is initiated in the larval epidermis, similar to the action of the natural hormone 20E. Because of their chemical properties, DAHs are not cleared efficiently from the larvae and the processes in the molt that require a decline in ecdysteroid signaling (such as ecdysis) are prevented. Thus, larvae treated with DAH agonists become trapped in the molting process and die over time from desiccation and starvation (characteristically with double cuticle).

Because of the lepidopteran EcR/USP complex is activated by DAHs very efficiently, lepidopteran larvae can respond very sensitively after their application. However, formulations of halofenozide have also been developed for control of ground-dwelling coleopterans (Retnakaran et al. 2003; Dhadialla et al. 2005; Nakagawa 2005; Smagghe et al. 2013).

10.3.1 Lepidopteran Larvae

After the screening of a collection of more than 150 DAHs and several AAKs in the lepidopteran cell lines Bm5 and Sl2 (Swevers et al. 2004; Soin et al. 2010a), selected compounds of high activity were tested for toxicity on lepidopteran larvae after topical application (Soin et al. 2010a). When tested on *Bombyx* larvae, a clear positive correlation was observed between activity in the EcR-reporter assay and larval toxicity (Fig. 10.2). For *Spodoptera littoralis* larvae, on the other hand, it was found that, with one exception, none of the tested experimental compounds showed toxicity (Fig. 10.2). By contrast, the commercial compounds tebufenozide,

methoxyfenozide, and chromafenozide were highly toxic, while lower levels of toxicity against *Spodoptera* larvae were also observed for halofenozide and the unsubstituted "mother" DAH compound, RH-5849 (Fig. 10.2; Soin et al. 2010a). Thus, for *S. littoralis* larvae, the capacity of DAH compounds to induce a lethal molt cannot be predicted in a straightforward manner from the in vitro screens that are based on the EcR-reporter assay and extensive in vivo validation assays remain necessary.

Only one new experimental compound, KU-106, which is also very potent in EcR-reporter assays, displayed toxicity against *Spodoptera* larvae that was similar to tebufenozide (Fig. 10.2; Soin et al. 2010a). However, KU-106 was not active against *Helicoverpa armigera* (cotton bollworm; Lepidoptera) larvae (in contrast to methoxyfenozide), further illustrating species-specific effects (Morou et al. 2013). Species-specific effects on lepidopteran larvae are also exemplified by the identification of three DAHs that are more than 1000-fold more toxic against *Bombyx* than *Spodoptera* larvae. This could be correlated with *Bombyx* being a domesticated species that has been artificially selected for silk production and may have lost particular detoxification mechanisms because of long-term rearing in protective environments. Reduced capacity of detoxification has previously been invoked to explain the sensitivity of *Bombyx* to chitin synthesis inhibitors with benzoylphenylurea structure (Nakagawa et al. 1992).

10.3.2 Dipteran Larvae

Although DAHs are much less active in EcR-reporter assays employing dipteran (*Drosophila* S2) cells, they surprisingly display considerable toxicity against midge and mosquito larvae (Fig. 10.5; Beckage et al. 2004; Boudjelida et al. 2005; Smagghe et al. 2002; Morou et al. 2013). During the exposure to DAHs, the ecdysone-responsive gene *hr3* was induced and larvae with double-cuticle phenotype were

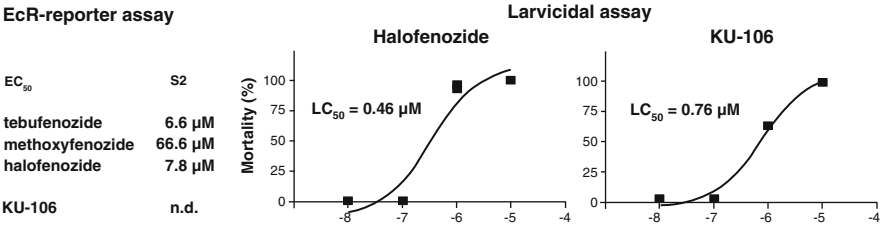

Fig. 10.5 Activity of selected DAHs in EcR-reporter assays using dipteran S2 cells (in vitro) and in larvicidal assays against *Anopheles gambiae* larvae (in vivo). Effective mean concentrations (EC_{50}) and mean lethal concentrations (LC_{50}) are indicated. Note that LC_{50}s are actually of higher potency than EC_{50}s which reflects the prolonged exposure (5 days) of mosquito larvae to the DAH compounds (accumulation of toxic effects) (Reprinted by permission from John Wiley & Sons Ltd (Morou et al. 2013))

observed, indicating that mortality was caused by lethal molt induction. Besides the commercial compounds (tebufenozide, methoxyfenozide, halofenozide), also the experimental compound KU-106, identified in the EcR-reporter screens mentioned above (Wheelock et al. 2006; Soin et al. 2010b), proved effective against *Anopheles gambiae* larvae (Morou et al. 2013). Because lethal effects could be caused at relatively low concentration (LD_{50} values in the submicromolar to micromolar range), the potential for DAHs to control mosquito larvae may be greater than expected and invites further experimentation in larvicidal assays.

10.3.3 Coleopteran Larvae

While DAHs in general do not have high potency or efficacy to activate the EcR/USP complex of Coleoptera (Soin et al. 2009; Ogura et al. 2012), halofenozide formulations nevertheless have been marketed to control coleopteran larvae in turf (Dhadialla et al. 1998, 2005; Nakagawa 2005; Smagghe et al. 2013). Larvicidal assays that employed coleopteran larvae have indeed revealed important differences regarding the physicochemical properties of DAH compounds to have toxic effects in coleopterans (Ogura et al. 2012).

As already mentioned above, DAH compounds have been identified that can bind to EcR/USP of *L. decemlineata* (Coleoptera) without activation of reporter activity, and this has been considered indicative of antagonist activity. In EcR-reporter assays employing lepidopteran cell lines, a systematic search for DAH antagonists (DAH compounds that prevent activation of EcR/USP by limiting concentrations of PonA or tebufenozide) was unsuccessful (Soin et al. 2010a). Although in some cases inhibition of reporter activity was observed, further examination revealed a nonspecific effect caused by general cellular toxicity of the compounds. While the evidence is limited, it suggests that DAHs can act as antagonists against EcR/USP in Coleoptera but not in Lepidoptera (while there is evidence that ecdysteroids could act as antagonist against EcR/USP in Lepidoptera; see further below).

While tebufenozide and methoxyfenozide are very hydrophobic compounds and have high activity against lepidopteran larvae, larvicidal assays have revealed that hydrophobicity is negatively correlated with toxicity in coleopteran larvae (Nakagawa et al. 1999). Results indicate that the efficiency to activate EcR/USP is a factor of moderate impact in larvicidal assays and that other factors that are related to the uptake and sequestration of DAH compounds play a greater role. To explain differences in insecticidal activity among DAHs, in vivo parameters that play a role at the organismal level are important, such as cuticular permeability for topically applied compounds, uptake and excretion by the gut for oral uptake, and metabolic detoxification activities. To predict the efficiency of ecdysone agonists such as DAHs to act as insecticides, cell-based assays can give only a minor indication and rather extensive larvicidal assays are necessary to uncover the physicochemical properties that are truly relevant. As larvicidal assays have shown, these properties can differ significantly among different insect groups and species.

10.4 Other Applications of In Vitro Screening Systems for Identification of Ecdysone Analogs

While the identification of ecdysone agonists can lead to direct applications such as their use as "molting-accelerating compounds" with insecticidal activity on insect larvae, the EcR-reporter assay can have also other practical applications, of which two are more elaborated: (1) the search for ecdysone antagonists and (2) the identification of endocrine disruptors in the environment.

10.4.1 The Search for Ecdysone Antagonists

In all screens that were performed with EcR-reporter assays, ecdysone mimics that were identified with certainty were ecdysone agonists. In a systematic screen with DAH, AAK, and THQ compounds using lepidopteran cell lines, no clear ecdysone antagonists were identified because inhibition of the EcR/USP complex was always associated with general toxicity (Soin et al. 2010a). Comparison of activity between biochemical ligand-binding assays and EcR-reporter assays indicated indirectly the possible existence of antagonists for coleopteran EcR/USP (Ogura et al. 2012), but clearly much more work is needed to obtain indisputable proof. However, the identification of ecdysone antagonists would be very valuable for both basic and applied research. In basic research, it would allow (reversible) blocking of EcR/USP signaling to analyze developmental processes. Up to now, this can be realized in *Drosophila* through inducible expression of dominant-negative EcR (Cherbas et al. 2003), but this technique is not feasible for other insects that are not easily amenable to genetic transformation. In applied research, ecdysone antagonists could be developed as insecticides by blocking physiological processes such as oogenesis and embryogenesis and by disruption of molting and metamorphosis.

Another recent study has also found more persuasive indication of the existence of ecdysone antagonists (Zotti et al. 2013). When the activity of three natural ecdysteroids was tested in EcR-reporter assays using dipteran S2 and lepidopteran Bm5 cell lines, only cyasterone showed ecdysone agonist activity at μM concentrations. Castasterone, on the other hand, showed no ecdysone agonist activity but could block activation by tebufenozide at <μM (S2 cells) and μM (Bm5 cells) concentrations (Zotti et al. 2013). Further evidence for interaction with EcR was obtained with in silico docking studies in the ligand-binding pocket and by normal mode analysis. When the protein flexibility of EcR was modeled, the pattern

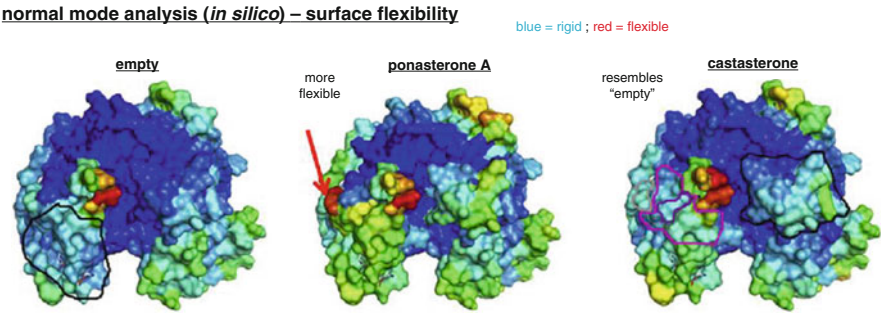

Fig. 10.6 Normal mode analysis of ligand-binding domains of EcR/USP heterodimer in the absence of ligand, or bound by the strong agonist PonA or the antagonist castasterone. Protein surface flexibility of castasterone-bound EcR/USP resembles EcR/USP without ligand in EcR (Reprinted by permission from Elsevier (Zotti et al. 2013))

observed for castasterone-bound EcR resembled that of apo-EcR, in contrast to PonA-bound EcR (Fig. 10.6). Thus, castasterone can interact with EcR in a manner that is different from the strong agonist PonA, which could reflect its action as an ecdysone antagonist (Zotti et al. 2013).

10.4.2 Identification of Endocrine Disruptor Activity

An important environmental concern is the accumulation of agricultural and industrial compounds in the aquatic milieu that affect the physiological and reproductive functions of animals. While most studies have focused on the effect of such "environmental disruptors" on vertebrates, more recently, it has been increasingly realized that invertebrates, most notably beneficial insects that act as pollinators (e.g., bees), can be severely affected as well, resulting in severe economic damage (Goulson et al. 2015). As an example, it has been argued that insecticides that act as "molting-accelerating compounds," such as the commercial DAHs tebufenozide, methoxyfenozide, halofenozide, and chromafenozide, can persist in the environment and potentially interfere with the life cycle of nontarget insects (De Wilde et al. 2013). Similarly, it has been realized that industrial compounds can interact with EcR/USP of crustaceans and insects to activate or inhibit gene activation. Thus, EcR-reporter assays provide important tools to investigate the endocrine disruptor activity of candidate compounds. Examples of industrial compounds that were identified in the EcR-reporter system include tributyltin, a biocide with potent antifouling activity, and bisphenol A, used in the manufacture of polycarbonate plastics, epoxy resins, and other products (Verhaegen et al. 2011; Kontogiannatos et al. 2015).

10.5 Conclusions

The EcR-reporter system is a useful tool for the identification of ecdysone mimics and can be used for the screening collections of chemical compounds and natural extracts. By using cell lines derived from different insect species, species- and insect order-specific effects can be rapidly evaluated. While it establishes quickly the potency and efficacy of candidate compounds to activate EcR/USP, it does not guarantee, however, that identified compounds will act as efficient insecticides in vivo. Considerable effort is needed to identify the parameters that determine the efficient delivery of the compounds to larvae through the integument or through feeding. These principles can only be established through rather large-scale larvicidal assays which is an expensive and time-consuming process. Validation of ecdysone agonists for insecticidal activity in larvae also establishes considerable species differences, even within a group of insects with minimal differences in activity in EcR-reporter assays (for instance, larvicidal activity in different species of Lepidoptera).

The use of the EcR-reporter system can have other applications, for instance the identification of ecdysone antagonists for basic research and the testing of industrial and agricultural compounds for interference with EcR/USP ("endocrine disruptor" activity).

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Chapter 11
Advances in Whiteflies and Thrips Management

Adi Kliot, Svetlana Kontsedalov, Galina Lebedev, and Murad Ghanim

Abstract The sweetpotato whitefly *Bemisia tabaci* and thrips species such as the tobacco thrips, *Thrips tabaci* and the western flower thrips *Frankliniella occidentalis* are major agricultural pests in various vegetable, ornamental and field crops. These pests cause extensive damage by a direct feeding on plants, reducing quality of the produce, secreting honeydew and transmitting economically important viruses. Management programs of both whitefly and thrips species rely on using chemical insecticides, resulting thereby in developing resistance to all major insecticide classes makes them a continuous problem in many cropping systems. Resistance monitoring is a major approach for the management and reducing resistance, and thus during the last decade *B. tabaci*, *T. tabaci* and *F. occidentalis* populations in Israel have been monitored for resistance to major insecticide classes. Monitoring results show that many *T. tabaci* and *F. occidentalis* populations were found to be resistant to major insecticides used for controlling thrips species, primarily spinosad, while whitefly populations were found to exhibit varying levels of resistance to neonicotinoids and other classes, depending on the whitefly biotype and other agricultural practices. The long-term monitoring results and their integration into resistance management programs will be presented in this review.

11.1 Introduction

The sweet potato whitefly *Bemisia tabaci*, the western flower thrips *Frankliniella occidentalis* and the onion (tobacco) thrips *Thrips tabaci* are agricultural pests causing heavy crop damages worldwide (Fig. 11.1) (Kirk and Terry 2003; Gill et al. 2015; Liu et al. 2012). Although these insect pests belong to different insect orders, all are highly destructive, small in size (1–2 mm long), highly polyphagous and are able to transmit plant diseases (Navas-Castillo et al. 2011; Riley et al. 2011; Ullman et al. 1995). In Israel, crop losses caused by thrips and whitefly species in vegetable

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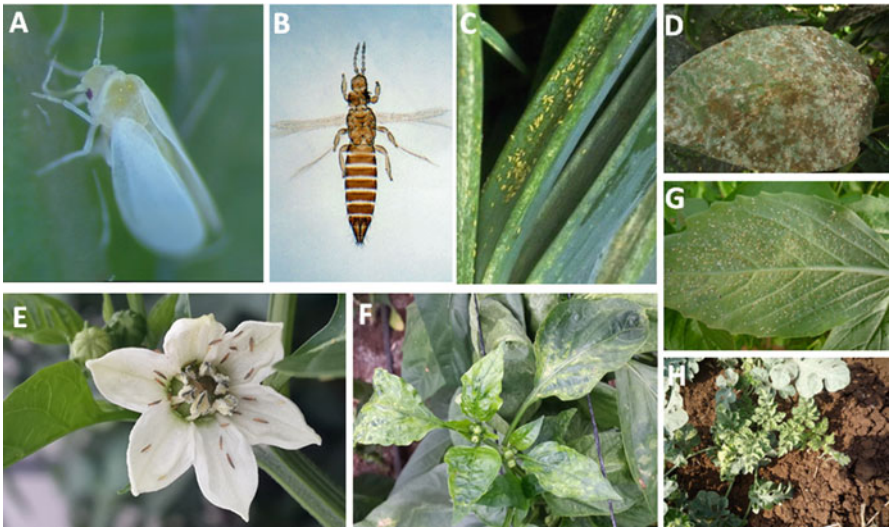

Fig. 11.1 Whitefly (**a**) and thrips (**b**) adults. (**c**) shows thrips nymphs on chives leafs the cosmetic damage. (**e**) shows thrips adults in a pepper plant flower and (**f**) shows the damage caused by *Tomato spotted wilt virus* (TSWV) in a pepper plant. (**d**) shows sooty mold fungus growth on a pepper leaf after infestation with the whitefly *Bemisia tabaci.* (**g**) shows a basil leaf infested with *B. tabaci* nymphs and (**h**) shows a watermelon plant infected with Squash leaf curl virus (SLCV) transmitted by *B. tabaci*

and field crops and ornamentals are very high, and sometimes they endanger the export of products to international markets because of the quarantine regulations imposed in many countries on these pests.

Bemisia tabaci is a small insect; adults measured 0.5–2 mm (Bedford et al. 1994). Thrips species are of similar size and both are cryptic, meaning all life stages tend to burrow into the leafy tissue they feed from and are thus hard to spot on the plant (Gill et al. 2015). These attributes made these insects highly invasive worldwide, both expanding to new places and countries, while human activities and international trade are the most common methods for expanding the distribution of these pests (Kirk and Terry 2003; Morse and Hoddle 2006; Liu et al. 2007). Both thrips and whiteflies develop through a very short generation time of 14 and 21 days accordingly. The adults live 30–40 days after they emerge from the pupa (Gill et al. 2015; Fekrat et al. 2009; Drost et al. 1998; Costa et al. 1991). Whiteflies and thrips are haplodiploid insects: the males are haploid and develop from unfertilized eggs while the females are diploid and develop from fertilized eggs. This mode of reproduction is also termed arrhenotoky, in which the females are able to control the males/females ratio (Gill et al. 2015; Denholm et al. 1998). Haplodiploidy is an efficient evolutionary characteristic and it was suggested that it contributes to the rapid development of resistance; however others have suggested that haploid male do not necessarily over express resistance genes because of the lack of one copy of the genome, on the contrary, this lack has a fitness cost and thus the haploid

males develop less resistance compared to diploid insects. The rapid selection for resistance in haploid males is suggested to occur by the positive selection of resistant mutations in the males; in the case of insecticide application only individuals with a resistance allele survive the selection pressure, regardless whether a dominant or recessive mechanism is existent. Females may be heterogeneous for resistant alleles, and thus may not express the resistance (and so remain susceptible to the applied insecticide) and pass it to half of their progeny. The surviving males, being haploids and hemizygous, will therefore pass the resistance allele to their progeny. This suggests that insecticide resistance can appear and rapidly spread within a few generations, and become fixed and irreversible within less than ten generations, assuming an equal female: male ratio and a dominant trait (Denholm et al. 1998). Unlike most whiteflies species, *B. tabaci* is highly polyphagous, and this is also the case for *F. occidentalis* and to a lesser extent for *T. tabaci*. This suggests that those insect species are able to evade insecticide applications by finding refuge on different host plants in a nearby sprayed field with insecticides. Although this strategy delays insecticide resistance development, it has the potential in causing damage to neighboring fields and diminishing the effectiveness of the insecticide applications (Morse and Hoddle 2006; Denholm et al. 1998).

Whitefly and thrips species cause cosmetic damage in ornamental and edible crops and can also transmit diseases (Fig. 11.1), thus, a very strict, low, pest tolerance threshold is required in the field. Thus far, limited numbers of commercially efficient biological-control agents are available for controlling whitefly and thrips species, because of their unique biology and ability to develop resistance, and because biological agents require conditions that do not always exist in the greenhouse. As a result, integrated management strategies are required, and those should include the mixing of different control strategies including chemical, biological, physical and cultural practices (Denholm et al. 1998). In Israel, such practices have been developed for whitefly and thrips species and are efficient in reducing the damage caused and are sufficient in many cases in delaying the development of resistance. Those practices include implementing results of long-term projects aimed at performing resistance monitoring and testing the efficacy of key insecticides in management. Those practices will be discussed herein for whitefly and thrips species.

11.2 *Bemisia tabaci* Biotypes and Damage

The whitefly, *Bemisia tabaci*, differs from other whiteflies species; it is highly polyphagous, spread worldwide and is now defined as a species complex comprised of over 37 different species, most of them only distinguishable using molecular markers while morphological markers are not available. The most invasive species worldwide, which are also considered the most devastating to agriculture are the MED (Mediterranean, previously termed Q) and MEAM1 (Middle East Asia Minor 1, previously termed B) species (Liu et al. 2012). In Israel, the B and Q biotypes

have been documented (Horowitz et al. 2013; Byrne et al. 1995). *B. tabaci* causes direct damage to the plant hosts by feeding on the plant phloem; secreting a sucrose-rich honeydew that attracts sooty molds, thus uglifying the plant leaves and damaging them (Byrne and Miller 1990) (Fig. 11.1). Some plants, primarily of the cucurbits, also show some physiological responses to certain *B. tabaci* species feeding by changing the shade of their leaves from green to light silver hence giving the MEAM1 species the name 'silverleaf whitefly' (Brown et al. 1995). The damage of *B. tabaci* is greater because it serves as efficient plant virus vector primarily it transmits begomoviruses of the Geminiviridae (vectored exclusively by *B. tabaci*). *B. tabaci* are vectors of criniviruses, ipomoviruses, torradoviruses and carlaviruses (Navas-Castillo et al. 2011). Almost 200 different Begomoviruses have been found thus far to be vectored by *B. tabaci*, some of which, like *Tomato yellow leaf curl virus* (TYLCV) and other begomoviruses in cucurbits such as *Squash leaf curl virus* (SLCV) and *Watermelon chlorotic stunt virus* (WmCSV), can cause complete crop loss before they are detected and managed (Navas-Castillo et al. 2011; Moriones and Navas-Castillo 2000). Other begomoviruses such as those causing the cassava mosaic disease (CMD) endanger the staple food source in Africa and other third world countries (Brown 2007). Begomoviruses such as TYLCV, SLCV and WmCSV are transmitted by *B. tabaci* in a persistent-circulative manner. Upon acquisition of the virus from an infected plant, the whitefly will be able to transmit it after a short latent period of 8–24 h for the rest of its life.

11.3 *B. tabaci* Management and Resistance Monitoring

11.3.1 Chemical Control of B. tabaci

Adult whiteflies are winged and are relatively good flying insects utilizing the wind for long distances movement and migration. The young developmental stages; from egg to pupa, are sessile and they do not move. Susceptibility to the same insecticide may vary between adults and other developmental stages. Management of *B. tabaci* relies heavily on using chemical insecticides, and it is known as one of the most devastating agricultural pests because its ability to develop resistance to almost all major insecticide groups (Horowitz et al. 2005). The Q biotype of *B. tabaci* was first recorded in Israel about 15 years ago (Horowitz et al. 2003), and since then has caused many losses, mainly due to the inability to chemically control this biotype with conventional and new insecticides (Kontsedalov et al. 2012). Several major classes of insecticides are still in frequent use against *B. tabaci* in Israel; and those include the neonicotinoids, juvenile hormone mimics and several other insect growth regulators such as the lipid synthesis inhibitors spiromesifen and spirotetramat (Elbert et al. 2008), diafenthiuron which interrupts the proper electron transfer during the cellular respiration and others. Classic insecticides that belong to the organophosphates and pyrethroids are still in use in some cropping systems, although high resistance levels have been documented (Gauthier et al. 2014).

Organophosphates, pyrethroids and neonicotinoids are all neurotoxins, and due to extensive frequent use, many cases of high resistance levels have been documented for all three classes among *B. tabaci* populations worldwide. The mutations causing resistance to organophosphates and pyrethroids are very well documented and have been pinned to point mutations in the acetylcholine esterase protein, the target sites of these insecticides (Gauthier et al. 2014). Resistance to neonicotinoids has been shown to be metabolically based, primarily on the expression levels of detoxification enzymes, such as the Cytochrome P450 family member Cyp6cm1 which was shown to be responsible for resistance against imidacloprid (Roditakis et al. 2011; Karunker et al. 2008). In spite of these resistance reports, these insecticides are still in use in Israel, however their effectiveness is always questioned, while Integrated Resistance Management (IRM) programs are sometimes implemented to keep some levels of effectiveness in using them.

11.3.2 Resistance Monitoring

During the last few years, we have monitored the resistance levels of *B. tabaci* populations that have been collected from different geographical locations and from different crops that were grown under different *B. tabaci* management regimes. The resistance monitoring we perform is based on rapid resistance tests in which populations that are collected from the field, are brought to the lab and exposed in the same day to the recommended insecticide concentrations by the plant protections and inspection services, in a leaf-detached bioassay that lasts 48 h, after which the mortality of the insects is calculated (Horowitz et al. 2005; Kontsedalov et al. 2012). Other monitoring methods include the collections of field populations, rearing them in the lab and building their mortality curves through a series of insecticide concentrations (Horowitz et al. 2005). Such methods were used between the years 1996 and 2003 to monitor resistance to pyriproxyfen and other neonicotinoids, and the results showed that susceptibility to pyriproxyfen remained relatively stable in Israel, though differences between early season and late season collections on cotton fields were evident, suggesting that the selection pressure leads to the development of resistant populations which are less susceptible toward the end of the season (Horowitz et al. 2005). Pyriproxyfen was released in the early 1990s and was reported to be effective against populations of the B biotype. However, since the first report of the Q biotype from Israel (Horowitz et al. 2003), high levels of resistance against this insecticide were reported, and its use gradually ceased over the years. Nowadays, pyriproxyfen is almost not in use for controlling *B. tabaci* populations in Israel. Another resistance monitoring project was conducted between the years 2008 and 2010 and tested three different neonicotinoids: imidacloprid, acetamiprid and thiamethoxam. Monitoring levels of resistance have been developed among many field-collected populations (Kontsedalov et al. 2012). The resistance problems with the three tested neonicotinoids were mostly associated with the Q biotype, which

was also shown to be mainly restricted to protected crops such as greenhouses and net houses, while the B biotype was mainly found in open field crops. The above two reports demonstrate the importance of such continuous field monitoring programs which provide growers with practical tools for the selection of efficient insecticides that should be used in management programs. In our monitoring program that we have performed during the last few years, we have tested several classes of insecticides for effectiveness against *B. tabaci* populations, which were also tested for biotype B and Q identification, and were collected in different locations in Israel (Fig. 11.2). Table 11.1 and Fig. 11.3 show monitoring results that were obtained in 2013 among *B. tabaci* populations collected across Israel. In general, both the B and Q biotypes were detected as was previously reported; however, sympatric populations were less abundant than was previously reported. Many of the collected populations in 2013 and in subsequent years show that most of the populations are composed of only one biotype, the B or the Q, and only some populations were sympatric. The reason for this change in populations is attributed mainly to the agricultural practices and the use of management programs that in many cases rely on chemical control, thus selecting for Q biotype populations. Less abundant usage of chemical control strategies will normally lead to the development of B biotype populations. Among the populations that were collected in 2013, some were subjected to laboratory resistance tests to determine the level of their susceptibility to some major insecticides used to control *B. tabaci* in Israel. As seen in the mortality results presented in Fig. 11.4, except diafenthiuron, dinotefuran and abamectin, all the other tested insecticides that included acetamiprid, bifenthrin, imidacloprid, thiocyclam hydrogen ***oxalate, pyrethrum and spirotetramat showed high to moderate levels of resistance*** (Fig. 11.4), while in some cases and among few populations the management was successful such as in the case of population 2 with acetamiprid. On the other hand, some insecticides, such as thiocyclam hydrogen ***oxalate***, failed to control any of the tested populations, suggesting that this insecticide is possibly not effective for use in Israel, and management programs should take that into account. These results clearly demonstrate that chemical control of *B. tabaci* is likely to lead to the development of resistant populations, and in the lack of resistance management programs, chemicals that face the development of resistance by target insects rapidly disappear from the market, while the development of new ones is usually much slower, leading to acute problems for farmers in managing insect pest problems.

11.4 Thrips Species, Management and Resistance Monitoring and Control

Some thrips species can be highly polyphagous and highly adapted to an invasive lifestyle – they are highly cryptic in their early, sessile stages making it easy for them to be transferred together with the host plant tissue. As adults they migrate from one plant to another easily with the wind and are also very attracted to yellow,

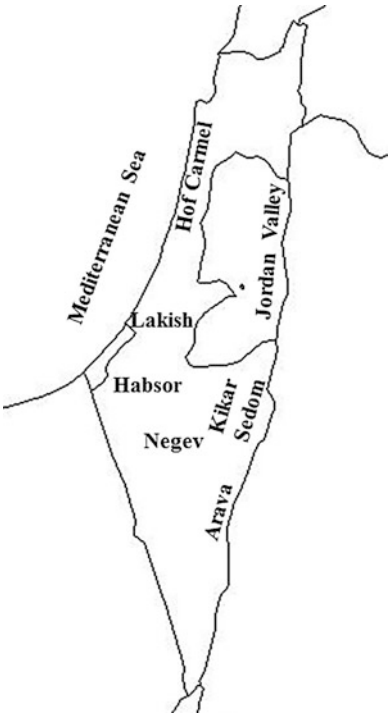

Fig. 11.2 Map of Israel showing the geographical locations where whitefly and thrips populations were collected and tested as detailed in Tables 11.1 and 11.2

white and blue colors, making it easy for them to be carried on human skin and clothes as a vector, thus making the colonization of new fields with thrips an easy task (Morse and Hoddle 2006). The western flower thrips exemplifies this invasive behavior; it has spread worldwide in merely 50 years, since being first described in the 1960s in Western United States (Kirk and Terry 2003). This thrips species was identified in Israel for the first time in 1987 and immediately became a major pest, found in protected open field crops (Chyzik and Ucko 2002). The western flower thrips and the onion thrips are highly polyphagous insects that can feed on a variety of plant families, with the western flower thrips having an amazingly vast host range spanning from annuals to fruit trees and it can feed on leaf, fruit and flower tissues and lacks an obligatory diapause (Kirk and Terry 2003; Gill et al. 2015; Morse and Hoddle 2006; Reitz 2009). The western flower thrips can develop in a wide range of temperatures; from 10° to 40 °C, thus enabling it to reproduce and develop outdoors year round in Israel, reaching high abundances in spring crops (March–April) during which time devastating loses are observed (Chyzik and Ucko 2002; Chyzik et al. 1995). Thrips species feed by injuring the plant tissue, exposing the mesophyll, excreting enzymes to digest the broken tissue and finally sucking the digested compounds. This feeding behavior creates silver leafing, significant reduction in photosynthesis and it primes conditions for pathogen development on the injured leafs (Gill et al. 2015). Thrips species prefer feeding on young tissue and thus can cause severe damages for the newly developing fruits and sometimes

Table 11.1 Field-collected *B. tabaci* populations for biotype and resistance monitoring during 2013

Population #	Collection location	Protected crop/open field	Crop	Collection date	Biotypes (%)		*n*
					B	Q	
1	Kikar Sedom	Open field	Watermelon	13.03.13	100	0	20
2[a]	Kikar Sedom	Greenhouse	Eggplant	13.03.13	100	0	20
3	Jordan Valley	Greenhouse	Basil	14.04.13	100	0	16
4	Jordan Valley	Nethouse	Basil	14.04.13	100	0	9
5	Jordan Valley	Nethouse	Basil	25.04.13	89	11	9
6	Jordan Valley	Nethouse	Basil	9.05.13	75	25	20
7	Jordan Valley	Nethouse	Basil	30.05.13	37.5	62.5	16
8	Jordan Valley	Nethouse	Basil	13.06.13	5	95	20
9[a]	Jordan Valley	Nethouse	Basil	23.06.13	0	100	20
10	Jordan Valley	Nethouse	Basil	18.07.13	0	100	15
10A	Jordan Valley	Nethouse	Basil	15.9.13	0	100	20
11[a]	Arava	Greenhouse	Squash	3.10.13	100	0	19
12[a]	Arava	Greenhouse	pumpkin	3.10.13	100	0	17
13	Arava	Open field	Melon	3.10.13	100	0	20
14	Arava	Greenhouse	Pumpkin	3.10.13	100	0	20
15	Arava	Greenhouse	pepper	3.10.13	100	0	17
16[a]	Bsor	Greenhouse	Rose	24.10.13	0	100	20
17[a]	Jordan Valley	Open field	Sage	7.11.13	0	100	20
18[a]	Jordan Valley	Greenhouse	Pepper	7.11.13	25	75	20
19[a]	Jordan Valley	Nethouse	Basil	7.11.13	0	100	19
20[a]	Jordan Valley	Nethouse	Basil	7.11.13	10	90	20
21[a]	Jordan Valley	Nethouse	Basil	7.11.13	0	100	20
22	Negev	Open field	Cotton	2.09.13	100	0	20
23	Negev	Open field	Cotton	3.09.13	100	0	20
24	Negev	Open field	Cotton	8.09.13	100	0	20
25[a]	Bsor	Greenhouse	Rose	12.12.13	0	100	19

[a]This population was tested for resistance
[n]Number of adults tested for biotype identification

abortion of fruits and flowers (Reitz 2009). In chives, the onion thrips feeding causes over 50 % of leaf loses, while in garlic and onions, its feeding on the leaves causes water loses, chlorophyll loses and general stress to the plants that leads to reduced bulbs size. The onion thrips also feeds on leaf and bulb tissues in post-harvest, leading to cosmetic damage of the crop (Gill et al. 2015). The western flower thrips causes large cosmetic damages in cut flowers and sunflowers, caused by feeding on young flower buds tissue. In sunflowers, this feeding also results in the development of defected kernels (Chyzik et al. 1995). Cosmetic damage to crops is caused not only from feeding but from oviposition leading to holes in the plant tissues, which can serve as an entrance point for pathogens. This can bring on a wound response in the plant resulting in spotting (Reitz 2009). However, by far, the most

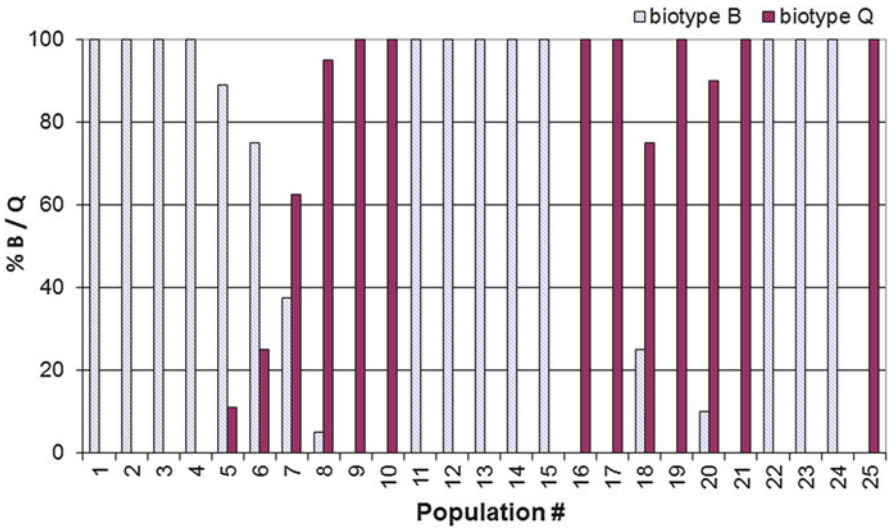

Fig. 11.3 The incidence of the B and Q biotypes of *Bemisia tabaci* populations in populations collected during 2013 in different locations in Israel. The locations and the crops from which these populations were collected are given in Table 11.1

devastating damage causes by thrips species are the viruses they transmit. Thrips are vectors of Tospoviruses such as the *Tomato spotted wilt virus* (TSWV) transmitted mainly by the western flower thrips and *Iris yellow spot virus* (IYSV) transmitted mainly by the onion thrips. Thrips species, especially the onion thrips, have been found to be able to transmit other viruses as well, but tospoviruses are the only significant group in terms of economic losses (Mumford et al. 1996). Tospoviruses are a small class of Bunyaviridae; only 20 viruses have been identified thus far and these are vectored by merely 14 thrips species (out of almost 2000 known thrips species) (Riley et al. 2011; Gera et al. 1998). In the USA alone, loses caused by tospoviruses are estimated at over 1.4$ billion in the years 2001–2011 (Riley et al. 2011). Tospoviruses are transmitted in a persistent-circulative and propagative manner; the virus propagates within the vector and can then be transmitted to healthy plants by the insect for the rest of its life. However, only acquisition of the virus by early larval stages of the thrips vector will result in transmission because the virus needs to cross important tissues along the thrips development and to replicate for reaching sufficient amounts required for transmission (Mumford et al. 1996). Late larval stages and adults may acquire the virus but will not transmit it (Ullman et al. 1995). Thus, management programs of thrips species that transmit viruses should take into account those facts of eradicating both early larval stages that acquire the virus, and adult stages that transmit it. TSWV was first identified in Israel in 1992 (Gera et al. 1998), and it can be vectored by both the onion thrips and the western flower thrips (Mumford et al. 1996). IYSV was described from onions in Israel in 2000 and is thought to have arrived as early as the 1997 and is transmitted by the

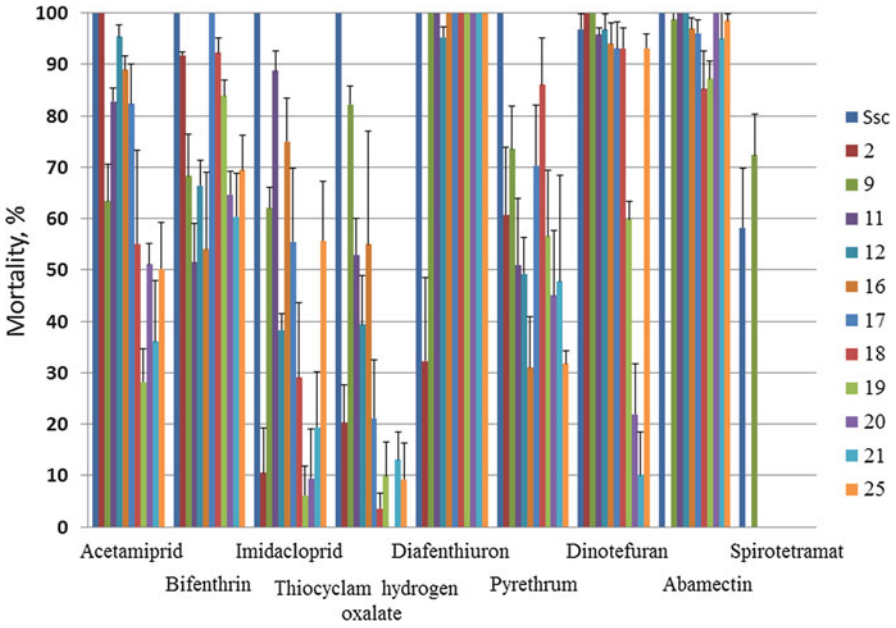

Fig. 11.4 Mortality levels of *Bemisia tabaci* populations collected in different locations in Israel, after exposure to different insecticides. Details regarding these populations are presented in Table 11.1

onion thrips (Gera et al. 1998, 2000). While adult thrips are mobile and can easily migrate between plant hosts and different plots and fields with the wind, younger stages are sessile and thus can be considered better targets for eradication. However the cryptic feeding behavior of these insects, burrowing into plant tissues and hiding within them makes it difficult to manage thrips with the classical ways of insecticide sprays.

11.4.1 Chemical Control and Resistance Monitoring of Thrips Species

Classical insecticide classes such as organophosphates, carbamates and pyrethroids are also in use for controlling many thrips species. However, recently, spinosyns and avermectins which were both isolated from natural soil-borne fungi are leading the insecticide classes used against several thrips species in Israel (Thompson et al. 2000; Ishaaya et al. 2002; Lebedev et al. 2013). Reports of resistance to almost all the above-mentioned insecticide classes were published over the years for either the onion thrips and the western flower thrips, while regarding spinosyns and avermectins, less resistance reports are available, mostly because these insecticide were

released only in recent years (Lebedev et al. 2013). Kontsedalov et al. developed a method in 1998 to rapidly examine resistance levels in thrips populations, using leaf dip applications of insecticides and the use of Münger cells (Kontsedalov et al. 1998). This method enables measuring thrips insecticide resistance levels within 48–72 h. The method was later employed in a field resistance monitoring research conducted in Israel between the years 2007 and 2011 for onion thrips populations. This research demonstrated for the first time high resistance levels to spinosad among many onion thrips populations in Israel and have demonstrated that populations collected in organic farms were always susceptible to it. The research further showed among many populations high resistance levels to emamectin benzoate and carbosulfan, the latter was recently banned for use in Israel. Interestingly, many of the tested populations also showed high resistance to the three tested compounds; spinosad, emamectin benzoate and carbosulfan, suggesting that those populations have developed cross resistance to the various tested compounds, or they bear different resistance mechanism that act in parallel. The resistance findings are imperative for designing pest management programs against thrips that take into account the status of field populations when selecting the insecticides to use. Finally, this research showed varying levels of resistance within the same populations over different seasons; populations collected during autumn and winter were more resistant than populations collected during the summer and spring seasons, when populations are at their highest peaks. This is partially attributed to the reduced efficiency of some insecticides when applied in field temperatures lower than 15 °C because the thrips populations reduce their metabolism and are thus able to better metabolize the insecticides (Lebedev et al. 2013). In the high temperatures seasons, the insecticides reach their target sites faster. The importance of this research and this method for field resistance monitoring is attributed to the speed and robustness of the results it produces, while the results can be communicated to the growers and help in better prevention and management of thrips outbursts. Using this method, a recent resistance monitoring survey was conducted by testing many onion thrips and western flower thrips populations collected in Israel (Fig. 11.2), and a list of insecticides used nowadays for controlling thrips species in Israel was tested. Some of the results are given in Table 11.2. The tested populations showed varying levels of susceptibility to the tested insecticides. Most notably it can be seen that populations tested with insecticides that are found in current use such as spinosad and abamectin show varying levels of susceptibility, suggesting the resistance still dynamic and depends on the location and management program, while insecticides that have been recently introduced such as spinetoram or that belong to classical classes that have not been in use for long time such as bifenthrin show high levels of efficacy against the tested populations. These results suggest that stopping the use of an insecticide for a certain length of time will in some cases reverse the resistance status of the populations, while newly introduced insecticides should be wisely included in management programs and alternated with other insecticides to prevent or delay the development of resistance.

In summary, *B. tabaci*, the onion thrips and the western flower thrips are serious, highly polyphagous pests posing serious challenges for growers in Israel. Those

Table 11.2 Mortality of filed-collected onion thrips and western flower thrips (WFT) populations for resistance monitoring during 2013–2015

Collection date	Thrips species	Collection location	Spinosad[1]	Formetanate[2] HCL	Abamectin[3]	Methiocarb[4]	Emamectin[5] benzoate	Spinetoram[5]	Acrinathrin[6]	Bifenthrin[7]	Spirotetramat[8]
1.13	Onion thrips	Arava	100						73 ± 14		
1.13	Onion thrips	Arava	100		74 ± 7		100	100	53 ± 17		
8.14	Onion thrips	Lakhish	42 ± 12				70 ± 11				
5.15	Onion thrips	Jorden Valley	70 ± 13	100							
3.15	Onion thrips	Jorden Valley	0	100	3 ± 3	81 ± 1					
3.15	Onion thrips	Jorden Valley	0	100	2 ± 2	7 ± 7					
2.15	Onion thrips	Jorden Valley	100	100	20 ± 6	90 ± 6	43 ± 18	100			
7.13	WFT	Hof Carmel	74 ± 9					90 ± 4			
7.14	WFT	Hof Carmel	85 ± 5					76 ± 3			
12.15	WFT	Hof Carmel		50 ± 11	97 ± 3 (P)		48 ± 9 (R)	93 ± 3 (M)		97 ± 3 (P)	17 ± 9 (M)
12.15	WFT	Hof Carmel		18 ± 9	100 (P)		78 ± 8 (R)	97 ± 3 (M)		100 (P)	72 ± 2 (M)
12.15	WFT	Hof Carmel		37 ± 3	86 ± 7 (P)		77 ± 12 (R)	97 ± 3 (M)		97 ± 3 (P)	60 ± 7 (M)
12.15	WFT	Arava		90 ± 10				68 ± 11			
12.15	WFT	Arava		100				97 ± 3		74 ± 3	

1 80 ml/dunam (1 ha = 10 dunams); *2* 150 g/donam; *3* 75 ml/donam; *4* 100 ml/donam; *5* 80 ml/donam; *6* 80 ml/donam; *7* 75 ml/donam; *8* 75 ml/donam, (P) These insecticides were mixed with Chlorfenapyr at 40 ml/donam. (R) This insecticide was mixed with Acrinathrin at 80 ml/donam. (M) These insecticides were mixed with Methriocarb at 100 ml/dunam

species are highly successful in developing resistance to all major insecticides, and the number of effective insecticides available against these pests is decreasing each year because of these resistance problems, and because of public health and environmental issues. The introduction of new chemicals should be accelerated, including biorational insecticides, and those based on natural resources such as plant extracts, green chemistries and others. The integration of such insecticides in IRM strategies, while performing monitoring for the development of resistance using the methods described above, will contribute to the sustainability of management programs for better, long-lasting control of these pests.

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Chapter 12
Resistance to Diamide Insecticides in Lepidopteran Pests

Ralf Nauen and Denise Steinbach

Abstract Diamide insecticides were first commercialised in 2006 by the launch of the benzenedicarboxamide derivative flubendiamide, followed by the anthranilic diamides chlorantraniliprole and cyantraniliprole. They are particularly active against a number of destructive lepidopteran pests and selectively activate insect ryanodine receptors (RyR), which are large tetrameric ryanodine-sensitive calcium release channels located in the sarco- and endoplasmic reticulum in neuromuscular tissues. Within a few years on the market, this class of insecticide chemistry gained blockbuster status by accounting for more than $1.2 billion of the 2013 global insecticide sales. On the downside, selection pressure on high-risk pests increased due to the frequent use of diamides, and high levels of field resistance to these insecticides have recently been reported in lepidopteran pests, such as diamondback moth, *Plutella xylostella*, and tomato leaf miner, *Tuta absoluta*. Here we briefly summarise cases of diamide insecticide resistance by analysing the underlying mechanisms of resistance compromising diamide efficacy in both laboratory- and field-selected strains of a number of lepidopteran pests. By far one of the most intensely investigated species, with respect to the underlying molecular mechanisms of diamide insecticide resistance, is diamondback moth. One of the major mechanisms of resistance including its underlying genetics yet identified is based on target-site mutations located in the transmembrane domain of the insect RyR. Possible fitness costs and metabolic mechanisms of resistance based on elevated levels of detoxification enzymes are not well studied yet. Finally we briefly discuss the general implications of the mechanistic findings gathered in several studies for the implementation of diamide resistance management programmes.

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12.1 General

The discovery, development and registration of novel chemical classes of insecticides with new modes of action, i.e. addressing a yet unexploited/underutilised target protein, or at least interfering with a new binding site on an established insecticide target, are major challenges in modern crop protection research. A challenge, which is – after consolidation of the agrochemical industry – pursued by a rather limited number of R&D based companies, particularly because of high budget needs for insecticide development and registration, often easily exceeding $200 million (Sparks 2013). Major drivers for the discovery and development of new chemical classes of insecticides are an increasing requirement for compounds with improved environmental and toxicological profiles, as well as the global spread of pest resistance compromising field efficacy of established insecticides and thus directly influencing yield and food supply. A recent survey revealed that in 2013 approximately 70 % of the global insecticide market was based on 5 out of about 55 different chemical classes listed in the insecticide mode of action classification scheme of the Insecticide Resistance Action Committee (IRAC), including neonicotinoids acting on nicotinic acetylcholine receptors (27 % market share), pyrethroids acting on voltage-gated sodium channels (16 %), organophosphates inhibiting acetylcholinesterase (11 %), diamides acting on ryanodine receptors (8 %) and avermectins acting on ligand-gated chloride channels (7 %) (Sparks and Nauen 2015). Out of these chemical classes, diamide insecticides represent the most recent class of chemistry introduced to the market approximately 10 years ago (Nauen 2006; Jeanguenat 2013).

12.1.1 Diamide Insecticides

Three diamide insecticides, i.e. the benzenedicarboxamide (or phthalic diamide), flubendiamide (Tohnishi et al. 2005; Hirooka et al. 2007; Hamaguchi and Hirooka 2012) and anthranilic diamides chlorantraniliprole and cyantraniliprole (Lahm et al. 2005, 2007, 2009), have so far been commercialised with a global turnover of >$1.2 billion representing approx. 8 % of the insecticide market in 2013 (Sparks and Nauen 2015). However, at least three more diamide insecticides, i.e. cyclaniliprole, tetrachlorantraniliprole and tetraniliprole, are currently under development and expected to be launched to the market within the next few years (Fig. 12.1), whilst other, more recently described chemical derivatives such as diamide sulfoximines have not yet revealed development candidates (Gnamm et al. 2012). The discovery and development of diamide insecticides has been recently reviewed by Jeanguenat (2013). Whereas flubendiamide and chlorantraniliprole are particularly active at low application rates against a broad range of lepidopteran and lepidopteran/coleopteran pests, respectively, cyantraniliprole – due to its systemic properties – also targets a number of sucking pests including aphids and whiteflies (Foster et al. 2012; Li et al. 2012; Gravalos et al. 2015). However, chlorantraniliprole also exhibits root-systemic properties and can therefore be used by systemic application but

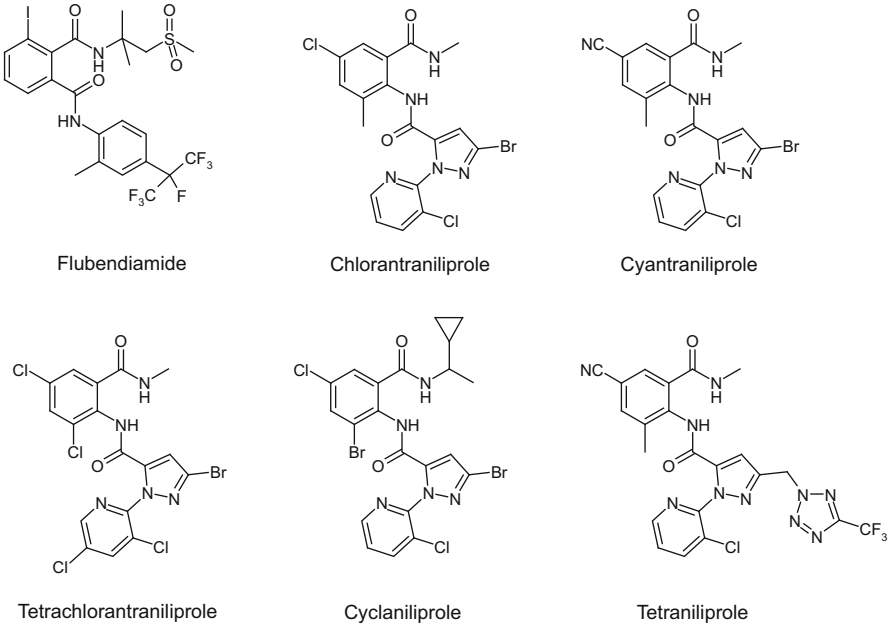

Fig. 12.1 Diamide insecticides acting as conformation-sensitive activators on insect ryanodine receptors. Flubendiamide (Nihon Nohyaku/Bayer), chlorantraniliprole and cyantraniliprole (DuPont) were launched in 2006, 2007 and 2012, respectively. Tetrachlorantraniliprole (Sinochem), cyclaniliprole (Ishihara) and tetraniliprole (Bayer) (ISO-proposed common names) are currently under development

mainly against foliar-feeding lepidopteran pests (Cameron et al. 2015). Diamide insecticides show low acute mammalian toxicity and a favourable environmental profile and are safe to beneficial insects and mites in many agricultural and horticultural settings investigated. When introduced to the market, diamides did not show any cross-resistance to existing chemical classes, as one would expect for a new chemical class of insecticides addressing a new binding site (mode of action) on a rather neglected molecular target, the insect ryanodine receptor (RyR). However, diamides are used to control a number of lepidopteran pests known to rapidly evolve resistance, including diamondback moth (*Plutella xylostella*) ranking number 2 among the globally most resistant arthropod pest species (Sparks and Nauen 2015).

12.1.2 Ryanodine Receptors and Diamide Mode of Action

Diamide insecticides were shown to act as conformation-sensitive activators of the insect ryanodine receptor (RyR), a large (homo)tetrameric calcium-channel located

in the sarco- and endoplasmic reticulum in neuromuscular tissues (Ebbinghaus-Kintscher et al. 2006; Cordova et al. 2006, 2007; Lümmen et al. 2007; Sattelle et al. 2008). RyRs are endogenously activated by calcium influx, mediated by voltage-gated calcium channels upon depolarization of the cell membrane (Lümmen 2013). By addressing a new binding site of the RyR, diamides cause a calcium-dependent calcium release resulting in the depletion of internal calcium stores which leads to uncontrolled muscle contraction, paralysis and eventually death as shown in lepidopteran larvae (Tohnishi et al. 2005; Cordova et al. 2006). Due to their new biochemical mode of action (MoA), diamide insecticides were classified by IRAC as ryanodine receptor modulators and assigned to a new main MoA group 28 (Nauen 2006). Whereas mammals possess three RyR isoforms localised in different tissues (Rossi and Sorrentino 2002), insects encode a single RyR gene with an open reading frame of >15,000 nucleotides translated into a protomer with a molecular weight of more than 5,000 kDa, as first described for *Drosophila melanogaster* (Takeshima et al. 1994). These protomers assemble to homotetrameric membrane proteins of >2 MDa forming the largest known ion channels (Hamilton 2005). RyRs were shown to be composed of six helical transmembrane spanning domains at the C-terminal end containing the calcium ion-conducting pore and a large N-terminal cytosolic domain (Lümmen 2013). A mammalian RyR1 structure determined by single-particle electron cryomicroscopy was recently published and provided interesting insights regarding its structural features as it resolves in total 70 % of 2.2 MDa molecular mass homotetrameric channel protein (Yan et al. 2015).

The RyR as an insecticide target-site has been utilised for decades and is named after the alkaloid insecticide ryanodine isolated from the South American plant species *Ryania speciosa*, known for its insecticidal properties for almost 200 years (Pepper and Carruth 1945; Rogers et al. 1948). A major problem of using ryanodine as an insecticide is its toxicity to both insects and mammals due to a lack of selective binding to RyRs (Lehmberg and Casida 1994); however, the synthesis of more selective and potent derivatives largely failed for various reasons (Waterhouse et al. 1987). The insecticidal properties of ryanodine were, however, rather limited under field conditions. Earlier work on both natural *Ryania* alkaloids and their semi-synthetic derivatives in order to increase their efficacy – including extensive structure activity relationship studies – failed to exploit this target to produce economically relevant insecticides (Jefferies et al. 1997, and references cited therein). Despite its limitations as an insecticide, ryanodine became a unique tool in the characterisation of RyRs owing to its binding specificity and high affinity for insect and mammalian receptors (K_D 5–15 nM). However, diamide insecticides address a different binding site on insect RyRs and act as positive allosteric activators as demonstrated by the increase of [^{3}H]ryanodine binding as a function of diamide concentration with an EC_{50} value in the nanomolar range to both insect thoracic microsomal membrane preparations as well as functionally expressed RyRs in insect cell lines (Ebbinghaus-Kintscher et al. 2006; Lümmen et al. 2007; Qi and Casida 2013; Steinbach et al. 2015; Troczka et al. 2015). Whereas diamides do virtually not bind to mammalian RyR isoforms (Ebbinghaus-Kintscher et al. 2006; Lahm et al. 2007), they show some species differences in

terms of selectivity among insects of different orders (Qi and Casida 2013; Qi et al. 2014). When utilising a photoreactive derivative of flubendiamide against a series of *Bombyx mori* RyR deletion mutants recombinantly expressed in HEK293 cells, Kato et al. (2009) concluded that the diamide binding site is likely to be located in the C-terminal transmembrane spanning domain, which was confirmed by studies on diamide-resistant diamondback moth strains carrying a target-site mutation in the transmembrane domain (Troczka et al. 2012; Guo et al. 2014a, b; Steinbach et al. 2015). Further evidence for a critical role of this transmembrane region for diamide binding was provided by a study replacing a 46 amino acid segment in the *Drosophila* RyR C-terminal domain by that of a nematode RyR which resulted in insensitivity to diamides (Tao et al. 2013). Since the introduction of diamide insecticides, several more insect RyR genes were cloned, sequenced and compared by phylogenetic means (Fig. 12.2), including those from lepidopteran pests such as diamondback moth (Wang and Wu 2012), which subsequently allows to investigate the implications of amino acid substitutions for diamide insecticide target-site resistance first described in diamondback moth (Troczka et al. 2012; Steinbach et al. 2015).

12.2 Diamide Insecticide Resistance in Lepidopteran Pests

Owing to their low application rates and high insecticidal efficacy, diamide insecticides were readily used right after their launch in 2006/2007 on a rather extensive scale for the control of several lepidopteran pests, especially in Southeast Asia and China. Meanwhile diamide insecticides are globally used both solo and in mixtures by millions of farmers for foliar, drench and seed treatment applications in a broad range of agricultural and horticultural cropping systems, thus facilitating the evolution of insect resistance due to increasing selection pressure, particularly on lepidopteran pests (Teixeira and Andaloro 2013). As a result of their frequent use and due to the lack of alternatives of similar efficacy, first cases of diamide field failure were reported only 2 years after launch in the Philippines and Thailand in cabbage against diamondback moth, *P. xylostella* (Troczka et al. 2012), a notorious lepidopteran pest in cruciferous vegetables. Subsequently high levels of diamondback moth resistance to diamides compromising the effectiveness of field recommended rates were confirmed in China (Wang and Wu 2012; Wang et al. 2013; Gong et al. 2014), Brazil (Ribeiro et al. 2014), Taiwan, India, USA, Japan, Korea and Vietnam (Steinbach et al. 2015). Lepidopteran pests other than diamondback moth which developed high confirmed levels of diamide resistance include tomato leaf miner, *Tuta absoluta* (Roditakis et al. 2015), and smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014). Whereas low to moderate resistance ratios in laboratory assays were reported for rice stem borer, *Chilo suppressalis* (Gao et al. 2013; He et al. 2014); beet armyworm, *Spodoptera exigua* (Lai et al. 2011; Che et al. 2013); oriental leafworm, *Spodoptera litura* (Su et al. 2012; Sang et al. 2015); rice leaffolder, *Cnaphalocrocis medinalis* (Zhang et al. 2014);

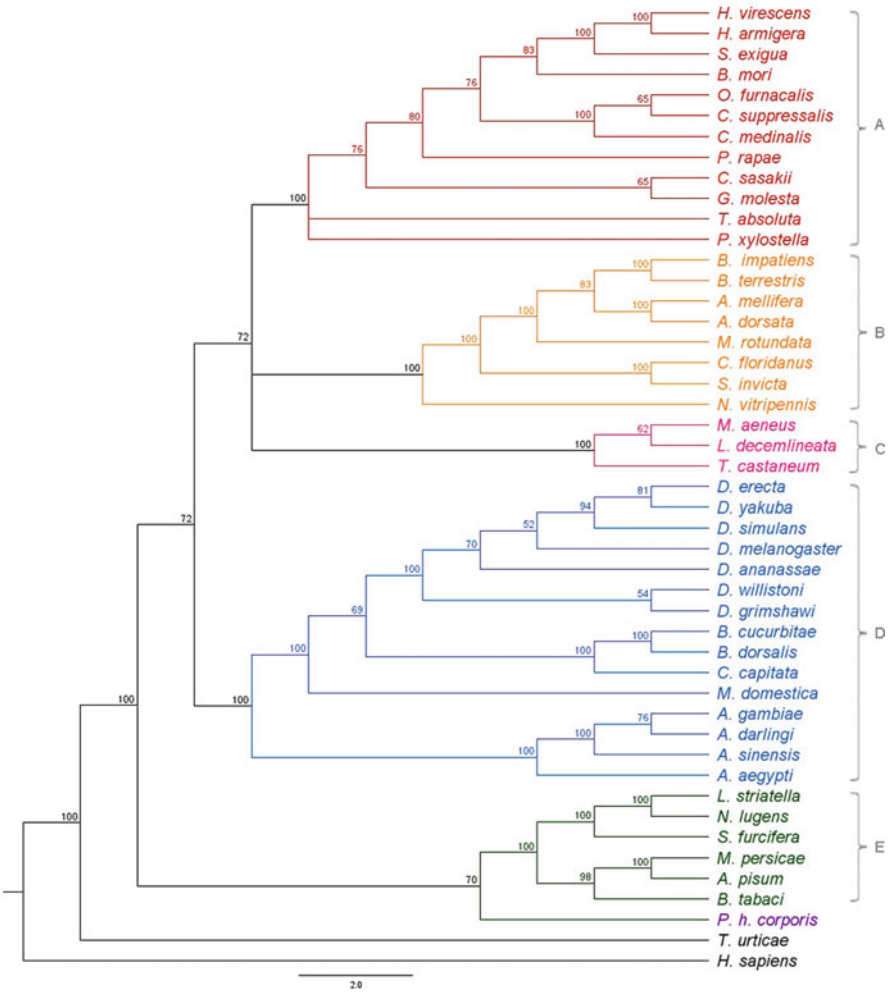

Fig. 12.2 Neighbour-joining phylogenetic analysis of the ryanodine receptor (*RyR*) of different insect orders and noninsect species. (**A**) Lepidoptera, (**B**) Hymenoptera, (**C**) Coleoptera, (**D**) Diptera, (**E**) Hemiptera. Root: *Homo sapiens*. The corresponding GenBank accession numbers are as follows: Coleoptera (*Leptinotarsa decemlineata*, AHW99830; *Meligethes aeneus*, unpublished (Nauen et al.); *Tribolium castaneum*, AIU40166.1); Diptera (*Aedes aegypti*, Q17EB5; *Anopheles darlingi*, W5JDV8; *Anopheles gambiae*, Q7PMK5; *Anopheles sinensis*, A0A084WAS3; *Bactrocera dorsalis*, A0A034W289; *Bactrocera cucurbitae*, A0A0A1WHX3; *Ceratitis capitata*, W8AL79; *Drosophila ananassae*, XP_001958793.1; *Drosophila erecta*, XP_001970412.1; *Drosophila grimshawi*, XP_001995333.1; *Drosophila melanogaster*, AFH07966.1; *Drosophila simulans*, XP_002080659.1; *Drosophila willistoni*, XP_002061506.1; *Drosophila yakuba*, XP_002089690.1; *Musca domestica*, XP_011296554.1); Hemiptera (*Bemisia tabaci*, I3VR33; *Laodelphax striatellus*, A0A059XRL5; *Myzus persicae*, A0A0A7RS32; *Nilaparvata lugens*, KF306296; *Sogatella furcifera*, KF734669); Hymenoptera (*Apis mellifera*, AFJ66977.1; *Apis dorsata*, XP_006622367.1; *Bombus impatiens*, XP_012250208.1; *Bombus terrestris*, XP_012175583.1; *Camponotus floridanus*, XP_011257849.1; *Megachile rotundata*, XP_003701507.1; *Nasonia vitripennis*, XP_008202582.1; *Solenopsis invicta*, XP_011158883.1);

soybean looper, *Chrysodeixis includens* (Owen et al. 2013); and the obliquebanded leafroller, *Choristoneura rosaceana* (Sial et al. 2011; Sial and Brunner 2012). Some lepidopteran pest species are known for their (geographic and intrinsic) variation in response to insecticides, and talking about resistance is misleading in those cases as one has to keep in mind that such variation is to some extent natural and not directly linked to resistance development based on selection pressure or cross-resistance issues. Such a variation in response was recently also confirmed in several baseline susceptibility studies with diamide insecticides, including high-risk pests, such as *Helicoverpa armigera* (Bird 2015), *C. suppressalis* (Su et al. 2014), *S. litura* (Su et al. 2012) and *T. absoluta* (Campos et al. 2015).

Diamide resistance ratios exceeding 1000-fold were yet only reported in diamondback moth and tomato leaf miner (Table 12.1), suggesting that some insect pests carry a higher potential to develop resistance to diamides than others. Whereas high levels of diamide resistance in diamondback moth is globally on the move as demonstrated by its documented presence in more than ten countries (Steinbach et al. 2015), highly resistant tomato leaf miner populations were yet only isolated from vegetable greenhouses in southern Italy (Roditakis et al. 2015). The molecular mechanisms conferring diamide resistance in *T. absoluta* are largely unknown and currently under investigation by research groups in Germany, the UK, Greece, Spain and Brazil. Diamondback moth is known as a notorious candidate for rapid resistance development to almost all chemical classes of insecticide introduced for its control, particularly in (sub)tropical areas with intensive use of crop protection products (Talekar and Shelton 1993; Teixeira and Andaloro 2013). For this reason it was not surprising that diamide (cross) resistance was first described in diamondback moth. The underlying mechanisms so far investigated are largely due to target-site mutations in the transmembrane domain of the RyR and not mediated by metabolic mechanisms such as overexpressed detoxification enzymes.

12.2.1 Target-Site Resistance

Early studies on the mechanisms of diamide resistance conducted in two diamondback moth strains collected in the Philippines and Thailand revealed an amino acid substitution G4946E in the C-terminal region of the *Plutella* RyR

Fig. 12.2 (continued) Lepidoptera (*Bombyx mori*, XP_004924916.1; *Carposina sasakii*, X2GG79; *Chilo suppressalis*, I3VR34; *Cnaphalocrocis medinalis*, I1XB02; *Grapholita molesta*, A0A089FYX0; *Helicoverpa armigera*, V5RE97; *Heliothis virescens*, DD408555.1; *Ostrinia furnacalis*, M4T4G3; *Pieris rapae*, R9R5D5; *Plutella xylostella*, AEI91094.1; *Spodoptera exigua*, A0A059XRP6; *Tuta absoluta*, unpublished data);Vertebrata (RyR 1) (*Rattus norvegicus*, F1LMY4; *Homo sapien*s, P21817; *Oryctolagus cuniculus*, P11716); others (*Pediculus humanus corporis*, E0VEK3; *Tetranychus urticae*, F5HSW9). The phylogenetic tree was generated using tree builder (Geneious 8.0) with 100 bootstrap replications. The scale bar represents 2.0 amino acid substitutions per site

Table 12.1 Selected studies of either field- or laboratory-selected (Lab) resistance to diamide insecticides in Lepidopteran pests

Species	Common name	Source	Diamide[a]	RR[b]	Mech[c]	Reference
Adoxophyes honmai	Smaller tea tortrix	Field	CPR	77	–	Uchiyama and Ozawa (2014)
			FLB	105		
Chilo suppressalis	Striped rice stem borer	Field	CPR	10	–	Gao et al. (2013)
		Field	CPR	15	M	He et al. (2014)
		Field	CPR	22	–	Su et al. (2014)
Choristoneura rosaceana	Oblique-banded leafroller	Field	CPR	4	–	Sial et al. (2010)
		Lab	CPR	8	M	Sial and Brunner (2012)
Chrysodeixis includens	Soybean looper	Field	CPR	6	–	Owen et al. (2013)
			FLB	9		
Cnaphalocrocis medinalis	Rice leaffolder	Field	CPR	9	–	Zhang et al. (2014)
Plutella xylostella	Diamondback moth	Field	CPR	>1000	T	Troczka et al. (2012)
			FLB	>1000		
		Field	CPR	>1000	–	Wang and Wu (2012)
		Field	CPR	>1000	M/T?	Lin et al. (2013)
		Lab	CPR	670	M/T?	Wang et al. (2013)
		Field	CPR	>1000	T	Gong et al. (2014)
		Field	CPR	>1000	–	Ribeiro et al. (2014)
		Field	CPR	>1000	T	Guo et al. (2014b)
		Lab	CPR	48	M	Liu et al. (2015a)
			CYA	3		
			FLB	7		
		Field	CPR	>1000	T	Steinbach et al. (2015)
			CYA	>1000		
			FLB	>1000		
Spodoptera exigua	Beet armyworm	Field	CPR	164	M?	Lai et al. (2011)
		Field	CPR	44	–	Che et al. (2013)
Spodoptera litura	Oriental leafworm	Field	CPR	24	–	Su et al. (2012)
		Lab	CPR	80	M	Muthusamy et al. 2014
		Field	CPR	15	M	Sang et al. (2015)
			CYA	16		
Tuta absoluta	Tomato leaf miner	Field	CPR	>1000	–	Roditakis et al. (2015)
			FLB	>1000		

[a]Diamide insecticides: *CPR* chlorantraniliprole, *CYA* cyantraniliprole, *FLB* flubendiamide

[b]*RR* resistance ratio; highest reported ratio of LC_{50} or LD_{50} of resistant strain/LC_{50} or LD_{50} of susceptible strain

[c]Mech = mechanism of resistance suggested in the study cited (if known): *M* metabolic, *T* target-site mutation, – unknown

(Troczka et al. 2012). The amino acid substitution was shown to have evolved independently in diamondback moth populations in the Philippines and Thailand by different non-synonymous single-nucleotide polymorphisms, i.e. GGG to GAA and GGG to GAG, respectively, both replacing a glycine by a glutamic acid residue. Subsequently other groups confirmed the presence of the G4946E mutation also in diamondback moth populations collected in China (Gong et al. 2014; Guo et al. 2014a, b; Yan et al. 2014) and other countries including India, Japan and the USA (Steinbach et al. 2015). Some studies also demonstrated that RyR transcript levels are either increased or decreased in addition to the G4946E mutation in diamide-resistant strains (Yan et al. 2014; Gong et al. 2014; Liu et al. 2015a). The fact that the G4946E mutation was found in populations from different geographies indicates once more that it evolved independently rather through migration of one population. The G4946E substitution is located in the RyR transmembrane domain approx. comprising 700 amino acids and suggested as crucial for the binding of diamides in earlier studies conducted with a photoreactive derivative of flubendiamide in RyR deletion mutants of *B. mori*, recombinantly expressed in human embryonic kidney cells (Kato et al. 2009). The RyR transmembrane domain is highly conserved among different insect taxa (Fig. 12.3), and homology modelling revealed that glycine 4946 is located at the interface between helix S4 and the S4–S5 linker (Steinbach et al. 2015), supposed to have a critical role in RyR gating by impacting the movement of pore-associated helices (Ramachandran et al. 2013). Phylogenetic analysis of the RyR of different insect orders reveal that lepidopteran species, which have >90 % homology in their amino acid sequence, share around 78 % homology to Coleoptera and Hymenoptera (Fig. 12.2). Other insect RyR isoforms, such as Diptera and Hemiptera, show a 75–77 % identity with Lepidoptera. As shown in Fig. 12.3, the C-terminal transmembrane part of the RyR is a highly conserved region especially in the transmembrane helices, whereas the cytoplasmic part of the protein has diverged during evolution (Lümmen 2013). The G4946E mutation was first described in 2012 and associated with a diamide-resistant phenotype of diamondback moth, but convincing functional evidence for its implications in diamide binding was only provided recently (Steinbach et al. 2015). It was shown in radioligand binding studies using thoracic microsomal membrane preparations of diamondback moth that the G4946E mutation has functional implications on both diamide-specific binding as well as on its concentration-dependent allosteric modulation of [^{3}H]ryanodine binding (Steinbach et al. 2015). In contrast to thoracic microsomal membrane preparations of a diamide susceptible strain, a diamide-resistant *Plutella* strain did not show specific saturable binding of a tritiated des-methylated flubendiamide analogue, [^{3}H]PAD1. The tritiated diamide radioligand showed nanomolar binding affinities to membrane preparations of susceptible diamondback moth (K_D-value 2.7 nM), but no conclusive equilibrium kinetics with membranes isolated from a resistant strain. Thus, Steinbach et al. (2015) provided for the first time functional evidence that the G4946E mutation confers RyR target-site resistance to diamide insecticides. The importance of the G4946E mutation for diamide resistance was confirmed in another study using clonal Sf9 cell lines stably expressing either the *Plutella* wild type or G4946E RyR (Troczka et al. 2015). It was shown that

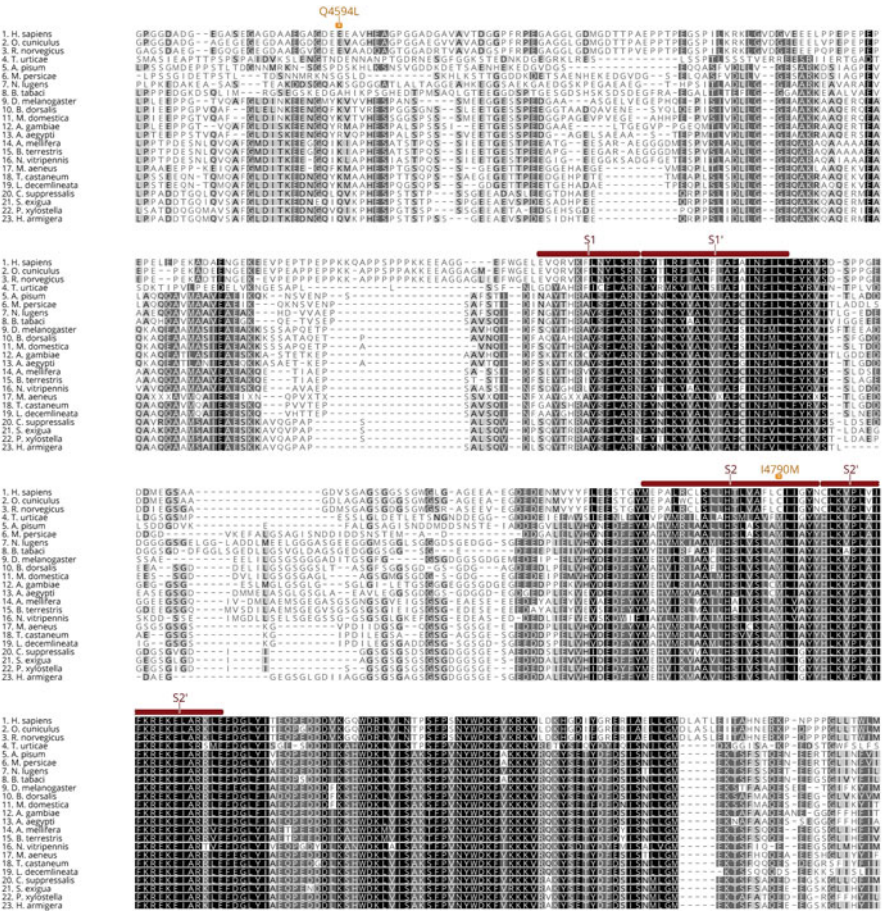

Fig. 12.3 Amino acid sequence alignment of the extended C-terminal transmembrane domain of ryanodine receptor (*RyR*) orthologues from mammals and arthropod species covering a broad phylogenetic range. Conserved amino acid residues across species are shaded in *black*. Secondary structural elements and domains are indicated above the alignment by coloured bars and based on a recently published rabbit RyR1 structure (PDB code: 3J8H) determined by single-particle cryomicroscopy (Yan et al. 2015). RyR mutation sites linked to diamide insecticide resistance in diamondback moth (*P. xylostella*) are located at positions Q4549L, I4790M and G4946E (numbering based on diamondback moth RyR). GenBank accession numbers are as follows: *Homo sapiens*, P21817; *Oryctolagus cuniculus*, P11716; *Rattus norvegicus*, F1LMY4; *Myzus persicae*, A0A0A7RS32; *Nilaparvata lugens*, KF306296; *Bemisia tabaci*, I3VR33; *Drosophila melanogaster*, AFH07966.1; *Bactrocera dorsalis*, A0A034W289; *Musca domestica*, XP_011296554.1; *Anopheles gambiae*, Q7PMK5; *Aedes aegypti*, Q17EB5; *Apis mellifera*, AFJ66977.1; *Bombus terrestris*, XP_012175583.1; *Nasonia vitripennis*, XP_008202582.1; *Meligethes aeneus*, Nauen et al. unpublished; *Tribolium castaneum*, AIU40166.1; *Leptinotarsa decemlineata*, AHW99830; *Chilo suppressalis*, I3VR34; *Spodoptera exigua*, A0A059XRP6; *Plutella xylostella*, AEI91094.1; *Helicoverpa armigera*, V5RE97

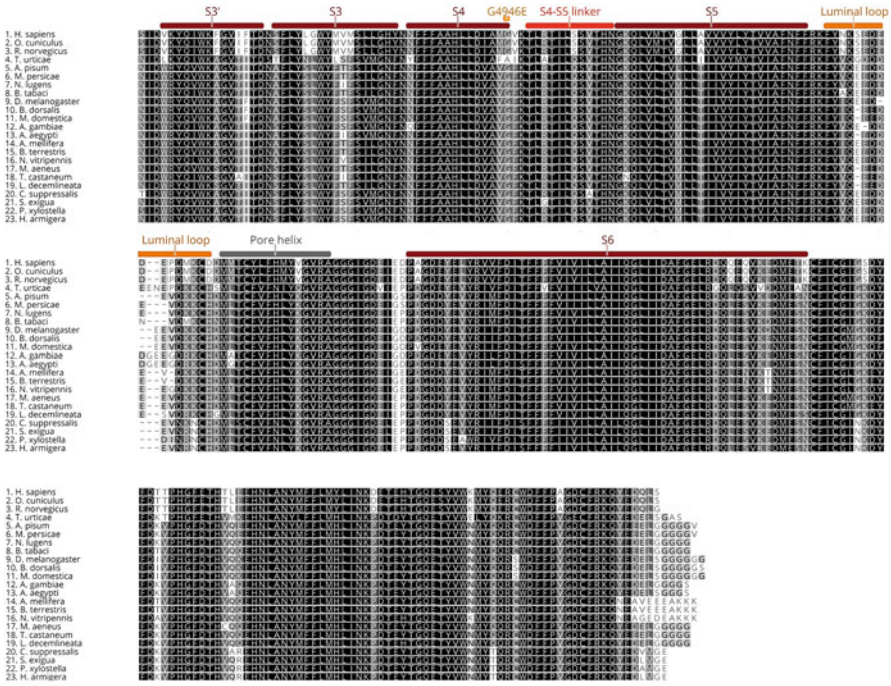

Fig. 12.3 (continued)

the binding of both phthalic and anthranilic diamides was dramatically impaired by the G4946E mutation in *Plutella* RyR recombinantly expressed in clonal Sf9 cell lines. Apart from the functional mutation G4946E, three more mutations, E1338D, Q4594L and I4790M, were recently identified in the RyR of a highly resistant *P. xylostella* strain from China and supposed to be involved in diamide resistance (Guo et al. 2014b). The critical role of the transmembrane domain at the interface between helix S4 and the S4–S5 linker for diamide binding seems obvious regarding the functional implications of G4946E in diamide binding. Interestingly the mutation site I4790M described by Guo et al. (2014b) in the upper helix S2 exhibits a greater diversity among insect taxa, but is located directly opposite of the G4946E mutation as shown in homology models of the diamondback moth RyR based on rabbit RyR1 (Steinbach et al. 2015). The distance between the respective Cα atom positions of the mutation sites is approx. 13 Å (Fig. 12.4). However, functional evidence showing the impairment of diamide insecticide binding by the presence of I4790M, either alone or in combination with G4946E, is still missing. On the other hand, it is tempting to speculate that differences in chlorantraniliprole and flubendiamide binding affinity (and selectivity) recently described in *Musca domestica* and *Apis mellifera* membrane preparations (both M4790) in comparison to Lepidoptera (I4790) (Qi and Casida 2013; Qi et al. 2014) are based on such less conserved residues rather than G4946. According to the recently published

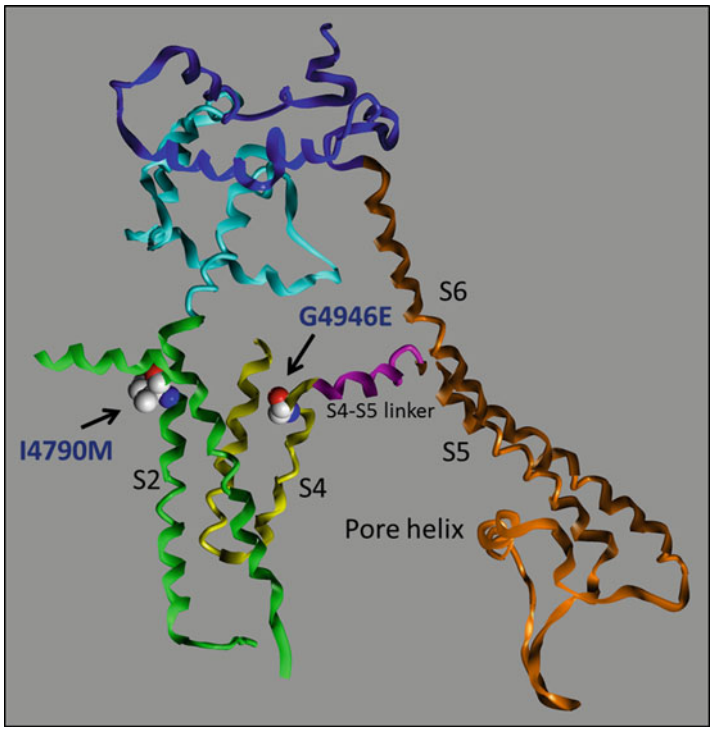

Fig. 12.4 Ryanodine receptor protomer modelling based on the recently published structure of rabbit RyR1 (PDB code 3J8H; Yan et al. 2015). Two mutations conferring diamide insecticide resistance in diamondback moth (Troczka et al. 2012; Guo et al. 2014a), G4946E and I4790M, are located in transmembrane domains S4 and S2 (Steinbach et al. 2015)

closed-state cryo-EM structure of rabbit RyR1 (Yan et al. 2015), the third mutation described by Guo et al. (2014b), Q4594L, is not located within the transmembrane domains, but in a region with several predicted EF hand domains (Takeshima et al. 1989). The implication of this mutation for diamide binding in lepidopteran RyRs also needs further investigation in the future, similar to E1338D which is located towards the N-terminus of *P. xylostella* RyR. Therefore, it is not in proximity to the other transmembrane-linked mutations (Guo et al. 2014b) and the putative binding site of diamide insecticides (Kato et al. 2009; Steinbach et al. 2015). In summary there is compelling evidence that the substitution of amino acid residue G4946 in RyRs plays a key role in diamide insecticide resistance, albeit its role in other species than diamondback moth yet needs to be explored. On the other hand I4790 is likely to be another important RyR mutation site possibly linked to diamide species specificity (and resistance).

See attached TIF files. The figure has been separated in two files, part 1 and 2.

12.2.2 Metabolic Resistance

Phase I metabolism of diamide insecticides in animals depends particularly on microsomal monooxygenases, i.e. cytochrome P450s. It has been reported that flubendiamide metabolism in rats is mainly driven by multistep oxidation of methyl groups (Justus et al. 2007), and a major metabolic pathway of chlorantraniliprole and cyantraniliprole in the goat and rat, respectively, was shown to be the hydroxylation of the *N*-methyl and methylphenyl carbons resulting in hydroxy metabolites (Gaddamidi et al. 2011; Yoshida and McGregor 2014). Virtually nothing has been published yet regarding the metabolic fate of diamide insecticides in target organisms such as lepidopteran larvae. Metabolic resistance can be characterised by the genomic changes that lead to amplification, overexpression and coding sequence variation in the three major groups of gene superfamilies encoding for metabolic enzymes such as cytochrome P450s, carboxylesterases and glutathione S-transferases (Li et al. 2007), thus allowing the insect to overcome the toxicity of the insecticide. Studies on synergism by co-applying inhibitors of major detoxification mechanisms usually provide a first line of evidence for the presence of metabolic resistance in resistant strains.

However, as major routes of detoxification in animals were shown to include oxidation, it seems appropriate to assume that cytochrome P450-driven metabolisation of diamides in pest insects may potentially mediate metabolic resistance if such enzymes are overexpressed due to prolonged selection pressure. However, even though diamides are used to control lepidopteran pests for almost 10 years, conclusive evidence of metabolic mechanisms of resistance compromising diamide efficacy at recommended field rates was not yet described. Field-collected strains of those species showing resistance ratios greater than 1000-fold, such as diamondback moth, were shown to express target-site resistance mediated by amino acid substitutions in the transmembrane domain of the RyR (Troczka et al. 2012; Guo et al. 2014b; Steinbach et al. 2015), or, such as tomato leaf miner, no concrete informations on the mechanisms of resistance were reported (Roditakis et al. 2015). Campos et al. (2015) tested both flubendiamide and anthranilic diamides against a number of field-collected strains of *T. absoluta*, and whilst the level of cytochrome P450 activity was significantly correlated with the variation in chlorantraniliprole and cyantraniliprole susceptibility, no such correlation was evident for the observed variation in flubendiamide efficacy. Though the observed overall variation in lethal concentration values among all tested tomato leaf miner strains against anthranilic diamides was low, it is interesting to note that those with the lowest LC_{50} values were also those with the lowest cytochrome P450 activity, a fact which suggests that oxidative metabolism determines at least to some extent the observed efficacy variation (Campos et al. 2015). The possible involvement of oxidative metabolism in diamide resistance was also suggested in a laboratory-selected Indian strain of *S. litura* exhibiting 80-fold resistance to chlorantraniliprole, but synergist studies using piperonyl butoxide (PBO) were not conclusive both in vitro and in vivo (Muthusamy et al. 2014). However, studies on Chinese *S. litura* strains failed to correlate low-level anthranilic diamide resistance with elevated levels of cytochrome

P450 activity (Su et al. 2012; Sang et al. 2015). Another noctuid species investigated for its capacity to develop chlorantraniliprole resistance after several laboratory selection cycles was *S. exigua* (Lai et al. 2011). Although elevated levels of cytochrome P450 and esterase activity were measured, their inhibition by synergists did not significantly increase diamide susceptibility in the selected laboratory strain. This is in contrast to diamondback moth where Liu et al. (2015a) demonstrated high PBO-mediated synergism of chlorantraniliprole activity in a moderately resistant strain selected for 52 generations under laboratory conditions, suggesting the involvement of increased oxidative metabolism, because the carboxylesterase inhibitor S,S,S-tributyl-phosphorotrithioate (DEF) failed to significantly synergise chlorantraniliprole, thus confirming earlier studies on a field-collected diamondback moth strain (Wang et al. 2013). In another study, laboratory selection of cyantraniliprole resistance in diamondback moth resulted in an increased cross-resistance to flubendiamide and chlorantraniliprole and could be synergised to some extent by PBO and diethyl maleate (DEM) (Liu et al. 2015b). A recent RNA-seq approach to investigate the transcriptome of three diamondback moth strains exhibiting low, moderate and high levels of chlorantraniliprole resistance revealed a correlation between the level of resistance and the up-regulation of a number of detoxification genes, such as cytochrome P450s, but also downregulation of RyR contigs (Lin et al. 2013), a phenomenon also described for other diamide-resistant diamondback moth strains (Gong et al. 2014). However, this is in contrast to other studies showing up-regulation of RyR transcripts to be involved in diamide resistance (Yan et al. 2014; Liu et al. 2015a). Strong synergism of chlorantraniliprole by PBO as well as DEF was recently described in a field-collected strain of a major rice pest, *C. suppressalis*, suggesting a role for both monooxygenases and esterases in the detoxification of chlorantraniliprole (He et al. 2014). Interestingly increased esterase activity was also found in a chlorantraniliprole-selected strain of *Choristoneura rosaceana* (Sial et al. 2011), and subsequent synergist studies principally supported the role of hydrolytic enzymes in chlorantraniliprole detoxification (Sial and Brunner 2012). In conclusion it seems fair to claim that most if not all studies on lepidopteran pests so far published failed to clearly demonstrate strong implications of metabolic mechanisms of diamide resistance causing field failure at recommended rates, but this may (will) change in the future. However, the growing tendency to utilise technologies such as RNA-seq for transcriptome assembly and expression analysis will for sure facilitate the identification of specific biochemical mechanisms and candidate genes to be principally capable to confer metabolic resistance to diamide insecticides in pest species under continuous selection pressure.

12.2.3 Genetics of Diamide Resistance

Among the few studies published to date of either field- or laboratory-selected diamide resistance high enough to compromise field efficacy, only some of those done on diamondback moth have examined the genetics of resistance to diamide

insecticides. To date, there have been a few different but highly resistant diamondback moth strains examined in these studies, and of these, all have suggested an autosomal incomplete to almost recessive mode of inheritance (degree of dominance D ranges from −0.13 to −0.81) based on reciprocal crosses of diamide-resistant and susceptible individuals (Wang et al. 2013; Guo et al. 2014; Steinbach et al. 2015; Liu et al. 2015a, b). Two of these studies tested the level of diamide resistance of backcrosses of the F1 progeny with the resistant parental strain and investigated whether the observed diamide resistance is conferred by a single or multiple genes (Steinbach et al. 2015; Liu et al. 2015a, b). For example, flubendiamide resistance (RR >10,000) in a field-collected Philippine strain of *P. xylostella* was found to be almost recessive (D −0.81) and near monogenic, based on the presence of a homozygous target-site mutation (G4946E) in the transmembrane domain of the diamondback moth RyR (Steinbach et al. 2015). The authors have shown that the frequency of the resistance allele is likely to be 100 % in their strain, which was maintained without selection pressure under laboratory conditions for more than 4 years. A second diamondback moth study found that cyantraniliprole resistance (RR >3000) in a field-collected Chinese strain selected for three generations under laboratory conditions was autosomal and incompletely recessive (D < −0.2), but controlled by multiple genes as shown by differences between expected and observed mortality figures in dose-response tests of the backcross of F1 progeny with the parental strain (Liu et al. 2015a, b). The authors have not analysed the molecular mechanisms conferring the high levels of cyantraniliprole resistance in their strain, but earlier studies on diamondback moth populations collected in the very same region, i.e. Zengcheng, Guangdong Province (southern China), revealed a high frequency of individuals showing a G4946E RyR target-site mutation (including heterozygotes) and diamide resistance levels greater than 2000-fold (Gong et al. 2014; Yan et al. 2014). However, one can only speculate that possibly a mix of diamide-resistant genotypes present in the cyantraniliprole-resistant strain (ZC, i.e. Zengcheng) investigated by Liu et al. (2015b) may have prevented to find a near monogenic resistance as well as an almost recessive mode of inheritance resulting in a heterozygously susceptible phenotype, as shown for a near-isogenic strain from the Philippines (Steinbach et al. 2015). Most of the available information on diamide resistance in diamondback moth seems to suggest a single, recessive gene, which is consistent with the presence of a target-site-based mechanism of resistance. Even when other (detoxification) genes may be involved, the known and well-described target-site-based resistance mechanism seems most important for diamide resistance in diamondback moth and possibly other pest insects exhibiting high levels of diamide resistance such as *T. absoluta* (Roditakis et al. 2015).

12.2.4 Fitness Costs of Diamide Resistance

The process of natural selection favours genes of phenotypes that show the highest fitness within a population (Holloway et al. 1990). As a result of the selection

pressure on insects, caused by extensive use of insecticides, the selection of alleles that confer an adaptation to this environmental stress factor is facilitated. Therefore, most insecticide resistance mechanisms are associated with fitness costs as these mutational changes often have deleterious effects on the overall fitness of a resistant insect compared to a susceptible counterpart. However, the costs caused by resistance are not fixed and are more or less dependent on environmental factors, such as temperature (Li et al. 2007), food quality (Janmaat and Myers 2005; Golizadeh et al. 2009; Farahni et al. 2011) and parasitism (Raymond et al. 2007). Furthermore, negative genetic trade-offs are often shown in the absence of the insecticide or in the presence of sublethal doses (Hoffmann and Parsons 1991; Ribeiro et al. 2014). When selecting for diamide resistance in *P. xylostella*, fitness costs were identified as a consequence of diamide resistance (Han et al. 2012; Yan et al. 2014), e.g. lower fertility in a cyantraniliprole-selected laboratory strain (Liu et al. 2015b). The overall fitness was strongly affected, showing a longer developmental time of larva as well as a decreased rate of pupation and adult emergence with a low relative fitness. When applying a sublethal concentration of chlorantraniliprole to a Brazilian field-evolved chlorantraniliprole-resistant diamondback moth strain (RR >27,000) and a susceptible reference strain, both strains were significantly affected in their fitness (Ribeiro et al. 2014). Moreover, the resistant strain had shown negative trade-offs, such as significantly reduced larval weight and fecundity, when chlorantraniliprole was absent. In other studies there was no significant effect on the longevity in *P. xylostella* and *S. exigua* when the insects were treated with a sublethal concentration of chlorantraniliprole (Lai et al. 2011; Han et al. 2012). In *Cydia pomonella*, it was shown that chlorantraniliprole exposure affected males more than females in terms of mating behaviour (Knight and Flexner 2007). Despite the fitness costs involved in diamide resistance, positive traits could be observed in diamondback moth, such as an increased larval survival, egg hatchability and male longevity (Ribeiro et al. 2014). This suggests that a physiological mechanism is present in order to compensate for associated fitness costs. However, Ribeiro et al. (2014) associated the reduced fitness in diamondback moth with the reversion of resistance to chlorantraniliprole as the resistant strain had shown a rapid decline in resistance without selection pressure. Most studies on fitness costs were yet conducted with diamide-resistant diamondback moth strains due to the fact that in most other lepidopteran targeted by diamides, resistance ratios so far reported are quite low and in most cases not compromising field efficacy.

12.3 Diamide Resistance Management

The development of field resistance depends on several factors including the genetic variability already present in a population of pests treated by consecutive applications with the same mode of action, thus facilitating the survival and reproduction of genotypes with a heritable ability to resist such applications at manufacturer recommended label rates. However, steadily increasing but still low levels of

resistance are often less obvious under field conditions in terms of initial efficacy, but can result in an incremental reduction of residual activity due to the capacity of selected genotypes to resist declining quantities of active substance which would still provide reasonable control of completely susceptible individuals. In order to prevent or – realistically spoken – delay such a process, resistance management strategies need to be implemented in order to sustain the efficacy of a mode of action or chemical class of insecticide. The introduction of diamide insecticides into global markets was accompanied by communication and educational activities mainly driven by an IRAC International Diamide Working Group as well as more than 20 different IRAC Diamide Country Groups, tying together knowledge including baseline studies on high-risk pests and suitable (regional) IRM strategies in a diverse range of cropping systems (Teixeira and Andaloro 2013). The main objectives of the established regional IRAC Country Teams were (a) the identification and prioritisation of high resistance risk pests and cropping systems; (b) the adaptation of the global IRM guidelines into appropriate regional resistance management strategies; (c) the development of communication strategies particularly facilitating product labelling (IRAC Group 28 insecticides), advertising and education; (d) the communication of IRM recommendations, rotation strategies and optimal number of applications per cropping cycle by a so-called window approach (Fig. 12.5); (e) the development of an extensive education and knowledge transfer programme to train influencers and growers utilising local industry and IRM experts; and, last but by no means least, (f) the implementation of IRM strategies through education and training programmes, both on a global and regional scales (Teixeira and Andaloro

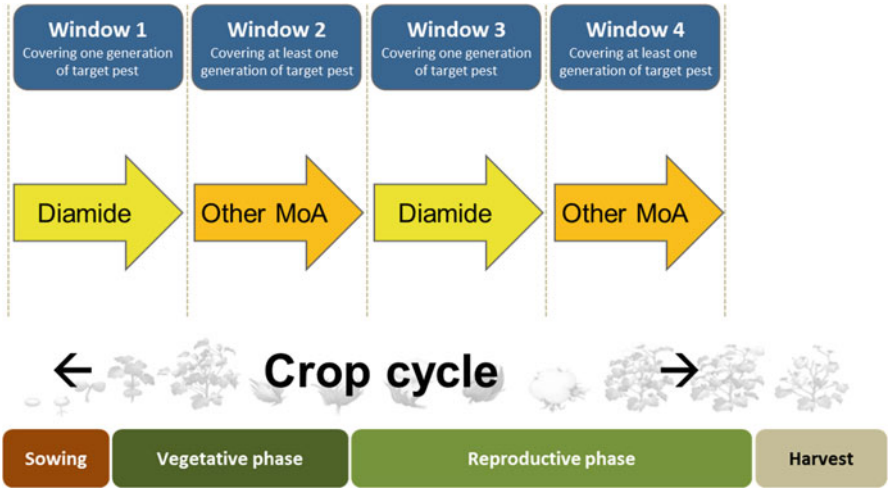

Fig. 12.5 Recommended insecticide mode of action rotation practice for resistance management by an application window approach to avoid exposure of consecutive pest generations to the same mode of action such as diamides acting on insect ryanodine receptors (IRAC MoA group 28; www.irac-online.org)

2013; refer also to www.irac-online.org for continuous updates on general IRM strategies, including diamides). A key point of established IRM strategies is the rotation of diamide insecticides between pest generations with other modes of action and to limit the number of applications throughout the cropping cycle by an IRM window approach (Fig. 12.5). In addition diamides exhibit some favourable application characteristics, such as low effects on populations of most beneficial insects, known to facilitate IRM within integrated pest management programmes. As diamides are distinct from all other chemical classes of insecticides (Sparks and Nauen 2015), they can principally be rotated with all those classes in IRM strategies. Currently, there is no metabolic detoxification mechanism described in any diamide-targeted pest conferring field-relevant cross-resistance to other Lepidoptera-active insecticides, rendering them highly valuable tools for both combining and alternating insecticide modes of action. The predominance and global spread of a target-site-based resistance mechanism in diamondback moth (Steinbach et al. 2015) – though recessive – should serve as a warning that resistance development may also easily extend to other pests if these are continuously selected by the treatment of consecutive generations, such as what recently happened for *T. absoluta* in southern Europe (Roditakis et al. 2015). However, cases of significant resistance to diamides under applied field conditions are so far regionally restricted to a few Lepidoptera species (Table 12.1), with the notable exception of *P. xylostella* (Troczka et al. 2012; Wang and Wu 2012; Gong et al. 2014; Ribeiro et al. 2014; Steinbach et al. 2015).

12.4 Conclusions

Diamide insecticides show a remarkable overall activity against lepidopteran pest species, and after 10 years on the market, this chemical class gained blockbuster status economically and considering its global impact in many agricultural and horticultural cropping systems. However, despite their widespread use, diamide resistance development compromising field efficacy is yet restricted to a few, mostly regional cases, except for diamondback moth. Investigations into the molecular mechanisms of diamide resistance in this pest revealed RyR target-site mutations with strong functional implications for diamide binding. This also facilitated fundamental research on the genetics of diamide resistance and associated fitness costs. However, the evolution of target-site resistance is definitely an unpleasant event from an applied perspective, but it also offers opportunities to extend our knowledge on the biochemistry of insect RyRs as insecticide targets, e.g. by contributing to the understanding of diamide selectivity (insects vs. mammals) and by mapping the elusive diamide binding site, possibly allowing the design of novel ligands overcoming target-site resistance. The fairly rapid evolution of this target-site resistance mechanism in diamondback moth, due to high treatment frequency in tropical conditions, suggests that other pests with a lower number of generations per year and thus less frequently treated are likely to follow soon, if no appropriate

IRM strategies as outlined above are implemented, helping to conserve diamide insecticides as a valuable chemical tool for sustainable agriculture.

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Chapter 13
Resistance Mechanisms of *Helicoverpa armigera*

Nicole Joußen and David G. Heckel

Abstract The cotton bollworm, *Helicoverpa armigera*, is one of the major agricultural pest species in the Old World and recently also of the New World. This noctuid moth species is highly polyphagous and possesses a huge geographical distribution and the ability to quickly evolve resistance to insecticides from different chemical classes. There are different mechanisms known with which insects combat insecticides. They are ranging from behavioral over morphological to physiological adaptations. These resistance mechanisms can occur alone or in combination and may change in the field according to changing selection pressures. A reduced penetration through the cuticle of *H. armigera* larvae is known which reduces the concentration at the target site. Also mutations of the target of pyrethroid insecticides, organochlorines, and oxadiazines, voltage-dependent sodium channels, were described that lead to high or lower resistance levels. Furthermore, both carboxylesterases and cytochrome P450 monooxygenases were investigated to determine their role in insecticide resistance. So far, only few enzymes were identified in *H. armigera* which were proven to metabolize and thus detoxify insecticides. Most studies deal with the resistance against pyrethroids. One important resistance gene is the chimeric P450 *CYP337B3* that is present in resistant and absent in susceptible individuals. The corresponding enzyme is capable of metabolizing fenvalerate and cypermethrin and thus confers resistance to *H. armigera* larvae. This new resistance mechanism by recombination seems to play an important role in *H. armigera* populations throughout the world.

13.1 The Noctuid Agricultural Pest Species *Helicoverpa armigera*

The cotton bollworm, *Helicoverpa armigera* (Hübner 1808) (Fig. 13.1a), is a noctuid moth that belongs to the subfamily Heliothinae. Another common name of *H. armigera* is Old World bollworm. This name describes its distribution area

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Fig. 13.1 *Helicoverpa armigera*. (**a**) Pictures of a female and a male moth, a fifth instar larva, and a pupa with still attached larval cuticle (© N. Joußen). (**b**) Schematic map illustrating the geographical distribution of *H. armigera*. The entire country is highlighted in *red*, even if the insect is only present in some part of the country. In summer 2015, one first male moth of *H. armigera* was collected from a field in Florida, USA

that covers almost all countries of the Old World across Africa, Asia, Oceania, and Europe (Fig. 13.1b). Furthermore, this common name distinguishes *H. armigera* from its close relative *Helicoverpa zea*, the New World bollworm or the corn earworm, which is morphologically almost indistinguishable from *H. armigera* and is native to America. In 2013, this geographical separation between both species was eliminated, when *H. armigera* was identified by molecular markers to be present in Brazil for the first time (Tay et al. 2013). It is still unclear from which country the first individuals originate that were most likely introduced by human activity (Tay et al. 2013). Since then, *H. armigera* invaded more and more countries in South America, namely, Argentina, Paraguay, and Bolivia (Murúa et al. 2014) (Fig. 13.1b) and reached Florida in the USA in summer 2015, where one male moth was collected in a pheromone trap (http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Plant-Industry-Publications/Pest-Alerts/Pest-Alert-The-Old-World-Bollworm). This is a severe problem, because *H. armigera* is one of the most significant agricultural insect pests worldwide due to its extremely wide geographical distribution (Fitt 1989; Tay et al. 2013) in combination with its high migratory ability (Feng et al. 2005), its highly polyphagous lifestyle (Fitt 1989), and its ability to quickly evolve resistance to many chemically distinct insecticides (www.pesticideresistance.org).

More than 200 wild and crop plant species are known to be host plants of *H. armigera* (Brun-Barale et al. 2010). Zalucki et al. (1986) reported *H. armigera* from 72 plant species in Australia that are distributed throughout 29 plant families including Fabaceae, Asteraceae, Malvaceae, Solanaceae, Brassicaceae, and Poaceae. Principal crop hosts are grain sorghum; pulses, including soybean,

chickpea, and groundnut; cotton; rape; and preferentially tobacco, sunflower, and maize (Zalucki et al. 1986; Firempong and Zalucki 1989). The feeding preference of the larvae for reproductive structures as buds, flowers, and fruits reduces the yield of infested crop plants directly (Fitt 1989). The moths are good long-distance migrants covering a distance of about 1,000 km (Feng et al. 2005) to colonize regions located so far north that overwintering is impossible.

Indirect costs are caused by pest management, as almost 30 % of all pesticides used worldwide are directed against *H. armigera* (Ahmad 2007). This has resulted in a high insecticide resistance of this species against 34 reported distinct insecticides that can be classified by their chemistry as pyrethroids (14 compounds without isomers), organophosphates (9), carbamates (3), organochlorines (1), cyclodienes (3), oxadiazines (1), macrocyclic lactones (2), and protein toxins (www.pesticideresistance.org, September 2015).

Most of these natural and synthetic insecticides target the central nervous system of insects by modulation and blocking of voltage-dependent sodium channels, inhibition of acetylcholine esterases, antagonism of GABA-dependent chloride channels, agonism of nicotinic acetylcholine receptors, and activation of chloride channels (Sparks and Nauen 2015).

13.2 Resistance Mechanisms Toward Insecticides

Resistance has been defined by the World Health Organization (WHO) in 1957 as follows: "Resistance to insecticides is the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species."

There are different mechanisms known through which insects can become resistant to insecticides, namely, behavioral, morphological, and physiological adaptations (Fig. 13.2). To this classical categorization based on physiological and biochemical criteria, Feyereisen et al. (2015) added a molecular genetic dimension classifying resistance by coding sequence mutations, gene duplications, conversions, and disruptions or differentially expression of genes. The genetic basis of the resistance mechanisms described in more detail below is given where known.

13.2.1 Behavioral Resistance Mechanisms

Insects can avoid insecticides and thus reduce their exposure by natural or developed behavioral characteristics. Feeding on the bottom side or inside of leaves or inside of stems or fruits is a natural behavior that besides protecting the insect from parasitoids and predators also reduces or avoids the exposure to insecticides. But because these are natural behaviors, they are not considered as resistance

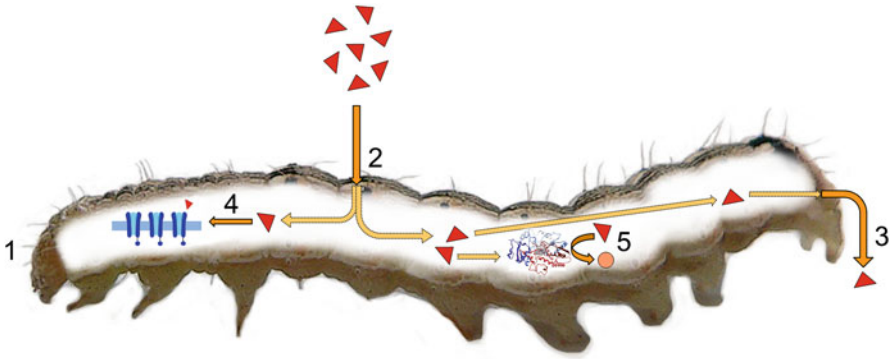

Fig. 13.2 Schematic diagram of resistance mechanisms occurring in insects. *1* behavioral resistance; *2* morphological resistance (penetration resistance); physiological resistance: *3* resistance by enhanced excretion; *4* target site resistance; *5* metabolic resistance. *Red triangles* symbolize insecticide molecules and a *pink circle* an insecticide metabolite

mechanisms. In 1957, the WHO defined behavioral resistance as "the development of the ability to avoid a dose (of toxicants) which would prove lethal".

Therefore, only the development of a behavior to avoid an insecticide is considered a resistance mechanism. There are several examples of developed behavioral resistance known from different insect species (Liu et al. 2006). Lockwood et al. (1985) described behavioral resistance from the horn fly, *Haematobia irritans*, where the resistant population was significantly more repelled and irritated by the pyrethroid insecticides fenvalerate and permethrin than the susceptible population (Liu et al. 2006). Silverman and Bieman (1993) reported a glucose aversion in the German cockroach, *Blattella germanica*, after the insects were exposed to a mixture of the electron transport inhibitor hydramethylnon and D-glucose in living spaces for 5 years. During this time the previous feeding stimulant D-glucose changed to a feeding deterrent thus reducing the intake of the insecticide. This glucose aversion was not determined as associative learning but as a result of a chemosensory mutation that might be located in a single major gene (Silverman and Bieman 1993).

No case of behavioral insecticide resistance of *H. armigera* has been described to date.

13.2.2 Morphological Resistance Mechanisms

One morphological mechanism described from different insect species is the reduced penetration of the insecticide through the cuticle of resistant individuals resulting in a lower concentration of the insecticide in the body and thus a lower concentration at the target site. This stronger cuticular protection was described for *Heliothis virescens*, another Heliothine moth species, as being due to a higher

protein and lipid content in the cuticle of resistant larvae and a higher degree of sclerotization (Vinson and Law 1971).

Gunning et al. (1991) determined the distribution of the ^{14}C-labeled pyrethroids fenvalerate and cypermethrin, respectively, after treatment of pyrethroid-resistant and susceptible Australian *H. armigera* by measuring the radioactivity in different larval body parts. Thus, they demonstrated that the pyrethroids persist longer on the cuticle of resistant than on that of susceptible individuals (Gunning et al. 1991). By rinsing larvae with acetone and measuring the radioactivity in these cuticle washes, they ended up with very similar results. After 4 h only about 10 % of fenvalerate could be recovered from the cuticle of susceptible larvae, whereas about 55 % were recovered from that of resistant ones assuming the rest entered the body tissue (Gunning et al. 1991). Penetration of esfenvalerate, the most toxic isomer of fenvalerate, at higher doses seemed to be facilitated in resistant larvae by piperonyl butoxide, a synergist of insecticides which inhibits cytochrome P450 monooxygenases and carboxylesterases (Gunning et al. 1995). Also horticultural mineral oil enhanced the penetration of the macrocyclic lactone insecticide abamectin through the cuticle (Wang et al. 2005). Penetration resistance was also reported from a pyrethroid-resistant Thailand strain of *H. armigera* against *trans*-cypermethrin (Ahmad and McCaffery 1999) and from an Indian strain (Kumari et al. 1995) and pyrethroid-resistant Chinese and Pakistani strains (Ahmad et al. 2006) against deltamethrin.

Although reduced penetration confers only low-order resistance of about 20-fold (Gunning et al. 1991), it is an important first line of defense. In *H. armigera*, Gunning et al. (1991) found three coexisting pyrethroid resistance mechanisms in the field in 1983, at the onset of pyrethroid resistance in Australia: reduced penetration, target site insensitivity, and increased metabolism with nerve insensitivity appearing to be primarily responsible for the high level of resistance observed.

13.2.3 Physiological Resistance Mechanisms

Physiological resistance mechanisms include enhanced excretion, target site insensitivity, and increased metabolism.

13.2.3.1 Resistance by Enhanced Excretion

Enhanced excretion reduces the exposure time of the insecticide in the body of the insect and thus at the target in the nervous system. Larvae of the resistant R-strain of diamondback moth, *Plutella xylostella*, excrete the organophosphate insecticide malathion in its unmetabolized form much faster and in higher amounts than the susceptible S-strain does (Doichuanngam and Thornhill 1992). Strycharz et al. (2013) demonstrated a fourfold higher excretion rate of the organochlorine DDT and its metabolites in the DDT-resistant *91-R* strain of *Drosophila melanogaster* compared to the susceptible *Canton-S* strain most likely due to the overexpression of

ATP-binding cassette transporters (ABC transporters), which are known to transport substrates across lipid membranes (Dermauw and Van Leeuwen 2014).

Excretion has not been reported as a specific resistance mechanism in *Helicoverpa armigera*.

13.2.3.2 Target Site Resistance

Target site resistance is caused by a modification of the target of the insecticide resulting in a reduced binding to the target and thus a lower toxicity. These targets are ion channels, receptors, or enzymes involved in the transmission of stimuli between nervous cells. Target site resistances can reach very high levels. The most common target site resistance reported from different insects is the knockdown resistance (*kdr*) (Dong 2007). This resistance is caused by one or more point mutations in the coding sequence of the *para* gene that encodes voltage-dependent sodium channels, the target of pyrethroids, organochlorines like DDT, and oxadiazines like indoxacarb. Insecticides of these classes lead to a delayed closing of the *m*-gate (activation gate) during an action potential leading to a prolongation of the influx of sodium ions inside the nerve cells. The most common *kdr* mutation is a leucine (L) to phenylalanine (F), histidine (H), or serine (S) substitution in segment 6 of domain II (IIS6) of the sodium channel that is associated with low levels of pyrethroid resistance (Dong 2007). The *kdr* L to H mutation was found in some pyrethroid-resistant strains of the Heliothine moth *Heliothis virescens*, a close relative of *H. armigera*, but some other resistant strains lack this mutation and exhibit instead a valine (V) to methionine (M) change at 421 in IS6 (Dong 2007). This V421M mutation reduces the pyrethroid sensitivity by tenfold when introduced into *Drosophila*, housefly, and cockroach sodium channels (Dong 2007). Highly resistant strains possess additional sodium channel mutations that coexist with the L to F mutation in IIS6 (Dong 2007). For example, *super-kdr* housefly strains (*Musca domestica*) exhibit an additional mutation to the *kdr* L1014F mutation in amino acid 918, which changes methionine to threonine in the linker connecting IIS4 and IIS5 (Dong 2007). Some mutations can also lead to completely insensitive sodium channels like the *super-kdr* mutation T929I in IIS5 along with the M827I and L932F mutations in pyrethroid-resistant head lice, *Pediculus capitis* (Dong 2007). *Kdr* and *super-kdr* mutations enhance the closed-state inactivation of the voltage-dependent sodium channel and thereby reduce channel opening, which is required for the action of pyrethroids that preferably bind to the activated (open) state of the channel and inhibit its deactivation (Dong 2007). Furthermore, these mutations also reduce the binding affinity of pyrethroids for open channels (Dong 2007).

Gunning et al. (1991, 1996) and Gunning (1996) reported a *kdr* and *super-kdr* nerve insensitivity toward pyrethroids from larvae of resistant Australian *H. armigera* demonstrated by nerve responses that were 100 to 500 times less in resistant than in susceptible strains. In 1983, this resistance mechanism conferred high resistance levels in Australian populations, but, as a consequence of severe

restrictions of pyrethroid use, lost its relevance by 1990 when no evidence for the strong *super-kdr* nerve insensitivity from 1983 was detected (Gunning et al. 1991, 1995). Ahmad et al. (1989) described a *kdr* resistance toward the pyrethroid *cis*-cypermethrin in *H. armigera* from Thailand in about 50 % of the individuals from the resistant population, whereas almost 70 % and over 95 % nerve-insensitive pyrethroid-resistant individuals were reported by McCaffery et al. (1997) from China and India, respectively. Sodium channels of a resistant strain of *H. armigera* from China selected by cyhalothrin showed a higher degree of insensitivity toward fenvalerate than cyhalothrin resulting overall in a higher resistance level (Ru et al. 1998). Two amino acid substitutions in the sodium channel conserved in both species were found in the nerve-insensitive JSFX strain of *H. armigera* that was developed from a pyrethroid-resistant Chinese field population and the NIR strain of *Heliothis virescens* that was developed from the putative nerve-insensitive NI1strain with both strains expressing target site resistance to *cis*-cypermethrin (Head et al. 1998). The first substitution is an aspartic acid to valine change at 1,561 and the second a glutamic acid to glycine change at 1,565, both located in the linker between domain III and IV, resulting in at least 100-fold less sensitive nerves compared to susceptible control strains lacking these mutations (Head et al. 1998). These substitutions lead to a net loss of two negative charges likely resulting in a conformation change of the channel (Head et al. 1998).

13.2.3.3 Metabolic Resistance

Metabolic resistance is mainly conferred by enzymes of the superfamilies of cytochrome P450 monooxygenases (P450s or CYPs), carboxylesterases, or glutathione *S*-transferases (GSTs). P450s and carboxylesterases directly act on xenobiotic molecules introducing or releasing new functional groups, thus increasing the hydrophilicity of the often lipophilic compounds. Furthermore, endogenous molecules like glucose can be conjugated by UDP-glycosyltransferases to hydroxyl groups of the xenobiotic molecule, or glutathione can be conjugated to the xenobiotic by glutathione *S*-transferases, thus further increasing the hydrophilicity of the molecule favoring its faster excretion. Besides the direct detoxification effect on xenobiotic molecules by metabolism, there are also indirect effects known by which enzymes bind to the insecticide without metabolizing it and thereby prevent the interaction between the insecticide and its target site.

Both, P450s and carboxylesterases, are discussed to be responsible for the metabolic resistance of Australian *H. armigera* toward pyrethroid insecticides.

Glutathione *S*-transferases

Glutathione *S*-transferases (GSTs) in insects are a large family of microsomal and cytosolic enzymes involved in cellular antioxidant defense against endogenous activated compounds, such as lipid peroxidation products and oxidized DNA bases,

and in detoxification of a wide range of xenobiotics including allelochemicals and insecticides (Li et al. 2007; Enayati et al. 2005). Generally, GSTs catalyze the conjugation of electrophilic compounds with the thiol group of reduced glutathione (Li et al. 2007; Enayati et al. 2005), an important antioxidant present in most organisms and a tripeptide synthesized from glutamate, cysteine, and glycine containing an uncommon gamma peptide linkage. The resulting conjugates are generally more water soluble and excretable than the parent molecules (Enayati et al. 2005). Some insect GSTs catalyze the dehydrochlorination of the organochlorine insecticide DDT to its noninsecticidal metabolite DDE by using reduced glutathione as a cofactor rather than a conjugate (Li et al. 2007; Enayati et al. 2005; Yang et al. 2004). Besides conjugation and dehydrochlorination reactions, GSTs are also involved in the intracellular and circulatory transport of endogenous lipophilic compounds, in xenobiotic binding, and in sequestration (Li et al. 2007). These direct or indirect functions of GSTs might be involved in the resistance of insects to organophosphates, organochlorines, and pyrethroids (Li et al. 2007).

Specific GSTs are described from *Musca domestica*, *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Nilaparvata lugens*, and *Plutella xylostella* (Li et al. 2007). Wee et al. (2008) identified by a cDNA-amplified length polymorphism (AFLP) approach among three other candidate genes the glutathione *S*-transferase *GSTX01* that was 1.9-fold upregulated in resistant individuals of the Australian AN02 strain of *H. armigera*. However, this gene was not linked to the fenvalerate resistance locus *RFen1* (Heckel et al. 1998) and therefore rejected to be responsible for fenvalerate resistance (Wee et al. 2008).

Thus, so far no GST was identified to be responsible for insecticide resistance in *H. armigera*, but several groups reported increased GST activity against model substrates in in vitro metabolism assays comparing enzyme extracts of resistant to susceptible populations (Martin et al. 2002; Yang et al. 2004).

Carboxylesterases

The second gene family encoding detoxifying enzymes in insects are carboxylesterases that are implicated in the resistance of insects to organophosphates, carbamates, and pyrethroids by binding to the insecticides (sequestration) or hydrolysis of the molecules (Li et al. 2007; Wheelock et al. 2005). Carboxylesterases are enzymes with the α/β hydrolase fold structure and catalyze the hydrolysis of carboxyl esters via addition of water (Wheelock et al. 2005).

Most pyrethroids contain chiral centers that greatly affect their metabolism and thus their toxicity. *Trans*-isomers of pyrethroids such as permethrin and cypermethrin are more rapidly hydrolyzed than the corresponding *cis*-isomers (Wheelock et al. 2005) that are more sensitive to oxidative metabolism by P450 enzymes (Shono et al. 1979). The most toxic (2*S*,α*S*)-fenvalerate enantiomer, also called esfenvalerate, is only slightly metabolized by carboxylesterases, while the less toxic (2*R*,α*R*)-enantiomer is rapidly hydrolyzed (Wheelock et al. 2005).

Gunning et al. (1996) described increased amounts of carboxylesterase proteins and also increased esterase activities against 1-naphthyl acetate by enzyme extracts from pyrethroid-resistant individuals of Australian *H. armigera*. Also a 1-naphthyl pyrethroid analog bound to *H. armigera* carboxylesterases separated by native polyacrylamide gel electrophoresis (PAGE), indicating that these carboxylesterases are capable of binding to pyrethroid-like substrates (Gunning et al. 1996). However, only poor catalytic activity of carboxylesterase extract toward the pyrethroid fenvalerate was detected suggesting that carboxylesterases might mainly act as insecticide sequestering agents (Gunning et al. 1996). In this case the high titer of carboxylesterases serves as an "insecticide sink" delaying or preventing the interaction between the insecticide and its target site (Wheelock et al. 2005). Srinivas et al. (2004) detected three additional carboxylesterase bands in resistant *H. armigera* from India compared to a susceptible strain by native PAGE using the substrate 1-naphthyl acetate. Also the overall carboxylesterase activity was ninefold higher in extracts of the resistant strain (Srinivas et al. 2004). Wee et al. (2008) identified, by a cDNA-AFLP approach (in addition to *GSTX01*), the carboxylesterase-like gene *ESTX018* as a candidate for the fenvalerate resistance of the AN02 strain of Australian *H. armigera*. This gene was 2.2-fold upregulated in resistant individuals compared to susceptible ones (Wee et al. 2008). But because this gene also did not map to the same linkage group as the fenvalerate resistance locus *RFen1* (Heckel et al. 1998), it was also rejected as the fenvalerate resistance gene (Wee et al. 2008). Furthermore, it lacks two of the three functional residues in the catalytic triad and thus was predicted to be inactive in cleaving ester bonds but might still bind insecticides (Wee et al. 2008). Using a proteomics approach, Teese et al. (2010) matched seven carboxylesterase sequences from a pyrethroid-resistant Australian *H. armigera* strain with their putative isozyme products of which four, CCE001a, CCE001i, CCE001g, and CCE006a, matched sequences from a zone of the native PAGE (relative mobility (Rm) of 0.36–0.49) which has been previously associated with organophosphate and pyrethroid resistance. Wu et al. (2011) detected by native PAGE a different carboxylesterase pattern between the Chinese fenvalerate-resistant YGF strain of *H. armigera* and a susceptible strain. Purified carboxylesterases were incubated with fenvalerate leading to a loss of fenvalerate by 19–43 % analyzed by gas chromatography (GC) (Wu et al. 2011), but no attempt was undertaken to determine hydrolysis products. The carboxylesterases were identified by mass spectrometry as CCE001a, CCE001d, CCE001i, and CCE001j that are located extracellularly in the insects (Wu et al. 2011). Their corresponding genes were amplified from fat body or midgut cDNA of the resistant YGF strain and were sequenced (Wu et al. 2011). The transcript level of all four genes was determined by reverse transcription-quantitative real-time PCR (RT-qPCR) revealing also a higher level in the resistant YGF strain (Wu et al. 2011). Teese et al. (2013) heterologously expressed 14 carboxylesterase genes cloned from a midgut cDNA library of the Australian susceptible GR strain of *H. armigera* in the baculovirus system. All 14 carboxylesterases were found to bind to model organophosphates and showed low hydrolytic activities. Higher activities were found against the pyrethroids esfenvalerate and the eight isomers

of cypermethrin showing some specificity for the different isomers tested (Teese et al. 2013). The most insecticidal isomers (1*R*)-*trans*-(α*S*)- and (1*R*)-*cis*-(α*S*)-cypermethrin were mainly cleared by CCE016a, CCE014a, and CCE017a and by CCE017a and CCE016a, respectively, and esfenvalerate was mainly cleared by CCE016a, CCE001g, and CCE014a (Teese et al. 2013). Only the substrate loss was determined by high-performance liquid chromatography (HPLC).

Two coding sequence mutations in carboxylesterases leading to altered amino acids and organophosphate resistance have been described from the blowflies *Lucilia cuprina* (G137D and W251L in the enzyme E3) and *L. sericata*, the housefly *Musca domestica*, the screwworm *Cochliomyia hominivorax*, and the parasitic wasp *Anisopteromalus calandrae* (Li et al. 2013). Li et al. (2013) introduced these mutations in vitro into eight *H. armigera* carboxylesterases and found increased activities for few mutants against model organophosphates, cypermethrin isomers, and esfenvalerate.

In conclusion, both overexpression and coding sequence mutations of carboxylesterases might contribute to organophosphate and pyrethroid resistance in *H. armigera*, but further studies are needed.

Cytochrome P450 Monooxygenases

P450s are likely the most important group of xenobiotic detoxifying enzymes. During their catalytic cycle, most P450 enzymes utilize molecular oxygen and reducing equivalents from NADPH provided by a redox partner like the NADPH-dependent cytochrome P450 reductase or cytochrome b_5 to introduce one oxygen atom into the bound substrate, producing besides water reactive oxygen species like hydrogen peroxide as by-products (Feyereisen 2012). P450s are capable of catalyzing a wide range of reactions like hydroxylation of aliphatic and aromatic carbons, epoxidation of double bounds, heteroatom (S-, N-, X-) oxygenation, heteroatom (O-, S-, N-) dealkylation, cleavage of ester bonds, dehydrogenation, reductive dehalogenation, nitro reduction, and isomerization (Zuber et al. 2002). Almost all organisms possess several P450 enzymes that are involved both in endogenous processes and xenobiotic detoxification. Insects encode from about 50 to more than 140 P450 genes that form four distinct clades, the CYP2, the CYP3, the CYP4, and the mitochondrial clade (Feyereisen 2006). The CYP3 clade contains enzymes of the P450 families CYP6, CYP9, CYP321, and CYP337 that are involved in the metabolism of host plant toxins and insecticides. Because of their genetic diversity, broad substrate specificity, and catalytic versatility, P450s and their associated NADPH-dependent cytochrome P450 reductase represent the only metabolic system that is capable of mediating resistance to all chemical classes of insecticides (Li et al. 2007).

All P450 enzymes that are proven or strongly suspected to be responsible for insecticide resistance in *H. armigera* are summarized in Table 13.1.

In *H. armigera*, there is conflicting evidence on whether CYP6B7 is involved in the detoxification of the pyrethroid fenvalerate. Ranasinghe et al. (1998) described an overexpression of *CYP6B7* mRNA in resistant Australian *H. armigera*. Also

Table 13.1 Cytochrome P450 monooxygenases that are proven or strongly suspected to be responsible for insecticide resistance in *Helicoverpa armigera* (www.pesticideresistance.org, September 2015)

Enzyme	Insecticide class	Mode of action	Insecticide	First report	References
Recombination					
CYP337B3	Pyrethroid	Modulation of voltage-dependent sodium channels	Fenvalerate	1984, Australia	Gunning et al. (1984)[a] and Joußen et al. (2012)[b]
			Esfenvalerate	2007, Australia	Gunning et al. (2007)[a] and Joußen et al. (2012)[b]
			Cypermethrin	1984, Australia	Gunning et al. (1984)[a] and Rasool et al. (2014)[b]
			α-Cypermethrin	1997, Pakistan	Ahmad et al. (1997)[a] and Rasool et al. (2014)[b]
Overexpression					
CYP6B7	Pyrethroid	Modulation of voltage-dependent sodium channels	Fenvalerate	1984, Australia	Gunning et al. (1984)[a], Ranasinghe et al. (1998)[b], and Zhang et al. (2010)[b]
CYP9A12	Pyrethroid	Modulation of voltage-dependent sodium channels	Esfenvalerate	2007, Australia	Gunning et al. (2007)[a] and Yang et al. (2008)[b]
			Deltamethrin	1984, Australia	Gunning et al. (1984)[a] and Zhang et al. (2008)[b]
			Cyhalothrin	1992, China	Cen (1992)[a] and Zhang et al. (2008)[b]
			Bifenthrin	1997, Pakistan	Ahmad et al. (1997)[a] and Zhang et al. (2008)[b]
CYP9A14	Pyrethroid	Modulation of voltage-dependent sodium channels	Esfenvalerate	2007, Australia	Gunning et al. (2007)[a] and Yang et al. (2008)[b]
CYP9A17(v2)	Pyrethroid	Modulation of voltage-dependent sodium channels	Deltamethrin	1984, Australia	Gunning et al. (1984)[a] and Zhang (2008)[b]
			Cyhalothrin	1992, China	Cen (1992)[a] and Zhang (2008)[b]
Coding sequence mutation					
CYP6B7	Pyrethroid	Modulation of voltage-dependent sodium channels	Fenvalerate	1984, Australia	Gunning et al. (1984)[a] and Zhang et al. (2010)[b]

[a]Reference for the first report of resistance
[b]Reference for the proven or strongly suspected resistance mechanism

CYP6B2 and CYP6B6, which genes occur in a tandem array with *CYP6B7* in the genome of *H. armigera* (Grubor and Heckel 2007), were investigated regarding their roles in insecticide resistance (Ranasinghe and Hobbs 1998; Grubor and Heckel 2007; Zhang et al. 2013; Xiao-Ping and Hobbs 1995). However, in 2007, Grubor and Heckel (2007) mapped the *CYP6B* cluster to AFLP linkage group 14, thus rejecting all three P450s as candidates for the semidominant fenvalerate resistance locus *RFen1* which was mapped to AFLP linkage group 13 (Heckel et al. 1998). Therefore, it seems unlikely that CYP6B7 is responsible for the pyrethroid resistance at least of the AN02 strain of Australian *H. armigera*. Also Zhang et al. (2010) reported an overexpression and three non-synonymous single nucleotide polymorphisms (SNPs) of *CYP6B7* in the fenvalerate-resistant Chinese HDFR strain of *H. armigera*. Tang et al. (2012) demonstrated a decrease in resistance to fenvalerate in larvae of the same strain after knockdown of *CYP6B7* and its electron donors NADPH-dependent cytochrome P450 reductase and cytochrome b_5 by RNA interference (RNAi). However, so far no studies were published in which the metabolic capability of the gene product of heterologously expressed *CYP6B7* proves or disproves its involvement in insecticide resistance.

Pittendrigh et al. (1997) found *CYP4G8* being 2.2-fold overexpressed in a fenvalerate-resistant Australian strain of *H. armigera* compared to a susceptible strain. Brun-Barale et al. (2010) reported multiple P450 genes being significantly overexpressed in one Spanish pyrethroid-resistant strain and several strains with different resistance levels from West Africa compared to a susceptible strain, altogether *CYP4L5*, *CYP4L11*, *CYP4M6*, *CYP4M7*, *CYP6AE11*, *CYP9A12*, *CYP9A14*, *CYP332A1*, and *CYP337B1*. No further evidence for the involvement of these P450s in the observed insecticide resistance of *H. armigera* was given.

However, Yang et al. (2008) demonstrated that CYP9A12 and CYP9A14 from the Chinese laboratory-selected pyrethroid-resistant YGF strain of *H. armigera* are capable of metabolizing esfenvalerate after heterologous expression in yeast. In the same year, Zhang (2008) showed that CYP9A12 and CYP9A17(v2) are capable of metabolizing the pyrethroids deltamethrin and cyhalothrin and that CYP9A12 additionally metabolized bifenthrin. Comparable to the majority of studies describing the metabolism by a particular P450 enzyme in a given insect species, in these studies (Zhang et al. 2008; Yang et al. 2008), a decrease in the parent compound was the only evidence of P450 metabolism; no attempt was made to detect and identify metabolites. Nevertheless, it is possible that all three P450s might be involved in pyrethroid resistance in the YGF strain of *H. armigera*.

In the same year, Wee et al. (2008) identified by cDNA-AFLP (in addition to *GSTX01* and *ESTX18*) two P450 genes, *CYP4S1* and *CYP337B1*, constitutively slightly (1.7- and 1.6-fold, respectively) upregulated in resistant individuals of the Australian AN02 strain of *H. armigera*. However, only *CYP337B1* was found to be tightly linked to the fenvalerate resistance locus *RFen1* (Heckel et al. 1998). Recently, Joußen et al. (2012) demonstrated that CYP337B3 but not CYP337B1 is involved in the metabolism of fenvalerate and thus confers resistance to *H. armigera*.

As RT-qPCR studies showing the overexpression of specific P450s or RNAi techniques demonstrating a decreased resistance level after reduction of the mRNA

level of a specific P450 could only hint at the involvement of one or more P450s in the insecticide resistance observed, in vitro metabolism studies with heterologous P450s followed by the determination and identification of metabolites by suitable analytical methods and controls and the overexpression of the resistance candidates in *Drosophila melanogaster* (Daborn et al. 2012) or in the native pest if applicable should be applied to reliably prove the involvement of particular P450 enzymes in the metabolism of insecticides and their role in insecticide resistance. Rasool et al. (2014) demonstrated by a RT-qPCR study comprising 58 P450 genes of *H. armigera* and by a subsequent linkage analysis using AFLPs that none of the P450s found to be overexpressed in the cypermethrin-resistant Pakistani strain FSD compared to a susceptible Australian line—even not the 147-fold overexpressed *CYP340G1*—are linked to cypermethrin resistance in that strain. However, the newly described *CYP337B3* was found to be highly linked to cypermethrin resistance (Rasool et al. 2014).

13.3 A Worldwide Resistance Mechanism by Recombination

The newly described CYP337B3 has an unusual origin: its gene is a chimera that arose by unequal crossing-over between the parental genes *CYP337B1* and *CYP337B2* (Joußen et al. 2012), which occur in a tandem array on chromosome 15 of *H. armigera* and most likely arose by an older gene duplication event. Their corresponding enzymes differ by about 25 % in amino acid sequence. *CYP337B1* and *CYP337B2* are allelic to *CYP337B3* so that homozygous individuals of *H. armigera* possess two copies of *CYP337B1* and *CYP337B2* or two copies of *CYP337B3*. Only heterozygous individuals possess all three genes in one copy each.

CYP337B1, *CYP337B2*, and *CYP337B3* were amplified from cDNA and genomic DNA of the Australian *H. armigera* strain TWB that was collected from the vicinity of Toowoomba, Queensland, in 2003 and is maintained in the laboratory since 2004. By sequencing, three alleles of both *CYP337B1* and *CYP337B2* were identified and one allele of *CYP337B3* (Joußen et al. 2012). *CYP337B3* shares its 5′ end with *CYP337B2* and its 3′ end with *CYP337B1*. Thus the corresponding CYP337B3 enzyme derived its first 177 amino acids including the first conserved region and the substrate recognition site 1 (SRS1) from CYP337B2, whereas the last 315 residues including four conserved regions and five SRSs came from CYP337B1 (Joußen et al. 2012) (Fig. 13.3). In these regions, a few amino acids differ between CYP337B3 and the respective parent enzyme, but except for valine in position 4, they lie within the variation of the CYP337B1 and CYP337B2 allozymes, respectively (Joußen et al. 2012). Thus, it was expected that the metabolic capacity of CYP337B3 would be very similar to that of CYP337B1.

All seven allozymes were heterologously expressed in the insect cell culture Ha2302, which is derived from hemocytes of *H. armigera* larvae, and were functionally characterized (Joußen et al. 2012). Only CYP337B3 was capable of efficiently metabolizing fenvalerate (Joußen et al. 2012), whereas only traces of the

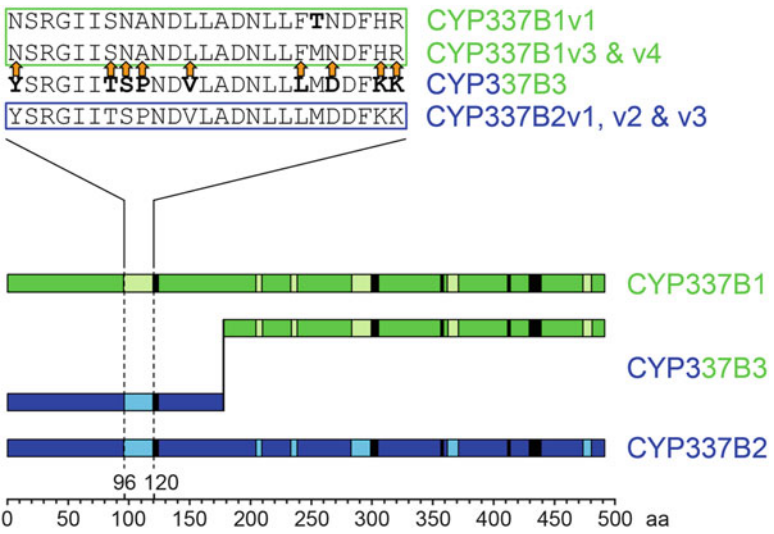

Fig. 13.3 Origin of the chimeric CYP337B3. Schematic diagram of the protein sequences of CYP337B1, CYP337B2, and CYP337B3. The chimeric nature of CYP337B3 is indicated by a *blue box* for the N-terminal part (aa 1-177) derived from CYP337B2 and a *green box* for the C-terminal part (aa 178-492) derived from CYP337B1. Five conserved regions of P450s are marked as black boxes: W-x-x-x-R motif in the C-helix, I-helix groove, E-x-x-R motif in the K-helix, "meander" located after the K′-helix, and heme-binding loop preceding the L-helix (from *left* to *right*). Six substrate recognition sites (SRSs) are highlighted in *light blue* and *light green*, respectively. SRS1 raging from amino acid 96 to 120 is shown in detail to highlight the main differences between the chimeric CYP337B3 and its more similar parent enzyme CYP337B1. Altered amino acids between the CYP337B1 allozymes and CYP337B3 are marked in bold type and are highlighted with *arrows* (Joußen et al. 2012)

metabolite were detected in in vitro metabolism assays with CYP337B1 and no metabolites were detectable in the case of CYP337B2. The metabolite was isolated and identified by gas chromatography coupled with mass spectrometry (GC-MS) and by nuclear magnetic resonance spectrometry (NMR) as 4′-hydroxyfenvalerate (Joußen et al. 2012).

To prove that the metabolism of fenvalerate by CYP337B3 is the resistance mechanism in vivo, the polymorphic TWB strain was separated into two lines by directed breeding. Therefore, the individuals were genotyped by PCR using specific primers for *CYP337B1* and *CYP337B2* located around the crossing-over position to detect the parent genes and a combination of the forward primer specific for *CYP337B2* and the reverse primer specific for *CYP337B1* to detect the chimeric *CYP337B3* (Joußen et al. 2012). Individuals possessing exclusively *CYP337B1* and *CYP337B2* were used to establish the *CYP337B1-CYP337B2* line and individuals possessing exclusively *CYP337B3* to establish the *CYP337B3* line (Joußen et al. 2012). Individuals from both lines and from crosses between these lines producing heterozygous individuals possessing all three P450s were tested

by topical application for their resistance level against fenvalerate. This bioassay clearly demonstrated that individuals carrying only *CYP337B3* were 42-fold more resistant against fenvalerate than individuals carrying *CYP337B1* and *CYP337B2* (Joußen et al. 2012). The resistance factor is comparable to that of 49 found by Forrester et al. (1993) in Australian field populations. Individuals carrying all three genes were 12-fold more and thus intermediate resistant (Joußen et al. 2012). This can be explained by the fact that heterozygous individuals with only one copy of *CYP337B3* compared to homozygous *CYP337B3* individuals possessing two copies of *CYP337B3* will produce only about half of the amount of CYP337B3 protein. Additional evidence for the hydroxylation of fenvalerate by CYP337B3 being the resistance mechanism in vivo is the fact that almost no toxicity of the metabolite 4′-hydroxyfenvalerate on susceptible *CYP337B1-CYP337B2* larvae was detectable (Joußen et al. 2012). Most likely 4′-hydroxyfenvalerate will be glycosylated both in susceptible and resistant individuals of *H. armigera*, thus increasing even more the hydrophilicity of the lipophilic and almost water insoluble insecticide and facilitating its excretion.

The differences in metabolic capacity of CYP337B3 and its more similar parent CYP337B1 are most likely caused by important regions of the N-terminal part of the enzymes that are differing between them. As mentioned before, this region comprises one conserved region and the SRS1 that CYP337B3 shares with CYP337B2. There are no amino acid substitutions in the conserved region between all CYP337B allozymes, but the SRS1 differs by 9–10 amino acids between CYP337B3 and CYP337B1 (Joußen et al. 2012) (Fig. 13.3). In docking models the amino acids threonine 102, serine 103, and leucine 114 located in the SRS1 of CYP337B3 and differing between CYP337B3 and CYP337B1 lay within a distance of 4.5 Å of the ligand fenvalerate, suggesting that one or more of the altered amino acids in SRS1 are crucial for fenvalerate recognition and binding in CYP337B3, thus explaining the differences in the metabolic capacity of CYP337B3 and CYP337B1 (Joußen et al. 2012).

It is assumed that *H. armigera* possesses different resistance mechanisms in different parts of the world based on differing patterns of overexpressed P450s between geographically distinct strains (Brun-Barale et al. 2010). However, the chimeric *CYP337B3* is not restricted to Australia but was also detected in the cypermethrin-resistant FSD strain from Pakistan (Rasool et al. 2014). Sequence analysis revealed 18 synonymous and three non-synonymous SNPs between the Pakistani and the Australian *CYP337B3* gene determining the Pakistani sequence as a new allele, *CYP337B3v2* (GenBank: KJ636466), which enzyme differs by three amino acids located in the C-terminus from the Australian CYP337B3v1 (GenBank: JQ284029, JQ995292) (Rasool et al. 2014). A more thorough nucleotide sequence analysis discovered a striking similarity between the *CYP337B1* part of the Pakistani *CYP337B3v2* allele and the Australian *CYP337B1v4* allele (GenBank: JQ284025), whereas the *CYP337B1* part of the Australian *CYP337B3v1* is more similar to the Australian *CYP337B1v1* (GenBank: JQ284023, JQ995291) or *CYP337B1v3* (GenBank: JQ284024) allele (Rasool et al. 2014). Furthermore, the sequence of the Pakistani *CYP337B3v2* resembles the sequence of *CYP337B2* (GenBank:

Table 13.2 Sequence identities between the introns of the *CYP337B1* alleles and these of the *CYP337B3* alleles of *Helicoverpa armigera*

Intron sequence identity	*CYP337B1v1* (921 bp)	*CYP337B1v3* (921 bp)	*CYP337B1v4-like* (1,060 bp)
CYP337B3v1 (921 bp)	99 %	99 %	85 %
CYP337B3v2 (1,017 bp)	86 %	86 %	92 %

JQ995291) over a longer range than the Australian *CYP337B3v1* does, indicating a possible shift of the unequal crossing-over position few nucleotides upstream of the position proposed for the Australian *CYP337B3v1* (Rasool et al. 2014; Joußen et al. 2012). Additionally, the length (Table 13.2) and the sequence of the intron differ between both *CYP337B3* alleles (Rasool et al. 2014). As the intron is located in the heme-binding motif in the *CYP337B1* derived part of *CYP337B3*, the intron should also resemble that of the corresponding *CYP337B1* allele. A comparison of the introns of the *CYP337B3* alleles with these of the different *CYP337B1* alleles is given in Table 13.2. Because of the lack of genomic DNA of the *CYP337B1v4* allele of the TWB strain, its intron sequence remains unidentified. However, it was possible to determine the genomic sequence of a *CYP337B1v4-like* gene from the Australian AN02 strain that exhibits in its coding sequence 18 synonymous and three non-synonymous SNPs compared to *CYP337B1v4* resulting in a sequence identity of 99 %. The high sequence identities between the Australian *CYP337B3v1* and the *CYP337B1v1/v3* alleles on the one hand and that between the Pakistani *CYP337B3v2* and the *CYP337B1v4-like* allele on the other hand (Table 13.2) in combination with the SNPs described in the coding sequence and the putative position of the crossing-over event support the hypothesis that both CYP337B3 alleles arose independently by unequal crossing-over between *CYP337B2* and two different *CYP337B1* alleles (Rasool et al. 2014).

Recently, Han et al. (2015) described two more *CYP337B3* alleles, *CYP337B3v3* and *v4*, at low frequencies from Chinese *H. armigera* populations which presumably also arose by independent unequal crossing-over events, resulting in non-synonymous SNPs, altered crossing-over positions, or different intron length compared to *CYP337B3v2* that is the major allele in China. Thus, *CYP337B3* seems to be a more common resistance mechanism than previously expected. However, Han et al. (2015) found no correlation between the resistance level against fenvalerate ranging from 256- to 1182-fold of field-collected strains and the allele frequency of *CYP337B3* varying from 96 to 100 % (Han et al. 2015). The resistant strains were compared to the SCD strain possessing no *CYP337B3* that was collected from Côte d'Ivoire in the 1970s provided by Bayer CropScience. The LD_{50} value—specifying the dose at which 50 % of the individuals of a specific population would die—was determined as 0.012 μg per larva for the SCD strain (Han et al. 2015), thus characterizing this strain as more susceptible than the before mentioned susceptible Australian *CYP337B1-CYP337B2* line with an LD_{50} value of 0.044 μg per larva (Joußen et al. 2012). Because the resistance factor is calculated by the division of the LD_{50} of the resistant strain by the LD_{50} of the susceptible

strain, either an increased LD_{50} of the resistant strain or a decreased LD_{50} of the susceptible strain will lead to an increased resistance factor. Consequently, the resistance ratio of the resistant Australian *CYP337B3* line would increase from 42- to 155-fold when it is compared to the SCD strain instead of the susceptible Australian *CYP337B1-CYP337B2* line, thus shifting its resistance level to the range of the Chinese field-collected strains described. Han et al. (2015) also claim that *CYP337B3* has a fitness cost leading to a decrease of the *CYP337B3* frequency during rearing of some field-collected strains without insecticide pressure, but this hypothesis is unproven so far. Actually, in three out of four strains, the *CYP337B3* frequency remained above 50 % (Han et al. 2015), thus contradicting a fitness cost.

The metabolic capacity of the CYP337B3 allozymes v1 and v2 is very similar with both enzymes being capable of hydroxylating cypermethrin in the 4′-position (Rasool et al. 2014) as described before for fenvalerate (Joußen et al. 2012). A minor metabolite remained unidentified. Toxicity bioassays revealed that both CYP337B3 allozymes contribute six- to sevenfold resistance to the cypermethrin resistance observed in *H. armigera* (Rasool et al. 2014). Also 4′-hydroxycypermethrin like 4′-hydroxyfenvalerate is not intrinsically toxic for susceptible larvae (Rasool et al. 2014).

Ongoing studies on this impressive chimeric CYP337B3 will identify amino acids that discriminate the metabolic capacity of CYP337B3 from that of its parent CYP337B1, thus explaining the important achievement of recombination that created this resistance gene. Furthermore, ongoing investigations of field populations of *H. armigera* regarding the frequencies of *CYP337B3* and its parents *CYP337B1* and *CYP337B2* using the described PCR screening system will give a full picture of the dimension of this new resistance mechanism and potentially also will elucidate its evolutionary history.

13.4 Status of the Knowledge About the Resistance Mechanisms of *H. armigera*

This review shows that so far only a few resistance mechanisms of *H. armigera* are understood. Most mechanisms described here deal with pyrethroid insecticides, but *H. armigera* is resistant to many different insecticides distributed in eight classes as described in Sect. 1. Even for pyrethroid resistance, only the tip of the iceberg has been seen. Han et al. (2015) and Rasool et al. (2014) pointed out that CYP337B3, even though it is clearly involved in the pyrethroid resistance of *H. armigera*, seems not to be the only player in the resistance to the pyrethroids fenvalerate and cypermethrin, respectively, in the strains studied. This also indicates that first, different mechanisms might be involved in the resistance of *H. armigera* toward even one insecticide and that, second, the relative contribution of the different mechanisms might vary between different populations as mentioned by Brun-Barale et al. (2010).

In conclusion, more research on this important crop pest is needed to discover additional resistance mechanisms that will provide the basis for new approaches in the management of *H. armigera*.

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Chapter 14
Advances in Managing Pest Resistance to Bt Crops: Pyramids and Seed Mixtures

Yves Carrière, Jeffrey A. Fabrick, and Bruce E. Tabashnik

Abstract Transgenic crops producing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) have been widely used for the control of insect pests during the last 20 years. Although Bt crops have provided significant environmental and economic benefits, sustainable use of these crops is threatened by the rapid evolution of resistance. The primary strategy for delaying pest adaptation to Bt crops has been to ensure that sufficient refuges of non-Bt host plants occur near Bt crops. Two relatively new approaches used with refuges are "pyramids", which are plants that produce two or more Bt toxins effective against the same pest, and planting random mixtures of Bt seeds and non-Bt seeds of the same crop within fields. Here we review theory and data about conditions favoring success of pyramids and seed mixtures for delaying evolution of pest resistance to Bt crops. Pyramids of structurally distinct toxins can be exceptionally effective under optimal conditions, particularly when pest populations are highly susceptible to all toxins in the pyramid. Seed mixtures eliminate the problem of farmers who fail to plant separate refuges of non-Bt plants, but may accelerate evolution of resistance when larval movement between plants or pollen-mediated gene flow between plants is extensive. In the many cases where pests are not highly susceptible to the toxins in Bt crops or other conditions are not optimal, we suggest that an effective refuge percentage of at least 20 % is required to substantially delay pest resistance, even when pyramids, seed mixtures, or both are used. We also recommend integrating Bt crops with other management tactics to delay resistance in pests with low susceptibility to Bt toxins.

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14.1 Type and Use of Bt Crops

Insecticidal crystalline (Cry) and vegetative (Vip) proteins from the soil bacterium *Bacillus thuringiensis* (Bt) are currently produced in virtually all crops genetically engineered for control of insect pests. The first Bt crops, Bt corn and Bt cotton, were introduced in the United States 20 years ago. In 1996, cotton-producing Cry1Ac was commercialized for improving control of the major lepidopteran pests *Heliothis virescens, Helicoverpa zea* and *Pectinophora gossypiella*, and corn producing the similar toxin Cry1Ab targeted *Ostrinia nubilalis* and *H. zea* (EPA 2001). Because Bt toxins have a narrow range of target specificity, these single-toxin crops did not affect corn rootworms (*Diabrotica* spp.) that are key coleopteran pests of corn in the USA (Gray et al. 2009) nor provide adequate control of several major lepidopteran pests of corn and cotton such as *Spodoptera frugiperda* and *H. zea*. To broaden and improve control of pests, Bt cotton and Bt corn producing more than one toxin were introduced in the USA 6 and 7 years after commercialization of single-toxin crops, respectively (Tabashnik et al. 2009; Carrière et al. 2015). For example, relative to non-Bt cotton, survival of *H. zea* was 56 % on cotton producing Cry1Ac and 4 % on cotton producing both Cry1Ac and Cry2Ab in North Carolina (Jackson et al. 2004). To delay evolution of resistance by pests, pyramided Bt crops produce two or more toxins that kill the same pest (Carrière et al. 2015).

Use of Bt crops has expanded considerably since their introduction. The cumulative worldwide total of Bt crops planted from 1996 to 2014 was 648 million ha, with corn and cotton by far the most abundant Bt crops planted to date (James 2014). Bt soybean was planted in Brazil in 2013 and 2014 on a cumulative total of 7.4 million ha and Bt eggplant was commercialized on a small scale in Bangladesh in 2014 (James 2014). In 2015, Bt corn accounted for 81 % of corn and Bt cotton 84 % of cotton in the USA (USDA ERS 2015).

14.2 Environmental and Economic Impacts of Bt Crops

Agricultural intensification based on increased inputs and breeding of high-yielding crops has generally been successful for increasing food and fiber availability (Godfray et al. 2010; Ray et al. 2012). Nevertheless, intensive agriculture also has negative consequences, such as increased pest pressure and use of insecticides (Meehan et al. 2011; Osteen and Fernandez-Cornejo 2013). Bt crops can increase or stabilize yield while reducing some of the negative impacts of agricultural intensification.

Yield gains provided by Bt crops are variable (Carpenter 2010; Shi et al. 2013). Data from farmer surveys indicate that Bt corn increased yield by 4 % (range −3 to 13 %) and Bt cotton by 7 % (range −8 to 26 %) in developed countries (Carpenter 2010). Consistent with these findings, 77–79 % of US producers stated that higher yield is their primary reason for growing Bt corn and Bt cotton instead of non-Bt alternatives (Fernandez-Cornejo et al. 2014). Across years and environments, yield

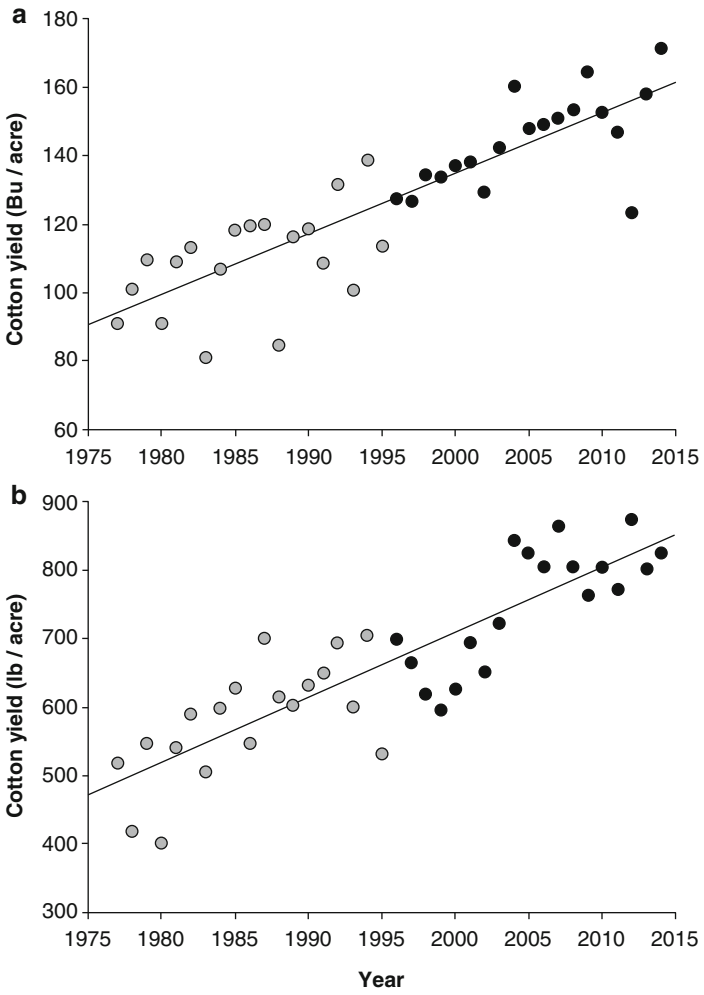

Fig. 14.1 Yield of (**a**) corn and (**b**) cotton 19 years before (1976–1995: *grey circles*) and after (1996–2014: *black circles*) commercialization of Bt crops in the U.S. (Data from USDA NASS 2015). The rate of yield improvement for corn or cotton (i.e., slope of the lines) did not differ significantly between these periods (covariance analysis, P values > 0.53), indicating that genetic engineering alone did not change the rate of yield improvement for these crops

was less stable for non-Bt corn than for Bt corn producing one or more toxins (Edgerton et al. 2012; Shi et al. 2013). Because pest pressure is often greater and pest management less effective in developing than developed countries, yield increases with Bt corn and Bt cotton have been higher in developing than developed countries (Carpenter 2010; Klümper and Quaim 2014).

Yield of corn and cotton has increased regularly during the last 40 years in the USA (Fig. 14.1). Nevertheless, genetic engineering alone did not affect the rate of yield improvement in corn and cotton (Fig. 14.1), indicating that changes in

biophysical or socio-economic factors occurring during the last 20 years in the USA (Ray et al. 2012) may have countered the positive impacts of Bt crops on yield. Crop breeding, changes in agronomic practices and biotechnology have contributed to yield improvement (Duvick 2005). Corn hybrids and cotton cultivars are not bred for yield improvement and other traits during the introgression of Bt traits, commercial seed production and after commercialization. Accordingly, newly commercialized Bt crops soon lag behind in terms of yield relative to continually improved hybrids and cultivars. This results in a high turnover rate of Bt crop varieties (ca. 7–8 years) in the USA (Edgerton 2009).

Relative to non-Bt crops, single-toxin and multi-toxin Bt corn and cotton can reduce use of conventional insecticides (Cattaneo et al. 2006; Hunt et al. 2007) and such reductions tend to be more important in developing than developed countries (Fernandez-Cornejo et al. 2014; Klümper and Quaim 2014). An analysis by Osteen and Fernandez-Cornejo (2013) shows the following trends in the USA between 1995 and 2010: The percentage of all corn hectares planted to Bt corn grew from 0 to 63 % and the percentage of all cotton hectares planted to Bt cotton grew from 0 to 73 %; the percentage of hectares treated with insecticides declined from 26 to 12 % for all corn and from 75 to 55 % for all cotton; the amount of insecticide active ingredients decreased by 88 % for corn and 66 % for cotton. These pesticide-use patterns were influenced by deployment of Bt crops but also by other factors, such as improvement of pest management and introduction of novel, low-rate insecticides (NRC 2010; Osteen and Fernandez-Cornejo 2013).

Relative to most insecticide sprays, Bt crops have less toxicity to arthropods not closely related to target pests (Cattaneo et al. 2006; Wolfenbarger et al. 2008; NRC 2010). Accordingly, reduced insecticide use associated with Bt crops can have favorable effects on beneficial arthropods and integrated pest management (Naranjo 2010; Lu et al. 2012). However, reduced insecticide use associated with Bt crops can sometimes increase the abundance of pests not killed by Bt crops that were previously controlled by insecticides (Lu et al. 2010; Naranjo 2010). This can lead to additional insecticide sprays targeting the pests not killed by Bt crops, thereby decreasing or eliminating one of the advantages of Bt crops.

In some cases where Bt crops are sufficiently abundant, the net reproductive rate of female pests targeted by Bt crops can be reduced enough to cause regional declines in pest abundance (Carrière et al. 2003). Regional declines in pest populations following deployment of Bt crops have been documented in several key pests of corn and cotton (Carrière et al. 2010; Naranjo 2010), including *P. gossypiella* in the USA and China (Carrière et al. 2003; Huang et al. 2013), *Helicoverpa armigera* in China (Wu et al. 2008) and *O. nubilalis* in the USA (Hutchison et al. 2010). Regional pest declines can increase yield and reduce the need for insecticides (Wu et al. 2008; Hutchison et al. 2010). For example, based on yield gains resulting from regional suppression of *O. nubilalis* by Bt corn over a period of 14 years, economic benefits were estimated to be >$4.3 billion for non-Bt corn hectares and $2.6 billion for Bt corn hectares in five Midwestern states of the USA (Hutchison et al. 2010). An eradication program based on the use of nearly 100 % Bt cotton together with mass releases of sterile moths in all cotton

fields and other tactics has virtually eliminated *P. gossypiella* from Arizona and has stopped use of insecticides targeting this pest (Tabashnik et al. 2010). The goal of this program, which entails collaboration between cotton growers, academics and state and federal agencies, is to eradicate this introduced pest from all cotton-growing areas of the USA and adjacent areas of northern Mexico (Naranjo 2010; Tabashnik et al. 2010).

Because yield gains and reduced use of insecticides in Bt corn and Bt cotton are often sufficient to compensate for technology fees, these transgenic crops can increase farmer profits (Fernandez-Cornejo et al. 2014; Klümper and Quaim 2014). However, environmental and economic benefits resulting from the use of Bt crops can be rapidly reduced or eliminated by the evolution of insect pest resistance to Bt toxins (Tabashnik et al. 2013).

14.3 Evolution of Resistance to Bt Crops

Field-evolved resistance to a Bt toxin is defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure to the toxin in the field (Tabashnik et al. 2013, 2014). Field-evolved resistance involves a statistically significant increase in the frequency of one or more alleles conferring resistance to Bt toxins but does not necessarily imply failure of a Bt crop (Tabashnik et al. 2013, 2014). Recognizing that resistance is not "all or none" and that various levels of resistance can have a continuum of effects on pest control, five categories of field-evolved resistance to Bt crops have been described (Tabashnik et al. 2014; Tabashnik and Carrière 2015). All five categories entail a statistically significant, genetically based decrease in susceptibility in field populations of pests, but only one category (practical resistance) indicates resistance is severe enough to generate reports of reduced pest control in the field. The five categories are: (1) incipient resistance: <1 % resistant individuals, (2) early warning of resistance: 1 to 6 % resistant individuals, (3) >6 % to 50 % resistant individuals, (4) >50 % resistant individuals and reduced efficacy expected but not reported, and (5) practical resistance: >50 % resistant individuals and reduced efficacy reported.

In a recent analysis of peer-reviewed publications, 12 of 27 cases examined (44 %) showed no significant increase in resistance after 2–15 years (median = 8 years) of exposure to Bt crops (Tabashnik and Carrière 2015). Of the remaining 15 cases, three were incipient resistance, four were early warning of resistance, one was >50 % resistant individuals with reduced efficacy expected but not reported, and seven demonstrated practical resistance. All seven cases of practical resistance involve resistance to single-toxin crops (Table 14.1).

Field-evolved resistance to Cry2Ab, which has been used in Bt crops only in combination with one or more other Bt toxins, has been reported as category 4 (i.e., >50 % resistant individuals and reduced efficacy expected but not reported) for *H. zea* in the United States (Tabashnik et al. 2009, 2013, 2014). The decreased susceptibility to Cry2Ab was first detected in 2005, when cotton producing this

Table 14.1 Seven cases of field-evolved practical resistance to single-toxin Bt crops (From Carrière et al. 2016)

Insect	Bt crop	Toxin	Country	Durability (years)[a]	Initial detection[b]
Helicoverpa zea	Cotton	Cry1Ac	USA	6	2002
Busseola fusca	Corn	Cry1Ab	South Africa	6	2004
Spodoptera frugiperda	Corn	Cry1Fa	USA	3	2006
Pectinophora gossypiella	Cotton	Cry1Ac	India	6	2008
Diabrotica virgifera virgifera	Corn	Cry3Bb	USA	6	2009
Diabrotica v. virgifera	Corn	mCry3A	USA	4	2011
Spodoptera frugiperda	Corn	Cry1Fa	Brazil	2	2011

[a]Years elapsed in the region studied between the first year of commercial use and the first year of field observations or sampling that yielded evidence of practical resistance

[b]First year of field sampling that provided evidence of practical resistance; publication of this evidence often occurred several years later. For example, evidence of *S. frugiperda* resistance to Cry1Fa in Brazil was published first in 2014 based on bioassay data from progeny of insects sampled from the field in 2011 (Farias et al. 2014)

toxin was uncommon (Tabashnik et al. 2013). This suggests that resistance to Cry1Ac caused some cross-resistance to Cry2Ab, which is consistent with data showing cross-resistance between these two toxins (Tabashnik et al. 2013; Carrière et al. 2015; Welch et al. 2015). In contrast, despite ≥85 % adoption of Bt cotton producing Cry2Ab and Cry1Ac in Australia since 2005, 8 years of monitoring data from the robust F_1 screen method show no significant increase in the frequency of resistance to Cry2Ab for either *H. armigera* (0.032 in 2007–08 to 0.021 in 2014–15) or *H. punctigera* (0.010 in 2007–2008 to 0.011 in 2014–2015) (Downes 2015; Tabashnik 2015). These results demonstrate that incipient resistance, which involved a statistically significant increase over time in the frequency of resistance to Cry2Ab in *H. punctigera* (Downes et al. 2010), does not always indicate that further increases in resistance are imminent (Tabashnik et al. 2013).

14.4 The Need for Resistance Management

The primary strategy used to delay pest resistance to both single-toxin and pyramided crops in the USA and elsewhere is the refuge strategy (Tabashnik et al. 2013; Carrière et al. 2015). Refuges are host plants that do not produce Bt toxins and promote survival of pests that are susceptible to Bt toxins (Fig. 14.2). Laboratory and greenhouse experiments, large-scale studies, and retrospective comparisons of patterns of field-evolved resistance show that refuges can delay resistance (Zhao et al 2005; Carrière et al. 2012; Tabashnik et al. 2013; Jin et al. 2015). Results from mathematical models indicate that, under some conditions, pyramids can delay resistance much more effectively than single-toxin crops (Roush 1998; Onstad and Meinke 2010; Tabashnik and Gould 2012). Nevertheless, both single-toxin and pyramided crops are vulnerable to resistance evolution, especially when conditions

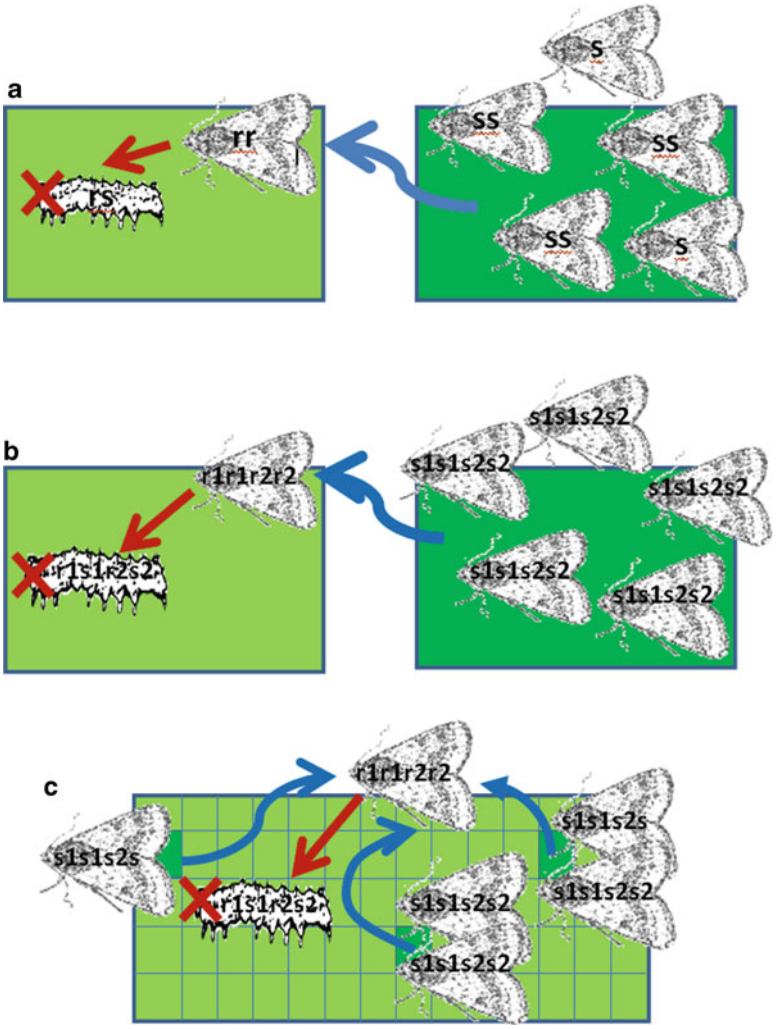

Fig. 14.2 The refuge strategy for delaying insect resistance to (**a**) single-toxin or (**b**) pyramided Bt crops with structured refuges, and (**c**) seed mixtures of pyramided Bt crops and non-Bt crops. In (**a**) and (**b**), each field contains either Bt plants (*light green*) or non-Bt plants (*dark green*). In (**c**), Bt plants (*light green squares*) and non-Bt plants (*dark green squares*) are randomly distributed within each field. Under ideal conditions, alleles conferring resistance (r) to Bt crops are rare and resistance is recessive so that heterozygotes carrying one allele for resistance and another for susceptibility (s) are killed by Bt crops. In principle, the relatively abundant homozygous susceptible moths (ss in A and s1s1s2s2 in B and C) in refuges mate with the rare homozygous resistant moths (rr in A and r1r1r2r2 in B and C; pointed to by blue arrows) surviving on Bt crops. The resulting offspring (caterpillars pointed to by red arrows) are heterozygous for resistance (rs in A and r1s1r2s2 in B and C) and are killed by Bt crops (red crosses), which delays evolution of resistance (From Carrière et al. 2016)

in the field differ from the ideal conditions assumed in some modeling studies (Carrière et al. 2010, 2015; Ives et al. 2011; Tabashnik and Gould 2012; Brévault et al. 2012, 2013; Devos et al. 2013; Santos-Amaya et al. 2015). Below we summarize recent developments in resistance management for Bt crops, focusing on refuges used together with Bt crop pyramids and planting random mixtures of Bt and non-Bt seeds.

14.5 Conditions Favoring Durability of Bt Crops

Five conditions promote the durability of both single-toxin and pyramided crops: (1) sufficient refuges are present, (2) alleles conferring resistance are rare, (3) resistance is recessive, (4) fitness costs are associated with resistance, and (5) resistance is incomplete (Tabashnik et al. 2013; Carrière et al. 2015). Fitness costs occur when fitness on non-Bt plants is lower for individuals with alleles conferring resistance than for individuals lacking such resistance alleles (Gassmann et al. 2009). Incomplete resistance occurs when resistant individuals have lower fitness on a Bt crop than on the corresponding non-Bt crop (Carrière and Tabashnik 2001; Carrière et al. 2010; Tabashnik et al. 2014). Retrospective analyses show that all cases of field-evolved practical resistance to single-toxin crops involve substantial deviations from one or more of the first three conditions (Tabashnik et al. 2013, 2014; Tabashnik and Carrière 2015). Furthermore, previous reviews concluded that fitness costs associated with resistance and incomplete resistance can increase the durability of Bt crops (Gassmann et al. 2009; Carrière et al. 2010; Onstad and Carrière 2014).

Here we review theory and evidence about three additional conditions that are important for the durability of Bt crop pyramids: (1) each toxin in the pyramid can kill all or nearly all susceptible insects, (2) no cross-resistance occurs between toxins in the pyramid, and (3) pyramids are not grown concurrently with single-toxin plants that produce one of the toxins in the pyramid (Zhao et al. 2005; Brévault et al. 2013; Tabashnik et al. 2013; Carrière et al. 2015). Conditions 1 and 2 favor redundant killing, which occurs when an insect resistant to one toxin produced by a pyramid is killed by another toxin produced by the pyramid (Brévault et al. 2013). If the concentration of each toxin in a pyramid is high enough to kill all susceptible insects and no cross-resistance occurs between toxins, complete redundant killing occurs because only individuals with alleles conferring resistance to all toxins in the pyramid will survive on the pyramid (Carrière et al. 2015).

The extent of redundant killing can be quantified using the redundant killing factor (RKF) = 1 – [(proportion survival on pyramid for insects homozygous resistant to one toxin) – (proportion survival on pyramid for insects homozygous susceptible to both toxins)] (Brévault et al. 2013). RKF varies from 0 (no redundant killing) to 1 (complete redundant killing), with values markedly lower than 1 projected to substantially accelerate the evolution of resistance (Brévault et al. 2013). In an analysis based on survival of three pests on different types of pyramids ($n = 12$ cases), RKF ranged between 0.81 and 1 (Carrière et al. 2015).

14.5.1 Each Toxin in a Pyramid Can Kill All or Nearly All Susceptible Insects

Results from a mathematical model indicate that the concentration of each toxin of a two-toxin pyramid must be high enough to kill at least 95 % of susceptible individuals for pyramids to be most effective (Roush 1998). Assuming that each toxin acts independently, two-toxin pyramids are thus expected to be most effective when they kill at least 99.75 % of susceptible insects (Carrière et al. 2015). In an analysis of nine pest-pyramid combinations, observed mortality on pyramids met this criterion in only half of the 18 observations (Carrière et al. 2015). Cases with <99.75 % mortality on pyramids relative to their non-Bt crop counterparts include *H. zea* and *H. armigera* on Cry1Ac + Cry2Ab cotton, sugarcane borer (*Diatraea saccharalis*) on Cry1A.105 + Cry2Ab + Cry1Fa corn, and western corn rootworm (*Diabrotica virgifera virgifera*) on Cry3Bb + Cry34/35Ab corn (Tabashnik and Gould 2012; Head et al. 2014a). These data indicate that mortality of susceptible insects on pyramids may often be too low for pyramids to be most effective. Across 18 cases, a significant negative association occurred between survival of susceptible insects on pyramids and RKF, showing that redundant killing generally declines as survival of susceptible insects on pyramids increases (Carrière et al. 2015).

It was proposed that assessment of relative mortality on pyramids in *D. v. virgifera* should consider that density-dependent mortality increases on non-Bt plants relative to Bt plants when larval population density is high (Hibbard et al. 2010). In five field experiments using natural infestations of *D. v. virgifera*, average relative mortality on eCry3.1Ab + mCry3A corn was 99.91 % (Hibbard et al. 2011). In six experiments unlikely to have been affected by density-dependent mortality because low densities of *D. v. virgifera* eggs were used to infest blocks of non-Bt and Cry3Bb + Cry34/35Ab corn, average relative mortality was 99.32 % (Head et al. 2014a). These data indicate that mortality of susceptible insects on pyramids may often be too low for pyramids to substantially delay evolution of resistance with small refuges (e.g., 5 or 10 % of the total area planted to Bt and non-Bt corn).

14.5.2 Cross-Resistance Between Toxins in the Pyramid Is Absent

Cross-resistance occurs when selection for resistance to a toxin causes resistance to a different toxin (Tabashnik et al. 2014), which can accelerate the evolution of resistance to pyramids (Caprio 1998). Strong cross-resistance between toxins consistently reduces redundant killing because individuals resistant to one toxin can also survive exposure to one or more other toxins in the pyramid. However, weak cross-resistance reduces redundant killing only for insects that do not have high

inherent susceptibility to the toxins in a pyramid. In cases where the concentration of each toxin substantially exceeds what is needed to kill susceptible insects, the slight decrease in their susceptibility caused by weak cross-resistance is not sufficient to increase their survival on the pyramid (Tabashnik et al. 2002). Thus, weak cross-resistance in such pests is not expected to accelerate evolution of resistance to pyramids. In contrast, weak cross-resistance is expected to accelerate evolution of resistance in pests with inherently low susceptibility to Bt toxins, because in such pests even some susceptible individuals survive on pyramids, implying that weak cross-resistance will contribute to survival on pyramids and accelerate the evolution of resistance (Carrière et al. 2010, 2015; Brévault et al. 2013; Welch et al. 2015).

A recent analysis of 80 cases involving 10 major pests and 7 sets of Bt toxins showed that cross-resistance between toxins used in pyramids is widespread (Carrière et al. 2015). This analysis considered related strains of pests selected or not with a toxin in the laboratory and subsequently tested for cross-resistance to other toxins used in pyramids. For each pair of strains, cross-resistance ratios were calculated for toxins not used for selection, by dividing the LC_{50} or IC_{50} (concentration killing or inhibiting growth of 50 % of tested insects, respectively) for the selected strain by the LC_{50} or IC_{50} of the unselected strain. This ratio is expected to be 1 without cross-resistance and >1 with cross-resistance. The cross-resistance ratio was >1 in 75 of the 80 cases (Carrière et al. 2015). Furthermore, for five of the seven sets of toxins examined (Cry1Aa and Cry1Ab; Cry1Aa and Cry1Ac; Cry1Ab and Cry1Ac; Cry1Ab or Cry1Ac and Cry1Fa; Cry1Ac or Cry1Ab and Cry2Ab), the average cross-resistance ratio was significantly greater than 1, demonstrating significant cross-resistance between toxins in these sets (Carrière et al. 2015). For two pairs of toxins (Cry1Ac and Cry2Aa; Cry1Ac and Vip3Aa), the average cross-resistance ratio was greater than 1, but statistical significance was marginal (Carrière et al. 2015). However, a subsequent analysis based on more data showed significant, but weak cross-resistance between Cry1A and Cry2A as well as between Cry1Ac and Vip3Aa (Welch et al. 2015).

Overall, the data indicate that cross-resistance is pervasive between toxins currently used in pyramids. The weak cross-resistance between Cry1A toxins and Cry2A or between Cry1Ac and Vip3Aa is most likely to reduce durability of pyramids only against pests that have low inherent susceptibility to these Bt toxins such as *Helicoverpa* species (Carrière et al. 2015; Welch et al. 2015).

14.5.3 Pyramids Are Not Grown Concurrently with Plants That Produce One of the Toxins in the Pyramid

Mathematical models and laboratory and greenhouse experiments indicate that resistance to pyramids evolves faster when single-toxin plants that produce one of the toxins in the pyramid co-occur with two-toxin plants (Zhao et al. 2005; Gould et al. 2006; Onstad and Meinke 2010; Santos-Amaya et al. 2015). For example, a strain of *S. frugiperda* with field-evolved practical resistance to Cry1Fa corn rapidly

evolved resistance to Cry1A.105 + Cry2Ab corn when selected for resistance in the laboratory (Santos-Amaya et al. 2015). Cross-resistance between the closely related toxins Cry1Fa and Cry1A.105 in *S. frugiperda* reduced the capacity of Cry1A.105 to kill insects resistant to Cry2Ab, which likely accelerated evolution of resistance to this pyramid (Santos-Amaya et al. 2015).

Because single-toxin crops can act as stepping-stones for resistance to pyramids, rapid replacement of single-toxin crops by pyramids benefits resistance management. For example, replacement of Cry1Ac cotton by Cry1Ac + Cry2Ab cotton was accomplished in a single year (2004) in Australia (Downes and Mahon 2012) and the percentage of resistant individuals remained <1 % for each toxin in both of the key target pests, *H. armigera* and *H. punctigera*, more than a decade after the pyramid was introduced (Downes 2015). In contrast, replacement of single-toxin cotton producing Cry1Ac by two-toxin cotton producing either Cry1Ac + Cry2Ab or Cry1Ac + Cry1Fa took eight years in the USA (Brévault et al. 2013) and was started after practical field-evolved resistance to Cry1Ac had occurred in the related pest *H. zea* (Tabashnik et al. 2009, 2013). Less than 3 years after the pyramid was introduced, the percentage of individuals resistant to Cry2Ab was >50 % in some populations of *H. zea* (Tabashnik et al. 2009, 2013). In India, replacement of Cry1Ac cotton by Cry1Ac + Cry2Ab cotton was still not completed after 9 years (Choudhary and Gaur 2015), increasing the risk that populations of *P. gossypiella* already resistant to Cry1Ac would rapidly evolve resistance to Cry2Ab (Dhurua and Gujar 2011). Indeed, practical resistance to Cry1Ac + Cry2Ab cotton was recently observed in the state of Gujarat (Kurmanath 2015). Replacement of Cry1Ac cotton by pyramided Bt cotton has not been initiated in China, despite the small but significant increase in *H. armigera* resistance to Cry1Ac from 2002 to 2013 (Jin et al. 2015; Gao et al. 2015).

Commercial release of three-toxin pyramided cotton such as Cry1Ac + Cry2Ab + Vip3Aa is anticipated for 2016 in Australia and the USA (Mahon et al. 2012; Carrière et al. 2015). This three-toxin pyramid is expected to be especially durable in Australia, where the frequency of resistance to all three toxins is relatively low in *H. armigera* and *H. punctigera* (Downes 2015). However, in some US populations of *H. zea* already resistant to Cry1Ac and Cry2Ab, the risk of resistance to this three-toxin cotton is high because it will function as a single-toxin crop. Similarly, the risk of rapid resistance to Cry1A.105 + Cry2Ab corn in *S. frugiperda* in Brazil is high because this pyramid is used remedially to counter practical field-evolved resistance to Cry1Fa, which is closely related to Cry1A.105 (Santos-Amaya et al. 2015).

Single-toxin corn hybrids targeting lepidopterans, coleopterans, or both are presently used concurrently with pyramided Bt corn hybrids in the USA (Table 14.2). Furthermore, some of these pyramids targeting lepidopteran pests are effectively single-toxin crops against important corn pests. For example, Cry1Ab + Vip3Aa corn is an effective pyramid for ear protection against *H. zea*, but functions as a single-toxin crop for ear protection against *O. nubilalis* (which is affected little by Vip3Aa) or for whorl protection against *S. frugiperda* (which is affected little by Cry1Ab) (Burkness et al. 2010; Niu et al. 2014). The evolution

Table 14.2 Twenty-one sets of one to five Bt toxins produced by Bt corn hybrids used in the United States (From Carrière et al. 2016)

Bt toxin(s)[a]	Single toxin against Lepidoptera	Single toxin against Coleoptera	Pyramid against Lepidoptera[b]	Pyramid against Coleoptera
Cry1Ab	X			
Cry1Fa	X			
Cry3Bb		X		
Cry34/35Ab		X		
mCry3Aa		X		
Cry1Ab + Cry3Bb [c]	X	X		
Cry1Ab + mCry3Aa [c]	X	X		
Cry1Fa + Cry34/35Bb [c]	X	X		
Cry1Fa + mCry3Aa [c]	X	X		
Cry1A.105 + Cry2Ab + Cry3Bb [d]		X	X	
Cry1Ab + Cry1Fa + Cry34/35Bb [d]		X	X	
Cry1Ab + Vip3Aa + mCry3Aa [d]		X	X	
Cry1A.105 + Cry2Ab			X	
Cry1Ab + Cry1Fa			X	
Cry1Ab + Vip3Aa			X	
Cry1Ab + Cry1Fa + Vip3Aa			X	
Cry1A.105 + Cry1Fa + Cry2Ab			X	
Cry1Ab + Cry1Fa + mCry3Aa + Cry34/35Ab [e]			X	X
Cry1Ab + Cry1Fa + mCry3Aa + eCry3.1Ab [e,f]			X	X
Cry1Ab + Cry1Fa + Vip3Aa + mCry3Aa + eCry3.1Ab [e,f]			X	X
Cry1A.105 + Cry1Fa + Cry2Ab + Cry3Bb + Cry34/35Ab [e]			X	X

[a]Relative to using pyramids alone, resistance in a particular pest evolves faster when plants that produce only one toxin effective against that pest are planted concurrently with crops that are pyramids against that pest

[b]Some plants producing two toxins are not pyramids against particular Lepidoptera when only one of the toxins is active against these species

[c]One toxin targets Lepidoptera and the other toxin targets Coleoptera

[d]Two toxins from the Cry1, Cry2, or Vip3 families target Lepidoptera and the other toxin targets Coleoptera

[e]Because resistance to Cry3Bb and mCry3Aa has occurred in *D. v. virgifera* in some regions of the USA, plants producing these two toxins do not act as pyramids against *D. v. virgifera* in these regions

[f]If strong cross-resistance occurs between mCry3Aa and eCry3.1Ab as expected (see text), then this combination of toxins will not act as a pyramid against Coleoptera

of practical resistance to mCry3Aa and Cry3Bb corn in *D. v. virgifera* in Iowa and Nebraska (Gassmann et al. 2014; Wangila et al. 2015) implies that all pyramids targeting this pest (Table 14.2) function as single-toxin crops in some regions of these states. Field-evolved practical resistance to single-toxin corn was also documented in other key pests in the USA (i.e., Cry1Ab corn in *H. zea* and Cry1Fa corn in *S. frugiperda*) (Dively 2014; Huang et al. 2014), which are targeted by several types of pyramided corn and cotton producing one of these toxins or a closely related toxin. Rapidly phasing out corn hybrids that function as single-toxin crops against lepidopteran and coleopteran pests is a priority to sustain effectiveness of Bt crops in the USA and elsewhere.

14.6 Linking Structure of Bt Toxins to Factors Affecting Sustainable Use of Pyramided Bt Crops

Several mechanisms of resistance to Bt toxins are known, but the most common and potent type involves mutations in receptor proteins that reduce the binding of Bt toxins to larval midguts (Ferré and Van Rie 2002; Caccia et al. 2010; Pardo-López et al. 2013; Wu 2014). Several receptor proteins bind to Cry toxins and confer susceptibility: cadherins, aminopeptidases (APNs) and alkaline phosphatases (ALPs) (Adang et al. 2014). It has been hypothesized, but not directly demonstrated, that ATP-binding cassette (ABC) transporter proteins also bind Cry toxins (Heckel 2012, 2015). Mutations or reduced transcription of cadherins, APNs, ALPs, and ABC transporters are associated with resistance to Cry toxins in numerous insects (Adang et al. 2014; Fabrick and Wu 2015; Heckel 2015; Tabashnik 2015). Alternative splicing and mis-splicing of cadherin RNA is also associated with resistance (Fabrick et al. 2014).

The amino acid sequence of toxins determines their structure and function (Adang et al. 2014). Accordingly, the amino acid sequence similarity between toxins in pyramids is expected to influence the durability of pyramids. Most toxins used in Bt crops share a similar three-domain structure, but others such as Vip3Aa and Cry34/35Ab do not, and are structurally distinct (Fig. 14.3).

Low survival of susceptible insects on pyramids is desirable for successful pest control and resistance management (Carrière et al. 2015). The toxins produced by pyramids can have antagonistic, independent, or synergistic effects on pest survival. Independent effects imply that survival on the pyramid is equal to the product of the survival on each of the single-toxin plants (Roush 1998). Relative to independent effects, synergy between toxins yields lower survival on the pyramid and antagonism increases survival on the pyramid (Carrière et al. 2015). The index of multiplicative survival (IMS) quantifies how the interaction between toxins

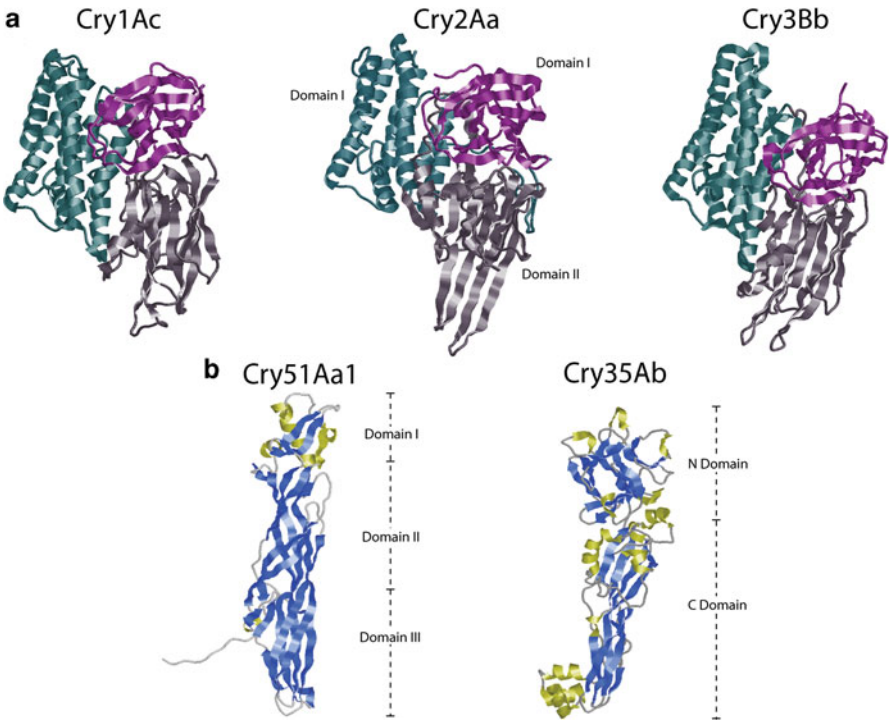

Fig. 14.3 X-ray crystal structures of (**a**) the three-domain crystal proteins Cry1Ac (PDB 4ARY), Cry2Aa (PDB 1I5P), and Cry3Bb (PDB 1JI6) and (**b**) the aerolysin-like toxins Cry51Aa1 (PDB 4PKM) and Cry35Ab (PDB 4JP0). Although the specificity of insecticidal activity for the three-domain toxins differs dramatically (Cry1Ac kills some Lepidoptera, Cry2Aa kills some Lepidoptera and Diptera, and Cry3Bb targets some Coleoptera), their three-dimensional structures share considerable similarity. Domain I (shown in *blue-green* in **a**) is comprised of a seven α-helix bundle that inserts into the insect midgut membrane to form a pore. Domain II (shown in *grey* in **a**) is a β-prism of three anti-parallel β-sheets involved in binding to midgut receptors primarily through the exposed loops. Domain III (shown in *purple* in **a**) has two anti-parallel β-sheets and contributes to receptor binding. The ETX-MTX (e.g., Cry51Aa1) and Bin-like (e.g., Cry34/Cry35Ab) protein families are β-pore forming toxins that share similarity with the aerolysin-type pore-forming toxins, but differ structurally from the three-domain Cry proteins. Cry51Aa1 (PDB 4PKM) is an ETX-MTX β-pore forming toxin with three domains. Domain I consists primarily of a hydrophobic core surrounded by several α-helical bundles, and domains II and III comprise the carboxyl-terminal tail and form β-sandwiches composed of anti-parallel β-sheets. The bin-like protein Cry35Ab (PDB 4JP0) has two domains, an amino-terminal β-trefoil domain (N Domain) and the carboxyl-terminal domain with extended antiparallel β-sheets (C Domain) similar to aerolysin folds. Toxins within these protein families often require the formation of binary interactions with other protein partners for toxicity (for example, Cry35Ab requires Cry34Ab to form the Cry34/35Ab complex that is toxic to some coleopterans) (Kelker et al. 2014). Interestingly, Cry51Aa1 and the close relative Cry51Aa2 do not require binary binding partners to be active against some coleopterans (both Cry51Aa1 and Cry51Aa2) and hemipterans (Cry51Aa2) (Xu et al. 2015; Baum et al. 2012). Monomers of Cry51Aa1 and Cry35Ab are shown with β-sheets in blue and α-helices in *yellow*

affects survival on pyramids (Carrière et al. 2015). IMS is calculated by dividing observed survival of susceptible insects on a pyramid by the product of their survival on single-toxin crops. Assuming the concentration of toxins is the same in pyramids as in the respective single-toxin crops, IMS is 1 with independent effects of toxins, >1 with antagonism and <1 with synergism. In an analysis of 17 cases from 14 pest-pyramid combinations, amino acid sequence similarity of domain III was positively and significantly associated with IMS, indicating that interactions were more antagonistic as similarity in domain III of toxins increased (Carrière et al. 2015). IMS was not associated with similarity of domain I or II (Carrière et al. 2015).

Several hybrid toxins produced by domain III exchange show increased potency against particular pests or a wider spectrum of activity compared to unmodified toxins (Adang et al. 2014). For example, the toxin Cry1A.105 is a chimeric protein containing domain I of Cry1Ab, domain II of Cry1Ac, and most of domain III from Cry1Fa (Huang et al. 2014). Domain III from Cry1Fa increases potency of Cry1A.105 relative to Cry1Ab or Cry1Ac against pests such as *Spodoptera* spp. (Huang et al. 2014). While this shows that specificity of individual toxins is affected by domain III, it remains unclear how domain III affects the interactions between toxins summarized above (Carrière et al. 2015). The formation of oligomeric structures is a key process affecting toxicity of three-domain toxins (Carmona et al. 2011; Pardo-López et al. 2013), suggesting that domain III could play a role in this process.

It is generally agreed that cross-resistance will be stronger between toxins that are structurally similar. Thus, among the Bt toxins used in transgenic crops, cross-resistance is likely to be stronger between the Cry1, Cry2, and Cry3 toxins that share a similar three-domain structure than between this set of toxins and those that do not have a three-domain structure such as Vip3Aa and Cry34/35Ab (Fig. 14.3). A more specific hypothesis is that cross-resistance is associated with similarity between toxins of domain II, because this domain plays a key role in binding of toxins to larval midgut receptors and altered binding is the most important mechanism of resistance (Tabashnik et al. 1996; Hernández-Rodríguez et al. 2013). This hypothesis was spurred by responses of a resistant strain of diamondback moth, *Plutella xylostella*, to 14 Cry1 and Cry2 toxins, including a hybrid toxin with domain III from Cry1C and domains I and II from Cry1Ab (Tabashnik et al. 1996). In this case and a recent study of *H. zea*, the association between cross-resistance and amino acid sequence similarity was stronger for domain II than domains I or III (Tabashnik et al. 1996; Welch et al. 2015).

A recent analysis of 80 cases evaluating cross-resistance in 10 major pests to seven sets of Bt toxins confirms this pattern and shows that amino acid sequence similarity of domain II, but not domain I and III, is associated with cross-resistance (Carrière et al. 2015). For example, cross-resistance was strong between Cry3Bb and mCry3Aa in *D. v. virgifera* (Gassmann et al. 2014; Wangila et al. 2015), which have 83 % amino acid sequence similarity in domain II (Carrière et al.

2015). In contrast, neither Cry3Bb nor mCry3Aa have structural homology with Cry34/35Ab (Fig. 14.3), and cross-resistance was much weaker between Cry3Bb or mCry3Aa and Cry34/35Ab (Gassmann et al. 2014; Wangila et al. 2015). The low but statistically significant cross-resistance seen between pairs of toxins that are not structurally similar and are unlikely to share high-affinity binding sites implies that mechanisms other than reduced binding can cause weak cross-resistance between unrelated Bt toxins (Carrière et al. 2015; Wei et al. 2015; Welch et al. 2015). The results summarized here suggest that choosing toxins that lack sequence homology and structural similarity in domains II can increase durability of pyramided Bt crops.

14.7 Seed Mixtures Versus Structured Refuges

The spatial configuration of refuges most likely to delay resistance remains controversial. "Structured refuges", which have been used extensively since 1996 in the USA, are blocks of non-Bt plants grown near blocks of Bt plants (EPA 2001). Starting in 2010, seed mixtures yielding a random mixture of Bt plants and non-Bt plants side-by-side within fields have been planted to manage resistance to pyramided corn (Fig. 14.2) (EPA 2011). Seed mixtures provide several advantages, including reducing the problem of farmer compliance with block refuge requirements (Head et al. 2014b). However, mathematical models show that seed mixtures can significantly accelerate resistance relative to block refuges when larvae move extensively between plants (Heuberger et al. 2011; Ives et al. 2011). Specifically, seed mixtures involving single-toxin crops or pyramids can accelerate resistance relative to blocks of Bt crops by reducing survival of susceptible insects and effective refuge size, or by increasing survival of heterozygotes and the dominance of resistance.

Laboratory and greenhouse experiments indicate that increased dominance in seed mixtures of single-toxin crops is most likely in pests with low susceptibility to Bt toxins. In a model system involving *H. zea* that has relatively low susceptibility to Cry1Ac cotton (Brévault et al. 2013), the dominance of resistance was significantly increased in a seed mixture relative to a block of Cry1Ac cotton, because survival of heterozygotes relative to susceptible individuals increased more in the seed mixture than in the block of Bt cotton (Brévault et al. 2015). In contrast, results from experiments with two pests (*P. gossypiella* and *P. xylostella*) that have relatively high inherent susceptibility to Cry1Ac suggest that the opportunity for individual larvae to eat both non-Bt and Bt plant tissues did not increase the dominance of resistance (Shelton et al. 2000; Heuberger et al. 2008). Pollen-mediated gene flow between Bt and non-Bt cotton yields bolls with various proportions of Bt and non-Bt seeds (Heuberger et al. 2010). However, in the seed-feeding pest *P. gossypiella*, the dominance of resistance did not vary significantly when Cry1Ac-susceptible, heterozygous, and Cry1Ac-resistant larvae fed in artificial bolls containing different proportions of Bt and non-Bt seeds (Heuberger et al. 2008). In a selection experiment involving a model system with *P. xylostella* and non-commercial Cry1Ac

broccoli, the percentage of larvae susceptible to Cry1Ac at the end of the experiment was not lower in seed mixture plots compared with plots containing separate blocks of Bt and non-Bt plants (Shelton et al. 2000), which indicates that seed mixtures did not increase the dominance of resistance. Empirical data are lacking to evaluate effects of seed mixtures of pyramided crops on the dominance of resistance.

Even without larval movement between plants, pollen-mediated gene flow could accelerate evolution of resistance in seed mixtures relative to structured refuges for insects that eat corn kernels (e.g., *H. armigera, H. zea, S. frugiperda*). Gene flow between Bt and non-Bt corn in seed mixtures produces a mosaic of Bt and non-Bt kernels in ears of non-Bt corn plants (EPA 2012; Yang et al. 2014). The Bt toxins in kernels of refuge plants in seed mixtures could accelerate resistance by killing susceptible larvae and reducing effective refuge size (Yang et al. 2014), increasing the dominance of resistance, or both. Empirical data are lacking to evaluate effects of gene flow on resistance evolution in seed mixtures.

14.7.1 Can Seed Mixtures Delay Resistance to Bt Crops in Diabrotica virgifera virgifera?

Many of the conditions underlying success of the refuge strategy for seed mixtures deviate from ideal in *D. v. virgifera*: (1) alleles conferring resistance are not rare; (2) resistance is not recessive; (3) fitness costs appear minimal; (4) cross-resistance occurs between some of the toxins in pyramids; and (5) pyramids are grown concurrently with plants that produce one of the toxins in the pyramid (Table 14.2) (Tabashnik and Gould 2012; Devos et al. 2013).

Field-evolved practical resistance of *D. v. virgifera* to single-toxin Bt corn producing either Cry3Bb or mCry3Aa has been documented in Iowa and Nebraska (Gassmann et al. 2014; Wangila et al. 2015). This is not surprising because *D. v. virgifera* rapidly evolved resistance to Bt corn producing either Cry3Bb or mCry3Aa in laboratory and greenhouse selection experiments (Tabashnik and Gould 2012; Devos et al. 2013). Because analogous experiments show rapid evolution of resistance to Bt corn producing Cry34/35Ab (Tabashnik and Gould 2012; Devos et al. 2013), the risk of evolution of resistance to Bt corn pyramids producing either Cry3Bb + Cry34/35Ab or mCry3Aa + Cry34/35Ab is high where this pest is already resistant to Cry3Bb and mCry3Aa. Cry3Bb and mCry3Aa are 83 % similar in domain II and cross-resistance occurs between them (Gassmann et al. 2014; Carrière et al. 2015). Furthermore, amino acid sequence similarity in domain II between mCry3Aa and eCry3.1Ab is 100 % (Carrière et al. 2015), indicating that they are structurally similar and cross-resistance between them is likely. Accordingly, the risk of evolution of resistance to mCry3Aa + eCry3.1Ab corn is also high.

Extensive larval movement between Bt and non-Bt plants occurred when *D. v. virgifera* were exposed to seed mixtures of non-Bt corn and a Bt corn pyramid producing Cry3Bb + Cry34/35Ab (Zukoff et al. 2012; Head et al. 2014b). Larval

movement in seed mixtures from Bt to non-Bt plants increased survival of susceptible larvae relative to their survival in blocks of Bt plants (Zukoff et al. 2012; Head et al. 2014b). Conversely, larval movement from non-Bt to Bt plants reduced survival of susceptible individuals relative to their survival on blocks on non-Bt plants (Zukoff et al. 2012; Head et al. 2014b). Larval movement could accelerate the evolution of resistance in seed mixture if it increases the fitness advantage on Bt corn for individuals with one or more resistance alleles relative to susceptible individuals. Because the risk of resistance to pyramids in seed mixtures is high in *D. v. virgifera*, integrating crop rotation with use of seed mixtures in regions where this pest remains susceptible to crop rotations could enhance resistance management (Devos et al. 2013; EPA 2015).

14.8 Conclusions

We have shown that some of the key conditions favoring durability of Bt crops are not met in many cases, especially for pests with inherently low susceptibility to Bt toxins. As pyramids are planted more widely, it will be increasingly important to develop resistance management strategies that address this challenge. Future insecticidal transgenic crops will use novel Bt toxins, modified Bt toxins, toxins from organisms other than Bt, and new ways to kill pests such as RNA interference (RNAi). These new transgenic crops will also target a broader range of pests such as the hemipterans *Lygus* sp. (Baum et al 2012). Nevertheless, about 12 years are currently needed to develop and implement novel insecticidal transgenic crops in the USA (McDougall 2011). This lengthy period increases the risk that some of the pests with low susceptibility to Bt toxins such as *S. frugiperda* and *D. v. virgifera*, which rapidly evolved resistance to Bt crops (Table 14.1), could overcome most or all transgenic insecticidal crops available to control them in the near future.

To sustain the efficacy of Bt crops against pests with inherently low susceptibility to Bt toxins, it will be essential to increase refuge size and to integrate Bt crops with other pest management tactics (Carrière et al. 2004; Fitt et al. 2004; Bates et al. 2005; Tabashnik and Gould 2012; Brévault et al. 2013; Andow et al. 2015; Welch et al. 2015; EPA 2015). In particular, in accord with previous recommendations (Tabashnik and Gould 2012), we propose that effective refuge percentage (Gustafson et al. 2006; Jin et al. 2015) must be at least 20 % to achieve substantial delays in the evolution of pest resistance when conditions are not optimal. Importantly, the relevant spatial scale must be considered when determining availability of effective refuges, because this scale can vary widely depending on the pest-crop combination (O'Rourke et al. 2010; Brévault et al. 2012; Onstad and Carrière 2014), and this can affect the evolution of resistance (Peck et al. 1999; Storer et al. 2003; Sisterson et al. 2004, 2005; Carrière et al. 2010; Onstad and Carrière 2014). We hope that the development of innovative resistance management strategies will continue to sustain benefits provided by transgenic insecticidal crops for the next 20 years.

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Chapter 15
Insecticide Resistance and Its Impact on Vector Control

Mark J. I. Paine and Basil Brooke

Abstract Insect disease vector control is primarily based on the use of synthetic insecticides that are used either for indoor residual spraying (IRS) or the treatment of fabrics, particularly bed nets. As yet, there are still only four classes of public health insecticides available for most insect vector-borne diseases including malaria: pyrethroids, organochlorines, organophosphates (OPs) and carbamates. Whilst extensive deployment of long-lasting insecticide-treated bednets (LLINs), which are dependent on pyrethroids, is a contributing factor in the dramatic spread of pyrethroid resistance across Africa, the implementation of front-line alternatives such as carbamates is already being affected by resistance. The limited numbers of insecticides available and the speed at which insecticide resistance can take hold lead to fundamental questions about mechanisms of resistance, impact on vector control and ways to overcome insecticide resistance. The global plan for insecticide resistance management in malaria vectors (GPIRM) is a rallying call from the World Health Organization (WHO) to tackle these questions. Great strides have been made in identifying enzymes associated with insecticide metabolism in mosquitoes and applying new technology for monitoring and predicting resistance. This chapter explores the impact of insecticide resistance on vector control and recent developments in resistance research.

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15.1 Introduction

15.1.1 The History of Vector Control

The control of malaria vectors is primarily based on the use of synthetically produced insecticides formulated either for indoor residual spraying (IRS) or the treatment of fabrics – especially bed nets. Indoor spraying using insecticides was first mooted in 1931 (see review by Coetzee et al. 2013b) as a species sanitation method because it specifically targets indoor-resting (endophilic) species. Indoor spraying was first conducted extensively in the Natal Province, South Africa, using pyrethrum dissolved in kerosene. This method proved especially effective at controlling a series of malaria epidemics that occurred in Natal during the 1930s (Coetzee et al. 2013b). The subsequent usefulness of dichlorodiphenyltrichloroethane (DDT) for the control of malaria and typhus first became apparent towards the end of World War II, and IRS campaigns using DDT and, to a lesser extent, dieldrin and carbamate insecticide formulations were fundamentally important during the Global Malaria Eradication Programme of the 1950s and 1960s (Mendis et al. 2009; Nájera et al. 2011). However, incidences of resistance to DDT were recorded in *Anopheles sacharovi* in Greece in 1951; in *An. stephensi* in Iran, Iraq and Syria in 1957; in *An. culicifacies* in India in 1959; in *An. gambiae* in the West African region in the late 1960s; and later in Central and East Africa (reviewed by Corbel and Guessan 2013; Ranson et al. 2011). Almost congruent with the development of resistance to DDT, increasing incidences of resistance in malaria vector species to dieldrin were recorded in the Middle Eastern region and the Indian subcontinent (Patel et al. 1958; Raghavendra 2002), Central America and the Caribbean (Gilotra 1965), and West Africa (Armstrong et al. 1958; Hamon et al. 1968; M.W 1960). Resistance to malathion was also recorded in various vector populations including *An. stephensi* in India (Raghavendra 2002).

By the 1980s, DDT usage, especially for malaria control, had largely been replaced by organophosphates, carbamates and pyrethroids. Pyrethroids have been used extensively for malaria vector control over the past three decades, and the net result is widespread resistance in malaria vector species, especially in sub-Saharan Africa (Knox et al. 2014; Ranson et al. 2009). Nevertheless, global malaria incidence has decreased by 40 % since 2000 which is attributable, at least in part, to a general intensification of insecticide-based malaria vector control, particularly the widespread distribution of pyrethroid-treated bed nets (Bhatt et al. 2015; World Health Organization. Global Malaria Programme 2012).

15.1.2 Current Status of Insecticide Resistance

The number of insecticides available for vector control is limited (Table 15.1). Only four classes of public health insecticides are available for the control of malaria and other insect vector-borne diseases: pyrethroids, organochlorines, organophosphates and carbamates. In the case of malaria, insecticide resistance in anopheline vectors has emerged to all four classes. The extensive deployment of long-lasting insecticide-treated bednets (LLINs), which are entirely dependent on pyrethroids, is seen as a major contributing factor in the dramatic spread of pyrethroid resistance across Africa (Mnzava et al. 2015). Currently, pyrethroid resistance has been detected in at least one vector species in 53 of the 65 countries that have been reporting surveillance data since 2010 (Kleinschmidt et al. 2015; Knox et al. 2014). The extent of the problem is illustrated in Fig. 15.1, leading to an urgent call to arms by the World Health Organization (WHO) for a 'global plan for insecticide resistance management in malaria vectors' (World Health Organization. Global Malaria Programme).

Insecticides for public health vector control are used predominantly for malaria, followed by dengue, leishmaniasis and Chagas disease (van den Berg et al. 2012). In 2009, the global use of vector control insecticides was dominated by DDT (dichlorodiphenyltrichloroethane) in terms of quantity applied (71 % of total) and by pyrethroids in terms of the surface or area covered (81 % of total) (van den Berg et al. 2012). These figures exclude the use of pyrethroids in long-lasting insecticide-treated bed nets (LLINs), which has increased dramatically in Africa. The use of carbamates and organophosphates is also on the increase as the insecticides of choice for IRS in response to escalating pyrethroid resistance (Akogbeto et al. 2010, 2011; CDC 2012; Fuseini et al. 2011; Thomsen et al. 2014). However, even as these

Table 15.1 Insecticide Resistance Action Committee (IRAC 2006) classification of insecticides used for mosquito control. Only groups 1, 2 and 3 are used or have been used in the past for the control of adult mosquitoes

Target site/action	Group	Chemical subgroup
Acetylcholinesterase inhibitors	1A	Carbamates
	1B	Organophosphates (OPs)
GABA-gated chloride channel antagonists	2A	Organochlorines, cyclodienes
	2B	Phenyl pyrazoles (e.g. fipronil)[a]
Sodium channel modulators	3B	Pyrethroids/pyrethrins/DDT
Juvenile hormone mimics	7A	Methoprene, hydropene
	7C	Pyriproxyfen
Nicotinic acetylcholine receptor agonists	5	Spinosyns
Inhibitors of chitin biosynthesis	15	Diflubenzuron
Microbial disruptors of insect midgut membranes	11	*Bacillus thuringiensis var israelensis*

[a]Fipronil has not been formulated for public health use to date

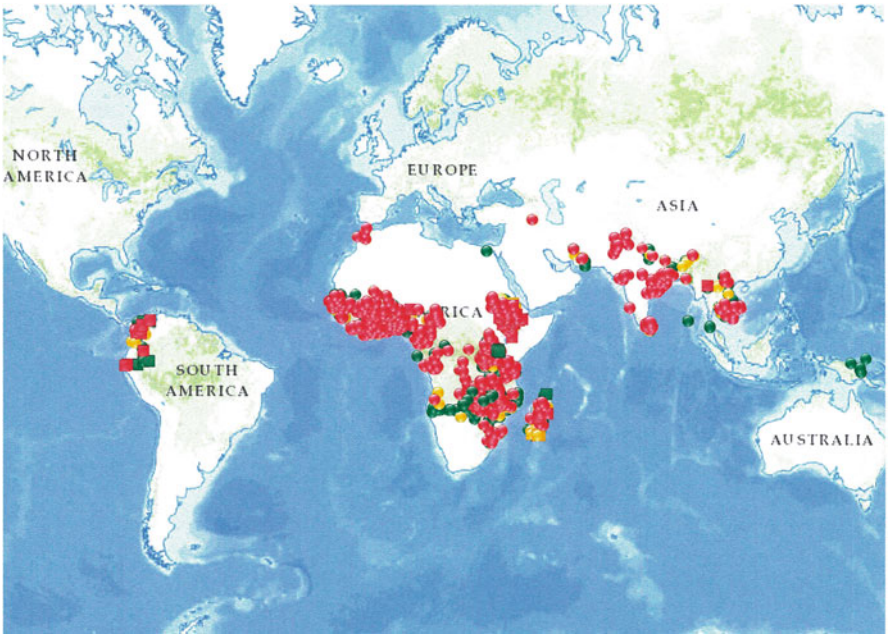

Fig. 15.1 Global extent of insecticide resistance/susceptibility for all malaria vector species across all classes of public health insecticides for the period 2000–2015. *Red* confirmed resistance, *yellow* possible resistance, *green* confirmed susceptibility. IR Mapper – accessed 18th Dec 2015

are being introduced as front-line insecticides, resistance to bendiocarb (a carbamate used for IRS) has already been reported in West Africa (Aïkpon et al. 2013; Edi et al. 2014).

The limited numbers of insecticides available and the speed at which insecticide resistance can develop lead to fundamental questions about mechanisms of resistance, impact on vector control and ways to overcome insecticide resistance. It is recognised that vector control has had a major impact in reducing disease prevalence. However, whilst great strides have been made in identifying enzymes associated with insecticide metabolism in mosquitoes and applying new technology for monitoring and predicting resistance, the impact of resistance on disease control is still not clear. This chapter explores recent developments and their potential impact on vector control, focusing primarily on malaria, which has been the focus for new technology development.

15.2 Types of Resistance

In mosquitoes, insecticide resistance is most commonly associated with insecticide target site modification and enhanced metabolism of insecticides. Cuticular resistance, associated with reduced insecticide penetration through cuticular thickening,

has also been implicated in Burkina Faso (Toé et al. 2015), adding further complexity to the resistance landscape. Target site resistance involves mutations leading to well-defined target site alteration and resistance to chemical insecticides (Donnelly et al. 2009). Metabolic resistance, on the other hand, involves more subtle alterations in the expression of a complex array of enzymes and detoxification pathways (Feyereisen 2005; Hemingway et al. 2004). Determining the mechanisms of resistance is further complicated by the extremely high frequency of single nucleotide polymorphisms (SNPs) in mosquitoes, whose genes average a SNP every 34 bp, approximately tenfold higher than humans (Wilding et al. 2009), and potential diel rhythmicity in some insecticide detoxification genes (Balmert et al. 2014). Environmental and biological factors may also impact insecticide resistance, although these are poorly understood.

15.2.1 Target Site

Mutational changes at position 1014 in transmembrane segment 6 of the sodium ion channel associate closely with resistance to DDT and, to a lesser extent, pyrethroid insecticides (Brooke and Koekemoer 2010; Hemingway et al. 2004). These are referred to as knock-down resistance (*kdr*) mutations and have emerged independently in both insect pests of agriculture and human disease. The L1014F *kdr* mutation (Martinez-Torres et al. 1998) was first described in *An. gambiae* (formerly the S molecular form – Coetzee et al. 2013a) and was later shown to have introgressed from *An. gambiae* into the very closely related *An. coluzzii* (formerly the M molecular form) (Weill et al. 2000). The L1014S *kdr* mutation was first identified in an *An. gambiae* population in Kenya (Ranson et al. 2000) and co-occurs, although rarely, with L1014F at some localities (Koekemoer et al. 2011; Verhaeghen et al. 2006, 2010). However, these two mutational events belie a greater complexity, because *kdr* haplotypes have arisen at least four times in *An. gambiae* (Pinto et al. 2007). Furthermore, *kdr* haplotype diversity indicates that the L1014F mutation shows the strongest selection imprint in association with insecticide exposure (Donnelly et al. 2009; Lynd et al. 2010; Jones et al. 2012a). As a result, *An. gambiae* populations generally show higher levels of resistance to DDT and pyrethroid insecticides than *An. coluzzii* populations. This variation is attributable, in part, to higher *kdr* frequencies in affected *An. gambiae* populations (Santolamazza et al. 2008). However, resistances to DDT and pyrethroids are almost certainly multifactorial, and *kdr*-associated resistance, especially to pyrethroids, is invariably also mediated by metabolic detoxification (Brooke and Koekemoer 2010). This is evident in an analysis of pyrethroid resistance in *An. coluzzii* in Burkina Faso in which the extent and intensity of resistance has recently escalated and has been linked to a suite of detoxification enzymes and other factors, with only a minor contribution from 1014F (Toé et al. 2015). Similarly, an analysis of multiple resistances in *An. gambiae* from Pointe-Noire in the Republic of Congo showed a complex interaction between metabolic detoxification and *kdr* resistance (Koekemoer et al. 2011).

The insect γ-aminobutyric acid (GABA) receptor Rdl is the target of cyclodiene insecticides including dieldrin and fipronil (Buckingham et al. 2005; Raymond-Delpech et al. 2005). An alanine to glycine mutation, A296G, located in transmembrane segment 2 (TM2) has been shown to underscore resistance to dieldrin, picrotoxinin and fipronil (Ffrench-Constant et al. 1993; Hosie et al. 1995). This mutation has been associated with cyclodiene resistance in *An. gambiae* and *An. arabiensis* (Du et al. 2005). The assortment of this locus and the persistence of the dieldrin/fipronil resistance phenotype despite a likely fitness cost in certain *An. gambiae* laboratory strains have been linked to the positive heterotic effect of the 2La inversion polymorphism (Brooke et al. 2000, 2002). Another mutation, V327I, has been discovered in dieldrin-resistant *An. funestus* and tends to be conserved with the TM2 A296G mutation in resistant individuals (Wondji et al. 2011). However, the actual impact of V327I on the resistance phenotype is currently unknown. Recently, A296G and a threonine to methionine (T245M) mutation were detected in Rdl of an *An. gambiae* dieldrin-resistant laboratory strain. These mutations also tend to be conserved in association with dieldrin resistance, but as with V327I, the effect of T245M on production of the resistance phenotype is currently unknown. Taylor-Wells et al. (2015) suggest that T245M may serve to offset the structural impact of A296G.

The neurotransmitter modulating enzyme acetylcholinesterase (AChE) is the target of carbamate and organophosphate (OP) insecticides. Alterations in acetylcholinesterase can lead to carbamate and organophosphate resistance (Hemingway et al. 2004). A glycine to serine amino acid substitution (G119S) of the *ace-1* gene has been associated with OP and carbamate resistance in several mosquito species including *Culex pipiens pipiens*, *Cx. pipiens quinquefasciatus*, *An. albimanus*, *An. arabiensis*, *An. sinensis*, *An. coluzzi* and *An. gambiae* (Djogbénou et al. 2007; Hemingway et al. 2004; Toé et al. 2015; Weill et al. 2004). In this instance, the replacement of the glycine residue, which is located at the base of the active site, causes substantially reduced substrate binding (Rivero et al. 2010). However, G119S alone induces a pronounced fitness cost (Djogbenou et al. 2010) which can be compensated by duplications of 119S alleles which co-occur with non-duplicated 119G alleles to produce an enhanced resistant phenotype against a background of wild-type functionality (Edi et al. 2014; Weetman et al. 2015).

15.2.2 Metabolic

Metabolic resistance occurs through increased biodegradation of the insecticide through overproduction of detoxification enzymes such as P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxyl/cholinesterases (CCEs) (Hemingway and Ranson 2000; Hemingway et al. 2004). Of these, P450s are the primary enzyme family associated with resistance to most insecticides including pyrethroids. The role of P450s in insecticide resistance has been extensively reviewed (David et al. 2013; Hemingway et al. 2004; Scott 1999). Elevated levels

of P450 activity are frequently observed in pyrethroid-resistant malaria vectors in Africa (Mitchell et al. 2012; Muller et al. 2008; Riveron et al. 2013; Stevenson et al. 2012; Wilding et al. 2012). Esterase hydrolysis of pyrethroids leading to detoxification is also believed to act as a cause of metabolic resistance in some instances (Djouaka et al. 2008; Hemingway and Ranson 2000), whilst GSTs are regularly found overexpressed in pyrethroid-resistant strains (Muller et al. 2008; Vontas et al. 2001). However, the contribution these enzymes make towards pyrethroid resistance and their biochemical relationships with P450-mediated resistance are still unclear, but increased GST activity is likely associated with protection against the damaging effects of oxidative stress induced by pyrethroid intoxication (Vontas et al. 2002).

15.2.2.1 P450s

Insects have evolved a large P450 repertoire that provides a chemoprotective shield against toxic compounds including insecticides. Since the onset of microarray-based studies, numerous P450s belonging to at least 11 families (CYP4G, CYP4H, CYP6N, CYP6M, CYP6P, CYP6Z, CYP9K, CYP12F, CYP314A, CYP325A and CYP325D) have been overexpressed in either DDT or pyrethroid-resistant mosquitoes (reviewed by David et al. 2014). Amongst these, CYP6M2 (Stevenson et al. 2011) and CYP6P3 (Muller et al. 2008) are most frequently found associated with insecticide resistance in *An. gambiae*, whilst their orthologues CYP6M7 and CYP6P9a are likewise most often overexpressed in pyrethroid-resistant *An. funestus* populations (Amenya et al. 2008; Irving et al. 2012; Riveron et al. 2013; Wondji et al. 2009). As well as pyrethroid resistance, CYP6P3 and CYP6M2 produce bendiocarb resistance via transgenic expression in *Drosophila* (Edi et al. 2014). CYP6M2 is also associated with DDT resistance (Mitchell et al. 2012), thus implicated in resistance to three distinct classes of insecticide. Most recently, elevated copy number at the CYP9K1 region in *An. gambiae* has been linked with insecticide resistance, although it has yet to be functionally characterised (Main et al. 2015).

Humans contain 55 individual P450s; however, 90 % of all drugs are metabolised by just six P450s (CYP1A2, CYP2C9, CYP2D6, CYP2C19, CYP3A4, CYP3A5). These represent a core set of enzymes for screening and investigating drug-P450 interactions (Guengerich 2005). Whilst mosquitoes contain over 100 P450s, several emerge frequently from on-going microarray comparisons of insecticide-resistant versus susceptible populations including CYP6P3 and CYP6M2 in *An. gambiae* (reviewed by David et al. 2013) and CYP6P9a, CYP6P9b and CYP6M7 in *An. funestus* (Amenya et al. 2008; Matambo et al. 2010; Riveron et al. 2013). These represent a core set of insecticide metabolising P450s that are often expressed at elevated levels in African populations of malaria transmitting mosquitoes. Since high levels of these P450s may influence the metabolism and disposition of any new insecticides introduced for vector control, they may be considered as targets for the development of diagnostics for insecticide resistance and insecticide development.

15.2.2.2 GSTs

Elevated GST activity has been implicated in resistance to several classes of insecticides in insects. Insect cytosolic GSTs comprise a least six classes, delta, epsilon, omega, sigma, theta and zeta (Hemingway et al. 2004), of which delta and epsilon classes are most commonly associated with insecticide resistance. GSTs detoxify compounds by conjugation with the reduced form of glutathione (GSH). GSTe2 has the most well-defined activity with respect to DDT resistance in *An. gambiae*, *An. funestus* and *Ae. aegypti* (Lumjuan et al. 2005, 2014; Prapanthadara et al. 2000; Ranson et al. 1997; Riveron et al. 2014). As well as elevated levels of gene expression, allelic variants have been identified in *An. gambiae* (Mitchell et al. 2014) and *An. funestus* (Riveron et al. 2013) with enhanced levels of DDT dehydrochlorinase activity. X-ray crystallography has revealed this may be associated with structural destabilisation increasing the local molecular dynamics of the DDT pocket facilitating substrate binding and/or product release (Mitchell et al. 2014), or enlargement of the GSTe2 DDT-binding cavity (Riveron et al. 2014), resulting in increased catalytic turnover.

In *An. funestus*, the single amino acid change L119F in *GSTe2* confers high levels of metabolic resistance to DDT in the malaria vector *An. funestus*, as well as cross-resistance to pyrethroids (Riveron et al. 2014) and strong geographical correlation with DDT resistance patterns across Africa, thus providing a potential DNA-based diagnostic marker to predict the spread of GSTe2-based resistance. Indeed, this is currently the only DNA-based marker for metabolic resistance, highlighting the complexity of uncovering genomic markers for metabolic resistance.

15.2.2.3 Esterases

Mutations altering the amino acid sequence of esterases and amplification of esterase genes have been shown to contribute to carboxylesterase (CoE)-based metabolic resistance to organophosphates in insects (Cao et al. 2008; Chung et al. 2009; Cui et al. 2007). CoEs can also confer resistance to carbamates and pyrethroids which are rich with ester bonds (Chouaïbou et al. 2014). In *An. arabiensis* from Sudan, an alteration in esterase activity conferred malathion resistance (Corbel and Guessan 2013; Hemingway 1983, 1985). Carboxylesterases have been implicated using biochemical assays in organophosphate, carbamate and pyrethroid resistance in several *Anopheles* species (Chang et al. 2014; Corbel et al. 2007; Hemingway and Ranson 2000; Vezenegho et al. 2009).

15.2.3 Cuticular Resistance

CYP4G16 has recently become associated with pyrethroid resistance as evidenced by overexpression in pyrethroid-resistant *An. gambiae* and *An. arabiensis* (Jones

et al. 2013; Müller et al. 2008). CYP4G16 does not metabolise pyrethroids but may play a role in enhancing cuticular hydrocarbon synthesis, similar to other members of the CYP4G family (Qiu et al. 2012). Other genes with putative roles in cuticular hydrocarbon synthesis including two elongases – 3-hydroxyacyl-coa dehydrogenase and fatty acyl-CoA elongase – are found overexpressed in pyrethroid-resistant *An. gambiae*, strengthening the support for the involvement of this pathway (Toé et al. 2015). Cuticular thickening has been linked to pyrethroid resistance in a laboratory strain of *An. funestus* originating from southern Mozambique and is likely a facilitatory mechanism to the primary mode of resistance which is based on metabolic detoxification (Wood et al. 2010).

15.2.4 The Pyrethrome

In humans, the routes by which drugs may be metabolised or biotransformed are well characterised. Drug metabolism is normally divided into two phases, phase I involving functionalisation reactions (e.g. oxidation) and phase II involving conjugation reactions such as glucuronidation or glutathione conjugation. Thus, phase I reactions prepare drugs for phase II reactions by generating chemically reactive groups that enable the conjugation reaction, which generally leads to a water-soluble product for excretion. Similar pharmacological principles apply for insecticide metabolism. There is accruing evidence that orchestrated interactions such as those between P450s and UDP-glucuronosyltransferases (UGTs), which lie opposite P450s on the luminal side of the ER, may facilitate the channelling and excretion of toxic and reactive intermediates (Hanioka et al. 2006; Jensen et al. 2011). This is supported by the pull down of networks of pyrethroid-metabolising enzymes by pyrethroid activity-based probes (PyABPs) (Ismail et al. 2013). Here, a deltamethrin mimetic ABP was able to identify active deltamethrin metabolising P450s along with related detoxification enzymes including flavin monooxygenases (FMOs), aldehyde reductases and UGTs in rat liver microsomes, leading to the suggestion of a network of associated pyrethroid-metabolising enzymes or pyrethrome (Ismail et al. 2013). Whilst overexpression of P450s, GSTs and CoEs is usually associated with metabolic resistance, aldehyde dehydrogenases (Lumjuan et al. 2014), aldoketoreductases (David et al. 2014; Strode et al. 2012) and UGTs have also been identified in microarray screening of pyrethroid-resistant mosquitoes, supporting a pyrethrome complex as illustrated in Fig. 15.2.

Analysis of deltamethrin metabolism by CYP6M2 shows several routes of metabolism. Whilst 4′-hydroxylation was the major route of metabolism, further sequential breakdown of 4′-hydroxydeltamethrin was evident demonstrating complex metabolism and multiple binding modes. The metabolism of pyrethroids by CYP6M2 is consistent with its overexpression in insecticide-resistant *An. gambiae*. CYP6Z2 and CYP6Z8, on the other hand, are commonly linked with pyrethroid resistance in *An. gambiae* and *Ae. aegypti*, respectively (David et al. 2013), yet they do not metabolise pyrethroids (Chandor-Proust et al. 2013; Mclaughlin et al.

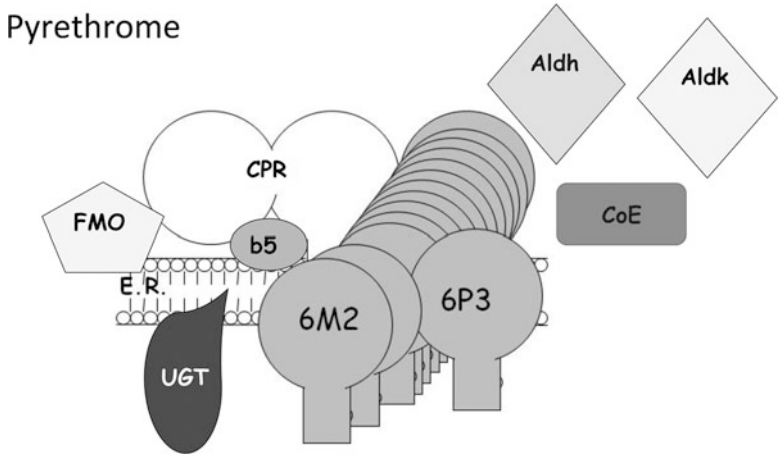

Fig. 15.2 Components of the putative *Anopheles gambiae* pyrethrome. Elements shown are derived from the protein networks identified in rats by PyABPs (Ismail et al. 2013). The two P450s illustrated (CYP6M2 and CYP6P3) are known to metabolise pyrethroids (David et al. 2013). *Abbreviations* are *FMO* flavin monooxygenase, *CPR* NADPH cytochrome P450 oxidoreductase, *b5* cytochrome b5, *UGT* UDP-glucuronosyltransferases, *Aldh* aldehyde dehydrogenase, *Aldh* aldoketoreductase, *GST* glutathione S-transferase, *CoE* carboxylesterase, *E.R.* endoplasmic reticulum

2008). However, they are able to metabolise the phenoxybenzyl alcohol aldehyde products of CoE hydrolysis. As well as showing that elevated levels of secondary metabolism are linked with resistance, this illustrates network associations indicative of 'pyrethrome'-mediated metabolism of pyrethroids.

15.3 Impact of Resistance on Vector Control

Resistance to insecticides is expected to have a dramatic effect on the efficacy of insecticide-based control interventions. This effect can usefully be measured using entomological indicators as a proxy (Strode et al. 2014), but is probably best measured in terms of epidemiological outcomes. However, natural cycles of disease transmission, bionomic factors including changes in vector species composition and abundance, climatic and environmental factors, migration, changing land use patterns and other control interventions are likely to confound evaluations of the actual effect of insecticide resistance on disease incidence. For example, variations in malaria transmission intensity in Kenya over the past four decades are most closely associated with rainfall and antimalarial drug resistance (Snow et al. 2015) even though insecticide resistance is prevalent amongst malaria vector populations in the East African region (Knox et al. 2014). Nevertheless, there are examples of where insecticide resistance can be causally linked to increased disease incidence, notwithstanding the confounding factors mentioned earlier.

Resistance to DDT in *An. stephensi* in Pakistan was associated with an exponential increase in malaria incidence in the early 1970s (Insecticide resistance action committee (IRAC) 2006; Metcalf 1989). Control was re-established using malathion, reinforcing the likelihood that the DDT resistance led directly to control failure resulting in an increase in malaria transmission. Added to these trends may be fluctuations in vector species composition and abundance in the affected regions because *An. stephensi* tends towards dominance where land use is characterised by intensive irrigation and consequent salinisation of breeding sites (Klinkenberg et al. 2004).

Resistance to pyrethroids was at least partially responsible for the malaria epidemic experienced in South Africa during the period 1996–2000. Prior to 1996, South Africa's IRS-based vector control programme was dependent on DDT. Whilst this regimen was generally sufficient for control (malaria incidence seldom exceeded 4000 cases per annum), sporadic outbreaks and more severe epidemics did occur, such as the 1971–1972 epidemic followed by the 1978 epidemic, both of which were congruent with widespread rains (Coetzee et al. 2013b). In 1995, a policy to move away from the use of DDT for IRS in favour of pyrethroids was adopted, largely because of mounting pressure against the use of DDT. Furthermore, an upsurge in cross-border migration from Mozambique coupled with good rainfall during this period coincided with a sharp rise in malaria incidence within South Africa, in which the number of cases rose from 8750 in 1995 to 27,035 in 1996 and peaked at 64,622 in 2000 (Maharaj et al. 2013). A primary cause of this epidemic was the resurgence of pyrethroid-resistant *An. funestus* following the introduction of pyrethroids for IRS (Hargreaves et al. 2000). Although the link between insecticide resistance and increased malaria incidence may seem tenuous based on these events alone, the reintroduction of DDT for IRS in South Africa post 2000 and the resultant substantial decline in malaria incidence to fewer than 10,000 cases per annum during much of the subsequent period strongly suggest that pyrethroid efficacy was severely undermined by the development of pyrethroid resistance in *An. funestus* and that DDT use, in conjunction with pyrethroids, was necessary to re-establish control (Coetzee et al. 2013b). However, the reintroduction of DDT for IRS also coincided with a change in antimalarial drug regimen from monotherapeutic sulfadoxine-pyrimethamine (SP) to artemisinin-containing combination therapy (ACT) (Maharaj et al. 2013). Although it is almost impossible to quantify the actual contribution of each intervention to the decrease in malaria incidence post 2000, the use of DDT dramatically decreased the abundance of *An. funestus* in South Africa to undetectable levels, leaving the less efficient vector *An. arabiensis* to maintain lower level residual transmission as a consequence of this species' behavioural plasticity and lower susceptibility to IRS (Hargreaves et al. 2003). Currently, DDT is used for spraying traditional structures, and pyrethroids are used for modern structures in South Africa's provincial IRS programmes, which conveniently amounts to a mosaic resistance management strategy as described in the global plan for insecticide resistance management (GPIRM) (World Health Organization. Global Malaria Programme et al. 2012).

An intensive malaria control programme was initiated on Bioko Island of Equatorial Guinea in 2004. This programme included a rotational IRS campaign using pyrethroids and carbamates and the distribution of long-lasting insecticide-treated bednets (LLINs) for vector control (Kleinschmidt et al. 2009). Entomological surveillance revealed substantial declines in the population densities of *An. gambiae*, *An. coluzzii*, *An. funestus* and *An. melas* following the first three spray rounds (Sharp et al. 2007). This programme succeeded in significantly reducing malaria morbidity and mortality within 5 years of inception although foci of high transmission persisted. A subsequent intensification and change in vector surveillance methodology revealed extremely high entomological inoculation rates (EIRs) in areas of high transmission, and these were correlated to high vector abundance and high sporozoite rates, especially in *An. coluzzii* (Overgaard et al. 2012). Interestingly, the inception of the indoor-based vector control interventions effectively eliminated *An. funestus* and *An. gambiae* from Bioko Island, leaving *An. coluzzii* as the primary vector in most of the remaining areas of high transmission and *An. melas* implicated in some areas. This likely occurred because of all the species/populations tested for *kdr* markers, only *An. coluzzii* showed appreciably high frequencies of the L1014F allele which is associated with resistance to pyrethroids and DDT. Unfortunately, assessments of actual association between *kdr* genotype and resistance phenotype were not conducted, but it is likely that insecticide resistance is at least partly responsible for the persistence of *An. coluzzii* on Bioko Island and therefore partly responsible for the intensive transmission experienced in some areas despite the control interventions in place. Another pertinent factor is the high incidence of outdoor feeding recorded in malaria vector mosquitoes on Bioko Island (Overgaard et al. 2012).

There is some evidence that increasing resistance to pyrethroids in *An. gambiae* in Dielmo village in Senegal led to a reduction in LLIN efficacy corresponding to an upsurge of malaria incidence during the period 2008–2010. During this period, the mass distribution of LLINs evidently led to the near elimination of *An. funestus* as well as an increase in the frequency of the L1014F *kdr* allele in *An. gambiae* from 8 % in 2007 to 48 % in 2010 as a consequence of intensive selection pressure. However, although the increase in pyrethroid resistance in *An. gambiae* is associative, it is not sufficient to indicate cause and effect. It is more likely that a decrease in protective immunity was primarily causative of the upsurge in malaria incidence and was congruent with a shift in the age groups most affected (Trape et al. 2011). A subsequent longitudinal study in Dielmo village spanning the period 1990–2012 showed that early detection and treatment of clinical cases using ACT coupled with mass distribution of LLINs was sufficient to maintain a low incidence of malaria despite insecticide resistance (Trape et al. 2015).

The meta-analysis of Strode et al. (2014) concludes that insecticide-treated nets (ITNs) can be an effective form of vector control despite insecticide resistance, as indicated by entomological outcomes. This is because ITNs are more effective in terms of reducing blood-feeding and killing mosquitoes than untreated nets even against a backdrop of insecticide resistance in target vector populations. This finding is reinforced by a recent assessment of the efficacy of ITNs on malaria prevention

which was conducted in the Machinga District of Malawi (Lindblade et al. 2015). The predominant malaria vector species during the course of the study were *An. funestus* and *An. arabiensis*, both of which showed moderate to high levels of pyrethroid resistance. The data indicate that ITN use significantly reduced malaria incidence in children less than 5 years of age despite the occurrence of pyrethroid-resistant vector populations, although the authors also concede that ITN efficacy may have been compromised at least to some extent by insecticide resistance. An assessment of the impact of pyrethroid resistance in *An. funestus* and *An. gambiae* in north-eastern Malawi was conducted during 2009 and again in 2010 following the widespread distribution of LLINs and the introduction of IRS into certain districts in 2009. Similar levels of reduction in *Plasmodium* infection prevalence in children 1–4 years old were recorded in the LLIN-only and IRS plus LLIN sites in 2010 compared to 2009, despite selection for resistance, particularly in *An. funestus*. Nevertheless, the introduction and scaling up of IRS did not enhance the reduction in infection prevalence beyond that induced by LLINs alone as was expected, suggesting that pyrethroid-resistant *An. funestus* were undermining the efficacy of IRS not unlike the situation recorded in the South African epidemic described earlier (Wondji et al. 2012).

There has been extensive research on insecticide resistance, although it has focused primarily on entomological outcomes that are sometimes contradictory. For example, regardless of pyrethroid resistance, ITNs are better than untreated nets in terms of mosquito mortality (Strode et al. 2014). The limited data available indicate that insecticide resistance, depending on intensity of phenotypic expression, can lead to vector control failure and thereby induce an epidemiologically significant effect on malaria incidence. The need for large-scale randomised control studies to quantify insecticide resistance in terms of disease impact is evident, particularly for bed nets, but is challenged by weak reporting systems, the logistics of field collections, and the fact that random allocation of exposure is not possible and randomising individuals receiving insecticide-treated or insecticide-untreated nets is unethical (Kleinschmidt et al. 2015). To address this, WHO coordinated multi-country prospective studies are underway in Benin, Cameroon, India, Kenya and Sudan to tackle the design and execution of robust studies to determine the impact of insecticide resistance on malaria vector control (Kleinschmidt et al. 2015).

15.4 Development of New Tools to Combat Resistance

15.4.1 Diagnosing Resistance

Insecticide resistance in a vector population can initially be detected and characterised using bioassays designed to determine whether particular insecticide-resistant phenotypes occur at any given time and can also be used to assess the intensities of expression of the phenotypes in question (WHO 2013; Bagi et al.

2015). Whilst these assessments can effectively be used to inform vector control interventions, more sophisticated assays are required to identify the underlying genetic causes or markers of resistance so as to track resistance dynamics and develop early warning systems and appropriate resistance management strategies.

Advances in genomics have led to the discovery of robust markers for target site resistance and strong candidate genes for metabolic and cuticular resistance in malaria- and dengue-transmitting species such as *An. gambiae* (Jones et al. 2012a, b; Mitchell et al. 2012; Muller et al. 2008; Toé et al. 2015) and *Ae. aegypti* (Faucon et al. 2015; Marcombe et al. 2012), respectively. Target site mutations for resistance to pyrethroids and DDT, organophosphates and carbamates are well known and have led to the development of PCR-based vector population monitoring tools (Bass et al. 2010). Likewise, elevated levels of individual P450s associated with insecticide metabolism or cuticular thickening have been identified, the principal ones being CYP6P3, CYP6M2 and CYP4G16 from *An. gambiae* (David et al. 2013; Toé et al. 2015) and CYPs 6P9a, 6P9b and 6M7 in *An. funestus* (Riveron et al. 2013). Whilst these have yet to be translated into diagnostic tools, they provide a wide coverage of appropriate markers for the future development of field technology capable of tracking insecticide resistance to support operational decision-making.

15.4.2 Predicting Metabolic Resistance

Currently, the only way to track metabolic resistance is after development, which is often too late to prevent resistance genes gaining traction and spreading throughout a population. In insects, P450s are the key enzymes involved in metabolic degradation but difficult to identify. Consequently, metabolic resistance probes are rarely developed. Developing probes that rapidly target the enzymes that metabolise specific insecticides would be a powerful diagnostic tool for metabolic resistance, particularly in identifying resistance-associated P450s in naïve populations of disease-transmitting vectors that metabolise target compounds, pre-empting their resistance activity.

The recent development of pyrethroid mimetic activity-based probes (PyABPs) offers the exciting potential to selectively label and identify P450s associated with pyrethroid metabolism (Ismail et al. 2013). The activity-based probes (ABPs) work in a mechanism-dependent manner to covalently label P450s, whereby the labelling events are detectable by adding a fluorescent reporter group via copper-catalysed azide-alkyne cycloaddition (‘click chemistry’) onto the probe – P450 adducts (Wright and Cravatt 2007; Wright et al. 2009). Furthermore, affinity tags can also be incorporated to pull down and identify probe-P450 adducts using mass spectrometry, allowing direct access to functionally relevant enzymes. Since the metabolism of insecticides by P450s is one of the principal mechanisms of resistance in most arthropods, it is hypothesised that activity-based probes could be used to profile P450s in disease-transmitting vectors before they become resistant. Thus far, this has been demonstrated using a rat liver model, where

PyABPs identified pyrethroid-metabolising P450s and associated networks of drug-metabolising enzymes in the pyrethrome (Ismail et al. 2013). This provides a new perspective on insecticide interactions, offering a powerful new tool for pre-emptive screening of enzymes associated with insecticide metabolism in other insect species that may lead to resistance.

15.4.3 Screening Resistance Liabilities

P450s are attractive targets for pharmacological intervention owing to their essential roles in the study of drug metabolism, pharmacokinetics and toxicity reactions. Hence, there is a great demand by pharmaceutical companies for in vitro enzyme assays to determine which drugs are metabolised by which P450s and to delineate potential drug-drug interaction liabilities of drug compounds. In vitro cytochrome P450 metabolism and inhibition data are useful in designing strategies for new drugs, which is facilitated by the ready production of recombinant P450s in *E. coli*, baculovirus, yeast or mammalian cells.

In the same way that liver P450s influence drug metabolism in humans, insect P450s control the metabolism and disposition of insecticides. Given that CYP6P3 and CYP6M2 are regularly found overexpressed in populations of *An. gambiae* across Africa (Abdalla et al. 2014; Djouaka et al. 2008; Mitchell et al. 2012, 2014; Muller et al. 2007, 2008; Toé et al. 2015) and metabolise a wide range of substrates, they are influential in the metabolism of insecticidal products. Thus, they may be viewed as resistance liabilities for new insecticides being developed in Africa. This has opened the door to developing recombinant P450-based prescreens as used for drug discovery. Panels of resistance-associated mosquito P450s are now commercially available (http://www.lite-testing-facility.com/our-services/mosquito-enzyme-panel/) and can be exploited by companies for insecticide screening, understanding insecticide interaction and guiding the development of vector control compounds.

However, whilst the identities of compounds that are metabolised by P450s associated with resistance can now be determined, an accurate assessment of resistance potential is greatly hampered by a lack of understanding of how individual rates of metabolic activity relate to insecticide clearance in vivo (i.e. what is the rate of metabolism that leads to resistance?). Unlike human drug metabolism, where the pharmacokinetics (PK) of drug metabolism and disposition are well defined, providing good correlative models for in vitro assays that describe the metabolic fate of insecticides in tropical disease vectors is poorly understood. This severely limits the predictive power of in vitro assays. Thus, there is a critical need to develop appropriate tools to measure the uptake and metabolic fate of insecticides.

15.4.4 Monitoring Insecticide Use

Insecticides only work if used correctly such that coverage is sufficiently extensive within an endemic area, and treatment is repeated when insecticide levels fall below the threshold concentration. As well as reducing impact on disease vectors, poor spraying with sublethal doses of insecticide is likely to speed the evolution of resistance, one of the biggest challenges in vector control. Indeed, large-scale, detailed quality assurance analysis of IRS in visceral leishmaniasis control programmes has demonstrated that less than 20 % of houses were being sprayed with the correct dose (1.0 g ai/m^2) (Coleman et al. 2015).

Whilst these results clearly demonstrate the need for routine monitoring of insecticide dosage, it is rarely done as the tools currently available for estimating insecticide amounts on treated surfaces are not practical for field use. These include cone bioassays requiring live insects, which are non-quantitative, and high-performance liquid chromatography (HPLC), which is reliant on sophisticated and expensive systems, severely limiting their application in resource-poor countries (WHO 2006, 2015). A range of new tests for insecticide quantification have recently been developed including biosensors and chemical tests for DDT and pyrethroid detection (Dowd et al. 2009; Green et al. 2009; Ismail et al. 2016; Morou et al. 2010; Russell et al. 2014) that are applicable in the field. These will enable vector control programmes to address key operational questions including verification that sprayers have sprayed a house correctly, evaluating the spray coverage in each house, verifying effective insecticide coverage rates and calculating and comparing the insecticide application rates for each area.

15.4.5 Some Alternative Methods/Technologies

The sterile insect technique is a proven insect control strategy. It is based on the use of laboratory-reared sterile males which are mass released into the natural environment of a target conspecific population. By successfully competing for mates, sterile males can sufficiently disrupt the production of progeny in the target population, leading to population suppression and possibly eradication (Alphey et al. 2008; Dame et al. 2009). The sterile insect technique was first used to eradicate the New World screw worm fly, *Cochliomyia hominivorax*, from the USA and Central America (Krafsur 1998). The use of SIT against mosquito populations has enjoyed limited success to date. However, recent advances in transgenic technology have resulted in renewed interest in SIT as a malaria control method (Oliva et al. 2014). Following the development of a genetic sexing strain of the major malaria vector *Anopheles arabiensis*, pilot projects are currently underway in northern Sudan and South Africa to ascertain the feasibility of using SIT to control populations of this species (Klassen 2009; Munhenga et al. 2011).

Although insecticide-based malaria vector control is principally based on IRS and ITNs, larvicides can also play an important role (Tusting et al. 2013). They can be incorporated into insecticide resistance management schemes and be used to target vector populations whose variable feeding and resting behaviours make them less susceptible to control by IRS and ITNs, such as *An. arabiensis* (Devine and Killeen 2010). Larvicides target the non-transmitting aquatic stages of mosquitoes and can therefore only affect disease transmission by significantly reducing the abundance of vectors. Identifying the most productive vector breeding sites is problematic because some vector species, including *An. arabiensis*, tend to breed in small, inconspicuous and widely dispersed pools of water (Gillies and Coetzee 1987; Oliva et al. 2014). Nevertheless, larviciding can be an effective and relatively inexpensive vector control option in situations where vector breeding sites have been identified.

15.5 Conclusion

The burgeoning incidence of resistance to insecticides in insect disease vectors, most notably malaria vectors, can lead to reduced efficacy of insecticide-based control interventions. The limited number of insecticides available for vector control and the need to maintain control efficacy have inspired a period of intense innovation in basic and applied research in this field. As a consequence, methods for identifying and characterising resistance have been developed and validated. These include bioassay, molecular, enzymatic and, most recently, partial followed by whole-genome analysis techniques that have led to a deeper understanding of how resistance phenotypes are constructed by mutagenesis. These techniques carry the promise of providing early warning and surveillance tracking systems, yet, strides still need to be made in implementing these tools in an effective manner to inform resistance management strategies. The need to develop alternative vector control products and technologies is also driven by burgeoning resistance as well as the realisation that effective vector control carries the greatest potential to reduce the incidence of malaria (Bhatt et al. 2015) and other vector-borne diseases.

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Chapter 16
Insecticide Resistance in Natural Enemies

Pablo Bielza

Abstract Pesticide resistance in pests has severe negative consequences but can be used as a positive trait for natural enemies as an opportunity to improve the simultaneous use of two very valuable tools in pest management: chemical and biological control. Biological control adoption is limited in some areas, crops, or seasons due to the imperative use of pesticides needed to control diseases and pests. Most studies on pesticides and natural enemies try to establish the degree of compatibility using only a population, not considering the natural variation in insecticide susceptibility. However, there is variation in the response to pesticides among populations of a beneficial species, similarly to the response in any pest species. Knowledge of the natural and potential variation in the tolerance of natural enemies to pesticides may improve the design of robust IPM strategies by extending the role of biological control in some agricultural systems and by increasing the number of available compounds to control diseases and key, secondary, and invasive pests. There are a number of excellent revisions on pesticide resistance in natural enemies. In the present review, new cases of insecticide resistance in natural enemies are discussed, as a better understanding of pesticide resistance in natural enemies will allow us to enhance the integration of chemical and biological tools in IPM programs.

16.1 Introduction

Pesticide resistance in arthropods is a worldwide concern in agriculture and public health (Sparks and Nauen 2014). Around 600 arthropod species have been reported exhibiting resistance to at least one pesticide (APRD 2015). Resistance has been reported to virtually all insecticide classes, from carbamates and organophosphates, through pyrethroids and neonicotinoids, to spinosyns and diamides.

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Beneficial insects and mites accounted for fewer than 3 % of the 447 species reported as resistant in the 1980s (Georghiou 1986). By 2015 resistance to at least one insecticide has increased to 593 species, with 13,627 cases (APRD 2015). Among them 336 are agricultural pests (8916 cases) and 38 are natural enemies (304 cases) (Figs. 16.1 and 16.2). Although the proportion of the number of species of natural enemies has now increased to 6.4 %, the number of cases of resistance has decreased, with only 304 cases, accounting for 2.2 % (APRD 2015).

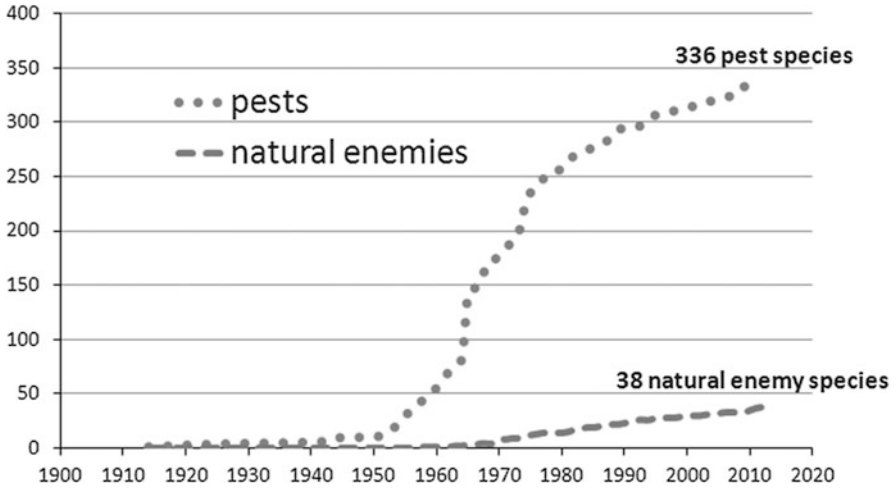

Fig. 16.1 Cumulative number of agricultural pest and natural enemy species resistant to at least one pesticide (Data adapted from APRD 2015)

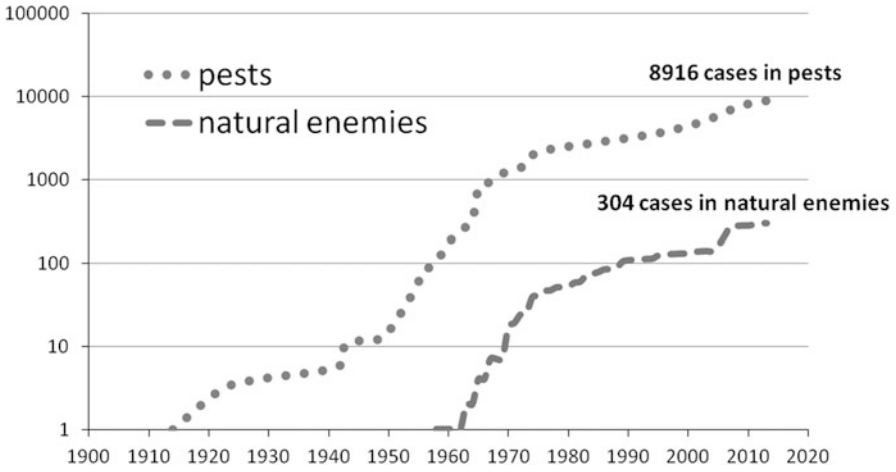

Fig. 16.2 Cumulative number of reported cases of resistance in agricultural pest and natural enemy species (Data adapted from APRD 2015)

The most accepted hypotheses to explain why the number of pesticide resistance cases in natural enemies pales in comparison with those in herbivore arthropods argue that natural enemies possess inferior detoxification mechanisms, suffer food shortage after selection, or that are simply less likely to detect when develop resistance (Tabashnik and Johnson 1999).

Insecticide resistance in pests has severe negative consequences but can be used as a positive trait for natural enemies. Therefore, this widespread phenomenon among insects and mites can be taken as an opportunity to improve the simultaneous use of two very valuable tools in pest management: chemical and biological control.

Natural enemies are the keystone of integrated pest management (IPM), especially when it is based on biological control as is widely undertaken in protected crops in Spain (Sanchez et al. 2000; Calvo et al. 2011). Even after insecticide use has been dramatically reduced by widespread adoption of biological control in Southeast Spain, some pesticides, mainly fungicides but also a limited number of insecticides and acaricides, continue to be applied against diseases, pest resurgences, and secondary pest outbreaks.

The most difficult aspect for an effective integration of pesticides and natural enemies is overcoming their incompatibility (Rodrigues et al. 2013a). Modern insecticides and acaricides are more selective and less harmful to beneficial organisms. However, in some crops and areas, broad-spectrum insecticides are still widely used (Sayyed et al. 2010). Most pyrethroids, organophosphates, carbamates, and neonicotinoids possess little selectivity toward beneficial insects. In order to guarantee the compatibility of pesticides with biological control agents (BCA), studies testing the acute toxicity are developed previous to their joint use in IPM programs. However, not only can pesticides affect natural enemies through lethal intoxication but also by alterations in behavior and fitness (Desneux et al. 2007). Biological control agents should be capable not only to survive an insecticide spraying but also to perform its beneficial activity (predatory or parasitism) under pesticide exposure. To this end more sophisticated approaches have been developed to determine threshold values for mortality and sublethal effects of pesticides on natural enemies (Hassan et al. 1985).

The vast majority of studies on pesticides and natural enemies try to establish the degree of compatibility between them using only a population, usually provided by a BCA supplier, not considering the natural variation in insecticide susceptibility (Bielza et al. 2009). Generalizations about the tolerance of beneficial pesticides based on testing one or very few populations may be inaccurate (Roush et al. 1990). Nevertheless, there is indeed variation in the response to pesticides among populations of a beneficial species, similarly to the response in any pest species (Espinosa et al. 2002; Fernández et al. 2009; Roditakis et al. 2013; Grávalos et al. 2014). Therefore, knowledge of the natural and potential variation in the tolerance of natural enemies to pesticides may improve the design of robust IPM strategies (Poletti and Omoto 2012) by enhancing the combined use of BCA and pesticides.

Biological control adoption is limited in some areas, crops, or seasons due to the imperative use of certain pesticides needed to control key pests and diseases. Improving the compatibility of pesticides and BCA will help to increase the role of biological control in IPM programs applied in some agricultural systems. Moreover, resistant strains of natural enemies may be selected to be released in the field (Hoy 1990; Hoy et al. 1990; Whitten and Hoy 1999).

The phenomenon of insecticide resistance among arthropod natural enemies received much attention in the 1970s and 1980s (Croft and Morse 1979; Tabashnik and Johnson 1999) due to the effort made for the wide adoption of the integrated pest management concept. There are a number of excellent revisions on pesticide resistance in natural enemies, to say a few, Croft and Brown (1975), Croft and Morse (1979), Theiling and Croft (1988), and Tabashnik and Johnson (1999). However, the last published to our knowledge dates back from 1999 (Tabashnik and Johnson 1999). In the present review, new cases of insecticide resistance in natural enemies are discussed, believing that a better understanding of the phenomenon of pesticide resistance in BCA will allow us to enhance the integration of chemical and biological tools in IPM programs.

16.2 Predatory Mites

The first observed evidence of pesticide resistance in natural enemies came among the phytoseiids (Acari: Phytoseiidae), predatory mites of plant-feeding mites and small insects, in the 1970s. *Neoseiulus* (*Amblyseius) fallacis* (Garman) was the first predator to be conclusively described having acquired resistance to the organophosphates azinphos-methyl and parathion (Motoyama et al. 1970) in apple orchards in the USA. Subsequently, more cases of OP and carbamates resistance were reported for this species (Croft and Meyer 1973).

Resistant strains OP, carbamates, and pyrethroids were reported for several species of predatory mites in the 1970s, 1980s, and 1990s, including *N. fallacis* (Motoyama et al. 1970; Croft and Meyer 1973), *Euseius* (*Amblyseius) hibisci* (Chant) (Kennett 1970), *Neoseiulus californicus* (*A. chilenensis*) (McGregor) (Croft et al. 1976; Sato et al. 2002; Poletti and Omoto 2012), *Galendromus* (*Typhlodromus* = *Metaseiulus*) *occidentalis* (Nesbitt) (Roush and Hoy 1980), *Metaseiulus* (*Typhlodromus*) *arboreus* (Chant) (Croft and Aliniazee 1983), *Phytoseiulus persimilis* Athias-Henriot (Avella et al. 1985), *Typhlodromus pyri* Scheuten (Hadam et al. 1986), *Amblyseius nicholsi* Ehara et Lee (Tang et al. 1988), *Neoseiulus* (*Amblyseius) womersleyi* (*Amblyseius pseudolongispinosus*) Schicha (Tang et al. 1988; Mochizuki 1994; Kawai 1997), and *Amblyseius andersoni* (Chant) (Dunley et al. 1991).

More recently high levels of resistance to pyrethroids and organophosphates were detected in populations of *T. pyri* and *A. andersoni* in grape crops in France (Bonafos et al. 2007). These resistant populations played a crucial role in the success of integrated pest management of tetranychid mites in commercial grape production regions (Bofanos et al. 2007).

Two field populations of *Typhlodromus exhilarates* (Ragusa) were tested against chlorpyrifos (Barbar et al. 2007), one collected from a vine crop in France and other from a very close unsprayed orchard (considered the susceptible strain). The LC50 values obtained for the two strains were not significantly different, but the LC90 values were. In addition, 20 % of the females of more resistant strain survived and reproduced at the recommended field rate of chlorpyrifos. The authors considered that the rate of survival would probably be higher under field conditions, and consequently this population could rebuild after a chlorpyrifos treatment.

Similarly low susceptibility to several pesticides has been reported in a population of *N. californicus* (Sato et al. 2002), being used in mite management programs in commercial strawberry fields in Brazil by integrating chemical and biological control (Sato et al. 2007). In the same way, populations of *Phytoseiulus macropilis* (Banks) with deltamethrin resistance ratios up to 3500-fold were detected in ornamental crops in Brazil (Poletti and Omoto 2012). Resistance to insecticides in *Kampimodromus aberrans* (Oudemans) was suspected from field studies (Posenato 1994) and was recently demonstrated in the laboratory from populations collected in vineyards and apple orchards in Italy, exhibiting extremely high resistance (145,000-fold) to chlorpyrifos (Tirello et al. 2012). A resistant strain of *K. aberrans* was successfully released in vineyards treated with fungicides and insecticides (Duso et al. 2009). Three populations of the predator *Neoseiulus* (*Amblyseius*) *longispinosus* collected from commercially grown vegetable crops in China were resistant to fenpropathrin, chlorpyrifos, and abamectin (Zhao et al. 2013).

Some intrinsic factors of phytoseiid mites such as their diet and mode of reproduction have favored their development of pesticide resistance (Poletti and Omoto 2012). Unlike pests, natural enemies evolve resistance with difficulty as the resistant individuals will die of starvation after an insecticide treatment due to the lack of food (Croft and Brown 1975; Croft and Morse 1979; Tabashnik and Johnson 1999) as the insecticide reduces the preys or hosts available for them. However, polyphagous natural enemies as phytoseiid mites, in which some of them also feed upon plants, pollen, leaf nectaries, etc., become resistant more readily than specialized monophagous natural enemies. Moreover, the type of feeding has been pointed out as the explanation for a higher tolerance to pesticides in the omnivorous predator *Euseius stipulatus* (Athias-Henriot) than in the specialized predators *Neoseiulus californicus* (McGregor) and *P. persimilis* in citrus orchards in Spain (Argolo et al. 2014). On the other hand, the reproduction system of phytoseiid mites, haplodiploidy, is advantageous for resistance evolution, since there is recombination in diploid females, and recessive resistant genes are exposed to selection in haploid males. Several haplodiploid pest species are among those developing more readily resistance, such as *Tetranychus urticae* Koch (Acari: Tetranychidae) (Van Leeuwen et al. 2010), *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Bielza 2008), and *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Fernández et al. 2009).

Nevertheless, a significant interspecies variability in the susceptibility to pesticides of phytoseiid mites has been observed. *N. californicus* was over 3000-fold more tolerant to deltamethrin than *P. macropilis* (Poletti and Omoto 2012).

Furthermore, considering the recommended field rates for deltamethrin, *N. californicus* would be compatible with the insecticide but *P. macropilis* would not. Other studies also indicate these differences in insecticide susceptibility among phytoseiid mite species. *T. pyri* were 300-fold more tolerant to deltamethrin than *P. persimilis* (Markwick 1986), *A. andersoni* was only fivefold more tolerant than *T. pyri* (Bonafos et al. 2007), *Euseius concordis* (Chant) was around 50-fold more tolerant than *Iphiseiodes zuluagai* Denmark and Muma (Poletti and Omoto 2005), and *N. californicus* more tolerant than *E. concordis* (Silva et al. 2011).

Predatory mites have exhibited a remarkable ability to develop strains resistant to pesticides (Croft and Meyer 1973; Croft and Van de Baan 1988; Hoy 1990). Apart from the numerous cases of resistance described in many species (see above), artificial selection of resistant strains has been successfully used to rapidly increase pesticide tolerance. After only seven generations of selection by deltamethrin, the resistance increased tenfold in a laboratory population of *P. persimilis* (Avella et al. 1985). Similarly a tenfold increase in cypermethrin resistance was found after a 12 months of selection in *P. persimilis* (Markwick 1986). Moderate resistance (33-fold) to the acaricide acequinocyl was reported after only six generations of selection from a susceptible population of *P. persimilis* (Yorulmaz Salman et al. 2015). After seven selections for resistance, an already resistant field population of *A. womersleyi* increased sevenfold its resistance to methidathion (Sato et al. 2006).

Among natural enemy species, predatory mites exhibit a higher variability in pesticide tolerance and a better potential for resistance selection. Among natural enemies, phytoseiids are the most cited for resistance to pesticides, with 17 species of 38 (APRD 2015), being the most promising to be used for selective breeding for pesticide resistance (Table 16.1).

16.3 Predatory Insects

There are a few reported cases of insecticide resistance in lady beetles (Coleoptera: Coccinellidae) (Table 16.2). The first published case was *Coleomegilla maculata* (De Geer) in cotton fields being resistant to DDT (15-fold), methyl parathion (11–29-fold), and monocrotophos (12-fold) (Head et al. 1977; Graves et al. 1978). Lately, there have been published several studies about insecticide resistance in lady beetles. *Stethorus gilvifrons* (Muls.) from apple orchards in Turkey was found to be 11-fold resistant to the pyrethroid bifenthrin (Kumral et al. 2011). A population of *Eriopis connexa* (Germar) collected in cabbage fields in Brazil was 20-fold resistant to lambdacyhalothrin relative to a susceptible population (Rodrigues et al. 2013b). Additionally, further selection of this population for 12 generations doubled the resistance ratio (RR). Moreover, according to the dose-mortality response curve provided by the authors, the recommended field rate of lambdacyhalothrin would kill only the 45 % of the specimens of the field-collected-resistant population and none of the lab-selected one.

Table 16.1 Top 12 resistant natural enemy species

Species	Order: family	Natural enemy type	# cases
Chrysoperla carnea	Neuroptera: Chrysopidae	Predatory insect	157
Neoseiulus (=Amblyseius) fallacis	Acarina: Phytoseiidae	Predatory mite	26
Galendromus (=Typhlodromus) pyri	Acarina: Phytoseiidae	Predatory mite	20
Galendromus (=Typhlodromus, =Metaseiulus) occidentalis	Acarina: Phytoseiidae	Predatory mite	10
Neoseiulus (=Amblyseius) longispinosus	Acarina: Phytoseiidae	Predatory mite	9
Neoseiulus (=Amblyseius) womersleyi (=Amblyseius pseudolongispinosus)	Acarina: Phytoseiidae	Predatory mite	9
Phytoseiulus persimilis	Acarina: Phytoseiidae	Predatory mite	8
Oomyzus sokolowskii	Hymenoptera: Eulophidae	Parasitoid	6
Coleomegilla maculata	Coleoptera: Coccinellidae	Predatory insect	5
Cotesia plutellae	Hymenoptera: Braconidae	Parasitoid	5
Diglyphus begini	Hymenoptera: Eulophidae	Parasitoid	5
Ganaspidium utilis	Hymenoptera: Eucoilidae	Parasitoid	5

Data adapted from APRD (2015)

Similarly, a population of another lady beetle species, *Hippodamia convergens* (Guérin-Méneville), collected in crimson clover in Georgia (USA) was found to be 220-fold more resistant to lambdacyhalothrin than a susceptible reference population (Rodrigues et al. 2013a). The calculated LC90 value was ten times greater than the maximum recommended field rate of lambdacyhalothrin. Even though, following the probit curve obtained in the study, a mortality of around 30 % is expected at that field dose for the resistant population. However, it would show 0 % survival for the susceptible population. In addition, a dose of the organophosphate dicrotophos as high as ten times field rate resulted in 0 % and 100 % mortality for the resistant and susceptible population, respectively (Rodrigues et al. 2013a), suggesting cross-resistance or multiresistance.

Another ladybird species, *Propylaea japonica* (Thunberg), has been found to be resistant to insecticides very recently (Tang et al. 2015). Populations collected from cruciferous vegetables in South China showed very low to low resistance (resistance factors ranging 1.0–6.2) to abamectin, imidacloprid, beta-cypermethrin, and chlorpyrifos, except one strain from Nanning which was moderately resistant to abamectin (10.1-fold). The strain from Guangzhou, with a low resistance factor

Table 16.2 Predatory insects reported to have developed insecticide resistance

Group	Species	Insecticides	Country	Reference
Ladybirds	*Coleomegilla maculata*	DDT methyl parathion monocrotophos	USA	Head et al. (1977) and Graves et al. (1978)
	Stethorus gilvifrons	Bifenthrin	Turkey	Kumral et al. (2011)
	Eriopis connexa	Lambdacyhalothrin	Brazil	Rodrigues et al. (2013b)
	Hippodamia convergens	Lambdacyhalothrin	USA	Rodrigues et al. (2013a)
	Propylaea japonica	Abamectin	China	Tang et al. (2015)
Lacewings	*Chrysoperla carnea*	Permethrin	Canada	Pree et al. (1989)
		Fenvalerate		
		Cypermethrin		
		Deltamethrin		
		DDT		
		Azinohosmethyl		
		Phosmet		
		Ethyl parathion		
		Malathion		
		Carbaryl		
		Methomyl		
		Chlorpyrifos	USA	Pathan et al. (2008)
		Profenofos		
		Lambdacyhalothrin		
		Alphamethrin		
		Deltamethrin	Pakistan	Sayyed et al. (2010)
		Alphamethrin		
		Lambdacyhalothrin		
		Chlorpyrifos		
		Profenofos		
		Emamectin benzoate	Pakistan	Mansoor et al. (2013)
		Spinosad	Pakistan	Abbas et al. (2014)

(4.1) to imidacloprid, was continuously selected over 20 generations for resistance to imidacloprid, increasing 9.4-fold the resistance.

Lady beetles tend to be more tolerant to insecticides than other aphidophagous insects, including lacewings, syrphids, hemipterons, and hymenopteran parasitoids (Hodek 2014). Sequentially, the historically intensive use of pesticides in the crop systems frequented by lady beetles would have put significant selection pressure for lady beetles populations to grow more resistant. The variability of tolerance to insecticides in lady beetles and the positive response to selection would allow for integration of these predators with the use of insecticides.

Lacewings are another group of predatory insects with documented cases of insecticide resistance (Table 16.2). *Chrysoperla carnea* (Stephens) has been reported to develop resistance to pyrethroids, organophosphates, emamectin benzoate, and spinosad (Pree et al. 1989; Pathan et al. 2008; Sayyed et al. 2010; Mansoor et al. 2013; Abbas et al. 2014). Field populations collected from regularly sprayed apple trees in Ontario (Canada) and from cotton in California (USA) were resistant to a wide range of insecticides, such as pyrethroids (permethrin 34–46-fold, fenvalerate fourfold, cypermethrin ninefold, deltamethrin 31-fold), DDT (11-fold), organophosphates (azinohosmethyl 17–33-fold, phosmet 62-fold, ethyl parathion 19-fold, malathion fivefold), and carbamates (carbaryl five- to sixfold, methomyl 20-fold) (Pree et al. 1989).

Recently, the toxicity to the main insecticides used in cotton fields in Pakistan, two organophosphates and three pyrethroids, was studied in *C. carnea* populations collected in five locations over three consecutive years (Pathan et al. 2008). The levels of resistance to chlorpyrifos (9- to 166-fold), profenofos (11- to 69-fold), lambdacyhalothrin (16- to 113-fold), and alphamethrin (11- to 88-fold) were moderate to high. However, deltamethrin resistance was comparatively lower (4- to 23-fold).

A field population collected in Pakistan from cotton showed a 47-fold resistance to deltamethrin, 86-fold to alphamethrin, 137-fold to lambdacyhalothrin, 76-fold to chlorpyrifos, and 110-fold to profenofos compared with a susceptible population (Sayyed et al. 2010). Additional selection for resistance to deltamethrin for four generations yielded a 30-fold increase of the resistance to deltamethrin and a fivefold cross-resistance increase to alphamethrin, but a nonsignificant change in lambdacyhalothrin, chlorpyrifos, and profenofos resistance. Using the dose-mortality curve provided by the authors, the recommended field rate of lambdacyhalothrin would kill only the 38 % of the susceptible population, but 0.5 % and 0.05 % of the field-collected-resistant population and the lab-selected one, respectively. According to these data, the authors conclude that resistant lacewings could be compatible with most spray programs (Sayyed et al. 2010).

Another field population collected from crop areas in Pakistan exhibited a 12-fold resistance to emamectin benzoate compared to a susceptible lab population (Mansoor et al. 2013). According to the data provided by the authors, less than 10 % of the susceptible population would survive a field rate of emamectin benzoate (15 ppm), but around 70 % of the field population would. This field population was further selected for resistance to emamectin benzoate for five generations (Mansoor et al. 2013). The resulted population was highly resistant to the avermectin insecticide, with a LC50 value of 1469.4 ppm, with a resistance ratio of 318 compared to the susceptible population. This lab-selected population would fully survive a field application of emamectin benzoate at the maximum recommended rate (15 ppm).

The level of resistance to spinosad was low (14-fold) for another population collected in crop areas in Pakistan (Abbas et al. 2014) compared to a susceptible population. However, the mortality at the maximum field rate of spinosad (120 ppm) would kill 73 % of the individuals of the susceptible population, but only 19 % of the

field population. After additional selection for five generations, the resistance ratio increased to 173-fold compared to the susceptible population (Abbas et al. 2014). This selected spinosad-resistant population would fully survive a field rate of spinosad.

Based on the abovementioned works, *C. carnea* seems to develop easily resistance to insecticides in the field when exposed to selection pressure. With 157 cases of resistance reported (APRD 2015), this species is by far the natural enemy most reported (Table 16.1), ranking the 22nd of any arthropod species, very close to very problematic pests such as *Aedes albopictus* (Skuse) (Diptera: Culicidae), *Panonychus ulmi* (Koch) (Acari: Tetranychidae), *Frankliniella occidentalis*, *Culex pipiens pipiens* L (Diptera: Culicidae), *Cydia pomonella* (L) (Lepidoptera: Tortricidae), and *Meligethes aeneus* F (Coleoptera: Nitidulidae). Moreover, such a resistance is high enough for the resistant populations to survive to field applications of insecticides. In that sense, lacewings would be a prime candidate for mass rearing and augmentative release of resistant-selected strains in suitable agroecosystems (Pathan et al. 2008; Sayyed et al. 2010).

Intriguingly, there is no a single reported case of insecticide resistance in any heteropteran predator. True bugs (Hemiptera) are the most widely used and commercially available insect predators in field and protected vegetable crops. Species of the family Anthocoridae (flower bugs), specially several species of *Orius* (minute pirate bugs) such as *O. laevigatus* (Fieber) in Europe and North Africa, *O. insidiosus* (Say) in America, and *O. strigicollis* (Poppius) in Japan, are mass reared and released to feed on a variety of small prey including thrips, spider mites, insect eggs, aphids, and newly hatched caterpillars. Miridae (plant bugs) including generalist predators such as *Macrolophus caliginosus* (Wagner), *Nesidiocoris tenuis* (Reuter), and *Dicyphus tamaninii* Wagner are important predators of whiteflies, mites, thrips, aphids, and caterpillars. The mirids are significantly used for biological control in tomato crops, as this crop is less suitable for establishment and foraging of mite and insect predator populations. Plant bugs successfully control pest populations in tomato crops such as *Bemisia tabaci*, *Trialeurodes vaporariorum* (Westwood), *Liriomyza* spp. (Diptera: Agromyzidae), and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Urbaneja et al. 2009; Calvo et al. 2012).

Most true bug predators are zoophytophagous to some extent. Flower and minute pirate bugs also feed on pollen in flowers when prey is not available. Mirids are omnivory and can feed on both plants and prey at the same developmental stage. As mentioned above, natural enemies develop resistance with difficulty due to the lack of preys after an insecticide treatment (Croft and Brown 1975; Croft and Morse 1979; Tabashnik and Johnson 1999). However, resistant polyphagous natural enemies, like phytoseiid mites and true bugs, can find food supply as they also feed upon plants and pollen. Therefore, omnivorous predators, like anthocorids and mirids, should become resistant more readily than more specialized predators such as ladybirds and lacewings. Several hypotheses might explain the scarcity of true bug predators showing resistance. Ecological, biological, and biochemical factors might be involved or simply a lack of specific studies (Tabashnik and Johnson 1999). A suggested explanation could be a lack of continuity in the selection pressure

over the same population. Vegetable crops, where these predators are normally used as biological control agents, are temporary, and consequently the potential resistant natural enemies selected in the season will migrate to other crops or wild plants, with different or none insecticide pressure. More importantly, these insect predators are mainly introduced by augmentative releases each season. Therefore, new mass-reared nonselected individuals are released in the crops, diluting the potential resistant genes selected previously.

16.4 Parasitoids

Considerably fewer cases of pesticide resistance have been reported for parasitoids. To our knowledge, the first reported case was a DDT-resistant strain of *Macrocentrus ancylivorus* Rohw. (Hymenoptera: Braconidae), a parasitoid of the larvae of *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), achieved by selective breeding (Pielou and Glasser 1952). Significant resistance to insecticides has been observed in a number of field populations of parasitoid wasps, such as *Bracon mellitor* Say (Hymenoptera: Braconidae) (Adams and Cross 1967), *Aphytis lingnanensis* Compere (Hymenoptera: Aphelinidae) (Havron et al. 1991a), *Ganaspidium utilis* Beardsley (Hymenoptera: Eucoilidae) (Rathman et al. 1995), *Diglyphus begini* (Ashmead) (Hymenoptera: Eulophidae) (Pollen et al. 1995), *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Baker et al. 1998), *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) (Perez-Mendoza et al. 2000), *Diadegma insulare* (Cresson) (Hymenoptera: Ichneumonidae) (Xu et al. 2001), *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) (Liu et al. 2007), *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Aphidiidae) (Wu et al. 2009), and *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae) (Zhuang et al. 2014).

Parasitoids seem to exhibit a higher susceptibility to pesticides compared to predators. A work on comparative selectivity of insecticides to the aphid *Brevicoryne brassicae* (L) (Hemiptera: Aphididae) and its three main natural enemies, the predatory coleopterans *Cycloneda sanguinea* (L) (Coleoptera: Coccinellidae) and *Acanthinus* sp. (Coleoptera: Anthicidae) and the braconid parasitoid *Diaeretiella rapae*, showed that overall the predators were more tolerant to the insecticides than was the parasitoid (Bacci et al. 2009).

The artificial selection for resistance in parasitoids has had a lower rate of success than in predators (Johnson and Tabashnik 1994). Laboratory-selected strains with resistance to different insecticides have been reported: *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) to carbaryl (Rosenheim and Hoy 1988), *Trioxys pallidus* (Halliday) (Hymenoptera: Aphidiidae) to azinphos-methyl (Hoy et al. 1990), *Aphytis holoxanthus* DeBach (Hymenoptera: Aphelinidae) to azinphos-methyl (Havron et al. 1991b), *A. lingnanensis* Compere (Hymenoptera: Aphelinidae) to azinphos-methyl (Javier et al. 1991), *Trichogramma chilonis* Ishii (Hymenoptera: Trichogrammatidae) to endosulfan (Jalali et al. 2006), and *Cotesia plutellae* to spinosad (Liu et al. 2007).

Table 16.3 Hymenopteran parasitoids reported to have developed insecticide resistance

Family	Species	Main hosts	Ecto/endo	Reference
Aphelinidae	*Aphytis lingnanensis*	*Aonidiella aurantii*	Ecto	Havron et al. (1991a)
		Aspidiotus nerii		
Aphidiidae	*Diaeretiella rapae*	*Brevicoryne brassicae*	Ecto	Wu et al. (2009)
		Other aphids		
Braconidae	*Bracon mellitor*	*Anthonomus grandis*	Ecto	Adams and Cross (1967)
Braconidae	*Cotesia plutellae*	*Plutella xylostella*	Endo	Liu et al. (2007)
Braconidae	*Habrobracon hebetor*	*Plodia interpunctella*	Ecto	Perez-Mendoza et al. (2000)
		Stored-product lepidoptera		
Braconidae	*Macrocentrus ancylivorus*	*Grapholita molesta*	Endo (Ecto)	Pielou and Glasser (1952)
Eucoilidae	*Ganaspidium utilis*	*Liriomyza* spp.	Endo	Rathman et al. (1995)
Eulophidae	*Diglyphus begini*	*Liriomyza* spp.	Ecto	Pollen et al. (1995)
Eulophidae	*Oomyzus sokolowskii*	*Plutella xylostella*	Endo	Zhuang et al. (2014)
Ichneumonidae	*Diadegma insulare*	*Plutella xylostella*	Endo	Xu et al. (2001)
Pteromalidae	*Anisopteromalus calandrae*	*Sitophilus oryzae*	Ecto	Baker et al. (1998)
		Stored-product coleoptera		

An explanation for the lower tolerance to pesticides in parasitoids compared to that in predators is that the latter are directly exposed to selection pressure, but the former have first to count on the host surviving the insecticide treatment. In fact, among the 11 parasitoid species reported to have developed resistance to insecticides (Table 16.3), seven are ectoparasitoids, which are directly exposed to pesticide applications.

The insecticide ingested by the host could be an important factor in the endoparasitoid's insecticide resistance development (Wu et al. 2009). Resistance has to evolve in the host as a condition for the parasitoid to develop resistance. It has been reported that exposure of parasitoids harbored by resistant hosts can effectively promote the selection of resistance in the parasitoid (Liu et al. 2007). In effect, three out of the four endoparasitoids having showed insecticide resistance (Table 16.3) parasitize *Plutella xylostella*, the second most resistant arthropod species (Sparks and Nauen 2014). Therefore, insecticide resistance is more likely to evolve in parasitoids which hosts readily develop insecticide resistance. Moreover, parasitism

may enhance the detoxification system in the host, and such improvement may increase the tolerance of the host to insecticides, helping both the host and the parasitoid evolve resistance to insecticides (Takeda et al. 2006; Liu et al. 2007).

16.5 Conclusions

The studies reviewed here show that there is a significant variability in response to insecticides among populations of natural enemy species. Therefore, most side-effect studies using only a population, usually provided by a BCA supplier, are not considering the natural variation in insecticide susceptibility. Consequently, on the one hand useful pesticides are being ruled out for some IPM programs, reducing the number of compounds available against several pests and diseases. This limited number of active ingredients favors the development of resistance (Bielza et al. 2008). As a result of resistance evolution, the pest or disease might be left without selective control tools, and the whole IPM program would break down. On the other hand, biological control cannot be implemented in many cropping systems as there are no options but applying noncompatible pesticides to control key pests.

As a consequence of that tolerance variation and pesticide pressure, development of insecticide resistance in natural enemies occurs in the field as there are reported cases for a wide range of species. In addition, resistance can be increased through artificial selection by mass rearing under selection pressure. Promoting BCA strains resistant to pesticides will broaden the range of scenarios (crops, agricultural systems, areas, seasons, etc.) where biological control can be used. The use of insecticide-resistant natural enemies can prevent secondary pest outbreaks and pest resurgence in many crops in which chemical management of pests is a common practice (Mansoor et al. 2013).

In summary, harnessing insecticide resistance in natural enemies may be an excellent opportunity to further integrate chemical and biological control by selecting strains of natural enemies resistant to pesticides.

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