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Biological Control of Pest and Vector Insects

Edited by Vonnie D.C. Shields

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Notice

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Meet the editor

Vonnie D.C. Shields, PhD, is currently a Full Professor in the Biological Sciences Department and the Associate Dean in the Fisher College of Science and Mathematics at Towson University, Towson, MD, USA. Dr. Shields' research explores gustatory, olfactory, and visual cues in insects. Her laboratory employs morphological, behavioral, and electrophysiological techniques to better understand sensory mechanisms by which larval and adult insects find host plants and detect plant-associated volatiles. Dr. Shields received both BS and PhD degrees from the University of Regina, Regina, Saskatchewan, CA. A portion of her PhD studies was carried out at the University of Alberta, Edmonton, Alberta, CA. After graduating, she accepted a research associate position to conduct postdoctoral studies at the Arizona Research Laboratories Division of Neurobiology, University of Arizona, Tucson, Arizona, USA, before she accepted a faculty position at Towson University.

Contents

Preface

This book contains chapters focusing on the central themes of biological control of insect pests, as well as insects as disease vectors. Chapter contributions fall under two main section headings: (I) “Strategies to Control Insect Pests" and (II) “Insects as Disease Vectors." In Section 1, the contributions of authors in this book reflect comprehensive reviews focusing on semiochemically based insect management techniques, the importance of sublethal effects of insecticides for integrated pest management, strategies for more efficient conservation biological control involving natural alternatives to chemical pesticide release with the use of natural enemies, gene silencing by RNA interference to provide alternative strategies to combat insect pests, various environmental factors and their influence in light-trap collection of insects, improving the efficacy of sex pheromonal components and attractants for capture of the cactus moth, management of pest pine weevil species involved in the damage of forest plantations in Poland, the use of some baculoviruses (i.e., nucleopolyhedrovirus (NPV)) and their identification via biochemical and biotechnological-based methods, and the activity of a pathogenic insecticidal activity of *Yersinia pseudotuberculosis* against the endangered Apollo butterfly. In Section 2, the authors present epidemiological features associated with the biting midges (*Culicoides*), with respect to their role as vectors of a number of arbovirus-causing diseases in domesticated livestock in Tunisia; transmission of arboviruses in Brazil by *Aedes* mosquitoes in humans; major human disease vectors, such as mosquitoes, tsetse flies, house flies, bed bugs, black flies, sand flies, and soft ticks in Tanzania; the impact of deer flies as pathogenic vectors to livestock and humans in Africa; a detailed investigation of the sexual reproduction in the blood-sucking insect, *Rhodnius prolixus*, with respect to population growth and spread of Chagas disease; and finally, development of transgenic and paratransgenic biotechnologies against pest and vector dipterans, including drosophilids, tephritids, mosquitoes, sand flies, tsetse flies, blow flies, and screw worms. These chapters represent recent contributions showing the diversity of ongoing research in this field of study. This book targets a wide audience of general biologists, as well as entomologists, ecologists, zoologists, virologists, and epidemiologists, including both teachers and students, in gaining a better appreciation of this rapidly growing field.

In Chapter 1, Section 1—“Strategies to Control Insect Pests," “Semiochemicals and Their Potential Use in Pest Management," El-Shafie and Faleiro begin by providing a general overview of insect semiochemicals, including definitions, classification, formulation, utilization in integrated pest management, as well as shortcomings of their application. The authors expound on insect management techniques, including mass trapping, mating disruption, and attract-and-kill methods.

In Chapter 2, “The Sublethal Effects of Insecticides in Insects," de França et al. consider studies focusing on the effect of insecticides on insect pests and nontarget organisms. Such infor-

mation is critical in accurately assessing lethal dose/concentration in comparing the toxicities of different active ingredients and different formulations of insecticides containing the same active ingredient. The authors define the sublethal dose/concentration as inducing no apparent mortality in the population and can result in reductions in life span, development rates, population growth, fertility, fecundity, changes in sex ratio, deformities, and changes in behavior, feeding, searching, and oviposition.

In Chapter 3, "Conservation Biological Control Practices," El-Wakeil et al. discuss conservation biological control practices in the preservation of natural enemies on target crops from pesticides and incompatible cultural practices. The authors explore preservation methods for maintaining natural enemies including the provision of habitat (i.e., greenhouse rearing), food resources (i.e., pollen, nectar, and plant sap) by growing flowering plants during non-crop periods, and topical applications of artificial or natural food supplements on crops, as the complete disappearance of natural enemies can occur over time due to agricultural practices involving the complete removal of plants after harvesting. In addition, factors associated with optimal rate, timing, and frequency of release of natural enemies are discussed, in keeping with the type of target pest, crop, and weathering conditions, among other factors.

In Chapter 4, "In Search of New Methodologies for Efficient Insect Pest Control: The RNAi "Movement," Kourti et al. evaluate an alternative method to replace conventional insecticides, namely, the use of silencing genes by RNAi (RNA interference) technology. The authors present an overview of the success of the main methods of RNAi delivery (i.e., injection and feeding), as well as alternative methods (i.e., naked dsRNA versus bacteria-, plant-, and virus-mediated RNAi) to achieve successful gene silencing. A discussion of the authors' research experiences using *Sesamia nonagrioides*, a lepidopteran pest, is provided.

In Chapter 5, "Light-Trap Catch of Insects in Connection with Environmental Factors," Nowinszky and Puskás analyze the connection between the light-trap collection of insects and environmental factors. The authors point out that fluctuations of light-trap collection results can be influenced by environmental impacts, for example, moonlight, as it has been found to reduce the efficiency of the light source. In addition to the effects of solar activity, data including other environmental factors, such as weather elements, are included.

In Chapter 6, "Reinvestigation of *Cactoblastis cactorum* (Lepidoptera: Pyralidae) Sex Pheromone for Improved Attractiveness and Greater Specificity," Cibrian-Tovar et al. address ways to improve the design and effectiveness of lures used to detect and monitor the South American cactus moth species, *Cactoblastis cactorum*, an invasive pest species of plants in the *Opuntia* genus (i.e., cactus family), distributed throughout the Caribbean, the United States, and Mexico. These authors discuss ways to improve the efficacy of lures (i.e., re-examine the composition and proportion of sex pheromonal components) using solid-phase microextraction (SPME).

In Chapter 7, "Insects Associated with Reforestation and Their Management in Poland," Skrzecz provides a comprehensive review of measures used to increase plant resistance in forest plantations from attacks by pine weevils (e.g., *Hylobius abietis, Pissodes castaneus, Cneorhinus plagiatus,* and *Brachyderes incanus*). As a measure to reduce the pollution of forest environments from the application of chemical treatments (e.g., pyrethroids and carbamates), a discussion of appropriate non-chemical control measures, including clear-cutting, the use of natural enemies (i.e., parasitoids), trap lures, as well as the incorporation of competitive fungi, botanical antifeedants, entomopathogenic nematodes, fungi, and viruses, is provided.

In Chapter 8, "Determination of Nucleopolyhedrovirus' Taxonomic Position," Nai et al. examine the use of various baculoviruses (i.e., insect-specific viruses), specifically nucleopolyhedrovirus (NPV), as showing promising results with respect to becoming commercialized as biopesticides. The authors discuss NPV identification via different genome sequencing technologies, as well as bioinformatics analyses approaches.

In Chapter 9, "Detection of *Yersinia pseudotuberculosis* in Apollo Butterfly (*Parnassius Apollo*, Lepidoptera: Papilionidae) Individuals from a Small, Isolated, Mountain Population," Łukasiewicz et al. investigate the pathogenic insecticidal activity of the bacterium *Yersinia pseudotuberculosis* against some insect hosts due to its ability to produce specific toxins. The endangered Apollo butterfly is discussed specifically, as populations of this species have declined drastically due to infection with *Y. pseudotuberculosis*, resulting in developmental abnormalities, such as deformation and reduction of wings.

In Chapter 10, Section 2—"Insects as Disease Vectors," "*Culicoides* spp. (Diptera: Ceratopogonidae) in Tunisia," Slama et al. review epidemiological features associated with *Culicoides* biting midges, important vectors of a number of arboviruses causing diseases in domesticated livestock in Tunisia. The authors discuss the development cycle, disease transmission, distribution, and tools for species identification.

In Chapter 11, "Transmission of Major Arboviruses in Brazil: The Role of *Aedes aegypti* and *Aedes albopictus* Vectors," Chouin-Carneiro and dos Santos provide a review of arthropod-borne viruses and describe major arboviruses currently affecting Brazil, focusing on Dengue, Zika, and Chikungunya, as they are transmitted between people by *A. aegypti* and *A. albopictus* mosquitoes. The authors discuss the *Aedes* mosquitoes' distribution, life cycles, reproduction, feeding behavior, breeding sites, and vector-virus interactions.

In Chapter 12, "Major Disease Vectors in Tanzania: Distribution, Control, and Challenges," Kweka et al. evaluate the roles of various disease vectors in Tanzania, namely, mosquitoes, tsetse flies, soft ticks, black flies, bed bugs, sand flies, and house flies, with respect to their distribution, abundance, control, and challenges associated with their eradication in Tanzania.

In Chapter 13, "The African *Chrysops*," Kouam and Kamgno examine the impact of these deer flies, namely, the species *C. silacea* and *C. dimidiata*, with respect to their ability to transmit many pathogens to both livestock and humans. The authors focus their review on the importance of these species with respect to medical and veterinary importance, hosts, classification, geographic distribution, morphology, life cycle, pathology, epidemiology, laboratory diagnosis, and control.

In Chapter 14, "Functional Anatomy of the External and Internal Reproductive Structures in Insect Vectors of Chagas Disease with Particular Reference to *Rhodnius prolixus*," Chiang and Chiang consider the anatomy and physiology of internal and external reproductive structures of the blood-feeding insect vector of Chagas disease, *Rhodnius prolixus*, as well as clarify our current understanding of the mechanics of egg laying, copulation, and the formation of the spermatophore. Chagas disease, also known as American trypanosomiasis, and caused by the protozoan parasite, *Trypanosoma cruzi*, is endemic to Central and South America.

In the last chapter, Chapter 15, "Developing the Arsenal against Pest and Vector Dipterans: Inputs of Transgenic and Paratransgenic Biotechnologies," Ogaugwu and Durvasula discusses transgenic and paratransgenic biotechnologies and how they have been applied to

develop and expand the arsenal against dipteran pests and disease vectors (i.e., drosophilids, tephritids, mosquitoes, sand flies, tsetse flies, blow flies, and screw worms).

I wish to thank InTech Open Access Publisher for initiating this book project and inviting me to serve as editor. I would like to recognize the Publishing Process Manager, Maja Bozicevic, assigned to the task of publishing this book, for guiding me through the process. I would like to acknowledge all the authors for their hard work in submitting and editing their contributions. Lastly, I wish to express a special thanks to my husband, Dr. Thomas Heinbockel, Professor and Director of Graduate Studies, Department of Anatomy, Howard University College of Medicine, and to our son, Torben Heinbockel, for their patience and understanding in the last year when I was working on this book project.

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Section 1

Strategies to Control Insect Pests

Chapter 1

Semiochemicals and Their Potential Use in Pest Management

Hamadttu Abdel Farag El-Shafie and
Jose Romeno Faleiro

Additional information is available at the end of the chapter

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Abstract

This chapter gives an account on the general concept of insect semiochemicals, their definitions, classification, formulation, utilization in integrated pest management programs, and the shortcomings of their application. The different semiochemically based insect management techniques, such as mass trapping, mating disruption, and attract-and-kill, are highlighted. The chapter also summarizes a case study from 7-year research on semiochemicals of the invasive red palm weevil, *Rhynchophorus ferrugineus*.

Keywords: olfactometer, mating disruption, chemical ecology, pheromones, kairomones

1. Introduction

Chemical communication plays an important and essential role in the survival of insects, which enable them to appraise immediate environment through modification of their behavior. Semiochemicals are organic compounds used by insects to convey specific chemical messages that modify behavior or physiology [1]. The term semiochemical is derived from the Greek word "semeon" which means sign or signal. Insects use semiochemicals to locate mate, host, or food source, avoid competition, escape natural enemies, and overcome natural defense systems of their hosts. Semiochemicals have the advantage of being used to communicate message over relatively long distances compared with other insect means of communication such as touch. Semiochemicals have different molecular weights depending on carbon chain. They are biologically active at very low concentration in the environment, thus their chemical characterization is complicated.

Expensive equipment items are needed for extraction and chemical characterization of semiochemicals. These equipment items include solid-phase microextraction (SPME), gas chromatography-electroantennography (GC-EAG), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) [2]. For the development of new synthetic pheromone blend, a lot of work on electrophysiological and behavioral bioassay is required [3].

Semiochemicals are species-specific and harmless to the environment. These advantages over conventional insect pest control agents make semiochemicals promising tools for the management of agricultural pests particularly under organic cropping systems.

2. Classification of semiochemicals

Semiochemicals are classified based on their effect or function and this should be taken into account because the same molecule could act as a pheromone for one insect species and as a kairomone or allomone for another species. Semiochemicals are divided into two broad groups: pheromones that mediate interactions among individuals of the same species (intraspecific reactions) and allelochemicals that mediate interactions among individuals of different species (interspecific interactions). According to the behavioral response, pheromones are further subdivided into primer pheromones that have long-term physiological changes and releaser pheromones that elicit short-term or immediate behavioral response. Allelochemicals are divided into kairomones that mediate interactions favoring the recipient, allomones, on the other hand, favor the emitter. Synomones favoring both the emitter and the recipient, and apneumones, which are substances, produced by nonliving material that elicit behavioral response favorable to the receiving organism but harmful to a second organism found on the nonliving material. Schematic diagram showing the classification of semiochemicals is shown in **Figure 1**.

2.1. Insect pheromones

Karlson and Lüscher [4] first proposed the term pheromone to describe chemical signals that mediate intraspecific interactions. The sex pheromone of the silkworm moth, *Bombyx mori* was the first pheromone to be chemically identified in 1959 and is considered as the most important semiochemical used in pest management. Other pheromones include aggregation pheromone, which are produced by males and attract both sexes of conspecific individuals. The sex pheromone of moths is the most studied and widely used in insect pest management than other pheromones [3]. One-day-old female moth emits the sex pheromone usually at a rate of a few tens of pictogram per second at a certain time of the day or night. It has a characteristic behavior of raising the abdomen and exposing the pheromone glands at the end of the abdomen, this behavior is termed as calling posture [3]. Male moths, on the other hand, synchronize their daily activity to calling females for mating to be successful. The males respond to pheromone by flying upwind in the plume from 10 to 100 m downwind to locate the source [3]. Insect pheromones diffuse from their source in the form of strands of odor that drift downwind and become stretched, twisted, and ripped apart into substrands, which interspersed with pockets of clean air to form odor plume that produce the sustained upwind flight or what is termed as attraction [5, 6].

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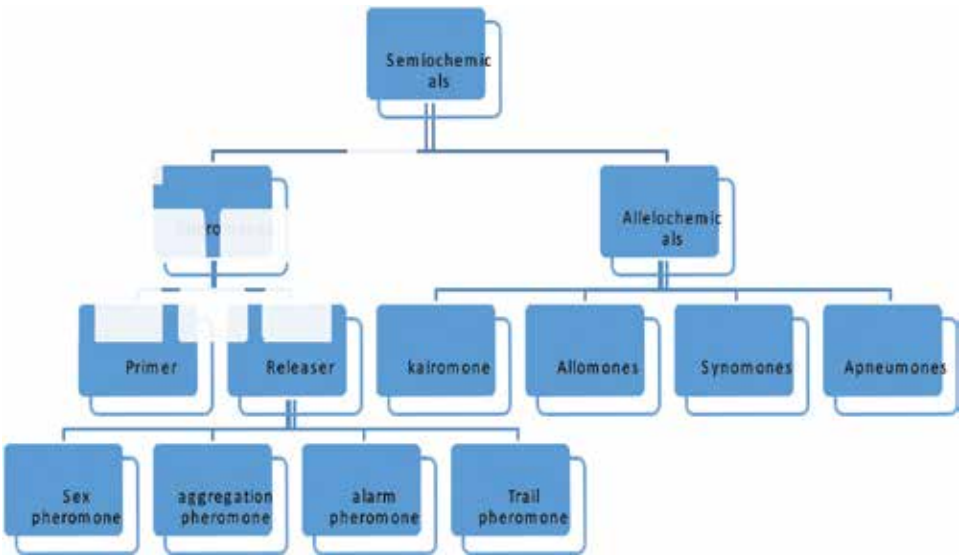

Figure 1. Classification of semiochemicals based on their effect and role in specific interactions.

2.2. Insect parasitoid kairomones

Semiochemicals play an important role in host-parasitoid relationship, which was categorized by [7] into three stages: habitat-location, host-location and host-acceptance, and oviposition. These semiochemicals included but not limited to aldehydes, alcohols, sulfur-containing compounds, esters, terpenes, alkanes, heterocyclic aromatic compounds, proteins, amino acids, triglycerides, and salts. He stated that semiochemicals identified in the habitat-location step were likely to be from the host-plant of the host insect, while in host-location and host-acceptance and oviposition steps, semiochemicals are predominantly from the host. Kairomones used by parasitoids to locate their hosts can be divided into two groups, external to the host, which are long-chain hydrocarbons, ketones of fatty acids, esterified cholesterols or proteins found in either host frass or glue used to attach eggs to a substrate [7]. The internal kairomones represented by amino acids and salts in the hemolymph, which have normally been sensed the ovipositors and serve as indicators for suitability of the host for the parasitoid offspring, a kind of maternity care found in many insect species.

3. Identification and behavioral characterization of semiochemicals

Identifying the range of volatiles that insects can detect in their environment is an important step toward understanding the role of olfaction in modulating insect behavior [8]. The process of semiochemical identification involves extraction or headspace collection, identification of active compounds, characterization of chemical composition of the identified compound, and elucidation of behavioral response of the insect to the active product. If the organ that produces the semiochemical is known, for example, the exocrine gland or the gut of the insect,

it can be extracted and identified. However, nonrelevant compounds can also be extracted, which complicate the identification process. Accordingly, headspace collection is preferred instead [2] where a charcoal-filtered air is passed over the insect or its organ in an isolated aeration chamber and the odor-laden air is withdrawn by vacuum for analysis [8]. The air containing a mixture of volatiles can be analyzed *per se* or after being absorbed by super Q using columns. In the 1950s, it has been discovered that a measurable voltage arised between the tip and base of the insect antenna when exposed to odors of biological significance for the insect. This antennal response to different odors is known as electroantennogram or EAG [9]. This voltage is thought to represent the summed potentials of multiple responding olfactory neurons within the antenna, and the amplitude of the voltage roughly corresponds to an insect's sensitivity to a particular compound. The EAG has been widely used in entomology for pheromones' identification. The electroantennogram was improved through time and the insect antenna has been used as a detector (EAD) for a capillary-column gas chromatography, which is coupled with the flame ionization detector (FID) that is sensitive to all organic molecules. The GC-EAD is gas chromatography coupled with electroantennographic detection of compounds present in complex mixtures. This analytical procedure allows for rapid and accurate identification of insect odors. It is widely used to discover and identify semiochemicals like insect pheromones and repellents [8, 10]. The apparatus consists of injector (heated chamber), the column (10–100 m long, 1 mm wide) lined with a semisolid wax or polymer, flame ionization detector, insect antennal preparation, and a monitor to display the voltage output of the detector and the insect antenna (voltage on the Y-axis against time on the X-axis). Retention time and peak of each molecule give the identity and amount of the compound in the mixture, respectively. The FID output can be used to confirm the presence, identity, and quantity of compounds exposed to the antenna while the antennal (EAD) output indicates the presence/absence of olfactory sensitivity to eluting compounds and provides a relative measure of the intensity of olfactory stimulation. The FID peaks of the test compounds can be compared with retention times of those of commercially available versions of the same compounds injected into the GC. Identifications can be confirmed by re-analyzing the extract with a coupled gas chromatography-mass spectrometer (GC-MS) using the same column and GC operating parameters as used in the GC-EAD analysis. Testing the behavioral activity of EAD-active volatiles is an important and complementary step in the identification of semiochemicals that modify insect behavior. This behavioral bioassay test can be carried out using wind tunnel or an olfactometer [11, 12].

4. Interaction of insects and plants semiochemicals

Insects live in an environment with many volatile compounds including insect herbivore, host plant, and insect carnivore semiochemicals. These volatile chemicals interact with each other and finally modify the behavior and the physiology of insect pest species. Some insects sequester or acquire host plant compounds and use them as sex pheromone or sex pheromone precursors [13]. Many butterflies, moths, beetles, grasshopper, and aphids used pyrrolizidine alkaloids from their host plants as strong feeding deterrents against their natural enemies or predators [14]. It has been shown that the oil palm *Elaeis guineensis*, the host plant of the

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African palm weevil *Rhynchophorus phoenicis* produce a mixture of volatile esters of which the ethyl acetate induces male weevils to release the aggregation pheromone E-6-methyl-2-hepten-4-ol or rhyncophorol [15]. The males of orchid bees collect a mixture of terpenoids from the orchids and use them as aggregation pheromone to induce the formation of leks or the sites where males compete for females [16].

5. Potential use of semiochemicals in insect pest management

Semiochemicals have been used for insect pest management more than 100 years ago [2]. Insect sex pheromones are the semiochemicals that are widely used for the management of insect pest particularly members of the order Lepidoptera. Aggregation pheromones from the order Coleoptera are also used for the management of agricultural insect pests of economic importance. Several serious agricultural pests including the carob moth *Ectomyelois ceratoniae*, the armyworm *Spodoptera frugiperda*, tomato leaf miner *Tuta absoluta*, fruit flies *Bactrocera* sp., mountain pine beetle (MPB) *Dendroctonus ponderosae*, Asian citrus psyllid *Diaphorina citri*, and the red palm weevil (RPW) *Rhynchophorus ferrugineus* have been successfully managed by using semiochemicals.

Semiochemicals are considered safe and environmentally friendly molecules due to their natural origin, low persistency in the environment, and species specificity, which attribute much to their harmless effect on nontarget organisms [17]. However, there are some difficulties in the practical applications of semiochemicals in pest management, and due to these challenges Semiochemically-based pest methods are still at the beginning[2]. Baker [3] mentioned the reasons that promoted or hindered the adoption of pheromones in the management programs of insect species as follows:

a. The biological differences in the mate-finding behavior of different species.

b. The chemistries of the pheromones used.

c. The successful engineering of the controlled-release dispenser and the use of proper trap design

d. The different political, economic, and use-pattern in different countries particularly the regulation of pheromones' application.

6. Semiochemical formulations

Strong plumes of the correct blend of pheromones that create the above-threshold plume strands for downwind of the pheromone source are the key points to the optimal use of pheromones in integrated insect pest management programs. To achieve this, a controlled-release system of pheromone dispenser that mimics the natural pheromone release by insect pest is required [3]. In addition, optimization of trap density, design, and trap position is essential to achieve trap efficiency.

Mafra-Neto et al. [18] listed the shortcomings in semiochemical formulations as follows:

a. High cost of semiochemicals in terms of method of deployment in the field compared to conventional synthetic pesticides. Most semiochemicals used in insect pest management are formulated in the form of devices which require manual application.

b. The physical limitations such as instability, volatility, and sensitivity of the active ingredient of the semiochemicals to environmental factors like temperature and light.

c. Inconsistency of semiochemical product to maintain release rate and short-field longevity.

d. Mechanism of behavior manipulation in some techniques in which the semiochemical is used (e.g., mating disruption formulation that acts by camouflage requires large quantity of pheromone to be deployed which means more application cost).

For the above-mentioned reason, ISCA technologies developed an innovative semiochemical application technique for agricultural and forest insect pests. The technique is called specialized pheromone and lure application technology (SPLAT®). It is an amorphous, flowable, and controlled-release emulsion, with chemical and physical properties that may be adjusted by small changes in composition in processing or application method. A shear-thinning thixotropic, non-Newtonian fluid enters a liquid state by agitation, but quickly solidifies when agitation is stopped. These physical characteristics give the formulation flexibility in application. SPLAT® can provide a continuous controlled-release of semiochemicals for a period ranging from 2 weeks up to 6 months [18].

Semiochemicals are utilized for the management of insect pests through the following tactics:

a. Detection of invasive species and in delimiting surveys.

b. Monitoring the populations of endemic species to synchronized the timing of insecticide treatments

c. Evaluation of the effectiveness of pest management tactics through post-application assessment.

d. Improvement of old method of insect counts used for decision-making.

e. Increasing the effectiveness of biological control by increasing the predation/parasitism rates of predators and parasitoids. Kairomones could be applied to plants to increase the rate of parasitization through increasing the search rate of *Trichogramma* sp. [19].

f. Reduction of pest population through mating disruption, attract and kill, mass trapping, and repellency techniques.

7. Semiochemical-based pest control techniques

7.1. Attract and kill (A&K)

The technique as the name implies simply use an attractant or semiochemical to lure an insect to a point source that contains a killing agent (insecticide, pathogen, or sterilant), hence the

technique is termed attract and kill, attract and infect, and attract and sterilize, respectively. The technique leads to the reduction of the insect population by killing the target insect or reducing its fitness and fecundity or disabling it by causing disease. For more information on this technique regarding the fundamental requirements for both the attractant and the killing agent, please see Mafra-Neto et al. [18].

7.2. Mating disruption

The technique is most commonly used in semiochemical-based pest management. It manipulates insect behavior in such a way that leads to population reduction. The environment where specific insect pest needs to be controlled is saturated with synthetic sex pheromones so that the abilities of males to locate the natural pheromone plume emitted by females are disrupted [18]. Mating disruption using synthetic pheromones or parapheromones does not completely shut off mating, but the delay in females mating may reduce their fecundity by approximately 50% [3]. Insect females have a critical time to mate and reproduce and any delay of mating may affect their fitness and their abilities to select the suitable sites for oviposition [18]. The mating system of some insects involves the transfer of certain peptides that trigger the egg laying behavior in the females. Four mechanisms were proposed to explain how mating disruption occurs, and these are:

a. Competitive attraction or false trail following
This happens when males respond to synthetic pheromone plumes produced by semiochemical dispenser rather than the natural plume emitted by the calling female [18]. This mechanism is density-dependent and decrease in efficiency as the population of pest increases.

b. Camouflage
This mechanism requires complete saturation of the environment with the synthetic pheromone. In this case, the male cannot locate the positions of the females and it is density-independent.

c. Desensitization
Adaptation of the male olfactory receptor system or habituation of the central nervous system may occur due to the overexposure to synthetic pheromone.

d. Sensory imbalance
Adaptation of the male olfactory receptor system or habituation of the central nervous system may occur due to the overexposure to synthetic pheromone.

7.3. Mass trapping

It is a pheromone technique commonly used for direct insect population suppression. The technique is defined as the deployment of sufficient high density of pheromone traps that eliminate enough adults from the population and thus reduce subsequent larval damage [3]. Pheromones for monitoring are usually used at low density and the trapped insects have no effect on reduction of the population [20]. Mass trapping is effective in the case of male-emitted pheromone system that attracts females such as weevils (red palm weevil) and snout beetle. In this system, females are trapped, thus mass trapping directly reduces egg laying. The technique

is effective with insects having relatively low population, live a long time before egg-laying, lay small number of eggs, and the emerging larvae cause considerable damage [21].

7.4. Repellents

A repellent is defined as a substance that deters or inhibits insects from finding, feeding on, or ovipositing on an attractive host substrate [18]. Several semiochemicals with repellent effect are available for the management of agricultural and forest pests. However, their practical application is limited due to the availability of cheap and effective pest control alternatives, lack of adequate formulations for delivery, and regulatory obstacles including registration [22].The repellent "verbenone" is now commercially available for the management of mountain pine beetle (MPB), *Dendroctonus ponderosae*. The repellents can be used alone or in combination with attractants for the management of insect pests as part of a push-pull strategy. Cook et al. [23] defined the push-pull strategy as the use of semiochemicals to make a protected resource an attractive or unsuitable for the pests (push) while luring them to an attractive source (pull) where the pests can be removed. Compared to other semiochemically based pest management techniques such as mating disruption or attract-and-kill, push-pull strategy requires more understanding of the chemical ecology of the insect pests [23].

8. A case study of the red palm weevil in date palm plantation

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Coleoptera: *Curculionidae*), is an invasive and destructive insect pest of date palm worldwide. RPW was first reported as a pest of coconut palm in India and since the 1980s, it has invaded many countries around the globe. The weevil is a relatively large insect exhibiting different color morphs and sexual dimorphism where the male can be easily distinguished by the presence of dense hairs on the rostrum which are absent in the female. The weevil is extremely difficult to manage because all the life stages are concealed inside the date palm tissues and are difficult to detect at an early stage of damage. Several tactics including preventive and curative measures are adopted to manage the weevil in date palm plantations [24]. However, RPW is currently managed in date palm groves through semiochemically based integrated management using male-produced aggregation pheromone or (4-methyl-5-nonanol) for both monitoring and mass trapping of adult weevils [25]. This pheromone was first identified and synthesized in the early 1990s and has since proven a valuable tool for the management of RPW [26]. The aggregation pheromone acts synergistically with 4-methyl-5-nonanone (ketone) and is more attractive to RPW when combined with kairomones or volatiles emitted from the host to increase the efficiency of the blend [25]. The RPW pheromone (Ferrolure +) is commercially available (a mixture of 4-methyl-5-nonanol and 4-methyl-5-nonanone with a ratio of 9:1) released at 3–10 mg/day. The addition of ethyl acetate (released at 200–400 mg/day), fermenting mixture of dates and water increases trapping efficiency. The pheromone/food-based trapping system is considered as an environmentally friendly approach compared to the use of insecticides, which is currently being applied for the control of RPW [27]. Ferrugineol is attractive to both sexes; however, several researchers reported that traps baited with this attractant tend to

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capture significantly more females than males, usually with a ratio of two females to one male [24, 28, 29]. In addition, the captured weevils were found to be young, gravid, and fertile, indicating significant impact of trapping on the population reduction of the weevil in a given locality [30, 31]. The advantages of semiochemicals over other methods of pest control are that they are naturally occurring substances with species-specific character. Semiochemicals, a major component of IPM strategy for the management of red palm weevil in date palm [32], are environmentally friendly and have no adverse effects on natural enemies and pollinators in the agro-ecosystem. The aggregated nature of RPW distribution in the field, the long-life cycle, adult longevity, reliance on aggregation pheromone and host kairomone, and the relatively low population make the use of semiochemicals ideal for the management of this notorious pest [33]. To have an efficient semiochemical-based management program for RPW, a highly optimized pheromone product is needed in terms of attraction to lure the weevil directly to the trap (point source). The weevil must successfully locate the trap, arrested and enter inside; otherwise, it will infest the palm, which should be avoided when using traps. The trap should be more attractive and arresting for the weevils than the natural kairomone emitted by the date palm in the field. Optimizing the RPW trapping system requires better understanding of semiochemical ecology of the weevil in date palm plantation. Accordingly, the authors conducted a series of laboratory and field experiments that expand for 7 years to understand the chemical ecology and semiochemicals of red palm weevil in the date palm plantations in order to optimize trapping efficiency for an effective semiochemically based integrated management of this notorious pest. A summary of the results of these experiments is given in the following paragraphs.

8.1. Research methodology

All laboratory experiments were conducted in the Date Palm Research Center of Excellence (latitude 25.16′6.9780″N, longitude 49.42′27.2772″E, and altitude 153 m), King Faisal University, Kingdom of Saudi Arabia. The field experiments were conducted in highly infested date palm groves selected based on data of trap catches obtained from the Directorate of Agriculture, Al-Ahsa, Ministry of Agriculture, and Kingdom of Saudi Arabia. Weevils used in olfactometer assays were obtained from a colony of RPW, which was established in the laboratory on bolts of the popular date palm cultivar "Khalas" that represent more than 85% of the cultivated date palms in Al-Ahsa oasis where the study has been carried out. To obtain virgin weevils, pupae were collected from the reared colony, and each pupa was kept separately in 20-ml-plastic jar with perforated lid. The jars were then kept in an incubator at a temperature of 30°C and 70% RH until adult eclosion. Emerged adult weevils were fed ad libitum on sugar cane for at least 3 days before being used for the different experiments.

8.1.1. Olfactometer bioassays

Olfactometers are used to gauge the odor detection threshold of substances. A four arm-choice olfactometer®, a custom-made by Analytical Research Systems, Inc., Florida (ARS Inc., Florida) (www.ars-fla.com/mainpages/Bio-Assay/4 & 6-choice.htm) was used to study the weevils' preference to different lures (**Figures 2–4**). This olfactometer was connected to a pump that maintained a constant flow of pure air through the four arms while at the same

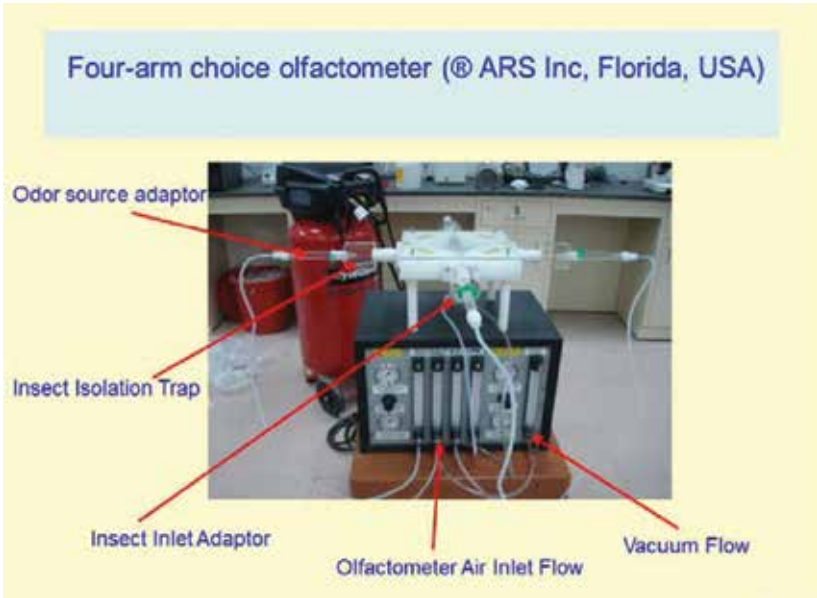

Figure 2. The layout of the four-arm choice olfactometer is shown. It consists of three units: a pump to provide purified air, clean air delivery system (vacuum), and odor exposure arena with polyethylene body and removable lid made of plexiglas.

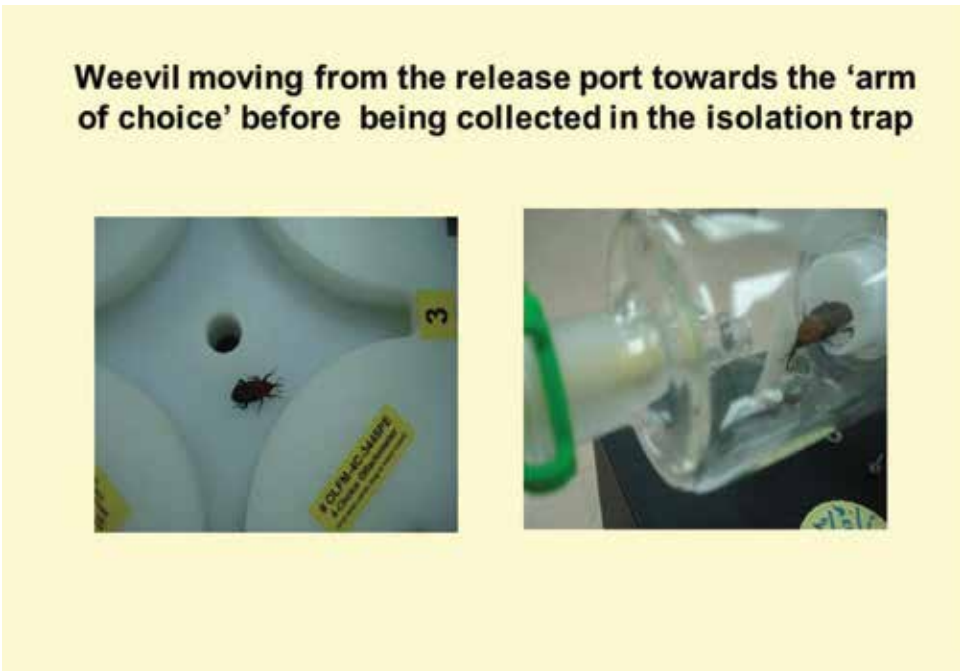

Figure 3. Dorsal view of the olfactometer showing insect inlet adaptor and isolation trap. An adult weevil moving upwind responding to odor source (*left*) before ending up in the insect isolation trap (*right*).

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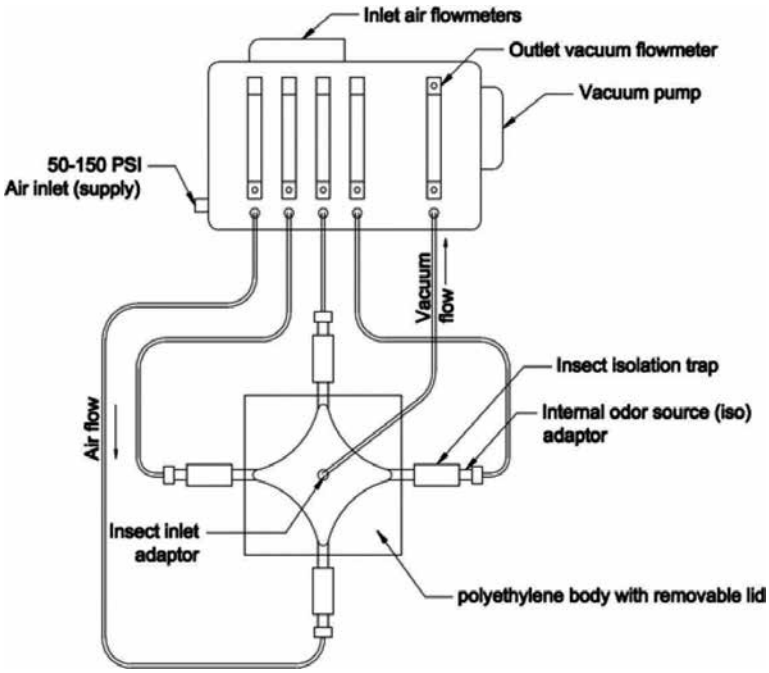

Figure 4. Schematic diagram of a four-arm olfactometer illustrating how it works. The olfactometer was connected to a pump that maintained a constant flow of pure air through the four arms while at the same time vacuuming out the odors emitted by the treatments through central suction. Four-flow meters controlled airflow in the olfactometer. Each of the four arms of olfactometer is provided with an odor source to be bioassayed, thus providing four odor fields for the test weevils to make a choice. Weevil directional preference to one of the four arms is used as an indicator to assess its behavioral response to that odor.

time vacuuming out through central suction the odors emitted by the treatments. Four-flow meters control airflow in the olfactometer. Each of the four arms of olfactometer is provided with an odor source to be bioassayed, thus providing four odor fields for the test weevils to make a choice. Weevil directional preference to one of the four arms is used as an indicator to assess its behavioral response to that odor. The apparatus is manipulated in such a way that other factors, which might affect the weevil response to the odor, are controlled. More details on the functioning of the olfactometer are given in Section 8.3 of this chapter. The olfactometer was calibrated before carrying out the experiments according to the specifications shown in **Table 1**.

8.2. Optimizing components of pheromone-baited traps

Several assays were carried out to evaluate the response of both male and female RPW adults to the aggregation pheromone (Ferrolure™) (El-Shafie and Faleiro, unpublished data). The test insects were (i) newly emerged unmated/mated insects and (ii) 1-month-old mated insects. Newly emerged insects were tested individually (20 replicates) while the 1-month-old adults were tested in batches of five weevils (10 replicates). The internal odor source (IOS) adapter of the olfactometer was moved from one arm to another sequentially, so that each arm of the

Inlet/outlet pressure and air flow rate	Test calibration
1. Olfactometer pressure (10–20 PSI)	15 PSI
2. Source inlet pressure (50–150 PSI)	60 PSI
3. Olfactometer vacuum: center suction (−5 to −22″Hg)	−10″Hg
4. Vacuum pump pressure (60 + PSI)	+60 PSI
5. Olfactometer air inlet flow (0–1.3 LPM)	0.9 LPM

ARS Inc, Florida, USA.
PSI = pounds/square inch; ″Hg = inch mercury; LPM = liters per minute.

Table 1. Calibration of the olfactometer® to study the response of the red palm weevil (RPW) to different RPW pheromone lures.

olfactometer had the same number of replicates thereby eliminating bias of the instrument and environment if any. Besides the commercial pheromone, the internal odor source of the instrument containing the lure was charged with 1 ml fermented date solution dispensed in a perforated tube. A 5-min period was allowed for the test insects to move toward the arms from the insect release tray (IRT). Weevils collected in the insect isolation trap (IIT) at the end of 5 min were recorded.

The results presented in **Figure 5** reveal significant and high attraction (>50%) to the aggregation pheromone in newly emerged unmated male and female weevils. Upon mating both newly emerged male and female weevils were significantly attracted to the pheromone but at a reduced (~30%) rate as compared to newly emerged unmated (virgins) individuals. With age (1 month old), the attraction to the pheromone reduced still further with only 14% of the female weevils responding significantly to the pheromone, whereas 1-month-old male wee-

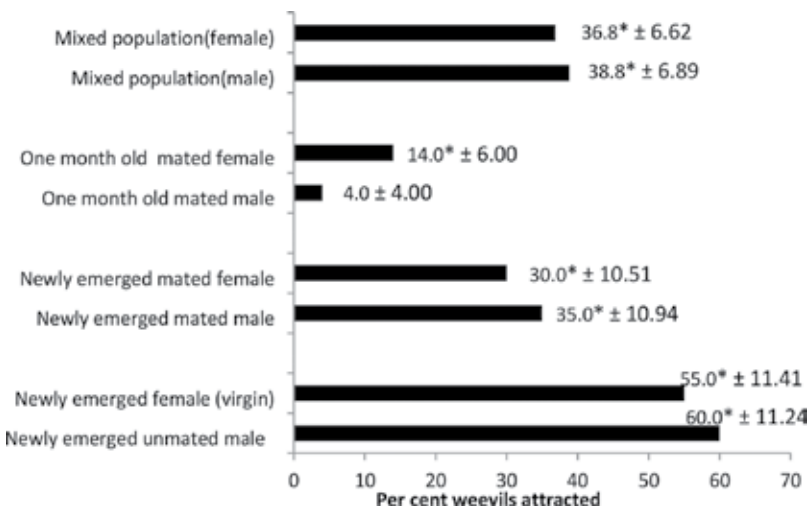

Figure 5. Response of *R. ferrugineus* to the aggregation pheromone in choice olfactometer assays (*t*-test; *significant at p = 0.05).

vil did not show significant attraction to the pheromone. In general, around 35% of the test insects (male and females) were attracted to the pheromone. The RPW uses the aggregation pheromone (ferrugineol) to recruit conspecific mates over long distance to colonize its host. This biological characteristic has been extensively utilized to manage this weevil through monitoring and mass trapping. Our results revealed that the response of RPW (males and females) to aggregation pheromone increased with mating as supported by the finding of Poorjavad et al. [34] who studied the effect of different doses of the aggregation pheromone on the diurnal response of virgin and mated males and females RPW under laboratory conditions using a two-choice pitfall static olfactometer. The authors concluded that in both sexes, the response to ferrugineol increased with mating. Mated females showed strong response to ferrugineol than unmated ones, due to the searching behavior of the former for egg laying sites.

Weissling et al. [35] stated that the optimal attraction of weevil to trap is affected by the proportional changes in volatile chemicals from fermentation overtime. These volatile chemicals or kairomones, as determined by GC-MS, included palm esters, ethyl acetate, ethyl propionate, ethyl butyrate, and ethyl isobutyrate [36]. Kumar et al. [37] tested different pheromone lures in India and reported differences in their efficiencies. This discrepancy could be attributed to difference in environmental conditions between India and Saudi Arabia where the present investigation was carried out. In this context, Faleiro and Chellapan [38] reported a difference in longevity of the lures in different seasons (winter and summer). They stated that the release of pheromone into the environment is faster in summer than in winter and that was attributed to higher temperature and day light. Thus, they recommended putting traps under shade conditions to sustain the efficiency of the trapping system. The fact that only 35% of the test weevil (males and females) responded to the aggregation pheromone, which is supported by El-Sayed et al. [27], require that the remaining population in the field have to be managed through other IPM tactics.

8.3. Determining the extent of attraction of weevils to date palm volatiles

Faleiro et al. [12] studied the mechanisms of resistance against RPW in seven major date palm cultivars of the Al-Ahsa oasis in Saudi Arabia viz. Khalas, Sheshi, Reziz, Khasab, Hatmi, Shahal and Gaar by determining the extent of attraction of female RPW adults to fresh palm volatiles emitted from date palm frond tissue through four-arm choice olfactometer assays. In each of the inlet odor source (IOS) adapters of the olfactometer, freshly cut palm petiole pieces (5 × 1 × 1 cm) of a single cultivar were placed. Two experiments were carried out wherein four cultivars were tested at a time with Khalas as the control treatment in both the experiments. Fifteen-day-old field collected gravid adult female weevils were used in the assays. Five female weevils were placed in the insect release device of the olfactometer. After 5 min, the number of adult female weevils collected in the insect isolation trap (IIT) was noted. Each experiment was replicated eight times. At the end of each assay (replication), palm tissue pieces and test insects used in the assay were discarded. New palm tissue pieces and test insects were used for every replication (assay). The IOS was moved sequentially to the next arm of the olfactometer at the end of each test replication so that every treatment was at the same arm of the olfactometer twice during each trial.

This was done to eliminate bias if any in the instrument and environment. Results revealed that the popular date palm cultivar Khalas had the least antixenotic effect on female RPW adults where a high degree of attraction to palm tissue volatiles was recorded which was statistically similar to the cultivars Reziz, Sheshi, and Hatmi. The cultivars Khasab, Shahal, and Gaar exhibited high degree of nonpreference (antixenosis) (**Figure 6**). Identifying the chemical components of tissue volatiles that trigger antixenosis in date palm to RPW will pave the way for future studies on chemical ecology of RPW and its interactions with date palm as a main host.

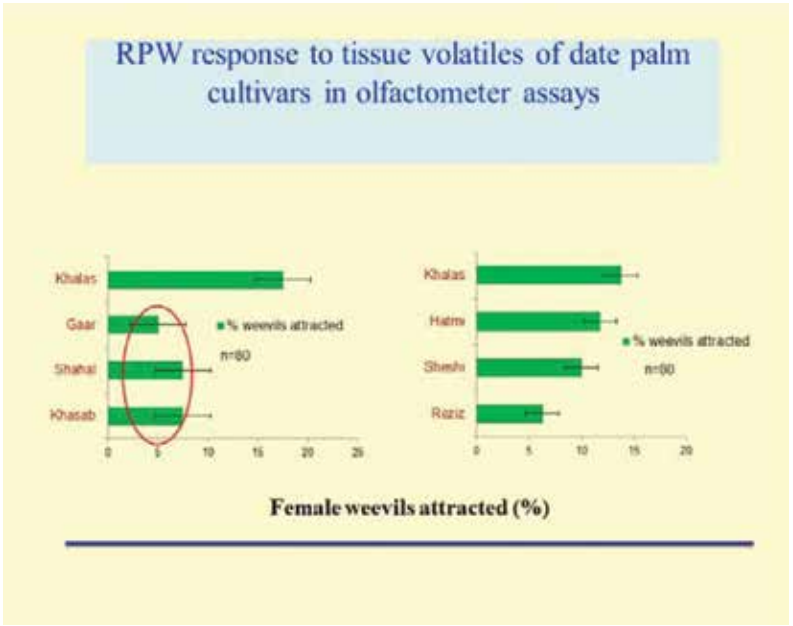

Figure 6. Red palm weevil response to tissue volatiles of date palm cultivars in olfactometer assays.

8.4. Testing the efficacy of Hook™ RPW versus food-baited pheromone traps

Food-baited pheromone traps (FBPTs) have been used to manage the red palm weevil in date plantations through monitoring or mass trapping techniques for population reduction. The FBPTs are simply made of plastic buckets baited with ferrugineol and fermenting dates as sources of kairomone (**Figures 7** and **8**). The main problem with FBPTs is that they require frequent service (replacement of food bait and water) which makes their utilization over large area laborious and expensive. ISCA technologies developed Hook RPW is an attract-and-kill formulation that combines the aggregation pheromone and the insecticide cypermethrin. The formulation does not require service once deployed in the field, thus it can drastically reduce application cost particularly in area-wide RPW integrated management.

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Figure 7. The standard RPW bucket trap showing the main components.

Figure 8. A marked RPW male walking toward the food-baited pheromone trap.

El-Shafie et al. [39] tested the efficacy of Hook™ RPW in an infested date plantation in Eastern province of Saudi Arabia. The product was applied in hundred 3 g dollops at an application rate of 250 dollops per ha. The area of treated plot was 0.4 ha containing ca. 60 date palms of the popular variety "Khalas." The number of weevils attracted to source point and killed was compared with the catches of the conventional FBPTs commonly deployed by the Directorate of Agriculture to manage RPW in date palm plantations. The data of weevils captured per week in the treatment plots was converted to number of RPW caught per ha per week to compensate for the discrepancy in trap density between the two trapping methods. Results revealed no significant difference between Hook RPW and FBPTs indicating that SPLAT formulation can be successfully used as a potent component in the integrated management of RPW. In a separate trial concerning bait-lure synergy, Hook RPW sustained the same level of attraction to PRW regardless of the presence or absence of food bait [39]. SPLAT for RPW proved to maintain field longevity and efficacy for 3 months under the dry summer conditions of Saudi Arabia.

A 10-fold increase in pheromone trap number in Al-Hasa date palm oasis during the period from 2007 to 2012 in Saudi Arabia decreased the RPW infestation in an area of 1104 ha by 86%. Likewise, the application of insecticides was reduced by 91% and the felling and eradication of infested date palm trees dropped by 89% [40]. Similar stories of successful management of RPW using semiochemicals in the Gulf and the Middle East are documented [28, 41]. The research on semiochemical repellents of RPW has been initiated and Guarino et al. [42] identified α-pinene and methyl salicylate for being potential repellents for RPW. This could open an avenue for the future use of push-pull strategy for integrated management of red palm weevil.

9. Conclusions

Semiochemicals have been exploited in several ways to manage insect pests. These include monitoring and detection, population suppression through mating disruption, mass trapping and attract-and-kill techniques. The male-produced aggregation pheromone is successfully used in food-baited traps for the area-wide integrated management of red palm weevil in date palm, coconut palm, and Canary Island palm plantations. Mated RPW females responded more to the aggregation pheromone than virgin females indicating that an egg-laying stimulus may be responsible for deriving these females to the aggregation pheromone. Large number of RPW adult weevils fly to traps located near to their colony to nearby traps and increasing distance to trap from infestation spot increase the probability weevil will not find the trap. This behavioral response of RPW to aggregation pheromone should be considered when specifying trap density per ha for mass trapping. Some date palm cultivars exhibited different kinds of resistances to RPW the mechanisms of which vary; however, antixenosis or egg-laying nonpreference is important. Future research on semiochemicals for insect pest management should focus on innovative formulation for field deployment as well as on optimization of controlled-release technologies and trapping efficiency. More research on the chemical ecology of target insect pest is of paramount importance for development of semiochemically based insect pest management programs.

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Chapter 2

The Sublethal Effects of Insecticides in Insects

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Additional information is available at the end of the chapter

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Abstract

Studies related to the effect of insecticides on insect pests and nontarget organisms, such as natural enemies, are traditionally accessed by the estimative of lethal effects, through mortality data. Due to the limitations of the traditional methods, recent studies in the past three decades are assessing the sublethal effects of insecticides upon several important biological traits of insect pests and natural enemies. Besides mortality, the sublethal dose/concentrations of an insecticide can affect insect biology, physiology, behavior and demographic parameters. In this chapter, many sublethal effects of insecticides were addressed for several chemical groups, such as botanical insecticides, carbamate, diamide, insect growth regulators, neonicotinoid, organochlorides, organophosphates, pyrethroid and others. An accurate assessment of these effects is crucial to acquire knowledge on the overall insecticide efficacy in the management of pest insect populations, as well as on their selectivity toward nontarget organisms.

Keywords: sublethal concentrations, pest insects, natural enemies, biological effects, physiological effects, behavioral effects, demographic studies

1. Introduction

Despite numerous novel control agents available at integrated pest management programs, insecticides remain as the most reliable method for insect control. The effects of insecticides and other toxicants on insect pests and other arthropods have been the subject of innumerous studies in the past several decades [1]. Methods to test the side effects of toxicants have been developed as a function of insect control evaluations. For a long time, the classical laboratory

method for estimating the side effects of insecticides on insect pests, natural enemies and beneficial arthropods was to determine the median lethal dose (LD_{50}) or lethal concentration (LC_{50}) [2]. The assessment of lethal dose/concentrations is a very useful tool to compare the toxicities of different active ingredients and different formulations of insecticides containing the same active ingredient. The lethal estimates may also be an important information when evaluating the development of resistant pest populations to insecticides.

Although the results of such estimates in laboratory have been extremely valuable, interpretation of the data is severely limited. In field crops, lower insecticide dose/concentrations usually occur after the initial application, as they degrade by several abiotic factors, such as rainfall, temperature and sunlight. In this way, under field conditions, insects can be exposed to sublethal doses/concentrations of insecticides and may experience related to sublethal effects [3].

Sublethal effects are defined as biological, physiological, demographic or behavioral effects on individuals or populations that survive exposure to a toxicant at lethal or sublethal dose/concentration. A sublethal dose/concentration is defined as inducing no apparent mortality in the experimental population [2]. In general, insecticide dose/concentrations under the median lethal (LD_{50}/LC_{50}) are considered to be sublethal. The sublethal effects may be manifested as reductions in life span, development rates, population growth, fertility, fecundity, changes in sex ratio, deformities, changes in behavior, feeding, searching and oviposition [4, 5]. Thus, toxicants can exert subtle as well as overt effects that must be considered when examining their total impact.

Due to the recognition of limitations associated with traditional methods for studying sublethal effects, a growing body of the literature has aimed at assessing insecticide sublethal effects on various important biological traits of pests in the past three decades. An accurate assessment of these effects is crucial to acquire knowledge on the overall insecticide efficacy in the management of pest insect populations, as well as on their selectivity toward nontarget organisms, such as natural enemies [6].

Sublethal effects were reported in several insect orders upon different biological, physiological, behavioral and demographic aspects, such as the effect of the aqueous extract of *Trichilla* sp. upon survival, development and larval and pupal weight of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) [7]. Physiological and behavioral effects were observed upon *Aphis mellifera* L. (Hymenoptera: Apidae) that when exposed to sublethal doses of permethrin exhibited lower learning response levels than untreated bees, but regained normal learning ability with time after the insecticide degradation [8]. Demographic parameters, such as net fecundity rate, intrinsic rate of increase (rm) and the intrinsic birth rate, were affected in *Brevicoryne brassicae* (Hemiptera: Aphididae) when exposed to sublethal concentrations of imidacloprid and pymetrozine [9]. In addition, natural enemies may be affected by sublethal dose/concentrations of insecticides, as *Catolaccus grandis* (Burks) (Hymenoptera: Pteromalidae), an ectoparasitoid of *Anthonomus grandis* Boheman (Coleptera: Curculionidae), developed no pupae from parasitism during a 24-h treatment period with malathion or spinosad [10].

Among the insecticides used in sublethal effect studies, the botanical and biological insecticides, organochlorides, organophosphates, carbamates, diamides, hydrazines, growth regulators, neonicotinoids and pyrethroids demonstrate several adverse effects presented throughout this chapter (**Table 1**). Therefore, we aim to discuss the importance of sublethal effects of insecticides for integrated pest management programs, through the effects upon pest insect biology, physiology, behavior, demographic parameters and natural enemies.

Chemical group	Active ingredient	Mechanism of action
Antibiotic insecticide (Spinosyn)	Spinosad	Nicotinic acetylcholine receptors and y-aminobutyric acid receptors
Botanical insecticides	Azadirachtin	Ecdysis inhibitor
	Essential oils and major compounds in general	Ecdysis inhibitor, acetylcholinesterase inhibitor, octopamine mimic
Carbamate	Methomyl	Acetylcholinesterase inhibitors
Hydrazines		
Bisacylhydrazine	Bisacylhydrazine (RH 5849)	Ecdysteroids agonists
Diacylhydrazines	Methoxyfenozide	
	Tebufenozide	
Diamide	Cyantraniliprole	Ryanodine receptors (affecting calcium channels in the sarcoplasmic reticulum)
	Chlorantraniliprole	
Insect growth regulators		
Juvenile hormone mimics	Pyriproxyfen	Inhibition of the development of insect adult characteristics
Urea derivatives	Hexaflumuron	Chitin synthesis inhibitor
	Lufenuron	
	Novaluron	
	Triflumuron	
Thiadiazines	Buprofezin	
Neonicotinoid	Acetamiprid	Acetylcholine mimic
	Clothianidin	
	Imidacloprid	
	Thiacloprid	
Organochlorides	Endosulfan	Interfere with the transmission of nervous impulses (flux of Na and K)
Organophosphates	Chlorpyrifos	Acetylcholinesterase inhibitors
Pyrethroid	Deltamethrin	Channel sodium modulators

Table 1. Chemical group, active ingredient and mechanism of action of the insecticides used in sublethal effects studies presented in this chapter.

2. Sublethal effects upon insect biology

The effects of insecticides sublethal doses/concentrations upon insect biology may present itself through reduced oviposition, increased development period of immature stages or decreased life span. Nevertheless, the effect of sublethal doses/concentrations of some neuro-

toxic insecticides upon insect fecundity and fertility may be related to behavioral changes, particularly during their reproductive stage [11]. Several biological effects are reported in the literature due to the use of sublethal dose/concentrations of insecticides, for example, the sublethal effects of the insecticides lufenuron, methoxyfenozide, spinosad, endosulfan, novaluron and tebufenozide upon *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), reducing the pupal weight, adult longevity and fertility [12]. The insecticide hexaflumuron decreased the total number of eggs, oviposition period, pupation and adult emergence of *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) [13]. The sublethal effects of cyantraniliprole on *Helicoverpa assulta* Guenée (Lepidoptera: Noctuidae) decreased the pupal weight and adult fecundity of the parental generation at LC_{30}. However, cyantraniliprole did not significantly affect the pupal period, the percentage of females and longevity of adults in other generations [14].

Several studies also report the sublethal effects of essential oils and their compounds upon insect biology. The insecticidal activity of essential oils is based on the high concentrations of major compounds that belong to the classes of terpenes, phenolics and alkaloids [15]. The essential oils of long pepper and clove demonstrated the activity of these substances on several biological parameters of *Spodopetra frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), such as reduced survival periods, alterations in larval and pupal weight and period, and decreased longevity, fecundity and fertility [16]. The essential oils of *Eucalyptus staigeriana* F. Muell. (Myrtaceae), *Ocimum gratissimum* L. (Lamiaceae) and *Foeniculum vulgare* Mill (Apiaceae) demonstrated several sublethal effects upon the biology of *S. frugiperda* reducing the larval and pupal weight in the sublethal doses of LD_{10}, LD_{20} and LD_{40} [17]. The neem oil (10 g L^{-1} azadirachtin A) presented different sublethal effects upon *Bonagota salubricola* (Meyrick) (Lepidoptera: Tortricidae), such as prolonged larval period, reduced pupal viability and fecundity [18]. In *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), an increase in the pupal period was reported, when the moths were exposed to the neem oil by ingestion [19].

The assessment of the sublethal effects of insecticides upon insect biology is of great importance for the integrated pest management programs, as sublethal doses/concentrations do not cause the insect death, but through the interference in biological traits may reduce the insect populations of next generations in the crops.

3. Insect behavior as a measurement of insecticide sublethal effects

The exposure to insecticide sublethal dose/concentrations may cause changes in several behavioral parameters of insects, such as food foraging, choice of oviposition sites, pheromonal communications and others. The production and emission of pheromone by females, males and its detection depend on complex physiological mechanisms involving hormones and neurohormones. Some insecticides that act on the endocrine system may also influence reproductive behavior.

Sublethal dose/concentrations of insecticides may change the chemical communication system and, therefore, decrease chances of reproduction in insects that largely rely on olfactory

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communication. For example, the effects of deltamethrin on the calling behavior and production of sex pheromone in *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) showed that *O. furnacalis* developed a compensation system in which males who survived the insecticide exposure present a low response to pheromone, while surviving females produces and releases more pheromone [20].

Besides adverse effects, the insecticides at sublethal doses/concentrations may cause positive responses at reproduction, known as hormesis and hormoligosis. However, there is still little information regarding the effects of sublethal dose/concentrations on insect behavior [21]. The sublethal doses of clothianidin on males of *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) presented a "biphasic effect" with increased or decreased male pherome response depending on the insecticide dosage [22].

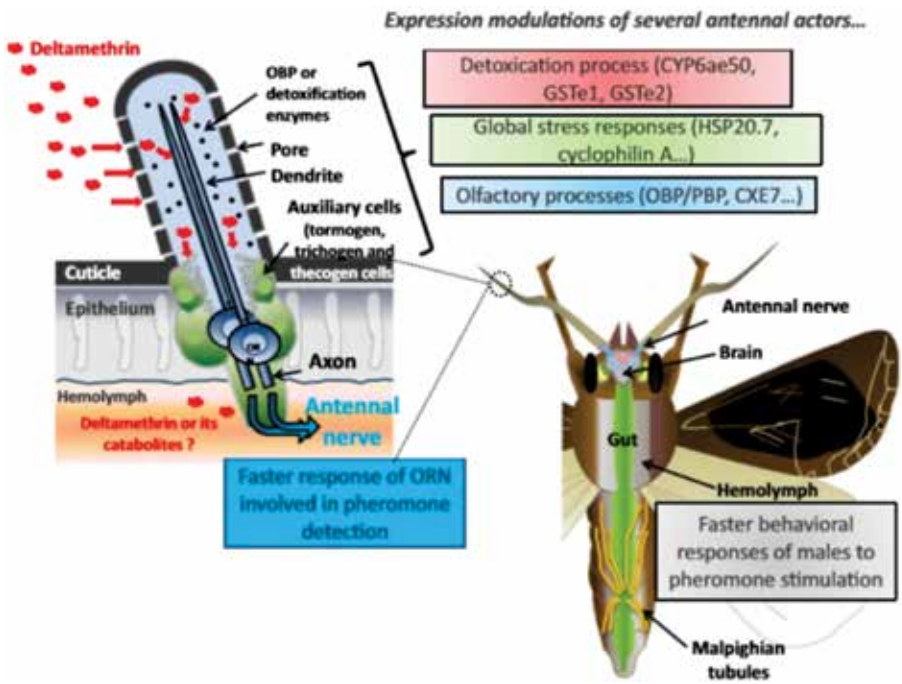

Figure 1. Proposed effect model of sublethal doses of deltamethrin upon *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) olfactory system [23].

Sublethal doses of deltamethrin on *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) antennae cause an increased male response to the sex pheromone (hormesis effect), but it did not affect the male response to the host plant. Sublethal doses of deltamethrin can change the expression of several antennal processes involved in insecticide detoxification. These changes may be observed on the transcription levels of several antennal genes involved in the detoxification system and in the odors recognition and transport, such as genes from odorant-binding proteins (OBPs). In *S. littoralis* males treated with deltamethrin, a significant increase in the

transcription levels of genes involved in insecticide detoxification was reported, such as P450 chromosome, glutathione S-transferases (GSTs) and esterase. The regular olfactory process was also affected, since the repolarization on the antenna was reduced, while a rapid response of the olfactory receptor neurons (ORN) was induced. Thus, a faster behavioral response of males to pheromone stimulation was observed (**Figure 1**) [23].

The sublethal dose LD_{01} of chlorpyrifos on *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae) males showed that the their response to the female sex pheromone was significantly decreased. On the other hand, when females were submitted to the insecticide, the response of males to the sex pheromone was slightly but significantly increased [24]. In addition, *T. brassicae* females which survived the exposure to sublethal doses of deltamethrin presented a lower parasitism rate of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs [25].

Altering plants-specific odor bouquet by nonspecific odors may cause oviposition sites rejection. In this way, insecticide sublethal doses/concentrations may present deterrent effect for insect oviposition and feeding. Sublethal concentrations of several essential oils caused the reduction in feeding and oviposition of *A. gemmatalis* by the presence of essential oil volatile components that modified the insect behavior [26]. Sublethal doses of methomyl promoted behavioral disruption of *S. littoralis* for food odors [27]. *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) exposed to *Cinnamomum zeylanicum* L. (Lauraceae) had their locomotor behavior affected, such as time, speed and distance of walk [28].

The use of behavioral control together with chemical control in the integrated pest management is recognized as a promising and efficient tool. For that, the evaluation of sublethal effects of insecticides in insect behavior is essential for the development of new strategies.

4. Physiological responses to insecticides sublethal doses/concentrations

Exposure to sublethal doses/concentrations of insecticides that attack the nervous system or disrupt the hormonal balance can affect insect physiology and reduce survival and reproduction [29]. Potentially, all classes of insecticides can affect insect reproduction through sublethal adverse effects on physiological parameters, such as egg fertilization, oogenesis, ovulation, spermatogenesis and sperm motility [11].

Insect growth regulators (IGRs) are ecdysone agonists and specific for Lepidoptera larvae, being effective against many important crop pests [30]. The HR 5849 bisacylhydrazine and the tebufenozide (RH-5992) IGRs insecticides adversely affect the development of male reproductive system and testicular volume of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) when the larvae were exposed to sublethal doses [31].

Studies with neonicotinoids, which act as agonist of acetylcholine receptor and disturb the neuronal cholinergic signal transduction, demonstrate that thiacloprid, imidacloprid and clothianidin can also interfere with the immune system of honeybees, affecting the total number of hemocytes, the encapsulation response and microbial activity in the hemolymph

[32]. Besides effects on the immune system, neonicotinoids such as imidacloprid have been found to reduce sperm viability by 50% in bees [33]. These factors may also affect disease resistance capacity [34].

Insecticides from the anthranilamide class, such as ciantraniliprole, target the rianodiana receptors in the muscles and the calcium channels [35, 36]. Ciantraniliprole demonstrated sublethal effects upon *A. ipsilon*, reducing nutritional parameters, including lipids, carbohydrates and proteins, affecting larvae development [37]. This same insecticide was also found to reduce the activity of esterase enzymes, glutathione S-transferase and oxidases of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) [38].

The amylase activity in the midgut of *Tribolium castaneum* (Herbst., 1797) (Coleoptera: Tenebrionidae) was reduced by sublethal concentrations of pyrethroids as dimilin and ambush [39]. However, sublethal doses of chlorpyrifos and methomyl did not induce changes in acetylcholinesterase enzyme activities of *S. littoralis* larvae [40].

Natural insecticides also demonstrate sublethal effects in physiological parameters of insects. *Artemisia annua* L. extracts decreased the amylase level of *Xanthogaleruca luteola* Mull (Coleoptera: Chrysomelidae) 24 h after treatment, but significantly increased it after 48 h [41]. The essential oil of *A. annua* significantly reduced protein, carbohydrates and lipids levels of *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) [42]. Changes in the embryonic development of *S. frugiperda* were verified by scanning electron microscope after exposure to sublethal concentrations of azadirachtin, lufenuron and deltamethrin. The changes consisted of undeveloped embryo, widely dispersed yolk granules, corium disintegration, unorganized blastoderme with the presence of vacuoles and amorphous cells in yolk region [43].

Other physiological parameters such as spermatogenesis and ovarioles histochemistry of *S. frugiperda* were affected by the essential oil of *Piper hispidinervum* C. DC. (Piperaceae) [44] and *Szygium aromaticum* (L.) (Myrtaceae) in sublethal concentrations [45]. The biochemical profile of *S. frugiperda* larvae was also affected when exposed to sublethal dose of *Cymbopogon winterianus* Jowitt (Poaceae), disturbing the insect reproductive histophysiology [46].

Understanding the physiological processes that affect insect life traits is an important step for the evaluation of the overall insecticide effects upon insect pest and natural enemies in integrated pest management programs.

5. Demographic studies for the assessment of insecticide sublethal effects

The use of ecotoxicology approaches is improving the evaluation of insecticides and other toxicants in integrated pest population control programs. The traditional lethal dose/concentration estimates are designed to measure one effect at a time [1]. Demography studies derive better estimates of insecticides impacts on insect pests and natural enemies, since it accounts for all effects a toxicant might have on a population including interactions that are not perceptible in short-term toxicity [47, 48].

The analysis of demographic parameters can evaluate sublethal effects well below the traditional dose/concentration-response curve, resulting in the assessment of population decline and extinction at doses/concentrations previously assumed to have few effects on individuals [49]. On the other hand, sublethal doses/concentrations of insecticides may also result in pest populations outbreaks mediated by reproduction stimulation [50, 51].

Demographic toxicological studies through fertility life table bioassays provide a measure of the insecticide effect upon the population growth rate. The sublethal effects on population growth rate after exposure to insecticides are highly influenced by the starting population structure. Because different insect stages/ages may present different susceptibilities to toxicants, it is essential to consider this factor to estimate the population susceptibility [52].

Life table response experiments are conducted by exposing individuals or groups to increasing doses or concentrations of a toxicant over their life span. Daily mortality and reproduction are recorded and used to generate life table parameters [1]. In the fertility life table study, the intrinsic rate of increase (r_m), the finite rate of increase (λ), the net reproductive rate (R_0), the mean generation time (T) and the doubling time (TD) are important parameters [53]. The major disadvantage to the use of demographic toxicology is that the development of life table data is expensive and time-consuming. One way to reduce cost is to use partial life tables [54] or another population growth rate method, such as the instantaneous rate of increase (r_i).

The instantaneous rate of increase is calculated by the following equation: $r_i = \ln (Nf/No)/\Delta T$, where Nf is the final number of insects, No is the initial number of insects and ΔT is the change in time (number of days the bioassay was run). Positive values of r_i indicate a growing population, $r_i = 0$ indicates a stable population and negative r_i values indicate a population in decline, toward extinction [1, 55]. Although this is not demography in the true sense, this approach does yield a measure of population growth.

These demographic approaches have been used in a toxicological context by several authors to assess the sublethal effects of synthetic. The use of fertility life table bioassays demonstrated that sublethal concentrations of cyantraniliprole decreased growth speed and reduced population reproduction of *A. ipsilon* [37]. Fertility life tables for the evaluation of sublethal concentrations of chlorantraniliprole also demonstrated prolonged larval duration and the pupal stages of lepidopteran pests such as *O. furnacalis* and *P. xylostella* [56, 57]. Demographic changes in multigeneration were observed in *P. xylostella* after exposure to sublethal concentrations of spinosad [58]. The sublethal effects of spinosad can affect *S. exigua* population dynamics by decreasing its survival, reproduction and delaying its development [59].

Aphis gossypii Glover (Hemiptera: Aphididae) exposed to botanical insecticides based on azadirachtin, aqueous extract of neem seeds, and castor oil presented negatives values for the instantaneous rate of increase (r_i) [60]. Negatives r_i for *A. gossypii* population were also observed with the use of the botanical insecticides Compostonat®, Rotenat® and Neempro® [61]. Negatives r_i and a decline in the population of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) were reported when using the botanical insecticide based on neem oil, NeemAzal T/S® [62].

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In this way, the study of sublethal effects of insecticides on insect pests and natural enemies through the use of demographic parameters is crucial for guiding the use of new toxicants, delaying the development of resistance and reducing the risk of pest resurgence.

6. Sublethal effects of insecticides on biological control

The studies of insecticide effects on beneficial insects, particularly natural enemies, have grown in recent years. These impacts are not limited to mortality, as they also present sublethal effects on insects that survive the insecticide exposure [2]. These effects may result, for example, in changes of biological parameters, reproduction (fertility, fecundity and sex ratio), development time, longevity and insect behavior [11, 63].

The sublethal effects upon natural enemies can be divided in two groups: physiological and behavioral. Among the physiological effects are changes in neurophysiology, development, adult longevity, fecundity and sex ratio [2]. Among the behavioral effects upon natural enemies are the changes in mobility of insects, although it is still little studied, changes in the ability to search for prey or host and changes in feeding behavior and insect oviposition.

Insect growth regulators (IGRs) may promote changes in the development of natural enemies by the interruption of the molting process and cuticle formation, besides acting upon the endocrine system of insects [2]. Fecundity and fertility reduction were observed as sublethal effects of insect growth regulators on the predator larvae of *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae) exposed to pyriproxyfen, tebufenozide, methoxyfenozide and buprofezin [64]. Pyriproxyfen is an insect growth regulator that mimics the juvenile hormone in some species. This insecticide significantly reduced the fertility of agricultural and urban pests and may also affect natural enemies [65]. In selectivity studies of insect growth regulators and neonicotinoids to *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae), novaluron insecticide was slightly harmful to the emergence of the F1 generation and acetamiprid, imidacloprid, lufenuron and triflumuron were harmless [66]. The insecticide acetamiprid upon immatures and adults of the ectoparasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) did not affect the development of the immature stages of the natural enemy until pupation [67]. The toxicity and sublethal effects upon fecundity and fertility of six insecticides upon on the natural enemies *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) and *Adalia bipunctata* (Linnaeus) (Coleoptera: Chrysopidae) demonstrated that deltamethrin affected the reproduction parameters (fecundity and fertility) of *C. carnea* adults, while caused the total mortality of *A. bipunctata* larvae and adults [68]. The lethal and sublethal effects of lufenuron insecticide on *Diatraea flavipennella* (Box) (Lepidoptera: Crambidae) and its parasitoid *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) were reported as a delay in the development period of *C. flavipes* when parasitizing *D. flavipennella* larvae that survived to sublethal exposure of the insecticide [69].

Not only synthetic insecticides are likely to affect the natural enemies but also botanical insecticides and essential oils. The effect of the neem-based botanical insecticide Azamax®, the aqueous extract of neem and the emulsifiable oil of *Ricinus communis* (Euphorbiaceae)

demonstrated an adversely affect upon the development of first and fourth instars larvae of the predator *Cycloneda sanguinea* (Linnaeus, 1763) (Coleoptera: Chrysopidae) [60]. Lethal and sublethal effects on *Eriopis connexa* (Germar, 1824) (Coleoptera: Chrysopidae) were also observed in laboratory when using neem seeds extract [62]. The evaluation of the effects of four botanical extracts upon the parasitoid *T. galloi* (Zucchi, 1988) (Hymenoptera: Trichogrammatidae) demonstrated that the bark extract of *Aspidosperma pyrifolium* (Apocynaceae) reduced the parasitism rate in *D. sacharallis* eggs (Fabr, 1794) (Lepidoptera: Crambidae) [70]. Several essential oils affected the reproduction of *Euborellia annulipes* (Lucas, 1847) (Dermaptera: Forficulidae), and the essential oils of *F. vulgare* Mill. and *Nicotiana tabacum* L. presented an inhibitory action upon the predator oviposition [71].

Sublethal doses/concentrations of insecticides can also affect beneficial insects such as bees, causing changes in development, behavior, morphophysiology and immune system, affecting the colony functions and decreasing the longevity of individuals [72]. The assessment of selective insecticides to natural enemies is of utmost importance for biological control on integrated management programs.

7. Conclusion

Studies on sublethal effects have been quite elucidated over the last decade, for synthetic and botanical insecticides effects upon pest insects and natural enemies (parasitoids and predators). However, this is still the beginning of the path of knowledge for this particularly area, since each individual and species may present a different response to each insecticide.

Overall, sublethal effects of insecticides may cause biological effects, disturbing the number of eggs, oviposition period, larval and pupal weight, development period, adult emergency, longevity and fertility; behavioral effects on feeding, oviposition, locomotor system and reducing or increasing the production and response to pheromones; and physiological effects upon reproductive and immune systems as well as upon the nutritional status of insects.

The use of demographic parameters in the assessment of sublethal effects came to extend the concept of the total effect of insecticides not only upon individuals, but also on insect populations. In addition, the assessment of sublethal effects upon natural enemies enables the development of integrated pest management programs with safer and effective combined use of chemical and biological control.

For future works, it is also important to target a broader look and observe the effect of sublethal doses/concentrations upon insects life history and expand this impact to a more widely perspective, such as communities and the ecosystem. The study of sublethal effects of insecticides upon insects is of great importance and need to be considered when accessing the total effect of a toxicant.

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Chapter 3

Conservation Biological Control Practices

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Additional information is available at the end of the chapter

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Abstract

Natural enemies are subjected to continuous deterioration in populations especially in modern agricultural systems characterized by complete removal of plants after harvesting as well as by insecticide applications. This complete removal of plants gives rise to disappearance of natural enemies after each crop season. Conservation biological control is the protection of NEs against adverse effects of pesticides and incompatible cultural practices and improving their efficiency via providing food sources. During non-crop periods, natural enemies may need of benefit from pollen and nectar. Preservation of natural enemies can be achieved by providing habitat and resources for NEs. This chapter aimed at discussing a suggested strategy for more efficient conservation biological control comprising collection, preservation and releasing the preserved natural enemies on target crops. The collection is mainly conducted before crop harvest and during winter from fruit orchards. Preservation greenhouses are dedicated for natural enemies rather than commercial production of crops. Natural enemies taken from preservation greenhouses are released in target crops during growing season. Different techniques used in collection, preservation and release of natural enemies are reviewed. Such a conservation biological control strategy might contribute to preserve the natural bio-diversity in the agricultural environment and provide natural alternatives to pesticides.

Keywords: natural enemies, biological control, collection, preservation, release, parasitoids, predators, insect pathogens

1. Introduction

Biological control is the regulation of pest populations by the activity of natural enemies (NE) (predators, parasitoids and pathogens) [1]. Natural enemies are periodically released in augmentative biological control of insect and mite pests [2]. In classical biological control, an NE is imported and released in a new area for regulating a specific pest [1]. Released and naturally occurred NEs are subjected to continuous deterioration in populations especially in modern agricultural systems characterized by complete removal of plants after harvesting. This complete removal of plants gives rise to disappearance of natural enemies after each crop season. Conservation biological control is the protection of NEs against adverse effects of pesticides and incompatible cultural practices and improving their efficiency via providing food sources [3–5]. Biological control of arthropod pests has used for a long time traditionally in different crops, therefore it should be used with other compatible integrated pest management methods [6]. Both the area on which it is used and the number of available biological control agents are still expanding [2, 7]. Natural enemies play an important role in limiting potential pest populations [8].

Conservation biological control is one of biological control main branches [8], which can be first realized by reducing the use of pesticides, use of selective pesticides, careful timing and placement of pesticide applications. We have seen what happens when insecticides destroy the natural enemies of potential pests. Insects that were of little economic importance may become destructive pests. When nontoxic control method is used natural enemies are more likely to survive and reduce the populations of pests.

During non-crop periods, natural enemies may need of benefit from pollen, nectar or honeydew (produced by aphids). Many crop plants flower for only short time, so flowering plants along the edges of the field or within the field may be needed for pollen and nectar [9]. Preservation of natural enemies can be achieved by providing habitat and resources for natural enemies [10]. They are generally not active during the winter. Unless they are re-released each year, they must have a suitable environment for overwintering [11, 12]. They usually pass the winter in crop residues, other vegetation or in the soil. Ground cover of fruit orchards, winter crops (like alfa alfa and breccias), usually provides shelter for overwintering natural enemies. Adding plants or other food sources for natural enemies must be done with knowledge of the behaviour and biology of the natural enemy and the pest [13–16].

It is widely known that the simplifications of agriculture systems towards monoculturing are mainly responsible for decreasing environmental quality, threatening biodiversity and increasing the possibility of insect outbreaks. Modern crops are often monocultures in highly specialized production units, where not only crop cultivation but also harvest and packaging techniques are specialized [17–19]. The development of farming systems (field or landscape) with greater dependence on ecosystem services, such as biological control of insect pests, should increase the sustainability of agro-ecosystems [20–22]. Farming systems like greenhouses, annual crop systems and other practices that end with removing the whole crop after harvesting, may give rise to elimination of biodiversity, and decreasing the population of natural enemies in the fields or in different agricultural environments [23, 24], as appeared in

Figure 1. Collection and transferring of natural enemies to environmentally controlled habitats could be useful in utilizing these natural enemies until releasing them in the next crop season.

Figure 1. Complete removal of maize may eliminate natural enemies (A) or after roses cutting (B).

Thus, they will try to contribute to preserve the natural biodiversity in the agricultural environment and provide natural alternatives to chemical pesticides. We concentrate here on the effects of conservative biological control on NE biodiversity and cleanliness of environment.

This chapter aims at discussing a suggested strategy for more efficient conservation biological control comprising of three main practices:

1. Collection of natural enemies before the end of crop season.
2. Preservation of collected natural enemies in special greenhouses during non-crop periods
3. Releasing the preserved natural enemies on target crops in the next growing season.

The sequence of these practices is illustrated in **Figure 2**.

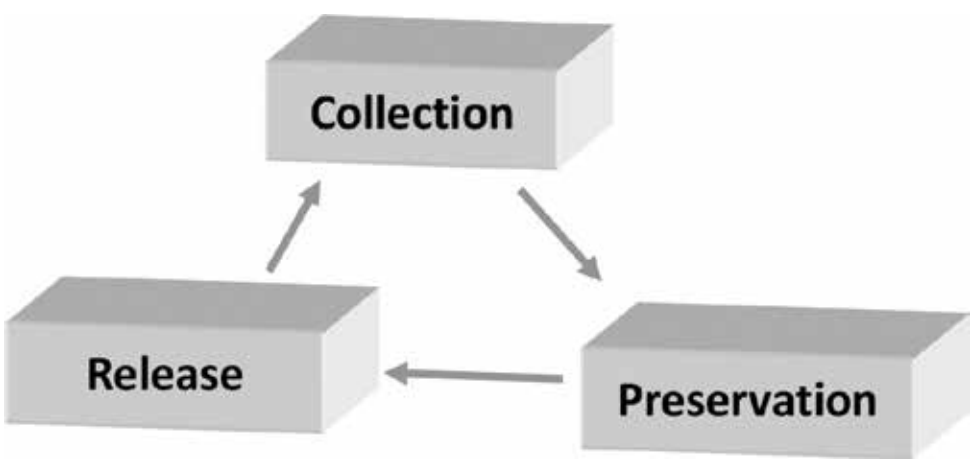

Figure 2. Logical practices diagram of conservation biological control.

2. Collection of natural enemies

The first step of the suggested strategy is collection of NE from fields shortly before the complete removal of plants and disappearance of occurring NEs. At the end of the crop season, the NEs are usually in their top population densities [1].

2.1. Collection time

Summer collection: High numbers of natural enemies may be found during the growing season on areas cultivated with some crops. These crops may not be in need for these natural enemies especially in absence of insect hosts or preys. For example, after heavy infestation of aphids to maize plants, high populations of aphid predators (lacewings and lady beetles) are built up. These predators could be mass collected and directly transferred to the preservation greenhouses or directly to other target crops that are in need for them.

Autumn collection: Before the end of most of annual crops, there are huge numbers of natural enemies which may be lost after harvesting and removing the plants. These NEs could be collected, preserved in greenhouses during non-crop periods then released in the next season.

Winter collection: In cases of permanent crops like fruit orchards and alfa alfa during cold weather in winter, many numbers of natural enemies may be lost as a result of absence of their hosts and preys, especially during non-suitable weather conditions. These natural enemies could be collected and transferred to greenhouses where maintained and improved them in numbers and quality control until release during the next crop season.

2.2. Collection sites

Natural enemies may be abundant in many sites around the year including landscape, fruit orchards, vegetable and field crops and ornamentals and others.

2.3. Collection techniques

Collection techniques differ according to the nature of natural enemies, crop, time and site.

The common collection techniques are vacuum collection, sweeping net, pitfall traps, manual collection etc. Example of collection techniques, sites and crops are assembled in **Table 1**.

Plant	Natural enemies	Pests	Technique	References
Mulberry trees	**Parasitoids**	*Brevipalpus* sp.	**Parasitoids:** Picking infested	Hendawy et al.
	Encarsia citrina	*Panonychus ulmi*	leaves containing parasitized	[25]
	Anagyrus kamali	*Thrips tabaci*	insects	
	Metaphycus sp.,	*Nezara viridula*	**Predators:**Individuals were collected by	
	Allotropa mecrida	*Bemisia tabaci*	beating tree	
	Scutellista caerulea	*Aphis gossypii*	branches in a suitable cloth bag	
	Chartocerus sp.	*Icerya aegyptiaca*		

Plant	Natural enemies	Pests	Technique	References
	Predators	*I. purchase*		
	Orius sp.	*I. seychellarum*		
	Coranus sp.	*Ceroplastes rusci*		
	Coccinella	*Coccus hesperidum*		
	undecimpunctata	*Saissetia oleae*		
	Cydonia sp.			
	Mantis religiosa			
Tomatoes	*Nesidiocoris tenuis*	*Tuta absoluta*	**Sweeping net**	Sayed [26]
	Chrysoperla carnea	*Phthorimaea*		
		opercullela		
		Bemisia tabaci		
Clover	*Bracon* sp.	*Phthorimaea*	Tomato or potato leaves were	ELbehery [27]
Tomatoes	*Coccinella*	*operculella*	collected in jars and kept in the	
Maize	*undecimpunctata*	*Spodoptera. littoralis*	laboratory until parasitoids	
Potatoes	*Hypera postica*	*Agrotis ipsilon*	emergence	
	Apanteles spp.	*Tuta absoluta*		
Mango trees	*Amblyseius* spp.	*Aulacaspis*	Infested small branches were	Mohamed and
	Oligonychus	*tubercularis*	collected in cloth bags and the predators	Nabil [28]
	mangiferus	*Kilifia acuminata*	were counted	
	Brevipalpus			
	obovatus			
	Cunaxa			
	capreolus			
Pineapple	*Pheidole*	**Mealybugs**	Infested small branches were	González-
	megacephala	*Dysmicoccus brevipes*	collected in cloth bags and the predators	Hernández et al.
	Ochetellus glaber	*D. neobrevipes*	were counted in the laboratory	[29]
	Lobodiplosis			
	pseudococci			
	Nephus bilucernarius			
	Sticholotis ruficeps			
	Anagyrus ananatis			
Sugarcane	*Tritaxys milias*	*Anoplognathus* spp.	**Direct collection of insect individulas:**	Sallam et al. [30]
	Cuphocera javana	*Dermolepida*	Insect larvae were collected and	
	Palexorista sp.	*albohirtum*	reared in the laboratory until emergence of	
	Dicamptus	*Lepidiota laevis*	parasitoids	
	fuscicornis	*L. sororia*		
	Zelomorpha sp.	*Athetis recluse*		
	Brachymeria sp.	*Leucania loreyi*		
	Lissopimpla scutata	*L. stenographa*		
	Lissopimpla	*Nodaria cornicalis*		
	Zosteria sp.	*Oncopera* sp.		

Plant	Natural enemies	Pests	Technique	References
Pine trees	**Predators:** *Chilocorus bipustulatus* *Cydoni avicina* *Pharoscymnus varius* *Paederus alfierii* *Calidomantis savignyi* *Embusa hedenberchii* *Hypsicorypha gracilis* *Iris oratoria* **Parasitoids:** *Aphytis spp.* *Encarsia* spp.	*Chrysomphalus aonidum* *Fiorinia fioriniae* *Lepidosaphues beckii* *Parlatoria proteus* *Cenopalpus fewstrii* *Coccus hespridium* *Chrysomphalus aonidum* *Aspidiotus nerii* *Leucaspis pini* *L. pusilla*	**Parasitoids:** Picking 20 leaves containing parasitized insects/tree **Predators:**Individuals were collected by beating tree branches in a suitable bags	González-Hernández et al. [29]
Pigeonpea (*Cajanus cajan*)	*C. septempunctata* *Andrallus spinidens* *Rhynocoris fuscipes* *Componotus* sp. *Mantis religiosa*	*Aphis fabae* *Oxyrachis tarandus* *Odontotermes obesus* *Nezara viridula* *Melanoplus bivittatus* *Sphenoptera indica*	**Sweeping net**	Sayed [26]
Abandoned orchards and Wild plants	*Lestodiplosis aonidiellae* *Ablerus perspeciosus* *Coccophagoides moeris*. *Chilocorus bipustulatus* *Cybocephalus fodori-minor* *Rhyzobius lophanthae* *Aphytis* spp. *Encarsia berlesei*	*Aonidiella aurantii* *Parlatoria oleae* *Lepidosaphes ulmi* *Pseudaulacaspis pentagona*	**Picking up:** Scale insect-infested plant parts were examined for collecting predators. **Aspirator:** Adult NEs were collected using an aspirator and dropped into jars.	Erler and Tunç [31]

Table 1. Examples of collection techniques of natural enemies.

Collection techniques depend on many factors like pest species, host plant, type of natural enemy, habit, time, weather and others.

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2.3.1. Picking up infested plant leaves

Plant leaves are picked up and transferred in cloth bags to the preservation greenhouses where emerged natural enemies could be classified and maintained. Infested leaves containing parasitized insects of mulberry trees were picked up and transferred to the laboratory; then the parasitoids were counted after their emergence [25]. Leaves of tomatoes or potatoes infested with leaf feeders *Phthorimaea operculella, Spodoptera littoralis, Tuta absoluta* and *Agrotis ipsilon* were collected then the parasitoids were counted after their emergence [26]. Immature predators were collected and transferred to the laboratory together with the plant material infested by their prey scale insects for rearing to the adult stage [27].

2.3.2. Beating tree branches in cloth bags

Leaves and/or branches (shoots) are picked up from trees and beaten in cloth or paper bags; then they were transferred to preservation greenhouses. Hendawy et al. [25] used this method for sampling predators and parasitoids of mealybug on mulberry trees. Small branches of pine trees were beaten in cloth bags and transferred in the laboratory for surveying mealybug natural enemies [28]. Also mango trees were sampled by the same methods for monitoring the natural enemies of *Aulacaspis tubercularis* and *Kilifia acuminata* [29]. Infested small branches were collected in cloth bags and predators were counted in the laboratory [27, 28].

2.3.3. Sweeping net technique

Sweeping net technique is a common technique for collecting parasitoids and predators such as Chrysopid, Syrphid and Coccinellid species from vegetable and field crop plants. Sayed [30], ELbehery [26], and Badr [31] used the sweeping net in tomato or potato fields, usually by 50 double strikes by walking diagonally across the experimental plots.

2.3.4. Direct collection of insect individuals

Parasitized caterpillars or white grubs infesting roots are directly collected and transferred to preservation greenhouses where emerged parasitoids could be classified and maintained until their releases in the next season. Sallam et al. [32] collected white grubs infesting sugarcane roots and reared until parasitoid emergence. Larvae of armyworms were collected in sugarcane fields and were taken to the laboratory and fed on pieces of cane leaves until parasitoid emergence.

2.3.5. Aspirator devices

Aspirator or vacuum devices are used for collecting flying natural enemies from trees, orchards, vegetable and field crops. Adult parasitoids and predators were collected using an aspirator and dropped into a jar. Erler and Tunç [27] used aspirator devices for collecting the predacious mites from orchards and wild trees.

3. Preservation of natural enemies

Preservation greenhouses are dedicated for natural enemies rather than commercial production of crops. Preservation practices represent the cornerstone of conservation biological control. Preservation practices could be applied individually or in combination to maintain and improve efficiency of collected natural enemies. The currently applied practices for preservation of natural enemies in different fields are summarized in **Table 2**.

Crop	Natural enemy	Pest		Practice	References
Sweet pepper, ornamental crops	Syrphids lacewings hoverflies Predatory mites, *Orius laevigatus, O. majuscules O. insidiosus*	White flies, thrips, aphids	**1**	Plants providing pollen and plant sap as food sources for natural enemies like sweet alyssum, coriander, *Ricinus communis* and flowering ornamental	Bozsik [33]; Coll and Guershon [34]; Symondson et al. [35]; Pineda and Marcos-Garcıa [36]; Igarashi et al. [37] Waite et al. [38]
Cucumber, chrysanthemum	predatory mites *Amblyseius swirskii* and *Euseius scutalis*,	Thrips, whitefly	**2**	Spraying or dusting artificial or natural food supplements onto the crop. i.e. corn pollen, apple pollen, *Typha latifolia* pollen	van Rijn et al. [39]; Hulshof et al. [40]; Wade et al. [41] Nomikou et al. [42]; Adar et al. [43, 44]; Delisle [45]
Cereal crops	Aphid parasitoids	Cereal aphids	**3**	Introducing non-crop plants harbouring the prey species	Arno et al. [46]; Frank [47]; Huang et al. [17]
Chrysanthemum	Phytoseiid predatory mites	Spider mite	4	Applying yeast and sugars for astigmatic mites that are suitable prey for phytoseiid predatory mites	Messelink et al. [48–50]
Sweet pepper	Predatory mites *P. persimilis*	Spider mite	**5**	Artificial field rearing sachets containing bran, sugars, starch, yeast and/or saprophytic fungi, for feeding preys	Kühne [51]; Sampson [52]; Wright [53]; Baxter et al. [54]; Bolckmans et al. [55]
Sweet pepper	predatory mites *P. persimilis*	spider mites	**6**	Inoculating plants with low levels of pests early in the season and release predators afterwards to help their establishment.	Markkula and Tiittanen [56]; Messelink et al. [57]
Ornamentals	*Orius insidiosus*	thrips	**7**	Mixed diet of prey, or mixes of prey and non-prey food sources	Butler and O'Neil [58]
Rose plants	predatory mites	Spider mites	**8**	Providing oviposition sites and shelters:	Walter [59]; Parolin et al. [60]

Crop	Natural enemy	Pest		Practice	References
				Sweet pepper was used by predatory mites for oviposition.	
Tomato, sweet pepper	General mired predators, *Orius* spp., lacewings	whitefly, leaf miners, *T.absoluta*	**9**	Planting suitable non-crop plants near fields that help natural enemies to migrate into fields	Thierry et al. [61]; Bosco et al. [62]; Perdikis et al. [1]; Ingegno et al. [63]
Cotton, wheat, tomato	*Orius* spp., lacewings, lady beetles	Aphids, thrips, leaf-feeders	**10**	Induced plant responses that attract and/or retain natural enemies	Pare and Tumlinson [64]; Turlings and Wäckers [65]; El-Wakeil et al. [66, 67]
Different crops	Aphid parasitoids, chrysopids	aphids	**11**	Applying semiochemicals for increasing efficacy of natural enemies	Glinwood et al. [68]; Kunkel and Cottrell [69]; Simpson et al. [70]; Kaplan [71]
Wheat	*Orius* spp., lacewings, lady beetles	Aphids, thrips, leaf-feeders	**12**	Mitigation of pesticide side-effects by selecting pesticides that are compatible with natural enemies	El-Wakeil et al. [72, 73]

Table 2. Examples of preservation practices of natural enemies in different crops.

Practices of preservation of natural enemies are many and vary according the types of natural enemies, the target pests, the plants and the ecological conditions.

3.1. Plant-provided food

Many plants can provide food sources for natural enemies like nectar, pollen and plant sap but the effect of these food sources depends on the type of predator/parasitoid. Specialist natural enemies reproduce only in the presence of their specific prey/host species. However, most other natural enemies are feeding on both plant resources and prey [34]. Wäckers et al. [9] stated that adults of parasitoids and gall midges can increase their longevity, flight activity and oviposition by feeding on nectar. General predators consume multiple prey types and may feed also on nectar and pollen provided by plants [9, 13, 34, 35, 37, 74]. Adding some flowering plants like sweet alyssum and coriander to a sweet pepper crop resulted in higher densities of hoverflies [36]. Plants that produce a lot of pollen, like *Ricinus communis*, provided more pollen to predatory mites [75]. Flowering alyssum provided food resources for the predatory bugs *Orius laevigatus* and *Orius majuscules* during times of prey scarcity [76–78]. Flowering ornamental pepper can support and increase populations of *Orius insidiosus* in ornamental crops [38]. Another approach can be to select crop varieties with increased levels of plant-provide food resources [79]. Thus, the availability of plant-provided food can be a driving force in biocontrol success program [80].

3.2. Food sprays

Artificial or natural food supplements can be sprayed or dusted onto the crop to support natural enemies in crops where nectar and pollen are absent or only present at low densities [41]. For example, pollen sprays can serve as food for predatory mites and enhance their efficacy against thrips and whiteflies on cucumber [39, 42]. Corn pollen is also suitable for increasing populations of *Amblyseius swirskii* and *Euseius scutalis*. These pollens could be mechanically collected in large quantities [43, 44]. Other types of pollen are commercially available for pollination, such as apple pollen and date palm pollen. Application of pollen on chrysanthemum plants increases the establishment of many natural enemies [45]. Studies with predatory mites showed that adding *Typha latifolia* pollen to a crop clearly enhanced the biological control of thrips, even though the pollen is edible for thrips itself [39, 40]. The development of inexpensive alternative food sources is one of the major opportunities and challenges for enhancing biological control in different crop [50].

3.3. Introducing non-crop plants harbouring the prey species

The use of alternative prey/host plant species for the preservation of released natural enemies in many crops has been of interest for biological control of insect pests [17]. A widely applied system in different crops has been the use of monocotyledonous plants with cereal aphids that serve as alternative hosts for parasitoids of aphids that attack the dicotyledon crop [17, 47]. Prey/host plants can also be established on the edges of the field to bridge non-crop periods and contribute to the preservation of natural enemies [46]. Some alternative prey species that are not harmful to the crop may support their natural enemies [11, 81–84]. Woody habitats (hedgerows, field margins) often provide a more moderate microclimate than the centre of fields, protecting natural enemies against extreme temperature variations [14, 85, 86].

3.4. Applying artificial food for natural enemies

The application of yeast and sugars in chrysanthemum maintained populations of astigmatic mites that are suitable prey for phytoseiid predatory mites [48, 49].

3.5. Artificial field rearing units

Rearing natural enemies in controlled conditions has been developed into artificial rearing units for some natural enemies. For example, rearing sachets containing bran with saprophytic fungi for feeding astigmatic mites (prey) were used for rearing predatory mites [51, 52]. Many modifications with different types of preys, predatory mites, food sources for astigmatic mites such as sugars, starch, yeast and types of sachets have been developed [53–55]. Such units may produce predatory mites for 3–6 weeks [54]. This could be optimized by balancing the rate of predator, prey and food in the rearing unit [55].

3.6. Inoculation with low pest levels

A risky method to support natural enemies is the release low levels of pest species into crops. Inoculating plants with a low level of spider mites early in the growing season and release

predators afterwards enhanced the establishment of predatory mites in the crop [56]. Currently, this method is mainly used in sweet pepper crops [50, 57]. Thus, allowing low levels of pests, in numbers insufficient to cause crop damage, might contribute to natural enemies preservation.

3.7. Supplementing mixed diet for natural enemies

The population of natural enemies in crops can be increased by providing mixed diets of prey and/or non-prey food sources. Survival and reproduction of *O. insidiosus* were enhanced when aphids with thrips were supplemented as a prey source [58]. Supplementing thrips with pollen increased egg production of *O. laevigatus* and predation rates of thrips larvae [87]. Thus, supplementing diets of single pest species for predators with alternative prey or food may increase predator population and enhance biological control.

3.8. Providing oviposition sites and shelters

Suitable oviposition sites are essential for reproduction of many predators. *Orius* spp. and *Mimulus pygmaeus* lay their eggs into soft plant parts and ovipositional acceptance of the host plant depends on the morphological characteristics such as epidermal thickness or trichome density [88–90]. The hard plant parts are not very suitable for oviposition behaviour of predators and may disrupt their establishment [91]. Cutting soft stems of flowers may remove a potential new generation of natural enemies from the fields [50]. The same problem can also occur on tomato with the de-leafing practice that has a strong negative effect on the development of mired predator populations [92, 93] and *Encarsia formosa* by removing parasitized whitefly scales [94]. These problems may be solved by adapting the de-leafing strategy or providing host plants with suitable oviposition sites for natural enemies.

A number of plants are considered as refuges for natural enemies [59, 95]. For example, the vein axils of sweet pepper plants are used by predatory mites for oviposition which reduced cannibalism and increased survival by providing such suitable microclimate [59]. Adding *Viburnum tinus* and *Vitis riparia* plants in roses enhanced mite control by predatory mites [60].

3.9. Planting suitable non-crop plants near fields

Mirid predators often migrate from non-crop plants into tomato fields, where they add to the control whiteflies, leaf miners and *T. absoluta* [1, 63, 96]. The natural existence of predatory bugs in tomato fields seems to be strongly related to the surrounding landscape. Migration of *Orius* spp. from neighbouring wild plants into sweet pepper fields may compete with populations of released *O. laevigatus* [62]. Many studies suggested that preservation biological control of predators can be enhanced by planting suitable non-crop plants near fields either to support migration into the crop or to provide a shelter when field crops are harvested and plants removed [1]. Field surroundings may also contribute to the migration of parasitoids into fields [97]. Providing overwintering shelters may enhance lacewings by providing diapausing adults with artificial overwintering chambers in greenhouses [61]. These methods may contribute to early establishment of natural enemies in new season in the spring.

3.10. Induced plant responses

Induced plant resistance against insects includes direct traits, such as the production of toxins and feeding deterrents that reduce survival, host preference, fecundity or developmental rate of pests and indirect traits, which attract and/or retain natural enemies [64, 65]. The latter contains traits such as the plant producing volatiles and floral nectar [98]. Insect-induced plant volatiles help natural enemies to detect their prey/hosts in a crop [23, 64, 99], whereas floral nectar production is increased in response to insect attack, guiding natural enemies to find their prey/hosts [100]. Preservation of natural enemies might be enhanced in different crops by breeding varieties that produce more volatiles and nectar [65, 101].

3.11. Applying semiochemicals

Behaviour of natural enemies is directed by semiochemicals. Attraction of natural enemies with synthetic compounds, similar to plant volatiles, is being tested in crops [71]. Natural enemies may also respond to odours that are produced by their prey/host species, such as sex pheromones or alarm pheromones. Sex pheromones are used either to monitor or mass trapping pest populations. However, volatiles for improving natural enemy performance are so far not applied in many crops. Glinwood et al. [68] mentioned that pheromones could be used to treat clusters of aphid infested plants in fields, which might increase efficacy of released parasitoids. Lures may also be used to attract released natural enemies in order to help them establish. Applying attractants in combination with food sprays may promote oviposition of released chrysopid predators into the target crop [69]. Hexane extract of corn borer larvae was applied on corn plants to enhance performance of larval parasitoid *Bracon brevicornis* adults against the corn borers *Ostrinia nubilalis* and *Sesamia cretica* [102].

3.12. Pesticide side-effects

Preservation of natural enemies should not be combined with pesticides, as most pesticides have lethal effects on NEs. Mitigation of side-effects on preservation of natural enemies can be realized by selecting pesticides that are compatible as possible with natural enemies.

Finally, with transfer of collected natural enemies into greenhouse with environmentally safe conditions, where these natural enemies can be fed on the pollen and nectar of flowering crops (clover and alfa alfa), these plants will provide shelter for the natural enemies. This procedure will be continued until the next crop season, where the proper site and time of release.

Balzan and Moonen [103] mentioned that studying field margin vegetation enhances biological control agents in addition to crop damage suppression from many insect pests in tomato fields. They suggested that these habitats may be important during early crop colonization by natural enemies. These results indicate that the inclusion of flower strips enhances the preservation of arthropod functional diversity in ephemeral crops, and that diverse mechanisms are important for controlling different pests. However, the efficiency of habitat management is likely to be better when it is complemented with the preservation of diverse seminatural vegetation in the pre-existing field margin. Therefore, the field margin should be considered and evaluated before the inundative release strategy [1, 74, 104, 105].

4. Release of natural enemies

Release techniques are varied according the type of biocontrol agents, host plants, weather conditions. For example, egg parasitoids are released as parasitized egg patches; larval parasitoids are released as adults. Predators are usually released in the pupal stage. Timing, rate and frequency of release are determined according to the nature of the target pests, natural enemies and crops. Pathogens like entompathogenic nematodes could be applied as sprays or injection [22, 106, 107]. Examples of cases of NE field releasing are summarized in **Table 3**.

Crop	Natural enemy	Pest	Release technique	References
Tomatoes	Egg parasitoids *Trichgramma* (29 starins)	*Tuta absoluta*	Paper cardboard or strips containing about 400 parasitized eggs of *Ephistia kuehniella* ready to emerge	Alomar and Albajes [108]; Cônsoli et al. [109]; Chailleux et al. [110, 111]; El-Arnaouty et al. [112]; Balzan and Moonen [103]
Cabbages	*Trichogramma*	*Pieris rapae*	Releasing *Trichogramma* to control *Pieris rapae*	Abbas [113]
Olive fields	*Trichgramma evanescens*	*Prays oleae*	a dose of 3000 wasps/card x 3 cards/tree was applied (8 releases)	Agamy [114]
Grape orchards	*Trichgramma evanescens*	*Lobesia botrana*	50 and 75 cards/ ha, each card contain 1000 parasitoids (5 release)	Ibrahim [115]
Cotton	*Trichogramma*	Bollworms	Releasing *Trichogramma* in cards, each contain 1000 parasitoid for several times	El-Wakeil [66]; Abdel-Hafez et al. [116]; Andrade et al. [117] Saad et al. [118]
Sugarcane fields	*Trichogramma*	*Chilo agamemnon*	30,000–120,000 parasitoids per Feddan were released (5 releases)	Abbas [119] Tohamy [120]
Rice	*Trichogramma*	*Chilo suppressalis*	Investigating performance of 4 Chinese *Trichogramma* species on *C. suppressalis*	Jiang et al. [121]; Yuan et al. [122]
Maize	Larval parasitoids *Bracon* spp	Corn borers	Larval and pupal parasitoids are released in the pupal stage on special carriers like talc powder	Zaki et al. [102] Loni et al. [123]; Ferracini et al. [124]; Zappalà et al. [125];Biondi et al. [126, 127]
Tomatoes		*Tuta absoluta*		

Crop	Natural enemy	Pest	Release technique	References
Tomatoes	Whitefly parasitoids *Encarsia* spp. and/or *Eretmocerus* spp.	Whitefly	237,000 *Eretmocerus siphonini* are released as parasitized pupae shortly before adult emergence	Abd-Rabou and Abou-Setta [128]; van Lenteren and Martin [129]; Gerling et al. [97] Abd-Rabou [130] Simmons and Abd-Rabou [131]
Tomato and cotton fields	*Eretmocerus mundus*	*Bemisia tabaci, B. argentifolii*	*Eretmocerus mundus* were released into cotton and tomato fields	Hoelmer [132]; Joyce et al. [133]; Gabarra et al. [134]
Cabbage, Faba bean, Oleander	Aphid parasitoids *Diaeretiella rapae*	*Brevicoryne brassicae, Aphis craccivora*	20 parasitoids/200 aphids per cage	Saleh [135]
Different orchards	Scale insect parasitoids *Coccophagus scutellaris*	Soft scale insects	About 953,000 *Coccophagus scutellaris* were released as parasitized individuals for controlling soft scale insects	Abd-Rabou [136–139]
Ornamental plants	Mealybug parasitoids *Anagyrus kamali* and *Gyranusoidea indica*	*M. hirsutus*	300,000 parasitoids in parasitized individual stage were released	Awadallah et al. [140]; Roltsch et al. [141]
Tomatoes	Insect predators *Nesidiocoris tenuis M. pygmaeus*	*Tuta absoluta*, whitefly	Predators release in pupal stages to control both insects	Gabarra et al. [142]
Tomotaoes and Pepper	Predacious mites phytoseiid predator	Spider mites whiteflies		El-Laithy [143];
			Predators release in pupal stages	Messelink et al. [49, 57]
Maize	Combination Trichogramma Entomopathogenic nematodes	Corn borers	20 and 30 cards (1000 parasitized eggs/ card)/ acre (3 releases) The infested plants were sprayed with (500 and 1000 IJs/ml) of *S. carpocapsae* and *H. bacteriophora*	El-Sherif et al. [144]; Kfir [145]; Saleh et al.1995 [158]; Ragab et al. [146]; El-Wakeil and Hussein [22]
Date palms	Entomopathogenic nematodes	Red palm weevil	Spraying EPNs around infested tree trunks	Saleh et al. [147]

Table 3. Release techniques regularly used for various natural enemies in different crops.

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4.1. Egg parasitoids

The common techniques of releasing egg parasitoids are paper cards or strips holding the parasitized eggs. Cardboard strips containing parasitized eggs in tubes were released in tomatoes for controlling *T. absoluta* [110, 112]. *Trichogramma buesi* was released against *Pieris rapae* eggs in cabbage fields [113]. A dose of 3000 *Trichogramma evanescens* wasps/card x three cards/tree was applied; each card contains three different ages of *Trichogramma* to keep searching adults continuously; 8–11 releases were performed per year at 2-week intervals against *Prays oleae* in olive fields [114, 148, 149]. Five releases of *Trichogramma* at two release levels (50 and 75 cards/ha, each contains 1000 parasitoids) were released in grape orchards for controlling *Lobesia botrana* [90, 115]. Over 100,000 parasitoids per Feddan were released against *Chilo agamemnon* in sugarcane fields; five releases were applied during season [120, 145].

Bollworms are causing highly infested boll in cotton; *Trichogramma* were applied for control them. Different releasing *Trichogramma* in cards, each contain 1000 parasitoid for several times [66, 116–118, 150, 151]. Four *Trichogramma* species (*T. japonicum, T. chilonis, T. dendrolimi* and *T. ostriniae*) was evaluated against *Chilo suppressalis* in rice fields. *T. chilonis* parasitized more eggs, while *T. dendrolimi* and *T. japonicum* performed the best [121, 122].

4.2. Larval parasitoids

Larval and pupal parasitoids are released in the pupal stage. Parasitized pupae just before emergence are carried on special carriers like talc powder and distributed in the target fields. Releasing *Bracon* spp to control corn borer larvae is one of the effective methods for controlling such insects [102]. Two ectoparasitoid species *Bracon* sp. and *Necremnus* sp. were released in tomatoes [152]. *Necremnus* sp. *Nrartynes* and other braconid species have already been proved to be potential key biocontrol agents of *T. absoluta* in tomato field [123–127].

4.3. White fly parasitoids

Encarsia spp. or *Eretmocerus* spp. are released as parasitized pupae shortly before adult emergence [153, 154]. Additional *Encarsia* species have been released against *Bemisia tabaci*; reached to 65% parasitized whiteflies [97, 130, 155]. Simmons and Abd-Rabou [131] confirmed that inundative releases of parasitoid *Eretmocerus mundus* against *B. tabaci* into tomato and cotton fields increased parasitization rates. Findings from their research may be useful in the enhancement and preservation of parasitoids of *Bemisia* [132, 133].

4.4. Aphid parasitoids

Aphid parasitoids are released as parasitized mummies of aphid host. Semi-field experiments were carried out to evaluate the performance of releasing parasitoid species *Diaeretiella rapae* for controlling *Brevicoryne brassicae, Aphis craccivora* and *Aphis nerii* infesting cabbage, faba bean and oleander plants. The highest percentage of parasitism was 92.20, 83.20 and 79.30% for *D. rapae* at 20 parasitoids/200aphids per cage in semi-field test *B. brassicae, A. craccivora* and *A. nerii*, respectively. The maximum numbers of mummies in the field were 185.60, 166.4 and 158.6

for *D. rapae* at 20 parasitoids per cage and minimum of 124.60, 97.40 and 83.0 mummies at five adults per cage [135].

4.5. Parasitoids of scale insects

Parasitoids of scale insects are released as parasitized host individuals. About 953,000 of *Coccophagus scutellaris* as parasitized individuals were released and evaluated for controlling soft scale insects *Ceroplastes rusci* on citrus, *Ceroplastes floridensis* on citrus, *Coccus hesperidum* on guava, *Pulvinaria floccifera* on mango, *Pulvinaria psidii* on mango, *Saissetia coffeae* on olive and *Saissetia oleae* on olive. The population of parasitoid *C. scutellaris* showed a significant correlation with the build-up of the population of the soft scale insects population in all of the release orchards studied [136–139].

4.6. Mealybug parasitoids

Parasitoids of mealybug are released as parasitized host individuals. *Anagyrus kamali* and *Gyranusoidea indica* were released at ten sites on ornamental plants. 300,000 parasitoids of *A. kamali* were released to control *Maconellicoccus hirsutus*. Population density of *M. hirsutus* was reduced by approximately 95% and *A. kamali* was the predominant parasitoid [140, 141].

4.7. Predators of *T. absoluta* and *B. tabaci*

General predators (lacewings and lady beetles) are released in the pupal stage with the suitable carriers. These general predators are used commercially for regulating many insect and mite pests. *Nesidiocoris tenuis* and *M. pygmaeus* were also released and caused a significantly reducing *T. absoluta* [155] and *B. tabaci* populations [142, 156].

4.8. Predacious mites

Individuals of predacious mites carried on special materials are released for regulating spider mites and whiteflies in tomato and pepper in the greenhouses [49, 57].

4.9. Combination entomopathogenic nematodes (EPNs) and egg parasitoid

Natural enemies may be released in integration with each other to regulate one or set of insect pests. Entomopathogenic nematodes (EPNs) and *Trichogramma* were used for *S. cretica, C. agamemnon* and *O. nubilalis*, respectively, in corn fields. The infested plants *S. cretica* were sprayed one time with 500 and 1000 IJs/ml of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. Three releases of *T. evanescens* were conducted to control *C. agamemnon* and *O. nubilalis* [22].

4.10. Entomopathogenic nematods application

Entomopathogenic nematods are injected in tunnels made by the red palm weevil larvae or sprayed around the trunks of infested trees to control the pest adults [147].

4.11. Evaluation of released natural enemies

Evaluation of preservation biological control practices varies according to the pest, natural enemy species and target crops. Evaluation items include crop assessment, crop damage, pest and natural enemy populations. These evaluation criteria may include natural enemy efficiency and persistence in the target fields, predation rates, parasitization rates and pest population reduction. For field experiments, the standard equation of Henderson and Tilton [157] will be used. This equation is applicable for evaluating insect and natural enemy population, damage level and yield.

5. Conclusion

Populations of natural enemies are subjected to continuous deterioration especially in modern agricultural systems characterized by complete removal of plants after harvesting. Conservation biological control is the protection of NEs against adverse effects of pesticides and incompatible cultural practices and improving their efficiency via providing food sources. During non-crop periods, natural enemies may be in need of benefit from pollen and nectar. Preservation of natural enemies can be achieved by providing habitat and resources for natural enemies. This chapter aimed at discussing a suggested strategy for more efficient conservation biological control comprising (1) collection of natural enemies before the end of crop season, (2) preservation of collected natural enemies in special greenhouses during non-crop periods and (3) releasing the preserved natural enemies on target crops in the next growing season. The collection is mainly conducted before crop harvest but also could be done during the growing summer season and during winter from fruit orchards and permanent crops. Collection of natural enemies may be done in annual crops, fruit and vegetable orchards, landscape, abandoned plants and bushes.

Preservation greenhouses are dedicated for natural enemies rather than commercial production of crops. Practices of preservation of natural enemies vary according to the types of natural enemies, the target pests, the plants and the ecological conditions. Many plants can provide food sources for natural enemies like nectar, pollen and plant sap but the effect of these food sources depends on the type of predator/parasitoid. Artificial or natural food supplements can be sprayed or dusted onto the crop to support natural enemies in crops where nectar and pollen are absent or only present at low densities. Introducing plants harbouring the prey species is essential for the preservation of natural enemies. The application of yeast and sugars in chrysanthemum maintained populations of astigmatic mites that are suitable prey for predatory mites.

Natural enemies taken from preservation greenhouses are released in target crops during crop growing season. Releasing technique, rate of release, timing and frequency of release depend on the type of target pest, the crop, the natural enemies, weather condition and others. The present chapter contains many cases of releasing NE for pest regulation. The common techniques of releasing egg parasitoids are paper cards or strips holding the parasitized eggs. Larval and pupal parasitoids are released in the pupal stage. Parasitized pupae just before

emergence are carried on special carriers like talc powder and distributed in the target fields. White fly parasitoids are released as parasitized pupae shortly before adult emergence. Aphid parasitoids are released as parasitized mummies of aphid host. Such a conservation biological control strategy might contribute to preserve the natural biodiversity in the agricultural environment and provide alternatives to chemical pesticides.

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Chapter 4

In Search of New Methodologies for Efficient Insect Pest Control: "The RNAi "Movement"

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Additional information is available at the end of the chapter

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Abstract

The development of insecticide formulations with new mechanisms of action (modes of action, MOAs) is a huge priority for pesticide industry. This priority has become apparent during the last few years after (a) the observed increase in insect resistance for the most widely used active substances and (b) the harmful effects of the excessive use of pesticides on human health, environment, beneficial insects and fish. Silencing of genes by RNAi (RNA interference) technology provides an alternative, selective to species level, environmentally friendly strategy to combat insect pests. Double-stranded RNA molecules (double-stranded RNAs, dsRNAs) targeting important developmental genes are taken up by the digestive tract of the targeted insect speciesand induce RNAi, which results in inhibition of growth, development and reproduction of the targeted insect species. After the rapid development of RNAi technology in the past 10 years, biotech industry is seeking for new applications aimed at producing environmentally friendly genetic insecticides or genetically modified plants (GMPs) that induce environmental RNAi in the targeted insect species. These technologies are expected on the market at the end of this decade. In this chapter, we exploit established methods involving recent initiatives of RNAi technology with respect to the development of new bio-insecticidal formulations.

Keywords: modes of action, RNA interference, double-stranded RNAs, genetically modified plants, agricultural biotechnology

1. Introduction

Eukaryotic cells are equipped with a conserved mechanism by which exogenously added or endogenously expressed RNA duplexes (double-stranded RNA or dsRNA) are directly

degradedin to their complementary endogenously encoded messenger RNA (mRNA), resulting in targeted gene silencing. This phenomenon is generally referred to as RNA interference (RNAi) [1, 2]. In plant and animal organisms, RNAi provides a line of defense against viruses and parasitic genetic elements (transposons) while a similar, but mechanistically separated, mechanism regulates tissue-specific gene expression at translational level [3]. Induction of RNAi begins when high-molecular-weight dsRNAs are selectively degraded by type III ribonucleases encoded by RNAse III-related genes known as Dicer or Dicer-like genes. The Dicer enzymes appear to specifically act on the dsRNAs leading to their degradation (digestion) by their 5'–3' ends resulting in the production of small 21–24 base (ds) ribonucleotides known as small interfering RNAs (siRNAs) [4]. The double-stranded siRNAs are incorporated into a multiprotein complex known as the RNA-induced-silencing complex (RISC) that is actually induced by the presence of the RNAs. In this complex, the "passenger" (sense) strand of siRNAs is degraded and the antisense or guide strand is directed to the mRNA target. The guide sequence enables Watson-Crick complementarity to the mRNA target which is enzymatically degraded by a family of proteins called "Argonauts" (Argonaute proteins), thereby preventing translation of the mRNA. Arguably, the mechanism of gene silencing is specialized and targeted at the nucleotide sequence level. The possibility of specific gene downregulation through RNAi has proven mainly in insects which lack methods for functional genomic analysis [5]. The extremely interesting observation that dsRNA molecules taken up through the digestive tract of the nematode *Caenorhabditis elegans* induce gene-specific downregulation [6, 7] created hopes that oral dsRNA uptake could regulate gene expression in other invertebrates as well, including insects. This technology could be used for the reasonable purpose of developing environmentally friendly pesticides to combat agricultural insect species.

The first-historical publications describing the use of RNAi technology in crop insect control were those of Baum et al. [8] and Mao et al. [9]. These researchers showed that genetically modified plants expressing dsRNAs of entomological origin could influence the development and growth of phytophagous insect species which were feeding on these plants. Corn plants expressing hairpin dsRNAs, which target the A subunit of the ATPase gene in *Diabrotica virgifera virgifera* (western corn rootworm, WCR), showed significant protection from the damage caused by this insect species [8]. Following previous discoveries, *Arabidopsis* plants expressing double-stranded RNA hairpins targeting the cytochrome P450 monooxygenase gene in the corn pest *Helicoverpa armigera* showed decreased resistance to the sesquiterpene gossypol [9]. The above developments in gene-targeting research gave birth to a new term known as environmental RNAi [10]. Environmental RNAi involves the phenomenon in which RNAi is induced after environmental exposure to insect dsRNAs by oral or topical administration. Successful environmental RNAi has been reported in a wide range of insect families belonging to the classes of Coleoptera, Diptera, Dictyoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera and Orthoptera [11]. The technology of environmental RNAi is the best candidate for replacing conventional insecticides and avoiding collateral damages to the ecosystem, beneficial insects and human health.

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2. dsRNA delivery systems

2.1. dsRNA injection

The most widely used methodology for administrating dsRNA molecules in insect cells is by injecting volumes of *in vitro* synthesized dsRNAs into their hemocoel. This methodology was mostly used for functional analysis of genes in model and non-model insects from the early 2000s till date. DsRNAs could be *in vitro* synthesized using different approaches (there are many alternatives but we describe the one which was extensively used in our laboratory). In this approach, the gene of interest should be cloned in both sense and antisense directions in plasmids flanked with at least one T7 promoter. Then, the plasmids are linearized with restriction enzymes and transcribed in two different reactions (using T7 RNA polymerase) to synthesize sense and antisense ssRNAs. The complementary RNA molecules are then mixed and annealed. For the annealing step, the two transcripts should be mixed in precisely equimolar amounts (**Figure 1**). We have been using this methodology for synthesizing a wide range of targets for functional analysis of genes of the lepidopteron *Sesamia nonagrioides*. For the juvenile hormone esterase-related gene of *Sesamia*, we have targeted different parts of the full gene transcript by synthesizing *in vitro* three different dsRNAs, corresponding to a 472-bp part of its 5′-translated region, a 1276-bp part of its central translated, 3′-translated and part of its 3′ -untranslated region and a 1725-bp part encompassing both of the above regions, which spanned 94% of the total cDNA. All transcripts resulted in gene-specific knockdown of *SnJHER* but only the 1725 bp dsRNA was able to cause a significant phenotype [12]. In order to silence the 1276-bp part, we ligated a SnJHER 1276-bp fragment into the MCS of the RNAi L4440 vector (**Figure 2A**). The L4440/ SnJHER1276 plasmid was then linearized with either XhoI or NcoI (to create sense and antisense plasmids) and used as template for RNA synthesis. After DNase treatment (to destroy the DNA templates), sense and antisense ssRNAs were annealed to form dsRNA and analyzed in agarose gels before injection to insects (**Figure 2B**).

In other insect species, a wide range of successful experiments of intra-hemolymph RNAi have already been published and some of them will be described below:

- In adult fruit flies, RNAi could be induced by injecting dsRNAs into the abdomen of anesthetized individuals targeting genes which were expressed in their central nervous system (CNS) [13].
- In *Bombyx mori*, injection of double-stranded RNA corresponding to the silkworm white gene (Bmwh3) into preblastoderm eggs of wild-type silkworm induced phenotypes similar to those observed with mutants of the white egg 3 locus. The induced phenotypes were characterized by the presence of white eggs and translucent larval skin [14].
- Additionally, parental silencing of the hemolin gene from the Giant silkmoth, *Hyalophora cecropia*, demonstrated that hemolin is crucial for the normal development of embryos. When RNAi females were mated, no larvae emerged from their eggs and when dissected, the eggs revealed malformed embryos [15].

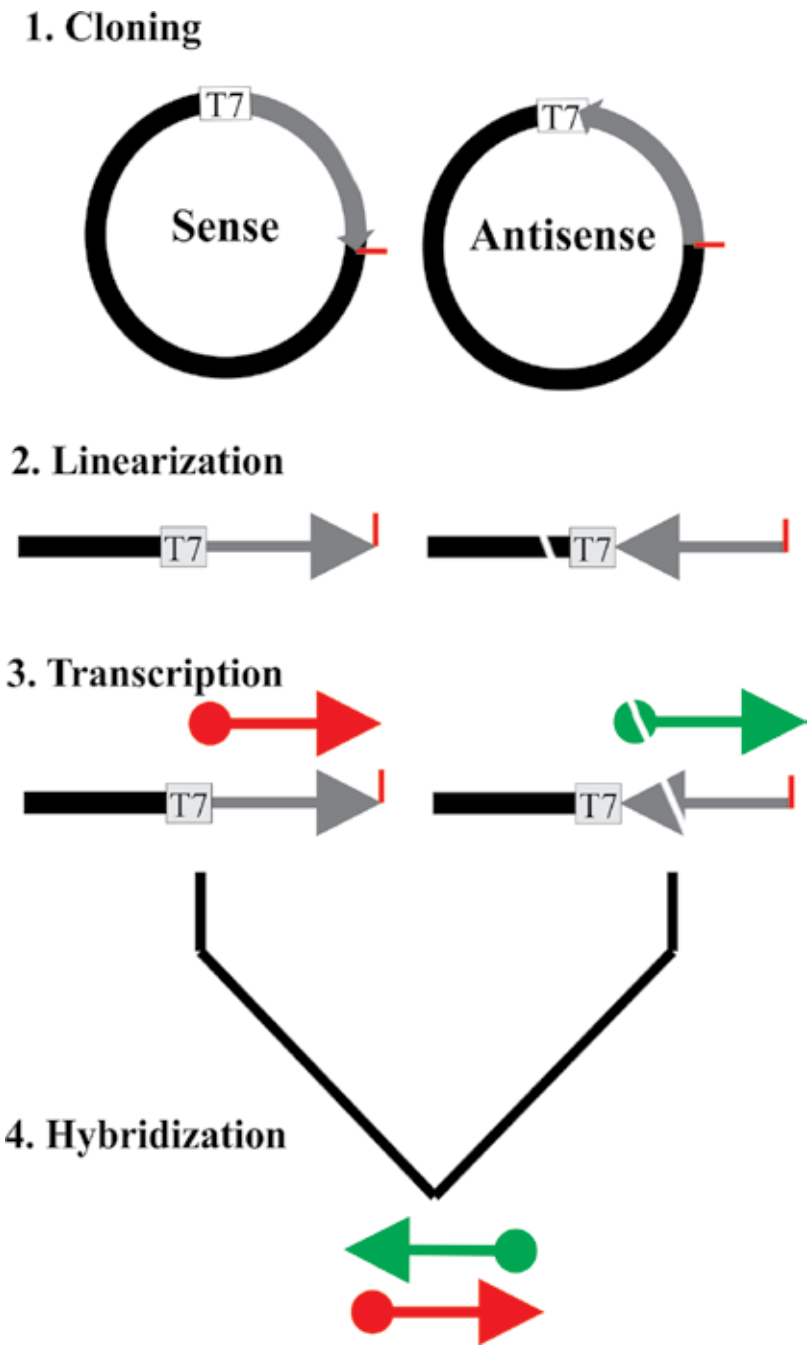

Figure 1. *In vitro* dsRNA synthesis strategy. Plasmid sequences are indicated in black while inserts for RNA productions downstream of the T7 promoter (cloned in two different orientations) are displayed in gray. Sense and antisense RNAs are indicated in red and green, respectively.

- In *Manducta sexta* injection of double-stranded integrin-beta1 RNA into larvae resulted in decreased integrin beta1 expression in plasmatocytes and significantly suppressed encapsulation [16].

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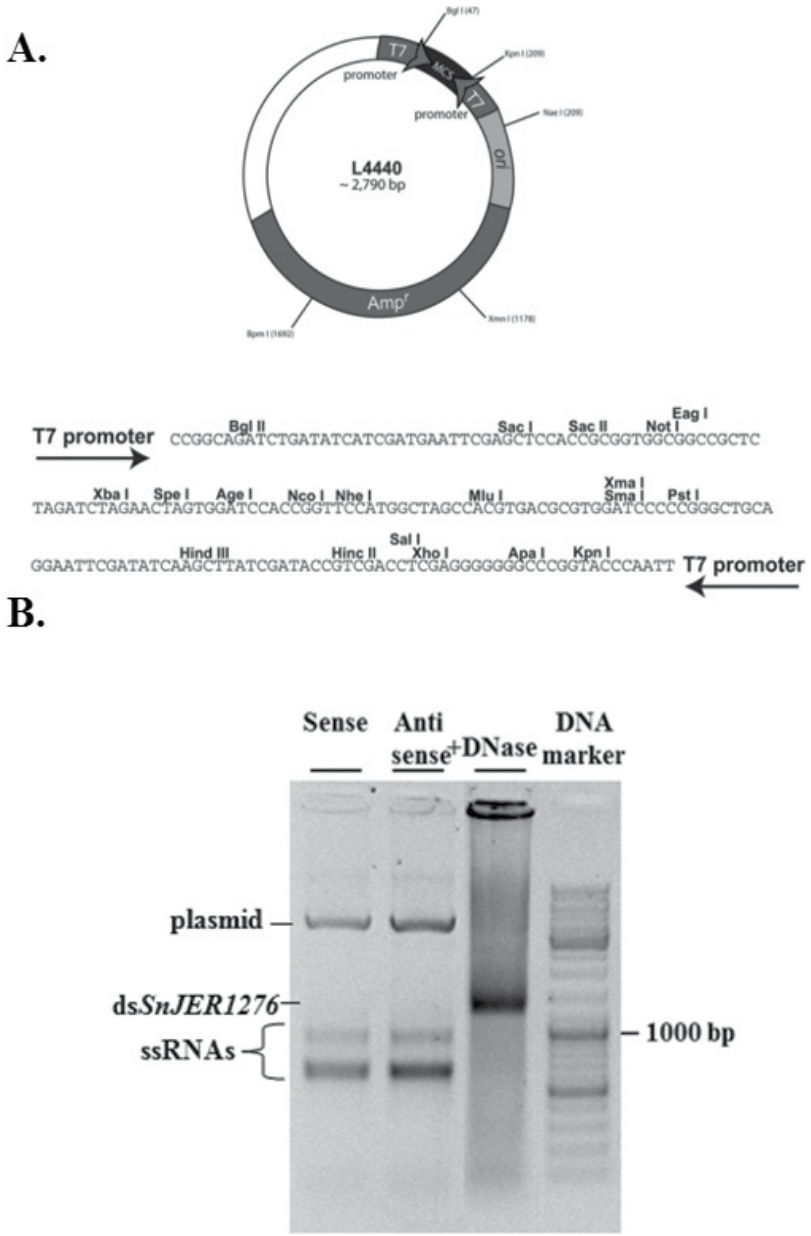

Figure 2. Production of dsRNA corresponding to the gene *SnJHER*. **A**. Schematic representation of L4440 plasmid and its polylinker for cloning gene fragments for dsRNA production (https://www.addgene.org/1654/). The polylinker is flanked on either side by a T7 RNA polymerase promoter for RNA synthesis. **B**. Synthesis of ds*SnJHER*1276. Sense and antisense ssRNAs running in 1% w/v agarose gel (first and second lanes counting from the left). The two ssRNAs were annealed and subjected to DNase treatment (third lane). DNA marker is being shown on the fourth lane.

- While in *Aedes aegypti* injection of double-stranded RNA corresponding to the broad gene (BR) isoform Z2 led to a significant decrease in the expression of the *Vitellogenin* (Vg) gene at 8 and 24 h post blood meal. Knockdown of Z1 or Z4 resulted in enhanced Vg expression beyond its normal expression time [17].

- Injection of both dsRNA/siRNA could induce the silence of chitin synthase gene A (CHSA), which is an important gene for the growth and development of cuticles and trachea in beet armyworm, *Spodoptera exigua*. The cuticle of CHSA-silenced insects was disordered and the epithelial walls of larval trachea did not expand. Moreover, injections significantly increased abnormalities relative to control larvae [18].
- In *Tribolium castaneum*, injection of dsRNA prepared using the common or isoform-specific regions of ecdysone receptor (EcR) and ultraspiracle (USP) genes as templates caused derailment of development [19].
- In *S. frugiperda*, silencing of the allatostatin AS-C-type (Spofr/Manse-AS) or the allatotropin AT 2 (Spofr-AT 2) genes after intra-hemolymph administration resulted in reduction of their transcript levels in brain and gut of last instar larvae and adults. This suppression led to an increased JH titer in larvae [20].
- In *Gryllus bimaculatus*, injection of the circadian clock gene period (*G. bimaculatus* period, Gb'per) dsRNA into the abdomen of third instar nymphs knocked down the mRNA levels to 25% of that in control animals. Most Gb'per dsRNA-injected nymphs lost their circadian locomotor activity rhythm, while those injected with DsRed2 dsRNA as a negative control clearly maintained the rhythm [21].
- The membrane-bound trehalase genes of *S. exigua SeTre-1 and SeTre-2* were analyzed by dsRNA injections. The RNA interference (RNAi) of either SeTre-1 or SeTre-2 was gene-specific and effective, with efficiency rates up to 83% at 72 h post injection. After RNAi of SeTre-1 and SeTre-2, significant higher mortality rates were observed during the larval-pupal and pupal-adult stages [18].
- In *D. virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), injection of laccase 2 DvvLac2-specific double-stranded RNA resulted in the prevention of post-molt cuticular tanning, while injection of chitin synthase 2 DvvCHS2-specific dsRNA reduced chitin levels in midguts. Silencing of both DvvLac2 and DvvCHS2 was confirmed by real-time polymerase chain reaction (RT-PCR) and quantitative RT-PCR [22].
- DsRNA-based gene silencing resulted in a dramatic reduction in the levels of the corresponding mRNA in the *Locusta migratoria manilensis* (Meyen) nymphs injected with dsRNA of chitin synthase 1 LmCHS1, or either of its two variants, LmCHS1A and LmCHS1B. mortalities of 95, 88 and 51% were observed in the locusts injected with the LmCHS1, LmCHS1A and LmCHS1B dsRNA, respectively [23].
- In *Leptinotarsa decemlineata*, specific interference of Ldace1, an ortholog of *Anopheles gambiae Agace1* by means of dsRNA injection, resulted in a reduction of AChE activity to an approximate 50% compared to control, while interference of Ldace2 reduced AChE activity to an approximate 85%. Interference of Ldace1 in CPB adults caused a significant increase in mortality (43%) as early as 3 days post injection (p.i.). Interference of Ldace2 also caused a significant increase in mortality (29%) compared to control, although at seven days p.i. [24].
- In *G. bimaculatus* and the firebrat *Thermobia domestica*, a dose-dependent effect of dsRNA was observed to achieve knockdown of clock genes. However, this effect was affected by the particular gene that was silenced and the insect species (*Gryllus* versus *Thermobia*) [25].

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- Injection of CHS dsRNA interfered with egg development in the ovary and the eggs that were laid were dark of color and not viable. Fluorescence microscopy demonstrated reduced deposition of chitin in previtellogenic and vitellogenic oocytes in the ovaries [26].
- In a later work, HSP70/HSC70 knockdown of *Rhodnius prolixus* insects showed lower resistance to prolonged starvation in comparison to appropriate controls, dying between 32 and 40 days after dsRNA injection. After blood feeding, the physiological effects of HSP70/HSC70 knockdown were more prominent and the insects died even earlier, within 14–20 days after feeding (21–27 days after dsRNA injection). These bugs showed impaired blood processing and digestion; reduced energetic metabolism and the midgut immune responses were compromised [27].

2.2. Oral delivery of dsRNAs

Oral delivery of dsRNAs aims to silence the selected gene after gut-mediated uptake and transport to the insect cells. If oral delivery is efficient, then much higher possibilities exist to formulate a dsRNA-based insecticide. For orally delivering dsRNAs, dsRNAs should be *in vitro* synthesized as described previously. Then, the dsRNAs are incorporated to the artificial diets of the insects or even sprayed in the plants which are used to be fed on. Important examples (highlights) from the literature are given below:

- Walshe et al. [28] first demonstrated specific gene knockdown by feeding in dipteran species. This was a first example of RNAi in a blood-sucking insect by including dsRNA in its blood meal. Delivery of dsRNA through the blood meal of *Glossina morsitans* was as effective as dsRNA injection with respect to the silencing of the midgut-expressed gene TsetseEP. By contrast, the gene 2A192 that is expressed in the fat body was only knocked down after dsRNA injection. Feeding of dsRNA reduced significantly the mortality rates of the flies compared with the injection treatment.
- Bautista et al. [29] silenced a P450 CYP6BG1 gene by dsRNA droplet feeding in *Plutella xylostella* in order to show its involvement in permethrin resistance. Quantitative real-time PCR showed efficient reduction of expression of CYP6BG1 transcripts in midgut and carcass after oral delivery, which was reflected in reduced total P450 activities of microsomal preparations and which resulted in significant reduction in resistance to the insecticide permethrin. The experiments indicate the participation of overexpressed CYP6BG1 in the resistance mechanism against permethrin [29].
- RNAi has been used successfully to silence endogenous honey bee genes by feeding [30]. This was the first successful large-scale real-world use of RNAi for insect-disease control. RNAi was shown to prevent bees from succumbing to infection from Israeli Acute Paralysis Virus, IAPV, under laboratory conditions. In this study, IAPV-specific homologous dsRNAs were used in the field, under natural beekeeping conditions in order to prevent mortality and improve the overall health of bees infected with IAPV [30].
- In the whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae), Lü and Wan [31] explored the role of heat-shock protein (Hsp) genes in both male and female sexes by dsRNA feeding.

- Chen et al. [32] examined the effects of using a feeding-based RNAi technique to target the gene trehalose phosphate synthase (TPS) in the brown planthopper, *Nilaparvata lugens*. DsRNA feeding resulted in rapid and significant reduction in expression levels of TPS mRNA and enzymatic activity. Developmental abnormalities were observed in dsRNA-fed *N. lugens* larvae which resulted in lethal effects.
- Hunt et al. [33] developed an RNAi-mediated bioassay to explore proposed connections between expression of hexameric storage proteins and worker versus gyne (potential future foundress) castes in naturally founded colonies of the wasp genus *Polistes* (*P. metricus*). They targeted the hexamerin 2 gene in fifth (last) instar larvae by feeding with double-stranded hexamerin 2 RNA directly to larvae in naturally founded colonies in the field. Their results pave the way for functional genomic research that can contribute significantly to learning the interactions between environment and development and its significance for paper wasp evolution and behavioral ecology [33].
- Luan et al. [34] developed a new and high throughput methodology to silence whitefly genes using a leaf-mediated dsRNA-feeding method. A leaf-mediated dsRNA-feeding method was developed to test silencing of whitefly genes in high-throughput format. While silencing of ecdysone biosynthetic and regulatory genes had little effect on survival and fecundity of adult whiteflies, reduced survival and delayed development were observed during the treatment of the nymphal stages.
- Moreover, Mao and Zeng [35] performed RNAi at the second instar stage to knock down *hunchback* (*hb*) expression in the pea aphid, *Acyrthosiphon pisum*. Continuous feeding of *Aphb* dsRNA mixed in their artificial diet led to reduction of *Aphb* transcripts and rise of insect lethality. Their results indicated that the gene *hunchback* was an efficient RNAi target for managing populations of aphids.
- Li et al. [36] introduced dsRNAs of P450 CYP6CM1 genes corresponding to the B and Q biotypes into the insect body of *B. tabaci* adults through membrane feeding. RNAi of the P450 CYP6CM1 gene reduced gene expression, increased mortality and inhibited the ability to detoxify a pesticide or a plant secondary metabolite in both biotypes of *B. tabaci*, with better efficacy in B biotype than in Q biotype.
- Finally, Abd El Halim et al. [37] evaluated oral delivery of dsRNAs targeting sodium ion channel paralytic A *(TcNav*) gene in *T. castaneum* as a viable means of controlling this insect pest. Oral delivery of dsRNA caused dose-dependent mortalities between 19 and 51.34% accompanied by a significant knockdown in gene expression following 3 days of continuous feeding. The majority of larvae injected with, or fed, dsRNA died during the final larval stage prior to pupation. This work provided evidence of a viable RNAi-based strategy for insect control.

2.3. Bacterial-mediated RNAi

Bacterial dsRNA administration is based on the observations of Timmons and Fire [6] which showed that ingestion of bacterially expressed dsRNAs could produce specific and potent genetic interference in *C. elegans*. This approach uses an RNase III-deficient *Escherichia coli*

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strain known as HT115 (DE3) [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase]. In this methodology, the gene of interest is being cloned between two T7 promoters on a special RNAi plasmid known as L4440 (T7p, T7p, lacZN, OriF1) (**Figure 2A**). The plasmid is being transformed in HT115 cells and dsRNA production is achieved after induction with IPTG. The induced cells are then introduced in the worm's growth media and RNAi is achieved after a short period of incubation.

Similarly in insects the IPTG-induced bacteria are incorporated in their artificial diets or they are even sprayed in plant organs that insects are feeding on and RNAi is induced after a period of continuous feeding (**Figure 3**).

The successful application of bacterial-mediated RNAi has been reported in several cases:

- Tian et al. [38] showed that the growth and development of *S. exigua* larvae fed with HT115 cells expressing dsRNAs of the chitin synthase gene A (SeCHSA) was disturbed, resulting in lethality. The survival rates of fifth instar larvae, prepupal and pupal stages were significantly lower than those of controls.
- Similarly, Li et al. [39] used genetically engineered HT115 *E. coli* cells. Engineered bacteria were generated that produce specific dsRNAs targeting several essential genes in *Bactrocera dorsalis*, such as the ribosomal protein Rpl 19, the type V ATPase D subunit, the fatty acid elongase Noa and the small GTPase Rab11. Quantitative real-time PCR indicated

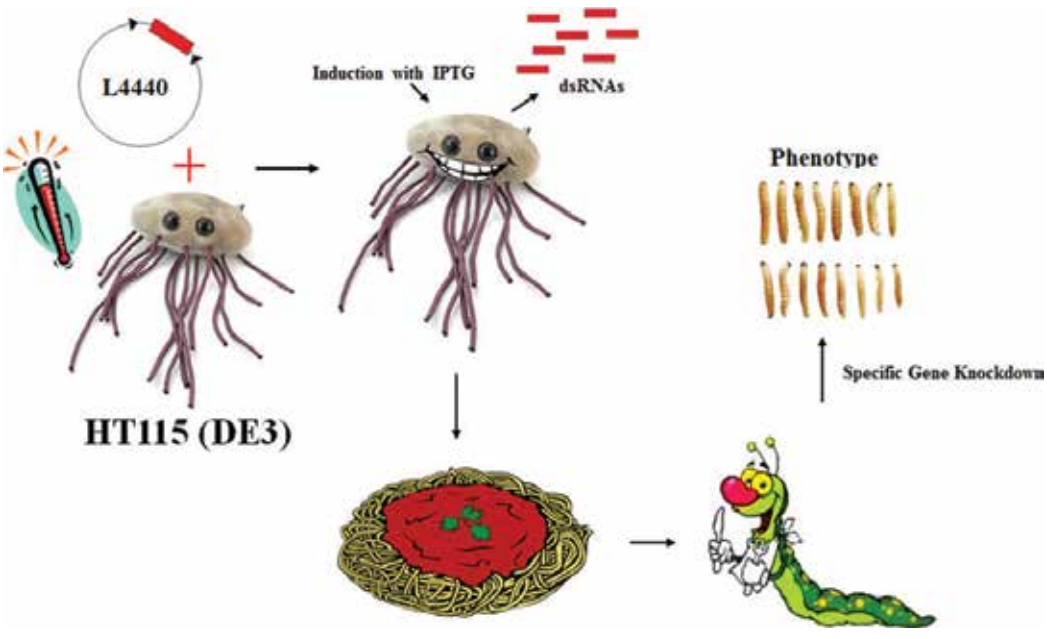

Figure 3. Strategy behind bacterial-mediated RNAi. The gene of interest is being cloned in the RNAi L4440 plasmid and transformed to competent HT115 cells. The transformed cells are then induced with IPTG and incorporated in insect's diet in order to induce RNAi.

that feeding of both engineered bacteria and isolated dsRNAs proved effective at silencing the four targeted genes when compared with nonspecific (EGFP) dsRNA.

- In the Colorado potato beetle, *L. decemlineata* (Say) (CPB), Zhu et al. [40] successfully triggered silencing of five target genes by dsRNA feeding resulting in significant mortality and reduced body weight gain in the treated beetles. These results suggested that the efficient induction of RNAi using bacteria to deliver dsRNA is a possible method for the management of CPB.
- Moreover, Zhang et al. [41] demonstrated the efficacy of RNAi in the cotton bollworm, *H. armigera*, using bacterial-mediated dsRNA expression of CYP6B6 gene. Gene and protein expression levels of CYP6B6 were reduced in *H. armigera* larvae fed with HT115 bacteria expressing CYP6B6 dsRNAs.
- Taracena et al. [42] silenced *R. prolixus* heme-binding protein (RHBP) and catalase (CAT) genes after feeding nymphs and adult triatomine insects with dsRNA expressing HT115 bacteria. The RNA interference effect was systemic and temporal. RHBP expression in the fat body was reduced by 99% three days after feeding and CAT expression was reduced by 99 and 96% in the ovary and the posterior midgut, respectively, 5 days after ingestion.

Essential for successful RNAi-feeding experiments is correct dsRNA induction before the oral administration step. A general protocol for bacterial-mediated dsRNA expression is given as follows. In this experiment, we cloned the 1276 bp part of *S. nonagrioides* juvenile hormone esterase-related gene in the L4440 plasmid and then we used it to transform HT115 (DE3)-competent cells. We used empty L4440-transformed HT115 cells as control. Single colonies of HT115/L4440-SnJHER1276 and HT115/L4440 cells were cultured in LB at 37°C with shaking at 220 rpm overnight. The cultures were diluted 50-fold in 100 ml LB supplemented with 100 μg/ml ampicillin plus 15 μg/ml tetracycline (Sigma) and cultured at 37°C to OD600 = 0.5. After induction of T7 RNA polymerase expression with 400 μM IPTG for 4 h with continuous shaking at 37°C, the bacteria were centrifuged (5000 g, 10 min) and the pellet was re-suspended with 0.5 ml of water. Total RNAs were extracted from bacterial cells using Trizol (Sigma). To remove excess ssRNA, RNA samples were treated with 1 μg of RNase-A (Sigma) in the presence of 0.3 M NaCl, which protects against digestion of dsRNA. The reaction occurred for 10 minutes at 37°C. The length and the quality of the produced dsRNAs were confirmed by electrophoresis on 1% agarose gel (**Figure 4**). As shown in **Figure 4** in the no RNase-A lane, no band was observed for the control L4440-transformed bacteria while a band running to ~1200 bp was observed in the L4440-SnJHER1276-transformed bacteria. After adding 1 μg of RNase-A, all RNAs were degraded leaving unaffected the DNA band. In the third lane after co-incubating dsRNAs with 1 μg of RNase-A in salinity of 0.3 M NaCl, only ssRNAs are degraded while dsRNAs remain unaffected. It was reported that RNase-A in high salinity buffers selectively digests ssRNAs leaving undigested the dsRNAs [43]. An alternative of the previous experiment was published in our laboratory by Kontogiannatos et al. [12]. In this experiment, we used a JHER hairpin expressed in pGEM T-easy plasmid which was used to transform HT115 cells and induce dsRNA expression.

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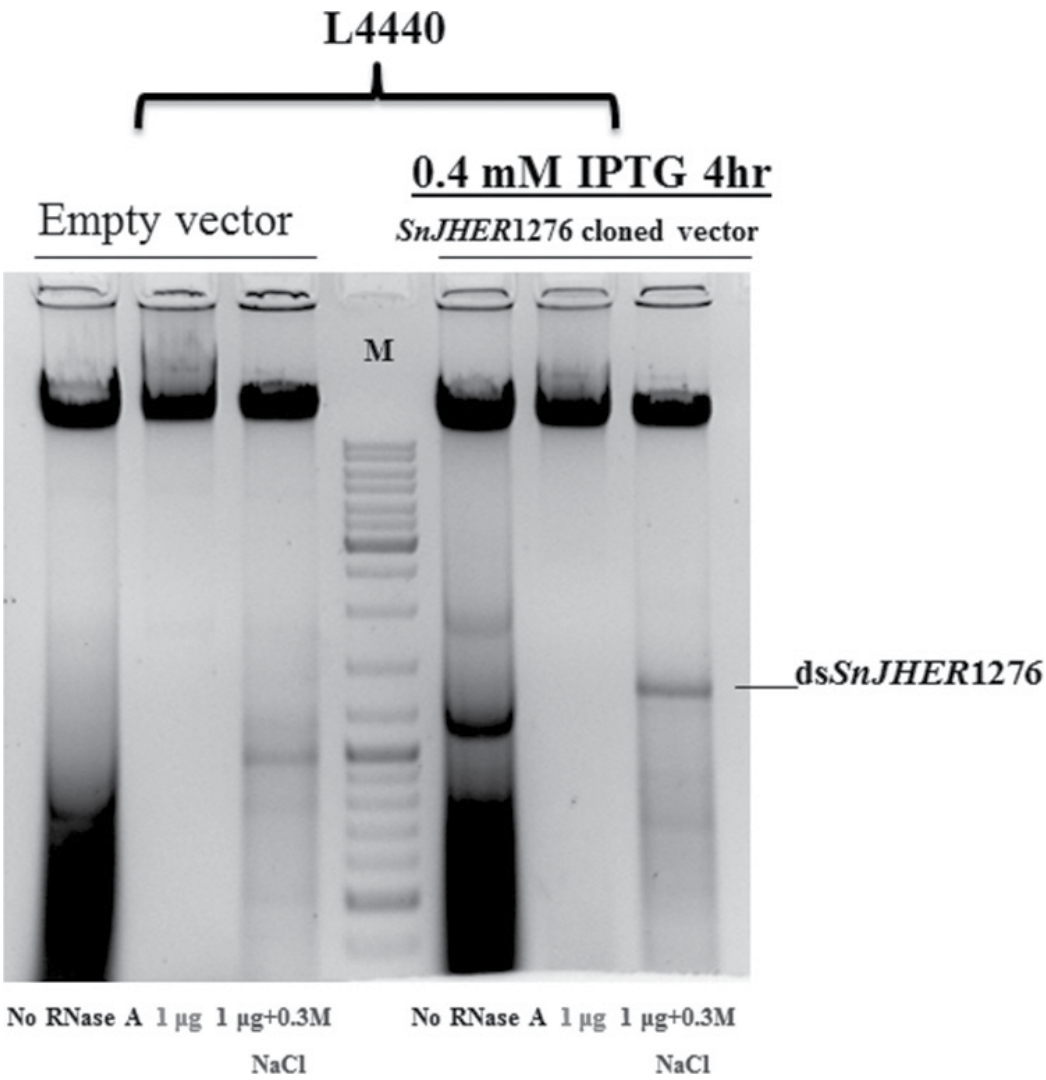

Figure 4. Bacterial-mediated production of SnJHER1276 in HT115 cells. Total RNA isolated from L4440 or L4440-SnJHER1276-transformed HT115 cells was analyzed in 1% w/v agarose gel. RNA extracts were treated with RNase A or RNase A+0.3 M NaCl. M: Molecular marker. Production of ds*SHER*1276 is indicated.

There is an alternative procedure for bacterial-mediated RNAi in which dsRNAs are first isolated from bacteria before injection into insects. In the following experiment, we silenced *S. nonagrioides ultraspiracle* gene after injecting bacterially expressed dsRNAs into its hemolymph. For doing that, we cloned a 689-bp fragment of *SnUSP* partial cDNA sequence (GenBank: JN704569) to the L4440 plasmid which was then used to transform competent HT115 cells. The protocol described previously was used to induce dsRNA expression. HT115 ds*SnUSP* expressing bacterial cells were Trizol treated and total RNAs were extracted and analyzed in 1% w/v agarose gels. In this case, we did not perform RNase-protection

assay since dsRNAs were clearly visualized (**Figure 5B**). Note that in **Figures 4** and **5B**, a 1000-bp band exists in the empty L4440-transformed bacteria which disappear when the gene of interest is cloned. Total RNA extracts were purified and injected in fifth instar larvae at day 3 (L5d3) and specific phenotypes were observed (**Figure 5C**). A proportion of ds*SnUSP689*-injected animals died presenting a large range of developmental abnormalities. Gene-specific downregulation was observed after RT-PCR analysis (data not shown).

2.4. Plant-mediated RNAi

The observation that genetically modified plants expressing dsRNAs targeting specific insect genes could induce RNAi in the insect pests was first reported in independent publications of Baum et al. [8] and Mao et al. [9]. Baum et al. showed that corn plants expressing hairpin dsRNAs that target the A subunit of ATPase gene in the western corn rootworm were sig-

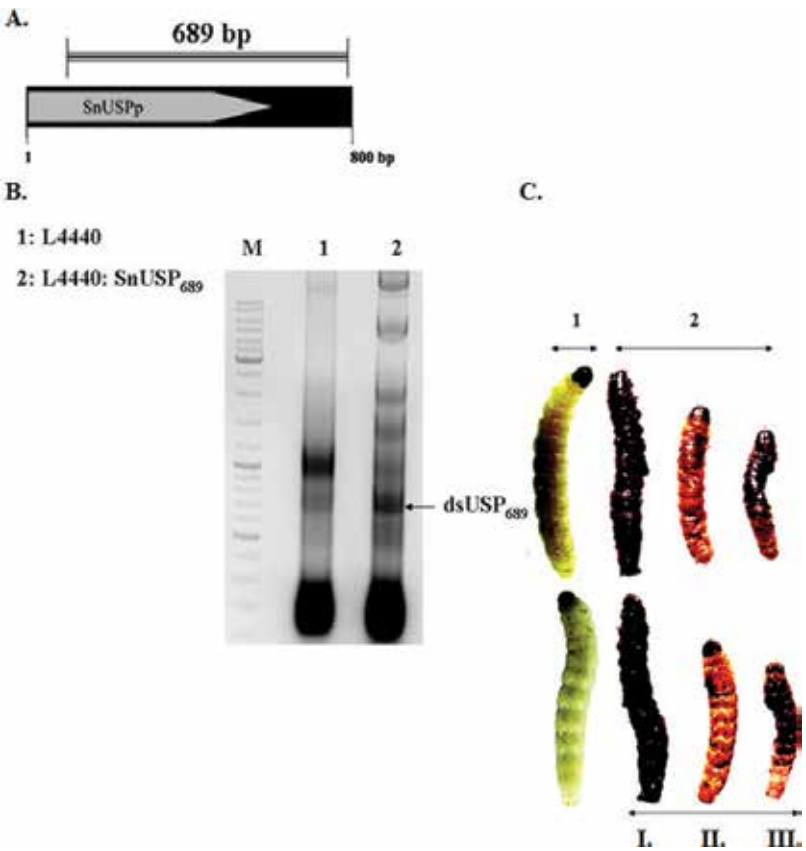

Figure 5. Phenotypes observed after knockdown of *SnUSP* by dsRNA produced in bacteria. **A**. Schematic representation of the partial cDNA sequence of *Sesamia nonagrioides ultraspiracle* gene (GenBank: JN704569). The bar above the sequence indicates the 689 bp fragment used for RNAi experiments. **B**. Confirmation of dsUSP689 synthesis in HT115 IPTG-induced bacteria. Total RNAs isolated from IPTG-induced HT115/L4440 (1) and HT115/L4440:SnUSP689 (2) bacteria were analyzed in 1% w/v agarose gels. C. Lethal phenotypes of L5d3-injected *S. nonagrioides* larvae injected with total RNAs isolated from the HT115/L4440:SnUSP689 (2) bacteria. Control HT115/L4440-injected animals are also indicated (1).

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nificantly protected by the damage caused by this pest [8]. Furthermore, *Arabidopsis* plants expressing dsRNA hairpins targeting the cytochrome P450 monooxygenase gene in the corn pest *H. armigera* led to decreased resistance to the sesquiterpene gossypol to the feeding insects [9]. Following these publications of proof-of-principle, several other reports have documented the successful application of plant-mediated RNAi:

- In *H. armigera*, a molt-regulating transcription factor gene was selected to be used in plant-mediated RNAi experiments. Four different fragments covering the coding sequence of *HaHR3* were initially tested by bacterial-mediated RNAi. The most effective fragment (in terms of RNAi efficiency) was used for *Nicotiana tabacum* agrobacterium-mediated transformation. When *H. armigera* larvae were fed the *E. coli* or transgenic plants, the HaHR3 mRNA and protein levels dramatically decreased, resulting in developmental deformities and larval lethality [44].
- Similarly, dsRNAs of the gap gene hunchback (hb) of *Myzus persicae* were overexpressed in transgenic tobacco. Continuous feeding of neonate aphids on transgenic diet reduced *Mphb* mRNA level in the fed aphids and inhibited insect reproduction [35].
- In the brown planthopper *N. lugens*, a common 360-bp fragment between ecdysone receptor (EcR) NlEcR-A and NlEcR-B genes was used to construct a transgenic RNAi rice line. After newly hatched nymphs of *N. lugens* fed on the transgenic rice lines, effective RNAi was observed. The NlEcR expression levels were decreased in all lines compared with the controls. In all lines, survival rates of nymphal stages were nearly 90%, but the average number of offspring per pair in the treated groups was significantly less than that observed in the control, with a decrease of 44.18–66.27% [45].
- Efficient RNAi after plant-mediated dsRNA delivery was reported in the *Triticum-Sitobion* system. *S. avenae* fed on transgenic *T. aestivum* lines expressing dsRNAs of its carboxylesterase (CbE E4) gene presented reduced CbE E4 gene expression. The number of aphids grown on transgenic *T. aestivum* lines was lower than the number raised on non-transgenic plants. CbE E4 enzyme isolated from *S. avenae* fed on dsCbE plants hydrolyzed only up to 20–30% Phoxim solution within 40 min whereas a solution of the enzyme from CbE E4 fed on control plants hydrolyzed 60% of Phoxim solution within 40 min [46].
- Efficient plant-mediated RNAi was also reported in *H. armigera* [47]. Researchers used this technology to silence the *arginine kinase* (*AK*) gene of *H. armigera* (*HaAK*), encoding a phosphotransferase that plays a critical role in cellular energy metabolism in invertebrate species. Transgenic *Arabidopsis* plants producing *HaAK* dsRNAs were generated by *Agrobacterium*-mediated transformation. The feeding bioassays clearly showed that resistance of transgenic *Arabidopsis* plants to *H. armigera* was improved and levels of *HaAK* transcripts were drastically suppressed.
- Parental RNAi after plant-mediated dsRNA delivery was observed in *M. persicae*. DsRNA producing *A. thaliana* lines were constructed to target genes with different functions in the aphid. RNAi-mediated knockdown in aphids was achieved independently of gene identity and function and could reduce original expression levels by 70% between 4 and 8 days after feeding on dsRNA-producing transgenic *A. thaliana*. Target genes were also

downregulated in nymphs born from mothers exposed to dsRNA-producing transgenic plants and the RNAi effect lasted twice as long (12–14 days) in these nymphs [48].

2.5. Virus-mediated RNAi

The use of viruses is a less common methodology to transfer dsRNAs into the insect tissues. Virus-mediated-RNAi involves the expression of a dsRNA transgene into a virus which is then used to infect the insect cell or a tissue in order to express dsRNAs intracellularly. This methodology has not been used extensively because of the general viral interference with normal cell physiology; for instance, baculoviruses cause high lethality and potential phenotypes could not be distinguished between dsRNA-producing and control viruses. In addition, viruses can produce inhibitors of RNAi, thereby lowering silencing efficiency [49].

In order to successfully distinguish effects of virus-mediated RNAi, wild-type viruses should be somehow inactivated or at least should not cause highly toxic effects in the insect host. The first report of successful viral dsRNA delivery was made by Hajos et al. [50]. In this paper, the researchers used a recombinant baculovirus, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), to express *Heliothis virescens* juvenile hormone esterase (JHE) gene in antisense orientation, driven by the viral p10 promoter. Infection with this recombinant virus greatly reduced the hemolymph JHE levels and resulted in aberrant morphogenesis of final-instar *H. virescens* larvae. This was the first time that baculovirus-mediated gene silencing could be accomplished and utilized to dissect insect development and to design a new class of baculovirus-based insecticides.

One of the most interesting virus-mediated RNAi reports is by Uhlirova et al. [51]. In this paper, researchers used a recombinant Sindbis virus as a tool to silence the gene encoding for the transcription factor Broad-Complex (BR-C) in *B. mori*. Sindbis virus with a BR-C antisense expression cassette reduced BR-C mRNA expression levels in infected tissues via an RNAi mechanism. BR-C silencing resulted in developmental arrest at the larval-pupal transition or in defective differentiation of adult compound eyes, legs and wings. Also the programmed cell death of the larval silk glands was prevented after RNAi of BR-C. *B. mori* nucleopolyhedrovirus (BmNPV)-mediated RNAi was demonstrated by our laboratory in a work by Kontogiannatos et al. [12]. Even if BmNPV's spectrum is extremely narrow, infection of the non-target species *S. nonagrioides* is feasible. In order to assess which kind of baculovirus is suitable for infection of *S. nonagrioides* larvae, we performed bioassays with genetically modified AcMNPV and BmNPV viruses expressing a GFP cassette under the control of *B. mori* actin promoter. Infected insects with 10^7 pfu/ml of the AcMNPV-BmA::GFP virus had significant higher mortality rates in almost 90% of the total injected animals. These animals presented all typical symptoms of polyhedrosis. In addition, the survived animals presented several developmental abnormalities, failing to complete normally their developmental cycle. In contrast to AcMNPV, infected insects with 10^7 pfu/ml of the BmNPV-BmA::GFP virus were able to proceed through their developmental stages and none of them presented signs of polyhedrosis. Both viruses were located mostly in the fat body tissues, in hemolymph (**Figure 6**), in epidermal cells and in tracheoles of the infected animals [12].

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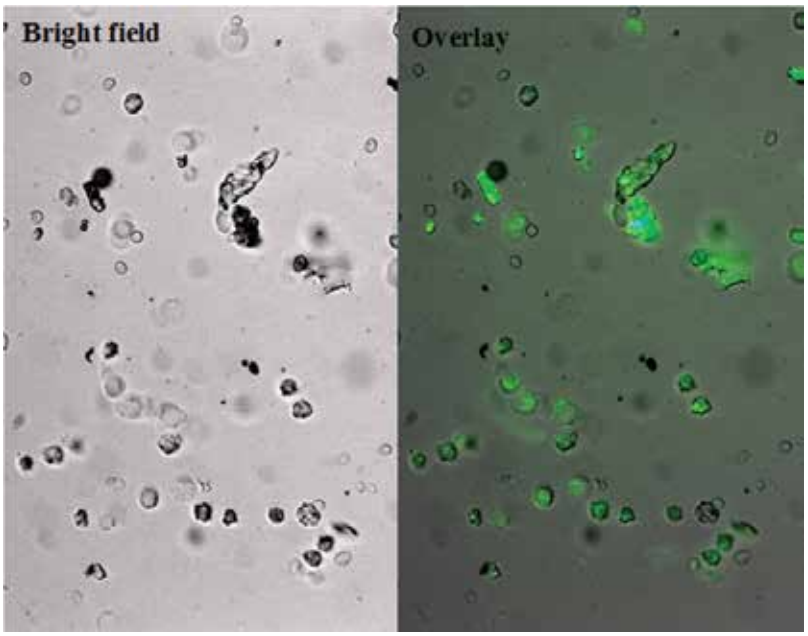

Figure 6. Hemolymph cells (20× magnification) of *S. nonagrioides* fifth instar larvae infected with the BmNPV-BmA::GFP virus, 7 days PI. Left. Bright field imaging of hemolymph cells infected with BmNPV-BmA::GFP. Right. Overlay of bright and fluorescence field images of infected *S. nonagrioides*' hemolymph cells.

The previous experiments showed that BmNPV should be the appropriate vector to transfer dsRNAs in *S. nonagrioides* cells. But this methodology should not be used for functional analysis of genes implicated in larval-pupal transformation since we observed that when insects were infected with either the AcMNPV-BmA::GFP or the BmNPV-BmA::GFP virus in the prepupal stage, larval-pupal transition was blocked while singular adults emerging from surviving pupae were also abnormal. Taking the above observations into consideration, it was nevertheless clear that the infection of *S. nonagrioides* larvae with a BmNPV-BmA::GFP-JHER472 hairpin-expressing virus resulted in specific gene downregulation with similar phenotypes than those after intra-hemolymph dsRNA administration [12].

Therefore, this methodology should be improved in order to create genetically modified baculoviruses that will cause even less physiological impact to the infected cells and to allow more clearly the distinction between infection-related effects and those caused by RNAi.

3. Two bottlenecks for efficient RNAi in insects: dsRNA stability and dsRNA uptake

Recently, several studies were published that focused directly on the causes for the variability of RNAi efficiency among different insect groups [52–54]. Beetles (Coleoptera) and cockroaches (Blattaria) are very sensitive to RNAi that is administered by injection or feeding;

locusts (Orthoptera) are sensitive to RNAi by injection but are refractory to RNAi by feeding, while caterpillars (Lepidoptera) are refractory to RNAi by both injection and feeding. A series of experiments with insects as well as derived cell lines investigated differences in cellular uptake of dsRNA as well as degradation of dsRNA among the different groups. One important finding was that dsRNA degradation correlated negatively with RNAi efficiency, that is, insects with low efficiency in RNAi (e.g. lepidopterans) degrade dsRNA faster in hemolymph and midgut than insects with high efficiency in RNAi (e.g. coleopterans) [52, 54]. The second important finding relates to the cellular uptake of dsRNA: while both lepidopteran and coleopteran cells can take up efficiently labeled dsRNA from the extracellular medium by endocytosis, this results in the production of siRNAs and silencing of target genes only in the coleopteran cells [52, 53]. The use of pH-sensitive dyes coupled to dsRNA molecules suggests that in lepidopteran cells endocytosis of dsRNA is followed by fusion of endosomes with lysosomes and subsequent degradation while in coleopteran cells presumably endosomal escape can occur and subsequent interaction of dsRNA with the RNAi machinery [53]. In lepidopteran cells, the core RNAi machinery works very efficiently [55] and the obstacle seems to be mainly the efficient arrival of the dsRNA trigger at the intracellular RNAi machinery.

These investigations indicate that the use of "naked" dsRNA to trigger RNAi by feeding will only work efficiently in beetles and cockroaches and that for other insects special delivery systems need to be developed. In the first instance, those delivery systems need to protect the dsRNA trigger from degradation in the midgut, an effort which is especially difficult to achieve for lepidopterans in which the alkaline gut content is prone to destabilize dsRNA even in the absence of nucleases [56]. Second, methods need to be developed for more efficient "functional" uptake of dsRNA in locusts and caterpillars in the sense that the internalized dsRNA is not degraded but efficiently presented to the RNAi machinery. Besides naked dsRNA, other delivery methods using bacteria, algae, plants, symbionts and viruses have been tested with variable efficiency [e.g. 36, 55, 57–59] but mechanistic details of how dsRNA escapes from the different vehicles to enter the cellular cytoplasm are lacking and should become an active area of research in the future.

Also synthetic nanoparticles are being tested for delivery of dsRNA in insects [23, 60]. Of note, chitosan dsRNA/siRNA nanoparticles have been reported to efficiently trigger RNAi in mosquito larvae [61, 62] and detailed protocols were published to achieve efficient gene silencing by this method [62]. In relevance to the discussion above, nanoparticles can be engineered to stimulate endosomal escape [63] and cell-penetrating peptides can be harnessed to deliver nucleic acid cargo directly to the cytoplasm [64, 65]. Moreover, fusions with viral capsid proteins have been used to deliver protein toxins to the hemocoel of insect pests [66]. Thus, research into the mode by which insect viruses can penetrate the midgut epithelium can lead to new biotechnological applications for efficient delivery of dsRNA/siRNA cargo to specific insect pests [67].

4. Commercialization of RNAi for crop protection

Because of the increasing burden of chemical pesticides (residual toxicity in the environment, pesticide resistance), interest for developing biological pesticides has expanded significantly during recent years. Biological pesticides are pesticides derived from natural materials such

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as animals, plants, bacteria and certain minerals [68, 69]. Biological pesticides include microbial pesticides (e.g. parasitoid wasps, predatory bugs), microbial pesticides (e.g. *Bacillus thuringiensis* (Bt) spores), semiochemicals (e.g. pheromones as attractants), natural products (e.g. fermentation products from bacteria such as *Streptomyces* and *Saccharopolyspora*) and plant-incorporated protectants (e.g. transgenic plants that produce Bt toxins). Advantages of biological pesticides are their reduced toxicity and reduced risk of persistence in the environment, increased specificity toward targeted pests and decreased risk for development of resistance. Biological pesticides often also represent a new mode of action and can thus be employed in combination with conventional pesticides to reduce their dose and environmental impact. The field of biological pesticides is now considered as a big opportunity for expansion since farming is becoming more and more environmentally responsible in the world. In the European Union, for instance, new guidelines are being developed for the promotion of the use of safer pesticides and biological pesticides are considered a major part for this solution [70].

RNAi technology is one of the most recent trends in the field of crop protection and conceptually approaches the "ideal" of the perfect pesticide: it targets only the intended pest and is predicted to have minimal impact on non-target organisms (pollinators, parasitoids, predators and vertebrates). Furthermore, it is biodegradable and therefore with minimal risk for human health and the environment. Because dsRNA is either produced enzymatically in vitro or in genetically transformed bacteria, it can be considered as a "natural product" (biological pesticide). However, RNAi technology, as with most biological pesticides, may be less effective than conventional chemical pesticides (slower killing may necessitate multiple applications) and associated with higher cost of production. Because of the "biological" nature, shelf life may be lower. Because of the novelty of the technology, delays can occur during registration for commercialization.

A major issue with RNAi technology so far concerns its efficiency. The success of RNAi to control pests seems to be mainly determined by the efficiency of delivery of dsRNA, the trigger of RNAi. Major obstacles in the success of RNAi are the uptake of dsRNA/siRNA by the cells and its stability (resistance to degradation) in the gut and the tissues [71–73]. Because of these issues, RNAi as an economically viable approach to control insect pests so far is limited to two coleopteran species, the western corn rootworm (*D. virgifera*) and the Colorado potato beetle (*L. decemlineata*), that are extremely sensitive to environmental RNAi [8, 74]. For other species, most notably non-coleopterans, it is perceived that the development of specialized "formulations" is necessary to deliver dsRNA with sufficient efficiency to cause an impact on larval growth and crop damage [75]. Specialized formulations could be tailored to the ecological and physiological characteristics of the targeted pests to stimulate oral uptake (e.g. food attractants), stability in the gut (e.g. chitosan nanoparticles) and interaction with gut epithelium (e.g. specific interaction with membrane proteins).

For the control of the two coleopteran pests mentioned above, two different strategies are used. A spray of dsRNA can be used to control infestation of potato plants by *Leptinotarsa*; this approach requires techniques for the (cheap) production of large amounts of dsRNA [74, 76]. For the control of *Diabrotica*, on the other hand, transgenic corn can be used that produces RNA hairpins targeting the genes of the pest [8]. Both approaches seem to be close

for commercialization. While RNAi technology could be used as a method to directly affect insect growth and mortality, its use as a "synergist" to enhance the effects of other pesticides also can have important applications. For instance, RNAi is proposed as a "pyramided" insect protection trait in genetically modified crops [49]. In "pyramided" protection traits, different insecticidal compounds are employed that act with an independent mode of action in insect pests. RNAi technology and Bt toxins provide such an example because their mode of action is entirely different and also the dynamics of their toxicity is complementary: while Bt toxins act fast but are less persistent, dsRNA achieves its strongest effects after extended periods. Because of the decreased risk of development of pesticide resistance, the combination of two independent protection traits in a crop can be considered as an alternative to the high-dose approach of a single protection trait and will reduce the refugee requirements for genetically modified crops.

RNAi technology can also be used to increase the efficiency of conventional pesticides [77]. RNAi can be used to decrease the expression of both the targets (for instance, acetylcholinesterase in case of organophosphates and carbamates) and the detoxifying enzymes (for instance, cytochrome P450 enzymes, carboxylesterases and glutathione-S-transferases) of chemical insecticides to increase their effectivity. In combination with RNAi, chemical pesticides might be employed effectively at lower doses, thereby increasing the safety of their applications. Thus, the employment of RNAi technology in pesticide formulations is an important area for further applied research with potential for commercialization.

5. Conclusion

RNAi has been successfully applied in entomological research to analyze gene function in homeostasis, development, immunity and reproduction of insects. Furthermore, the potential of RNAi to control agricultural insect pests and vectors of human disease was revealed. In this review, an overview was presented of the success of the main methods of RNAi delivery (injection and feeding) and the use of different delivery vehicles was discussed (naked dsRNA versus bacterial-, plant- and virus-mediated RNAi) together with highlights of our own experiences with the lepidopteran pest *S. nonagrioides*. Our experiences and a survey of the literature indicate the usefulness to try different approaches for the delivery of dsRNA to achieve successful gene silencing. Regarding injections of dsRNA, we observed much more efficient gene silencing and phenotypic effects in the prepupal stage than in the larval stage and this effect was dependent on the length of the injected dsRNA fragment [12]. Thus, the success of the method of dsRNA injection may be dependent on the size of the dsRNA and the developmental stage of the insect. For feeding of bacteria expressing dsRNA, silencing of targeted genes was observed but phenotypic effects were limited, indicating insufficient delivery of the dsRNA trigger in our experimental system. Finally, in our work [12], we have pioneered the method of virus-mediated RNAi. Baculoviruses that express an RNA hairpin directed against the *JHER* gene could induce specific developmental phenotypes during metamorphosis in *Sesamia*. This approach, however, needed large groups of animals for evaluation since common effects on metamorphosis were observed after infection with

baculoviruses that express specific (targeting *JHER*) and non-specific (targeting *luciferase*) hairpins. Engineered baculoviruses that are deficient in genes targeting the insect hormonal system (e.g. ecdysteroid glucosyltransferase) could be tested in future experiments to allow easier evaluation of phenotypes.

While clear successes in RNAi-mediated gene silencing were achieved and commercial application is expected for pest control in a few instances before the end of the decade, it is equally clear that much needs to be learned about the RNAi process in insects. Further research should focus on the deeper understanding of the process of RNAi in insects, especially with respect to the uptake of dsRNA from the environment. Recent research indeed indicates that efficient delivery of dsRNA is essential for robust RNAi-mediated-silencing effects. Improvements in RNAi should focus on increased stability of dsRNA in the environment, gut content and insect tissues and the development of efficient vehicles for effective release of dsRNA in the cytoplasm (as opposed to endocytotic vesicles) of the targeted cells. Only detailed understanding of the process of RNAi in insects, taking into account their different physiology and ecology, will allow us to develop the tools for robust triggering of gene silencing and to realize its full potential for insect pest control.

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Chapter 5

Light-Trap Catch of Insects in Connection with Environmental Factors

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Additional information is available at the end of the chapter

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Abstract

This chapter deals with the connection between the light-trap collection of insects and the environmental factors that influence the trapping. These factors are as follows: the solar activity and its effects on the Earth (solar activity featured by *Q*-Index and the 2800 MHz radio flux, ionospheric storms and atmospheric radio noises, the interplanetary magnetic field sector boundaries, UV-B radiation of the Sun and geomagnetic indices), the moon phases and the polarized moonlight, the weather (macrosynoptic weather situations, weather fronts and air masses, weather events, weather elements), and air pollutants. The presented results show that these all modify the volume of captured insects.

Keywords: light trapping, solar activity, Moon, weather, air pollutants

1. Introduction

Since the mid-1930s, following Williams' [1] experiments, known now as classical experiments, light trapping developed into the most general method of collecting nocturnal insects throughout the world.

In Hungary, this was followed from 1952, by the introduction of an internationally unique network of traps established on an initiative by academician Jermy [2].

The Hungarian national network is uniformly outfitted with Jermy-type light-traps. The traps of the research and plant protection institutions work from 1 April to 31 October, while those of the forestry establishments are operational from 7 p.m. to 5 a.m. every night of the year, regardless of weather, or the time of sunrise and sunset.

After the beginning of the regular light-trap collections the researchers experienced that the fluctuations of the daily catch results do not follow exactly the swarming of species. These fluctuations are obviously caused by environmental impacts. First, the influences of meteorological elements were studied. These research studies continued soon with the examination of the influence of the moonlight as well. The essence of light trapping comes from the fact that the moonlight reduces the efficiency of the light source.

There was a light-trap network in operation in Hungary since the last six decades. This network gave an inestimable substance with a scientific value to the entomology researches. Nowinszky and his colleagues examined the influence of the environmental factors onto the light trap catch since the last four decades. This enormous amount data made it possible to study the influence of more environmental factors that were not examined by researchers or only some of them made such examinations. The results of this work are discussed in this chapter.

Researchers have examined the influence of the various weather elements on collection by light-trap all over the world. Williams [3] published a fundamental study.

Williams [1] found a lower catch at a Full Moon. He thought it was because of the smaller gathering distance or because moonlight had a direct influence on activity and reduced the number of insects in flight. After several decades, there is still no valid answer to this question.

Williams et al. [4] offered two possible explanations:

- Moonlight reduces insect activity.
- Accompanied by moonlight, lamplight collects from a smaller area.

The collecting distance as a function of changing moonlight has been calculated by a number of researchers [5–8].

Baker and his coworkers verified that the tethered and free-flying moths of the Large Yellow Underwing (*Noctua pronuba* Linnaeus) and Heart and Dart (*Agrotis exclamationis* Linnaeus) fly to the artificial light from the close quarters vicinity of lamp, only a few meters found that the insects reacted to artificial light from the amazingly short distance of 3–17 m, depending on the height of the light source. These authors ruled out the possibility of moonlight exerting any influence on the collecting distance. They hold that the growing intensity of light slackens flight activity.

In an earlier study [9], we detected the abundance of catch in the First and Last Quarters can be explained with the high ratio of polarized moonlight.

In clear moonlit nights, a band of highly polarized light stretches across the sky at a 90° angle from the Moon, and it was recently demonstrated that nocturnal organisms are able to navigate based on it [10].

In Hungary, the geomagnetic data measured at one single observatory supply sufficient information for the whole country [11].

Tshernyshev [12] found a high positive correlation between the horizontal component and the number of trapped insects.

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Our study [13] deals with the modification of the catch of a dozen Caddisfly (Trichoptera) species by light trap in the region of the Tisza and Danube rivers in connection with the *H*-index (geomagnetic horizontal component). It demonstrates that in parallel to increasing values of the *H*-index the catch of 9 of the 12 species increased as well, but that of two species declined instead.

We did not find any previous studies in the literature dealing with those environmental factors that were investigated in our study. Therefore, we can cite only our own studies.

2.The Solar activity and its influence on the Earth

2.1. Solar activity featured by *Q*-index

Kleczek [14] was the first researcher, who introduced the concept of *Q*-index ($Q = i \times t$), to use the daily flare activity through quantification of the 24 h of the day.

The daily activity of the flares is characterized by the so-called *Q*-index that, used by several researchers, considers both the intensity and period of prevalence of the flares [15, 16]. Solar flares are most powerful and explosive of all forms of solar activity and the most important in terrestrial effects. This idea led solar physicists to assess the daily flare index [17]. Most authors have used *Q*-index to characterise daily flare activities, which also expresses the significance of flares by their duration. It is calculated by the following formula:

$$Q = (i \times t) \tag{1}$$

where i = flare intensity, t = the time length of its existence.

2.2. Solar activity featured by 2800 MHz radio flux

Solar flux from the entire solar disk at a frequency of 2800 MHz has been recorded routinely by radio telescope near Ottawa since February 1947.

2.3. Solar activity featured by ionospheric storms and atmospheric radio noises

The ionospheric disturbances caused by corpuscular radiation appear during the solar flares when the Sun emits a large amount of electrically charged and uncharged particles that enter the atmosphere of the Earth and change the conditions of the ionospheric layers. Among them, the most important is F_2 layer at night.

2.4. Interplanetary magnetic field sector boundaries

Besides studies of the longer cycles, emphasis has more recently shifted to research on the short-term atmospheric phenomena that also result from changes in the solar activity. These include the passing of the Earth through interplanetary magnetic field boundaries roughly once in every 8 days [18].

2.5. UV-B radiation of Sun

The UV-B range is especially detrimental in large quantities to living organisms. Our studies could not be related with the studies of other authors, dealing with the effect of the Sun's ultraviolet radiation and light and pheromone trapping of insects. Therefore we studied light-trap catch of insect species and pheromone trap catch of moth (Lepidoptera) species on the nights following days with a different solar activity. Low sunspot activity leads to a thinner ozone layer and thus higher surface ultraviolet (UV)-B radiation [19].

The light-trap success of European Corn-borer (*Ostrinia nubilalis* Hbn.) was examined by Puskás et al. [20] at those nights when during the previous day the UV-B radiation had a different intensity.

2.6. Geomagnetic indices

Becker [21] has found that certain species of Isotermes, Coleoptera, Diptera, Orthoptera and Hymenoptera are guided in their orientation by the natural magnetic field. Mletzko [22] carried out his experiments with specimens of ground beetles in the Moscow botanical garden. The insects flew in a given direction with an accuracy of +5° at daylight and +60° at night. The author assumes that orientation is guided by geomagnetism. Iso-Ivari and Koponen [23] studied the impact of geomagnetism on light trapping in the northernmost part of Finland. A weak but significant correlation was found between the geomagnetic parameters and the number of specimens of the various orders of insects caught. Studying the few Spotted Ermel (*Yponomeuta rorrella* Hbn.), Pristavko and Karasov [24] revealed a correlation between the C and ΣK values and the number of individuals caught. In a later study [25], they also established that at the time of magnetic storms ΣK has a greater influence on the flying activity of the above species. Tshernyshev [26] found a high positive correlation between the horizontal component and the number of trapped insects. Later, however, he reported that while light-trap catches of some Coleoptera and Lepidoptera species increased, that of other Lepidoptera and Diptera species fell back during magnetic perturbations [27].

Examinations over the past few decades have also confirmed that in the case of some Lepidoptera species, such as Large Yellow Underwing *(Noctua pronuba* L.) [15], both the Moon and geomagnetism guide their orientation and they can even integrate these two different types of information [28]. We have investigated the light trapping of Turnip Moth (*Agrotis segetum* Den. et Schiff.) Heart and Dart (*Agrotis exclamationis* L.) and Fall Webworm (*Hyphantria cunea* Drury) in relation with the *H*-index of the geomagnetic field strength using hourly data from the Kecskemét fractionating light trap [29].

2.7. The moon phases and the polarized moonlight

We summarize the known facts from the literature about the relationship between the Moon and light-trap catch, without our own results.

Williams [3] has published fundamental studies in this field. According to Williams [3] and El-Ziady [4], the smaller catch can be explained by the following reasons.

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- The activity of the insects may be reduced by the light of the Moon; therefore, the active proportion of the population affected by the light trap can be smaller.
- It is possible that insects like to fly rather at shady places, than at clear areas, and probably in higher altitudes at a Full Moon.

No scientist could give a provable answer to this question in recent decades, most have not even tried. Some authors find an explanation by accepting the theory of the impact of a collecting distance, others refer to decreased activity.

2.8. Moonlight decreases the distance of collecting

Luminous intensity of the artificial light source (candela) is theoretically constant. Theoretical collecting distance has been calculated by several authors, for different light trap types and lunar phases [5, 7, 9]. The authors cited above did not as yet have considered light pollution. The actual collection distance may differ significantly from the theoretical one, because much abiotic and biotic factors influence it. These are summarized in Nowinszky's [8] work.

2.9. Moonlight inhibits flight activity

Bowden and Morris [7], discovered that the catch of most taxa changes in a 2:1 or 3:1 ratio between New Moon and Full Moon. However, for some taxa the trap catches more at a Full Moon. Thus, this study confirms both hypotheses, also the one asserting that insects are more active at a Full Moon, because the catch [30] is higher than what could be expected due to the decreased efficiency of the trap. From their studies [31–33], it is hypothesised that moonlight cannot have an influence on the collecting distance.

2.10. Height of flight

El-Ziady [34] believes in the likelihood of insects flying higher at the time of a Full Moon. Danthanarayana [30] came up with a theory that the three-peak lunar periodicity of the flight of insects might be related to migration. In these periods, insects fly in the higher layers of the atmosphere, reaching heights where they are further transferred by streams of air in a horizontal motion.

In a Macrolepidoptera material caught at heights of 2 and 10 m, respectively, by light traps working with 125 W mercury lamps as the light source in a forest environment the authors determined the number of species and individuals in connection with migration and moon phases [35].

3. The weather

3.1. Macrosynoptic weather situations

We can mention our own studies only in this topic.

We examined the effectiveness of the light trap catch in connection with Péczely- and Hess-Brezowsky macrosynoptic weather types in our previous studies [36].

3.2. Weather fronts and air masses

We examined from these factors the influences of the weather fronts and air masses.

3.3. Weather events

The light-trap collecting results—showing its flight activity—of Turnip Moth (*Agrotis segetum* Den. et Schiff.) were examined in connection with the instability line, the convergence zone, the cyclogenesis, the country-wide rain, the cold and warm weather fronts, the maritime- and continental moderate, arctic and subtropical air masses [37].

3.4. Weather elements

In Szombathely (47°14′01″N; 16°37′22″E), within the premises of the Kámon Botanic Garden, the Forestry Research Institute kept a Jermy-type light-trap in operation between 1962 and 1970, which has about 2 km in a straight line the local weather observatory, which operated in airport. As the insects are poikilotherm creatures, therefore it is understandable; their body temperature is always the same as the temperature of the environment.

4. Material

The data of environmental factors were downloaded from yearbooks other publications and NASA's website.

The collecting data of investigated Lepidoptera, Coleoptera and Heteroptera species were copied off the light-trap diaries. The Trichoptera individuals were collected by Ottó Kiss and we processed them in our previous joint studies.

4.1. Solar activity featured by *Q*-index

Data used in this study were calculated by T. Ataç and A. Özgüç from Bogazici University Kandilli Observatory, Istanbul, Turkey.

4.2. Solar activity featured by 2800 MHz radio flux

Data used in this study were from the Quarterly Bulletin of Solar Activity (Zürich-Tokyo) and the Journal of Geophysical Research.

4.3. Solar activity featured by ionospheric storms and atmospheric radio noises

The data we needed for our calculations (border frequency of the F_2 layer of the ionosphere (f_0F_2) and the atmospheric radio noise at 27 kHz (SEA)) were provided by publications released by the Panská Ves Observatory of the Geophysics Research Institute of the Czechoslovak Academy of Sciences.

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4.4. Interplanetary magnetic field sector boundaries

Data for the transition of interplanetary magnetic field sector boundaries have been taken from the studies of Wilcox [18].

4.5. UV-B radiation of Sun

UV-B data used for the study come from measurements in the Keszthely observatory of the Hungarian Meteorological Service [38]. Daily totals given in MED/day are calculated by totalling hourly values.

4.6. Geomagnetic indices

For our present work, we downloaded the earth's magnetic x and y data from the World Data Centre for geomagnetism, Kyoto's website (http://wdc.kugi.kyoto-u.ac.jp/hyplt/). These values were calculated on the horizontal component of the formula, according to the advice of Mr. László Szabados Tihany Geophysical Observatory:

$$H = \sqrt{x^2 + y^2} \tag{2}$$

We used the values of H-index over 2150 nT.

Catch effectiveness was examined in connection with the H-index and Quarters of the Moon.

4.7. The moon phases and the polarized moonlight

Data on the illumination of the environment were calculated with our own software. This software for TI 59 computer had been produced by the late astronomer György Tóth specifically during our joint study [39]. The software was transcribed for modern computers by assistant professor Miklós Kiss. The illumination of the sky with stars, the moonlight and the Sun at dusk—all in lux—on any day and time, summarized or separately, for any given geographical location. Cloudiness is also calculated, anddata were provided by the Annals of the Hungarian Meteorological Service Data are recorded on every third hour in okta. We used the value obtained in a given hour for the following 2 h.

4.8. The weather

4.8.1. Macrosynoptic weather situations

The Péczely-type macrosynoptic weather situations was worked out by Péczely [40] who identified and characterized 13 types of daily macrosynoptic weather situations for the Carpathian Basin taking into account the surface baric field. Since 1983, typifying has been continued and Károssy [41] has published the daily code numbers.

The catalogue of Hess-Brezowsky [42] based on baric circumstances of Central Europe, distinguishes four zonal, 18 meridional and seven mixed types of weather situations, maintaining one type for unclassified baric areas. The codes which were necessary for these investigations are taken from publication of Hess and Brezowsky [42].

4.8.2. Weather fronts and air masses

We got the meteorological data measuring hourly in Budapest by National Meteorological Service. We categorized the weather fronts, discontinuity surfaces, the surface and upper air masses after Berkes [43]. We determined the upper air masses according to the measuring of radiosondes giving information about the cross-section in time. We used for our examinations the data of the Heart and Dart (*Agrotis exclamationis* L.) adults getting from the light-trap network in Hungary. The different air masses were classified into 22 classes, the weather fronts in turn into 20 classes [44].

4.8.3. Weather events

We used the meteorological data that was published in 'Calendar of weather phenomena' between 1967 and 1990 by Hungarian Meteorological Service for the examination of weather events.

4.8.4. Weather elements

The measurements of the weather elements made every 3 hours were collected from the 'Yearbook of the Central Meteorological Institution of the National Meteorological Service'. We used the whole Macrolepidoptera data for the investigation of the number of species and individuals in connection with daily temperature range [45]. The caught individuals and species were investigated separately according to each aspect: spring, early- and late- summer and autumn [46]. Our study [47] deals with the effect of weather conditions on the light-trap catch of two Caddisfly (Trichoptera) species.

The values of atmospheric electricity given in V/m are measured at the Sopron-Nagycenk Observatory of the Geodetic and Geophysical Research Institute of the Hungarian Academy of Sciences and are published in the yearbooks of the Institute.

4.9. The air pollutants

We analysed the ozone data registered at K-puszta between 1997 and 2006 (http://tarantula.nilu.no/projects/ccc/emepdata. hzml/) for the examinations of light-trap catch in connection with the ozone pollution.

We have downloaded the ozone content data ($\mu g/m^3$) from the website of Norsk institutt for luftforskning (Norwegian Institute for Air Research (NILU) (http://tarantula.nilu.no/projects/ccc/emepdata. hzml/).

For the recent study, the values of the chemical air pollutants: SO_2, NO, NO_2, NO_x, CO, PM10 and O_3 (in milligram per cubic meter) were measured in nearest automatic measurement station at Székesfehérvár (47°17'45"N and 18°19'59"E).

5. Methods

The number of individuals of a given species in different places and years is not the same. Therefore, we calculated relative catch (RC) values. This is for a given sampling time unit

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(one night) and the average number individuals per unit time of sampling, the number of generations divided by the influence of individuals. RC values were placed according to the features of the given day, and then were summed up and averaged. We arranged the catch and environmental data pairs of in classes, and then averaged them. Regression equations were calculated for RC of examined species and environmental factors data pairs.

Data on the environmental factors were arranged into classes according to the Sturges' method [48]. The relative catch values were assigned into the classes of the environmental factors belonging to the given day and then they were summarized and averaged.

6. Results

6.1. The solar activity and its influence on Earth

6.1.1. Solar activity featured by Q-index

The paper of Nowinszky and Puskás [49] deals with connections between the solar flare activities and light-trap collection of Horse Chestnut Leaf Miner (*Cameraria ohridella* Deschka et Dimić 1986). It was confirmed from their data that the *Q*-index significantly modified the daily catches, thus expressing the different intensities and duration of the solar flares. It was noticed that some of the Caddisfly species (Trichoptera) collected by Nowinszky et al. [50] showed the increase of the high values of the *Q*-index, but in other species there was a decrease in the *Q*-index. In case, the value of the *Q*-index is high, there is an increase of the catch after a decrease, which can be observed in some cases. We found an increasing tendency in nine species, if the *Q*-index value was in an increasing period. A decrease could be seen in the case of 14 species and increases after decreasing in the case of seven species if *Q*-index was increasing.

6.1.2. Solar activity featured by 2800 MHz radio flux

Tóth and Nowinszky [51] found that a moderate increase of solar radio flux measured at 2800 MHz in the preceding day coincided with an increase, however, a slight decrease or marked increase of the radio flux with a decrease in the light-trap catches of the Turnip Moth (*Agrotis segetum* Den. Et Schiff.) on nights following the solar H-alpha flares of importance (class) 2 and 3, the yield of light-trap catches also decreased.

6.1.3. Solar activity featured by ionospheric storms and atmospheric radio noises

We found in one of our previous study [52] that at the time of negative ionospheric storms (ΔKf_0F_2) the light-trap catch of Winter Moth (*Operophthera brumata* Linnaeus) and Scarce Umber (*Agriopis aurantiaria* Hübner) decrease. However, during positive ionospheric storms the catch of these two species was low. The catch of Turnip Moth (*Agrotis segetum* Denis et Schiffermüller) increases in connection with the strengthening atmospheric radio noises (SEA).

6.2. Interplanetary magnetic field sector boundaries

Light-trap catches of all the six pestilent species decrease in the neighbourhood of the sector boundaries of the interplanetary magnetic field. The minimum catch of the four winter geometrid moth species (Winter Moth (*Operophthera brumata* L.), Mottled Umber (*Erannis defoliaria* Cl.), Scarce Umber (*Agriopis aurantiaria* Hbn.) and Feathered Moth (*Colotois pennaria* L.) occur on or following the day of the event [53]. It is remarkable, however, that in contrast to the results reported by Wilcox et al. [54] confirming a fallback of the vorticity area index 2 days prior to the event, in the case of winter moths collected by light-trap, there is a significant decrease in the number of individuals only on or 1 day after the event. This fact contradicts the findings of Wilcox et al. [54] who could not prove a modification of the cyclone activity in connection with the sector boundaries in the summer half-year.

6.2.1. UV-B radiation of Sun

In the majority of examined swarming, the solar UV-B radiation increases the catch initially; at higher values of UV-B radiation the catch is lower. Ten of all swarming was obtained in this result, regardless of the trapping method and location of the taxonomic classification of species. Three times we experienced continuous elevation in swarming, though a decrease in one casedecrease when the value of UV-B radiation was increasing. In our recent study (in press), we show the catch increases earlier and afterwards a decrease can be found in two Caddisfly (Trichoptera) species at higher UV-B radiation values. There was an increase at the catch of the third species, but there was decrease in case of the fourth one at higher values of UV-B radiation [55].

6.2.2. Geomagnetic indices

The results of our calculations have shown that in the period of the New Moon, when there is no measurable moonlight, the higher values of the horizontal component are accompanied by an increase in relative catch. In the First Quarter and the Last Quarter, growing values of the horizontal component (*H*-index) are accompanied by a decreasing catch [56].

At New Moon, when there is no measurable moonlight, decreasing relative catch can be found with higher values of vertical component. At the time of other moon phases, in surrounding of First Quarter, Full Moon and last Quarter when there is no moonlight, the relative catch increases linearly with the increasing values of horizontal (*H*) component. The geomagnetic field intensity can increase the insects' flight activity, but the light stimulus is most important factor in orientation, so trapping is more successful. During New Moon when insects cannot get any light from the Moon for their orientation for the whole night, it can be supposed the increasing geomagnetic field intensity plays a bigger part in the orientation in contradiction to light stimulus, which increases the safety of orientation [15, 31, 57].

6.2.3. The moon phases and the polarized moonlight

Based on our knowledge acquired from the research studies of other scientists and our own findings described above, we summarize the effect of the Moon and moonlight on light trap collection in the following way [8]:

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6.3. Lunar phases and the efficiency of light trapping

Lunar phases affect catch result on the different days of lunation considering all light trap types and all species under examination.

Deviations may vary between species; the behaviour of different species may be similar or different,

The catch of certain species may be different or similar when the volume of catch at two distant periods of time is compared.

The catch of the same species might be different in the same period of time and geographical locality, when different types of light traps are used. However, the collecting efficiency of some light traps is almost the same.

In the case of light trap types and all the species under examination a minimum catch is recorded in the presence of a Full Moon.

Maximum catches rarely occur exactly on a New Moon, rather in the First and/or the Last Quarter, or in the phase angle divisions between a New Moon and the Quarters. This might be explained by the joint effect of an already relatively large collecting distance and the high ratio of polarized moonlight characteristic for this period. Consequently, the effect of high polarization that intensifies activity is added to the effect of the collecting distance in increasing the catch.

The influence of the lunar phases in modifying the catch may be detected not only during moonlit hours, but also in those without moonlight. This seems to prove a statement by Danthanarayana [30] claiming that lunar influence is independent of the visibility of the Moon.

Thus, we have to distinguish lunar influence and the influence of moonlight.

6.4. Collecting distance and the efficiency of light trapping

We have to draw a line of distinction between the concept of theoretical and actual collecting distance. The actual collecting distance is, in most cases, much shorter than the theoretical one calculated on the basis of the level of illumination in the environment,

The constant change of the theoretical and actual collecting distance used to play an important, but not exclusive role in the efficiency of collecting. Due to light pollution, the difference between the theoretical and actual collecting distance has become basically balanced out. Consequently, the catch of certain species is practically equal at a Full Moon and at a New Moon.

The actual collecting distance—just like the theoretical one—varies by light trap types and taxa, but in the case of 100 W normal bulb traps it was approx. 90 m for many species.

If a catch minimum can be detected at a Full Moon also in the catch data of recent years, the reason for this should be found in other lunar influences.

We find the correction of catch results—applied earlier by more authors– acceptable, even in case of data dating back several decades, only if it happens based on an actual collecting distance. We find a similar correction of recent data perilous.

6.5. Illumination from the Moon and the activity of insects

Generally, illumination by the Moon does not hamper the flight activity of insects. Besides the points made by Dufay [5], the following facts prove this theory. It is a justified fact, that certain insects use polarized moonlight for their orientation. It is unthinkable that the activity of these insects would decrease when polarized moonlight is present in a high ratio. Our investigations have also proved the catch to be higher in case of higher polarization.

In moonlit hours, we observed a higher catch on more occasions than in hours without moonlight. Based on data on the rising and setting of the Moon in the period close to the Last Quarter, Reddy et al. [58] determined whether each flight occurred only if the Moon was above the horizon before midnight, the period when this species is active.

The relatively strong illumination by the Moon cannot be the reason for a minimum catch recorded at a Full Moon. Most insects start to fly in some kind of twilight. And illumination at twilight is stronger by orders of magnitude than illumination by moonlight.

Suction trap studies by Danthanarayana [30] have not justified the decrease observable with light traps at a Full Moon.

Observation claiming that insects spend less time in flight during a Full Moon should be compared with similar observations for a New Moon. High standard scientific investigation is needed to study both periods.

Not even on the basis of the relative brightness of the Moon do we find a correction of the catch data acceptable, as this method does not consider the role of polarized moonlight and it is not effective throughout the whole lunar month.

6.6. The certainty of the orientation of insects

Moderate catch results recorded at a Full Moon may be explained by the better orientation of insects. This hypothesis attributes low catch results to negative polarization typical for the period immediately before and after a Full Moon, possibly enabling insects to distinguish the light of the lamp from moonlight and thus avoid the trap. Our findings force us to reconsider this hypothesis, as we could not detect any difference between the catch during positive and negative polarization. Still, Jermy's [2] assumption might be true. The experiments by Dacke et al. [59] allow us to presume that the high ratio of polarized moonlight provides more information for insect orientation, than the smaller ratio of positive or negative polarized moonlight in the vicinity of a Full Moon. This might be the reason for high catches recorded in the First and the Last Quarter, and the low ones at a Full Moon. It is derived from the observation that insects use sources of information other than moonlight for their orientation in the vicinity of a Full Moon. Such sources may be the polarization pattern of the sky, lines of geomagnetic force or certain objects in the field. However, in this case orientation relies on light stimuli to a much smaller extent, thus the certainty of orientation might increase. For the nocturnal species, the sensitivity of the optical polarization compass can be greatly increased without any loss of precision [60].

In the last few years, we proved that the polarized moonlight plays a deciding role in the effect of the Moon [16, 61–65].

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Comparing the catch results of the different migrant types with those of full lunation (lunar month), the following can be established:

The higher trap catches a smaller number of specimens of the non-migrant species in the First Quarter and at a Full Moon, but there is no observable difference between the different quarters in the catch of the lower trap,

In the case of migrant species, significant differences can be observed in the catch of the lower trap. Collecting is least successful at a New Moon and in the Last Quarter, when the catch is minimum even in the higher trap.

Vertical migrants can be caught with little success in the higher trap in the First Quarter and at a Full Moon, while in the catch of the lower trap no difference can be detected.

There is no significant difference in the catch results of the proposed migrant species, either in the higher or in the lower trap. The development of the number of species and the number of specimen caught of the different migrant types and lunar phases is practically the same [35].

The catching peak of ten harmful Microlepidoptera species is in First Quarter, another ten species have the peak in the First Quarter and Last one, and only in two cases, the catching peak is in Last Quarter [57, 58]. This fact in these Moon Quarters attributes to the high-polarized moonlight. This confirms the results of previous studies given in references [9, 30, 62, 66, 67], which have already established that the polarized moonlight helps the orientation of insects.

6.7. The Weather

6.7.1. Macrosynoptic weather situations

The flying activity of Turnip Moth (*Agrotis segetum* Den. et Schiff.) during the change of macrosynoptic situations classified due to Péczely is investigated by the numbers of captures of this kind of moths by light traps. It can be shown that the flying activity is high during periods of fundamental changes in the weather situation and the activity resumes low if there is no change in the atmospheric circulation regime. At times of changes and/or existences of these types, the light-trap catches of two insect pests have been investigated [68], Fall Webworm *(Hyphantria cunea* Drury) and the Gipsy Moth *(Lymantria dispar* L.). We publish in this paper the favourable and unfavourable meteorological situations to trap the two given species.

The authors have established that from the various 29 types of Hess-Brezowsky's macrosynoptic weather situations, if they are continuous, which one are favourable or unfavourable from the point of view of collecting the moths, moreover how the species investigated react to the change of the weather situations [36].

6.7.2. Weather fronts and air masses

A few number of individuals were caught by the light-trap if the cold air mass was near the surface. The collecting is successful if there is warm air mass above the surface.

We found the effectiveness of subtropical air masses in increasing flight activity and of course, light-trap catching. We found high catch in that cases, when the arriving cold front brings temperate maritime air in place of Saharan air coming from the Mediterranean Sea which has the strong activity of spherics (electromagnetic radiation) [69].

6.7.3. Weather events

The instability line decreases alone the number of caught specimen only at that case, when it repeats during some days. If other meteorological events are involved, the influence is disadvantageous or inefficient for the catching result. The next day the amount of the collection increases only if a subtropical air mass also arrives. The convergence zone is inefficient on its own, but in case of cyclogenesis, the number of collected moth decreases compared with the results of the day before. There is a disadvantageous influence if a moderate maritime air mass is involved from the previous day to the next. The collecting results are small in number on the previous day if cyclogenesis is the only influencing factor. On the day of arrival, it is also low when it is combined with any other meteorological events. In case of country-wide rain, the catching is low even on the next day. It is noticeable that country-wide rain on its own is favourable before and after the event for the success of the catching, but if it comes with any other meteorological events, it is unfavourable for the catching. For a cold weather front arriving on its own on, the previous day of its arrival is advantageous for collecting, but it is unfavourable on the day of arrival and the following one. It is also disadvantageous if it is combined with a moderate air mass, and the collecting results are higher in number in case of an arctic air mass, but they decrease on the next day. A warm weather front arriving combined with a subtropical air mass is favourable for the catching on the day of arrival and the previous one, but it is unfavourable if the warm front combines with moderate maritime air mass. The number of moths caught is low on the day of arrival and the following one if there is a moderate maritime air mass and it is independent from whether it is combined with any other meteorological events or not. The number of the catching is not very high—except if it is combined with other meteorological events—on the previous day of the arrival of a moderate continental air mass, but it is high on the following days. If the instability line on the previous day is followed by a moderate continental air mass with a cold front on the day of arrival, the catching of the previous night is high in number, but it is low on the following one. If the instability line on the previous day is followed by a moderate maritime air mass with a cold front on the day of arrival, a low number of the collection can be detected on that day, but it is increasing on the following one. Subtropical maritime air masses—arriving on their own, with the instability line and a cold front—are disadvantageous, but they are favourable on the previous and following days. If these sorts of air masses combine with a convergence zone and cyclogenesis, the number of the collection is less on the previous night.

Subtropical maritime air masses—arriving with a warm weather front—are advantageous for the success of collecting on the previous day and also on the day of arrival. The number of moths caught showed a decrease on the day of the arrival of a subtropical continental air mass and the trend was the same on the next day. The number of moths collected is lower on the day of the arriving of subtropical continental air masses and the following days. The catching

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is high in number on the previous day and the day of the arriving of an arctic air mass combined with a cold weather front, but there is a decrease on the following day [70].

6.8. Weather elements

Temperature may have an important part from the point of view of insects' flying activity. The given temperature requirements of insects can be explained by the fact that their body mass is very small compared to both its surface and the environment. That is why the temperature of their body, instead of being permanent and self-sufficient, follows the changing temperature of the environment. This is because the ratios of the body mass and surface of insects determine the difference between the inner heat content and the incoming or outgoing heat. The heat content of the body is proportionate to its mass, while, on the other hand, the heat energy intake or loss is proportionate to the size of the surface of the body. Therefore, an external effect makes its influence felt as against the inner, small heat content of a relatively small mass. The speed as well as the size of the impact brings on the ratio between the mass and surface of the body of the insect [71]. So the temperature value always exerts a substantial influence on the life processes of insects. The chemical processes described as metabolism that determine the life functions of insects always follow the temperature changes in the direct surroundings. Naturally, the activity of the organs of locomotion also depends on the temperature of the environment, which explains why we can expect a massive light-trap turnout by what is an optimal temperature for the given species [72]. Southwood [73] on the other hand, is of the view that the flight of insects has a minimum and maximum temperature threshold typical for each species. The insect flies if the temperature is above the minimum and below the maximum threshold and becomes inactive when the value is below the minimum or above the maximum threshold. According to him, there are other reasons for the fluctuations in the number of specimens experienced in the interval between the low and high threshold values. However, research in Hungary has proved that in the context of a single species, too, a significant regression can be established between the temperature values and the number of specimens collected by light-trap [47, 74, 75].

The high values of air temperature vapour pressure, saturation deficit and the height of cloud base increase the catch of *Rivula sericealis* Scopoli, and on the contrary, the wind velocity, relative humidity and amount of cloud decrease it.

The decreasing clouds, and thunder and lightning preceding thunderstorms also increase the flight activity. Modifying effect of precipitation has become more accurate as well. The effect of rain in hindering the catch is well known, but the fact that the hindering effect remains after the rain has stopped is a new finding.

Our results demonstrate that low temperature minima depress both the number of species and individuals in all aspects. In contrast, higher than the minimum value can rise in number of caught species and individuals. The daily temperature ranges—the 24-hour period noted between the highest and lowest temperature difference—in the temperate zone are more important than in the tropics, as activity of insects is strongly dependent on the daily temperature range in the temperates than in the tropics [76].

We found that the light trap catch of both Caddisfly (Trichoptera) species increased when the daily maximum temperature, minimum and average values of temperature were higher. The results can be written down with second- or third-degree polynomials. The fluctuation in temperature had no clear influence on the catch. The hydrothermal quotient has a strong influence on the catch of both species. Precipitation has no significant influence on the catch of the tested species [47].

The study of Nowinszky and Puskás [77] examines the efficiency of light-trap catching of Turnip Moth (*Agrotis segetum* Den. et. Schiff.) and various values of atmospheric electricity. The number of specimens trapped is the largest near the region close to 0 V/m. The rising positive values have a slight effect on the catch, while negative values of the atmospheric electricity are extremely disadvantageous for trapping.

6.9. The air pollutants

We established that the light trapping of European Cockchafer (*Melolontha melolontha* L.) is most effective if the ozone concentration is high. As opposed to this, low ozone concentration reduces the success of the catch [78]. We established that the light trapping of this Scarce Bordered Straw (*Helicoverpa armigera* Hbn.) is most fruitful when the ozone content of the air exceeds the 80 $\mu g/m^3$ value. As opposed to this, the low ozone values reduce the success of the catching to a moderate level. Our results suggest that the flying activity of the European Cornborer *(Ostrinia nubilalis* Hbn.) increase when the ozone content is high. The light-trap catches verify this fact [79]. In a recent study, the light-trap catch of three beetle species (Coleoptera) in connection with the everyday function of the chemical air pollutants (SO_2, NO, NO_2, NO_x, CO, PM10, O_3) has been examined.

We found that the behaviour of the studied beetle species can be divided only into two types: as the air pollution increases the catch either increase or decrease [80].

7. Discussion

Based on our studies, the examined species are of three types: ascending, descending, ascending then descending. The increase or decrease in the catch can be explained by our previous hypotheses. There is always a correlation between low relative catch values and environmental factors in which the flight of insects is reduced. However, high values cannot be interpreted easily. Major environmental changes lead to physiological transformations of insect organisms. The imago is short-lived; therefore adverse conditions endanger the survival of the given specimen and the species as a whole. According to our hypothesis, the individual may adopt two different strategies to evade the impacts hindering its normal functioning. It may either display more activity by increasing flying intensity, copulation and egg-laying activities or take sanctuary against environmental factors of an unfavourable situation. In accordance with what we have found, we might say that both high and low catch can occur in case of unfavourable environmental factors [16]. It can be explained on the basis of our hypothesis of the first rising and then falling catch results. However, the answer is in the passivity for the additional increase of the radiation.

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Chapter 6

Reinvestigation of *Cactoblastis cactorum* (Lepidoptera: Pyralidae) Sex Pheromone for Improved Attractiveness and Greater Specificity

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Additional information is available at the end of the chapter

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Abstract

Cactoblastis cactorum (Berg.) is recognized as an invasive species in the Caribbean, the United States, and Mexico. Prior work using hexane extracts of sex glands showed that the sex pheromone of this species has 54% of (Z, E) -9.12 -14: acetate, 42% of (Z, E) -9.12 -14:OH and 4% of Z9-14: Ac. Although traps baited with this mixture are effectively to attract males of the cactus moth, it is necessary to determine whether the pheromone can be optimized and to determinate if female diet may impact pheromone composition. Experiments with insects were made at the USDA-ARS Crop in Tifton, Georgia, where there is a colony maintained on cactus and another on an artificial diet. Solid-phase microextraction (SPME) was used to collect pheromones in the headspace above a single calling female and by rubbing the excised female sex gland with SPME fibers. Rubbing the gland directly with SPME fiber revealed that the pheromone consists of the compounds cited above plus Z9-14:Ac. With dynamic aeration and capture of volatiles with fiber only captured two compounds. In addition, our results indicated that natural or artificial diet does not influence the composition of the sex pheromone.

Keywords: cactus moth, sexual pheromone gland, cactus, Mexico, rubbing gland

1. Introduction

The South American cactus moth, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae), was used success fully for biological control for several invasive *Opuntia* species around the world

[1–3]. However, it is also recognized as an invasive species in the Caribbean, the south eastern USA, and Mexico. This followed its release on some Caribbean islands beginning in 1957 [4], its subsequent spread to most other islands in the Caribbean, its attack of most of the 20 *Opuntia* species native to the region [5], and its eventual detection in the Florida Keys in 1989 [6, 7]. *C. cactorum* now threatens more than 80 species of the economic and ecological native and cultivated *Opuntia* species in the United States and Mexico [8, 9, 3, 10]. As *C. cactorum* spread throughout most of the Florida peninsula and along the Atlantic coast to South Carolina and the Gulf coast of Louisiana [11], Mexico developed an awareness/preventative campaign which included monitoring and sampling in commercial and wild areas [12].

Lures used in this work were formulated with the putative sex pheromone components elucidated by following solvent extraction of excised sex glands [13]. Pheromone components were a three-component blend of 54% (Z, E) -9.12 tetradecadien-1-ol acetate (Z9, E12-14: Ac), 42% (Z, E) -9.12 tetradecadien-1-ol (Z9, E12-14: OH) and 4% Z9-tetradecen-1-ol acetate (Z9-14: Ac). This blend when formulated on rubber septa was found to effectively attract male moths; however, changes in the ratio of these components had little effect on lure efficacy. Also, Heath et al. [13] reported that in wind tunnel bioassays the percentage of male moths landing on the odor source was higher when live females were used as a lure than for any of the synthetic pheromone blends. They surmised that, even though the lure was effective in monitoring populations of *C. cactorum* males, identification of additional components was needed to elucidate all the pheromonal components used by *C. cactorum* females.

Although there are some concerns about the efficiency and selectivity of this sex pheromone lure, it was used successfully to detect and monitor populations of the cactus moth in the United States and Mexico. This lure was especially helpful in detecting and monitoring *C. cactorum* populations during the outbreaks of *C. cactorum* in wild cactus (*Opuntia strictadelini*) in Isla Mujeres on August 10, 2006, and in Isla Contoy on May 4, 2007, both located in Quintana Roo, Mexico. With actions implemented immediately by the Mexican phytosanitary authorities, populations were eliminated through the use of trap monitoring, host plant removal, sanitation, and the sterile insect technique [14]. Eradication was declared for *C. cactorum* in Isla Mujeres [15] and Isla Contoy [16] in 2009 in accordance with the model proposed by Tassan et al. [17]. Mexico continues to monitor throughout the country for early detection of new incursions.

While the usefulness of the commercial pheromone has been demonstrated, several factors suggest that there may be missing components or that the blend of components could be optimized. As noted, Heath et al. [13] found that changes in the ratio of the three components in the pheromone blend had little effect on lure efficacy. Also, they found that in wind tunnel bioassays the percentage of male moths landing on the odor source was higher for live females than for any of the synthetic pheromone blends. Another concern is that, unlike traps baited with virgin female moths, traps baited with the synthetic pheromone lure often capture large numbers of "non-target" moth species. Not only does the capture of non-target species suggest a lack of specificity for the pheromone lure, it creates the need for additional labor to examine survey traps for the presence of *C. cactorum* males when the traps contain numerous other moth species.

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In this study, we re-examined the composition and proportion of sex pheromone components of *C. cactorum* by analysis of pheromone gland volatiles using solid phase microextraction technique (SPME). This was done by capturing volatiles within the headspace surrounding calling females and by direct contact of SPME fibers with excised moth sex glands. SPME has been used in this manner to elucidate moth pheromone chemistry in numerous studies [18, 19]. We also used SPME to compare the sex pheromone produced by female moths reared on a meridic diet devoid of Opuntia plant components to the sex pheromone produced by female moths reared on Opuntia cladodes. Overall objectives of this study were to improve the efficacy of the sex pheromone lure, to reduce the number of nontarget moths collected in traps baited with the synthetic sex pheromone, and to compare the quality of sex pheromone produced by female moths reared on host plants and meridic diet.

2. Materials and methods

2.1. Insects and rearing

All *C. cactorum* used in this study originated from the laboratory colony at the USDA-ARS Crop Protection and Management Research Unit Laboratory, Tifton, Georgia. This colony was established from multiple collections of *C. cactorum* larvae from infested *Opuntia* spp. along the Florida Gulf Coast during 2002 and 2004, and from nearly 10,000 eggs collected from *Opuntia* spp. plantations near Craddock, South Africa, and shipped to Tifton, Georgia in 2002. Insects were reared continuously either on a meridic diet devoid of Opuntia plant material [20] or on *Opuntia ficus-indica* cladodes using the protocols described by Marti and Carpenter [21]. Diet-reared and Opuntia-reared pupae were separated by gender and placed in separate containers under the same condition until emergence.

2.2. Collection of volatiles with SPME

To sample headspace volatiles we used 35 mm polyethylene film containers with an orifice in the center of the lid of 2 mm in diameter. In this hole was inserted a rubber septum 10 mm outside diameter (Sigma-Aldrich Z553921) with the large opening to the outside. Within this container was placed a female cactus moth 2–4 days of age 1 h before the calling period. At the calling period, the metal sheath of the SPME assembly was inserted through the rubber septum once inserted and the fiber (Supelco 57300-U) was extended and held in the exposed position by 10 min. The fiber was then withdrawn into its sheath and the assembly placed in 15-mL glass centrifuge tube sealed with a Teflon-lined screw cap. The fiber was then transported to the analytical laboratory for gas chromatography-mass spectrometry (GC-MS). There were a total of five repetitions. Fibers were preconditioned by holding in the 250°C injection port of a gas chromatograph for 1 h prior to each use.

2.3. Collection of volatiles by SPME fiber contact with pheromone glands

Sex pheromone glands were obtained from 2- to 4-day-old females after 8–9 h PM after lights were turned off. Females were most frequently observed to take a calling posture at this time.

The tip containing the pheromone gland was excised when it was exposed with fine tweezers and rubbed repeatedly with the exposed SPME fiber SPME for 15 s. The fiber was withdrawn into its metal sheath and stored in a sealed centrifuge tube prior to GC-MS analysis: Frerot et al. [22] were the first to report the use of this approach to lepidopteran pheromone analysis.

2.4. GM/MS

Each SPME fiber was thermally desorbed in the inlet of a Thermo Finnigan DSQII (San Jose, CA, USA) gas chromatograph-quadrupole mass spectrometer. The inlet temperature was 220°C with desorption for 1 min during split less injection. The instrument's oven was fitted with a 30 × 0.25 mm (i.d.) J+W DB-WAX-fused silica capillary column (Agilent, Santa Clara, CA, USA). The column liquid film thickness was 0.25 μm. Helium carrier gas flow was maintained at 2.0 mL min^{-1}. Following injection, the initial oven temperature, 60°C, was held for 1 min. The temperature was then increased to 240°C at 5°C min^{-1} and held for 4 min. Ionization use, the mass spectrometer was tuned to meet manufacturer performance criteria for perfluorotributylamine. Authentic standards of a mixture of (Z9,E12)-tetradecadien-1-ol acetate, (Z9,E12)-tetradecadien-1-ol, (Z9)-tetradecen-1-ol, and (Z9)-tetradecen-1-yl acetate obtained from Bedoukian (Danbury, CT, USA) were dissolved in methylene chloride analyzed by split less injection into the GC/MS to confirm structural assignments.

2.5. Chemicals

(Z9, E12)-tetradecadien-1-ol acetate, (Z9,E12)-tetradecadien-1-ol and Z9-tetradecenyl acetate were obtained from Bedoukian Co. The purity of compounds was as follows: Z, E-9, 12-14: Ac 93% (Cat Bedoukian P6050-93), Z, E-9, 12–14: OH 93% (Cat Bedoukian 6051 93) and Z9-14: Ac 95% (Cat Bedoukian P6030-95).

2.6. Lure formulation

Rubber septa with a 10-mm outer diameter (Sigma-Aldrich Cat Z553921) were loaded with one milligram of different proportions of the components of the cactus moth sex pheromone. Each septum was held for 24 h in a fume hood to allow evaporation of the solvent. Septa containing the commercial pheromone (Suterra, Inc.) or empty septa were used as controls.

2.7. Field tests

Field tests were conducted in Pampa Muyoj, in Argentina from 1 to 25 March 2011 within a 100-ha cactus plantation. Baited Pherocon 1-C Wing traps (Trécé) were used in all field tests. Tests were conducted during peak flight periods. Treatments were traps baited with various release rates and/or ratios of the putative pheromone components found in this study and tested in comparison with traps baited with commercial pheromone and two virgin females (**Table 1**). Females were 1–2 days old when placed in the traps and were replaced after each sample period. Synthetic lures were replaced after 2 weeks. A number of males captured were determined every 3–4 days. The ratios of a components blend were tested at doses of 1 mg per septa. Trap location within a replicate was randomly selected and randomized each day. Traps were wired onto cactus pads 0.5–1.0 m above ground along cactus rows. Each replicate

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set was separated by at least 20 m, and each trap within a replicate was 3–5 m apart. The number of males captured in each trap was determined daily. All treatments were replicated five times. Control traps were baited with septa treated with the same amount of hexane and in some experiments, we used virgin females 2–4 days old.

2.8. Data analysis

All counts were converted to a number of insects per trap, and data were analyzed using analysis of variance (ANOVA), with the minimum variance unbiased quadratic estimation PROC MIXED MIVQUE0 [23]. MIVQUE0 provides reliable estimates of parameters for data with a non-normal distribution, large numbers of zero values, and unequal variances. Weekly capture data from each trap are used as replicate data for individual traps in the statistical analyses. Results on the graphs are presented as means (±SEM) across all trapping periods.

Treatment	Virgin females	Commercial pheromone	Z,E-9,12-14: Ac	Z,E-9,12-14: OH	Z9-14: Ac	Z9-14: OH	Z9-16: Ac	Tetradecanoic acid
1	*							
2		*	52	44	4			
3			52	44	4			
4			38	44	4	4		4
5			41	56	4			
6			38	44	4	4	4	4
7								
8			60	40				
9			40	60				

Table 1. Combinations of the sex pheromone components of the cactus moth, evaluated in the field at Pampa Muyoj, Argentina, 2011.

3. Results and discussion

3.1. Pheromone identification

Each SPME analysis involved a use of a single calling female. Ion current chromatograms following SPME "headspace" sampling and direct contact with the moth sex gland are shown in **Figure 1**.

The ion used to construct these chromatograms, m/z = 67+, is the base peak of spectra of the mono and di-unsaturated alcohols and acetates that were detected [24]. The four compounds positively identified by comparison of retention times and full scan spectra to authentic

standards were (Z9, E12)-tetradecadien-1-ol acetate, (Z9, E12)-tetradecadien-1-ol, (Z9)-tetradecen-1-ol, and (Z9)-tetradecen-1-yl acetate. Chromatographic data showed that two sampling methods provided matching results. Means of the percent composition of pheromones identified by headspace and contact sampling were 45, 50, 3, and 2 and 47, 47, 3, and 3%, respectively (**Figure 2**). Significant differences were not indicated when peak to peak comparisons were made.

In addition, SPME results were in close agreement with results reported by Heath et al. [13]. Their data which were obtained following solvent extraction of sex glands closely match our findings using SPME sampling (**Figure 2**). The minor exception was that we detected (Z9)-tetradecen-1-ol. This compound was not reported by Heath et al. [13].

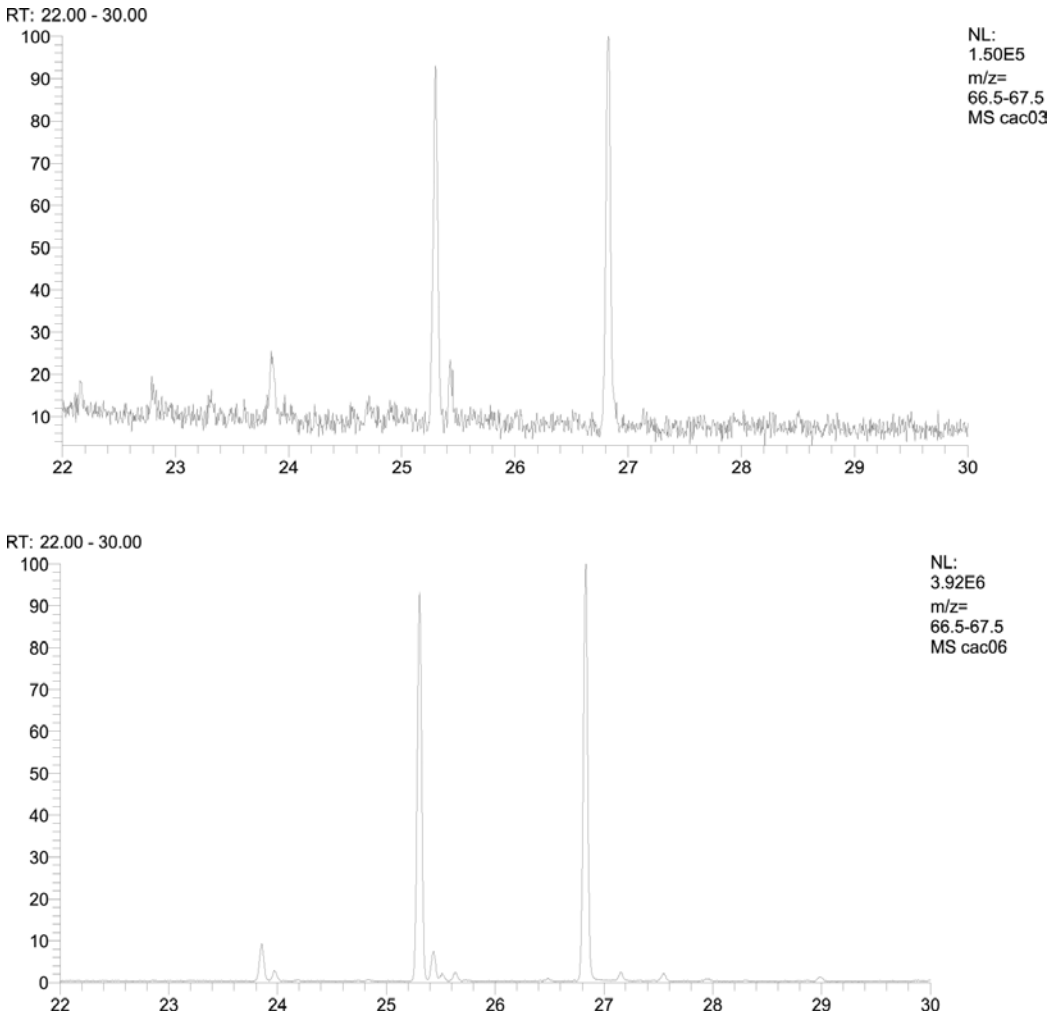

Figure 1. Ion current chromatograms (m/z = 67+) using SPME to sample the headspace above a single cactus moth calling female and by direct contact of the SPME fiber with a single moth sex gland.

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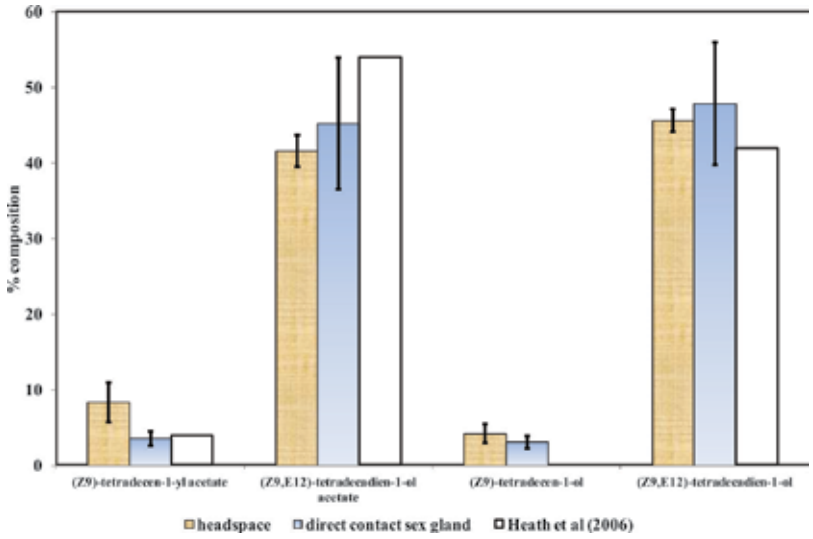

Figure 2. Cactus moth pheromone percent composition by SPME sampling of headspace above single calling females ($n = 3$) and by direct contact of SPME fibers with a moth sex gland ($n = 3$) compared to results obtained flowing solvent extraction of sex glands reported by Heath et al. [13].

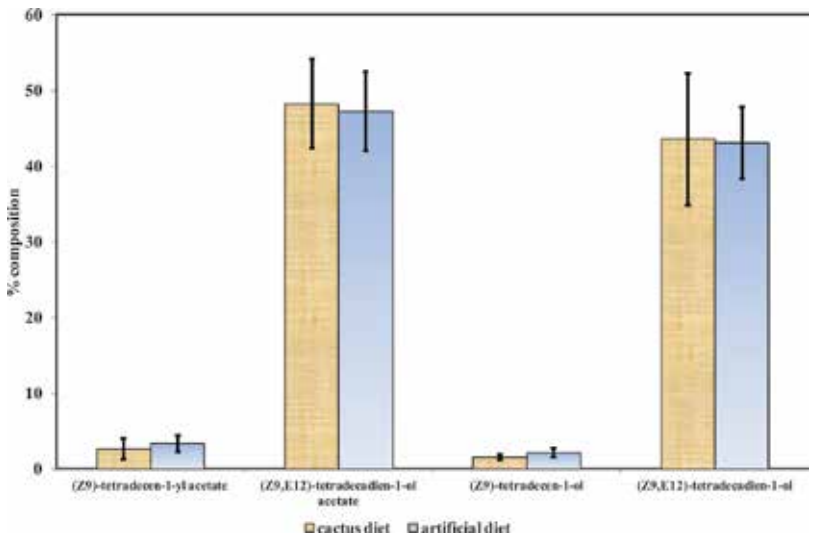

Figure 3. Percent composition of pheromone in cactus moths reared on artificial and cactus diets measured by SPME contact of sex glands of single calling females.

Finally, our analyses did not indicate that diet had an impact on pheromone composition. Only minor differences were detected when pheromones from moths raised on the artificial and cactus diets were analyzed and the percent composition was not significantly different (**Figure 3**). Dietary fatty acids commonly serve as precursors for pheromone biosynthesis in

Lepidoptera [25]. This suggests that the diets used in our study both provided these dietary precursors and as a result differences in pheromone composition were not found.

The results of catches of male cactus moth and other lepidopteran species with different blends and virgin females in Pampa Muyoj, Argentina, are shown in **Figures 4** and **5**. The number of males captured of the cactus moth by the various treatments was statistically different (F = 2.56, P value = 0.02). Treatment of only two compounds (Z9-E12-14: Ac-E12 and Z9-14: OH, T8) in the ratio of 60:40 captured a greater number of the male moths, followed by the reverse ratio of 40:60 (Z9-E12-14: Ac and Z9-E12-14: OH, T9). It is clear that using fewer bait compounds this will be cheaper long as you maintain the same efficiency that comparable lures. Virgin females (T1), the commercial pheromone (T2), commercial pheromone prepared in our laboratory (T3) and remaining mixtures caught similar numbers of males but different from the control (**Figure 4**).

Adding Z9-14: OH (T4) did not improve the efficiency of the bait. A similar effect was noted with the addition of Z9-16: Ac and tetradecanoic acid (T4 and T6), respectively. It is clear that the combination of di-unsaturated acetate and the corresponding alcohol play a key role in the communication system of this species, as in *Copitarsia decolora*, showing a similar effect with only two compounds [26, 27]. Treatment number nine had lower capture of males (although not statistically different from T8). One possible explanation is that the perception range of *C. cactorum* male is relatively broad as shown in *C. decolora* [27]. In this experiment, and in others, it became evident that the pheromone produced in our laboratory with proportions similar to the commercial pheromone (Suterra), captured a smaller number of males (although not statistically different with the commercial). Further chemical analysis showed that both septa are equal, the proportions are similar, but in the septum with the commercial pheromone, we found the anti-oxidant 2, 6-Di-tert-butyl-4methylphenol. This compound may prolong the lifetime of the compounds (date). A similar effect was observed in the mixture of 60:40, perhaps with the addition of the antioxidant would be more efficient in capturing males.

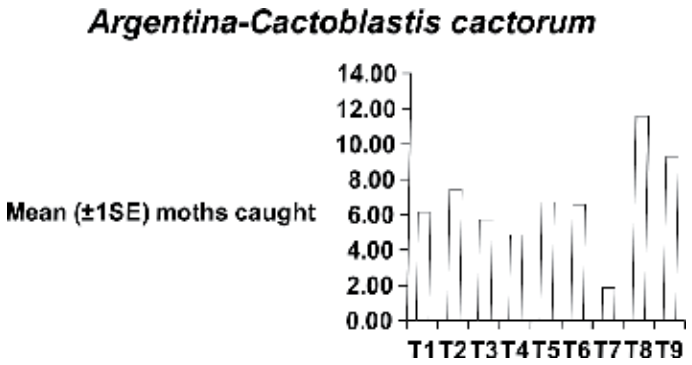

Figure 4. Average (mean ± SEM per trap) of catch per trap of male cactus moth, baited with different blends and virgin females, Muyoj Pampa, Argentina, 2011. Same letters above the bars indicate no statistical difference.

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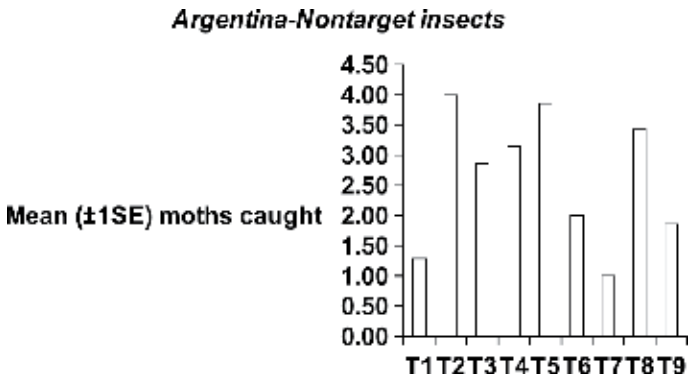

Figure 5. Average (mean ± SEM per trap) trap catches from non-target species, baited with different blends and virgin females, Pampa Muyoj, Argentina, 2011. Same letters above bar indicate no statistical difference.

An important aspect in the use of pheromones is to avoid catching nontarget species [28, 29]. Reducing the capture of nontarget insects increases the efficiency of the trap, reduces the potential impact on endemic species [30], and facilitates inspection of the trap [31]. In the present study, the evaluated mixtures showed no statistical difference in the capture of non-target insects (F value = 1.45, Pr > f = 0.2030, **Figure 5**). Apparently, the evaluated mixtures did not influence decisively in some of the treatments on insects caught. Perhaps, a contributing factor in that no significant difference is due to the low number of non-insects captured by treatments. Probably the type, color of the trap, and the presence of more compounds (T2–T6) could influence the capture of these species. Most nontarget Lepidoptera captured was identified to family. These corresponded to the family Pyralidae and Noctuidae who share with the cactus moth at least one of the compounds tested and that could influence the capture of nontarget males captured [32].

Sex pheromone traps are an important tool for monitoring activity of cactus moth in the Mexican border, and interpretation of data derived from these traps is important for making pest management decisions. Understanding factors that may affect interpretation of data are important in efforts to design better baits and optimize efficiency of monitoring efforts. Bait designs with low capture efficiency pose the risk of underestimation of pest presence and, thus, the unexpected pest introduction. Conversely, designs that are overly attractive to insects can cause inefficiency of monitoring efforts due to saturation by nontarget insects (such as other lepidopterous species) [33]. Optimally, traps used for efficient pest monitoring should be attractive to pests while being unattractive to nontarget species. For cactus moth, the 60:40 mixture data indicate that this is preferable to other sexual pheromone component combinations.

4. Conclusions

The sex pheromone of cactus moth is composed of (Z, E) -9.12 tetradecadien-1-ol acetate (Z9, E12-14: Ac), (Z, E) -9.12 tetradecadien-1-ol (Z9, E12-14: OH) and Z9-tetradecen-1-ol acetate

(Z9-14: Ac). Rubbing the gland directly with SPME fiber was an appropriate technique for recovering sexual components of sex gland females. Our results indicate that natural or artificial diet does not influence the composition of the sex pheromone. Of the eight mixtures evaluated in the field, more moths were captured with binary mixtures of the di-unsaturated acetate and di-unsaturated alcohol in 60:40 proportions. Using two-component mixtures as bait will likely make traps less expensive while providing capture efficiencies that are equal to or greater than commercial traps that are currently available that use four components and therefore more expensive. Currently, the mixture of the main compounds in 60:40 proportions is used in 1400 wing traps that are changed and checked every month along the Gulf of Mexico to detect the entry of the cactus moth to Mexico. In the near future, the next step will be to study the volatiles of *Opuntia* species and determine if the female of the cactus moth uses them to find their host plant. Thus, we would have a pheromone for capturing males and an attractant for capturing females.

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Chapter 7

Insects Associated with Reforestation and Their Management in Poland

Iwona Skrzecz

Additional information is available at the end of the chapter

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Abstract

Weevils (Coleoptera: Curculionidae) are the most important pest insects of forest plantations established on clear-cut areas, and *Hylobius abietis* is a pest insect of great economic importance in Europe. *Pinus sylvestris* plantations and thickets established on sandy soils or postfire areas can be severely impacted by *Cneorhinus plagiatus* and *Brachyderes incanus*. Young pine forests weakened by biotic and abiotic factors are particularly susceptible to *Pissodes castaneus*. Buds and shoots of *P. sylvestris* trees are mainly damaged by Lepidoptera larvae. For many years, chemical treatments have been the main way of protecting forests against insects. At present, to reduce the pollution of forest environments with insecticides, the strategy of integrated pest management (IPM) was put into practice. It involves prophylactic measures to increase plant resistance to insect attacks and to select appropriate control methods based on a multistep decision support system (DSS). Nonchemical control measures aim at collecting pest insects in traps fitted with attractants and biological methods, mainly based on entomopathogenic nematodes (EPNs) and wood-decomposing fungi. Chemical insecticides are used only in cases of high threats to reforestation stands. This paper presents the state of knowledge concerning pest insect management in forest plantations in Europe, with particular emphasis on insects occurring in Poland.

Keywords: forest plantations, *Hylobius abietis*, *Pissodes castaneus*, *Brachyderes incanus*, protection, IPM

1. Introduction

In Poland, forests cover a total area of around 9.2 million hectares, taking up 29.4% of the land area [1]. Poland is therefore one of the countries with the largest forest areas in central Europe. The main forest type is coniferous forest, accounting for 70%, with Scots

pine (*Pinus sylvestris* L.) as the dominant species, especially in the center and the northern parts, where it takes up to 58.5% of the forest area. Norway spruce (*Picea abies* (L.) H. Karst) and European beech (*Fagus sylvatica* L.) prevail in the South, mainly in the mountains. Each year, the share of deciduous trees has been increasing, and oaks (*Quercus* spp.), due to their high ability to adapt to various habitats, now belong to the most common trees in Polish forests (8%) [1].

Monolithic species composition, even-aged forest structure, is a result of reforestation of thousands of hectares destroyed during World War II, and unfavorable atmospheric conditions resulting from influences of maritime and continental climates are the causes of the susceptibility of some stands to a variety of harmful biotic and abiotic factors. Among European forests, the Polish forests belong to the ones which are most threatened by biotic factors, mainly insects and pathogenic fungi occurring cyclically in the forms of mass outbreaks or epiphytotics and affecting thousands of hectares. In the years 2011–2013, the areas threatened by pest insects exceeded more than 4.2 million hectares each year, representing more than 23% of the total forest area [1].

Current problems of forest protection concern weakness of forest stands caused by climatic changes, which intensify previously infrequent phenomena such as extreme heat and droughts and violent storms, often accompanied by powerful hail, hurricane winds and whirlwinds, as well as floods. Repeated influence of these forces weakens forest stands, which are subsequently attacked by pests or colonized by fungal pathogens. Long-lasting droughts, which became more common during the last two decades, were one of the major factors which started the process of large dieback of Norway spruce forests in the mountains intensified by the outbreak of European spruce bark beetle *Ips typographus* (L.) and pathogens from the genus *Armillaria* [2]. In pine stands, disruption of water balance can become a major factor leading to dying of Scots pine forests due to the diseases caused by *Gremmeniella abietina* (Lagerb.) M. Morelet, *Cenangium ferruginosum* Fr., and *Sphaeropsis sapinea* Fr. Fungi. Water-related stress leads to weakening of broadleaved, especially oak *Quercus* spp. stands, which are being attacked by *Agrilus* spp. beetles and pathogens from the genus *Phytophthora* [3]. It is possible that long-lasting droughts initiated the development of infectious ash disease caused by *Chalara fraxinea* fungi, which resulted in dieback of *Fraxinus* spp. forests throughout Europe [4]. Hurricane winds in lowlands and in the mountains cause the damage to coniferous forests by pulling and breaking the trees which provide a place for development of secondary pests, mainly from subfamily Scolytinae [5]. Hail storms as well as heavy snow falls combined with glaze ice on pine branches lead to damage in a form of broken and twisted trees, which are often attacked by weevils *Pissodes* spp. [6]. In addition, root systems damaged by drought, sudden freezes, or torn as a result of hurricane winds become a "gateway" for infection fungal pathogens initiating a multistage disease of stands, involving harmful insects. Moreover, climate warming increases probability of arrival to Central Europe of new insect and fungal species, which are more common in areas with higher air temperature. The presence of such species in Poland could be of an invasive form, and therefore setting up of continuous monitoring of such organisms' presence is essential.

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Forests can be susceptible to insect attacks at all stages, and forest plantations newly established on clear-cuts left after harvesting of old stands facilitate the concentration of insects associated with specific stand ages (**Photo 1**). In Poland, weevils (Coleoptera: Curculionidae) represent the most important group of pest insects of 1–5-year-old forest plantations established on clear-cuts [7–9]. The aim of this paper is to present the most important insect species damaging forest plantations and their management, including methods to estimate and reduce their numbers.

Photo 1. Typical *Pinus sylvestris* plantation in Poland.

2. Pest insects in forest plantations

2.1. *Hylobius abietis*

The large pine weevil *Hylobius abietis* L. is one of the pests with the greatest economic importance in Europe [10, 11]. The spruce weevil *Hylobius pinastri* Gyll. is another species damaging young forest plantations, but it occurs only occasionally and has a lower impact than *H. abietis*. In Poland, both species have been recorded every year throughout the whole country. Over

the last twenty years, the area of their occurrence has decreased from more than 40,000 ha in 1995 to just about 10,000 ha in 2015.

During the growing season, two distinct periods of increased occurrence of *H. abietis* in reforestation areas can be clearly defined [12–14]. The first period of pest mass occurrence, representing a significant threat, usually appears in May due to the migration of beetles from adjacent stands attracted to the monoterpenes emanating from the resin of fresh stumps left after harvesting of old coniferous trees in the reforested areas. These volatiles include α-pinen and 3-carene, which show synergistic effects with ethanol [15, 16]. These compounds are also used in practice as kairomones in bait traps to attract and collect weevils. The studies of Azeem et al. [17] showed that *H. abietis* beetles are the vectors of fungi *Ophiostoma canum* (Münch), *Ophiostoma pluriannulatum* (Hedqc.) Syd. and P. Syd., and yeast *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij., which produced methyl salicylate that strongly reduced the large pine weevil's attraction to the *P. sylvestris* volatiles. The second period of mass occurrence takes place in August or September as the result of hatching of the second generation developed from eggs laid in the spring of the same year.

The first appearance of beetles on clear-cuts depends on the weather conditions, especially on air temperature. Similar to observations made in Norway [18, 19], in Poland, weevils leave their wintering places when air temperatures exceed 10°C, which is usually at the turn of April and May. The beetles move on foot or fly from adjacent stands, attracted by volatiles emanating from the resin of fresh woody debris left after harvesting [11, 12]. They can fly in May and June [11]. Not much is known about the distance they can cover, but in Poland, marked insects were found at a distance of 2 km from the place of release [20]. In a study in Sweden, the range of weevil flight oscillated between 80 and 100 km [21]. It is assumed that in one day, beetles can fly a distance of 10 km, while they can walk a distance of 50 m. However, questions remain concerning the period of the development cycle in which beetles lose their ability to fly. Nordenhem [22] observed young and mature beetles, which have already copulated, flying. This view is supported by Korczynski [20], who stated that the beetles lose their ability to fly in a certain period of the growing season, possibly due to temporary weakness of the muscle wings.

In Poland, the large pine weevil population reaches its maximum of abundance in the second half of May [23]. In addition to young beetles, the population also consists of older individuals that have wintered two to three times. Generally, beetles that have wintered in warmer positions appear first, followed by those which have wintered in colder areas [24, 25]. The beetles avoid reforestation areas with high humidity [26]. Analysis of changes in the spatial distribution of the seedling damage caused by the large pine weevil showed that initially, beetles accumulate on the edge, making their way into the central zone of the forest [27].

According to Korczynski [27], feeding activity peaks in the evening hours, while Christiansen and Bakke [19] observed highest feeding activities at night, when air temperatures oscillated between 19 and 28°C. These results were partially supported by Fedderwitz et al. [28], who observed that most of the beetles under laboratory conditions were feeding in the second half of the dark phase and in the first hours of the subsequent light phase. They also showed that weevils spend only 6% of their time feeding. Temperatures above 30°C cause the disappearance of the activity of the insect [29].

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The seedlings of all conifer and some deciduous (e.g., *Betula* spp. or *Quercus* spp.) tree species can be damaged by *H. abietis*. The weevils chew patches in the bark of stems and lateral shoots, causing their deformation and even death [30–32]. The large pine weevil also feeds on bark and needles of young shoots in older stands, including trees left on the clear-cuts for natural regenerations. Experiments on food selectivity showed that species of the genera *Pinus*, *Picea* and *Larix* spp., especially *P. sylvestris*, *Pinus strobus* L., *P. abies*, and *Larix decidua* Mill., are the most attractive food sources for *H. abietis* beetles [31, 32].

After supplementary feeding, the beetles copulate, and at the turn of May and June, the females start to lay eggs on the roots of stumps or on course woody debris such as soil branches and piles of bark remaining after tree debarking. According to Bylund et al. [33], *H. abietis* female lays approximately 70 eggs during the first season. In Poland, Korczyński [34] observed that during the growing season, one female laid up to 100 eggs, mainly in the second half of June.

Fresh stumps of coniferous trees and their roots are the most important breeding bases for *H. abietis* development. Experiments conducted in Sweden showed that monoterpenes α- and β-pinen, 3-carene, and terpineol, secreted by the roots of stumps, attract the beetles to the breeding bases [35]. The stumps remain suitable as breeding sites as long as the cambium remains in good condition. According to a study conducted by von Sydow and Birgersson [36] on Scots pine and Norway spruce, during the first months after cutting, a number of chemical and physical processes get activated in the stump, followed by a decrease of stump humidity, a reduction of the number of living wood cells, and a decline of ethanol concentrations, attracting species of the family Curculionidae. The studies estimated the attractiveness of various coniferous species as breeding material for the large pine weevil and showed that stumps of *P. sylvestris*, *P. abies*, and *L. decidua* are more often colonized by the pest than stumps of other species [37]. Based on laboratory tests, Nordenham and Nordlander [38] found that females can lay their eggs directly on the ground. In a similar study, Pye and Claesson [39] showed that about 90% of females lay eggs at a depth of 5–10 cm near fine the roots distributed around the stem base. Once the larvae have hatched, they chew tunnels down the roots, reaching a length of up to 1 m. Skrzecz [40] analyzing colonized *P. sylvestris* stumps found most of the larvae on roots with a diameter of 2–4 cm and reaching a depth of 0.5 m. In the case of *H. abietis* larvae wintering in stumps, they were found in roots with a diameter of up to 2 cm. Most likely, such behavior protects the insects against low winter temperatures when soils are frozen. According to Eidman [41], the development of eggs lasts from 12 to 16 days at temperatures oscillating between 20 and 28°C. After oviposition, the females do not die, but feed and spend the winter in the forest litter; in the following year, they oviposit again after supplementary feeding in spring.

The length of larval development depends mainly on the temperature. In Poland, the large pine weevil develops one generation yearly. Dominik [42] stated that in shaded places under the canopy, the development can be extended, leading to a 2-year generation. At the same time, this author demonstrated that the sunlight, influencing soil temperature, is the main factor impacting *H. abietis* development. These results were confirmed by Kuziemska-Grzeczka [43], who observed faster development of this pest insect in sunny areas than in shaded ones. Eidman [41] reported that under laboratory conditions, the larvae develop within 97 days at a temperature of 11°C, while at 25°C, development is completed within 42 days. Temperatures

below 20°C can cause a diapause of the last instar larvae lasting from 60 to 220 days. The larvae pupate in the pupal chambers where they remain for one to five weeks. The young beetles stay in the pupal the chambers up to three weeks and hatch in August or September of the same year. Some of the beetles overwinter in the chambers and leave them in the spring of the following year. Despite many studies on the biology of *H. abietis*, we do not know much about the influence of temperature on the development of these insects, especially in the context of global warming. Daegan et al. [44] studied the effect of temperature on the development and life cycle regulation of the large pine weevil in the aspect of projected climate warming, i.e., an increase of mean temperatures in the UK by the 2080s. They confirmed a linear relationship between temperatures and *H. abietis* development rates, concluding that the predicted increase in average temperatures may result in the development of two generations during one year, even in northern European countries. In connection with climate change, which also affects the distribution of insects, Barredo et al. [45] proposed to establish an open European database of geo-referenced insect pest distributions, including that of *H. abietis*.

2.2. *Pissodes castaneus*

The banded pine weevil *Pissodes castaneus* (De Geer) is one of most dangerous pest insects in forest plantations and thickets weakened by biotic factors, mainly pathogenic fungi and deer, as well as abiotic factors, including drought, hail, and fire [46]. It is a species commonly found in Europe, especially in northern Italy, Austria, Germany, the Asian part of Russia, and Turkey, as well as in North Africa [47, 48]. In 2001, it was introduced to South America, where it was initially described in Brazil, Argentina, Uruguay, and Chile [49]. In South America, it damages *Pinus taeda* L. and Douglas fir *Pseudotsuga menziesii* (Mirb.) Franco, while in Europe, many species of pines, primarily *P. sylvestris*, *Pinus pinaster* Aiton, and *Pinus pinea* L., are affected. In Poland, *P. castaneus* is commonly found in *P. sylvestris* plantations and thickets (**Photo 2**). From 2000 to 2015, the area of its occurrence increased in Europe, including Poland, to over 8000 ha per year.

In central and southern Europe, *P. castaneus* develops two generations per year, whereas only one generation is observed in northern European countries. The beetles leave their wintering places in the first half of April and then feed on the buds and young shoots of *P. sylvestris*, which is usually insignificant, but in the case of mass occurrence, it can lead to severely inhibited shoot growth. In May, the females lay their eggs on the lower parts of Scots pine stems, generally between the root collar and the second whorl of branches. Alauzet [50] found that under laboratory conditions, the females can produce over 500 eggs in their lifetime. After 8–10 days at 22–23°C, the larvae hatch and start to excavate galleries under the bark of stems, causing dieback of infested trees [47]. The constructed galleries end with pupal chambers in which pupae can be found between May and July. The beetles of the second generation hatch in late June and early July and start feeding immediately; in July and August, the females oviposit. The first larvae can be observed from the second half of August. During warm summers and autumns, the larvae develop to pupae or beetles and then overwinter. In the case of a cold spring or autumn (air temperature <10°C), the development of the first and, consequently, the second generation is longer, and the insects overwinter as larvae, pupae, or rarely as beetles [51].

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Photo 2. *Pinus sylvestris* seedling with the characteristic symptoms of the colonization by *Pissodes castaneus*: leaks of resin on a stem, hanging top shoots.

2.3. *Cneorhinus plagiatus*

Very young (1–2-year-old) Scots pine plantations and thickets established on previous fire areas, especially on poor, sandy soils, can be heavily affected by weevils of the species *Cneorhinus plagiatus* Shall. These beetles occur in reforestation areas in April and May and feed on the buds, needles, and bark of *P. sylvestris* seedlings during the night. Mass appearance of both species may lead to severe seedling damage or even death within a relatively short time. During the day, beetles stay in the soil close to the root collars of the seedlings. The insects copulate in May and the females oviposit 30–50 eggs into the soil. The larvae feed on the roots of herbaceous plants. Pupation and overwintering take place in the soil. In Poland, *C. plagiatus* is currently not of economic importance as it is only recorded in less than 10 ha per year.

2.4. *Brachyderes incanus*

The weevil *Brachyderes incanus* L. mainly attacks newly established *P. sylvestris* plantations on postfire areas [52]. Although this insect is also present in plantations on depleted post-agricultural land, it is characteristic for large areas damaged by fire. In Poland, the area of mass occurrence of this insect has reached over 20,000 ha of postfire land since the 1990s but does not exceed 20 ha per year. The beetles usually feed on *P. sylvestris* needles, but during mass appearance, they can also cause damage to *Picea* or *Larix* needles and even to the bark of young *Betula* or *Quercus* trees.

The insects feed on needles of the two highest whorls of branches. Although they can damage up to 95% of these needles, the infested trees have not died because one-time feeding is not detrimental to growing trees. However, repeated feeding can lead to growth inhibition and significant weakening, resulting in death in some cases.

The insect produces one generation per year. The beetles overwinter in the forest litter and start to feed in April–May; at the beginning of June, the females oviposit eggs into the soil. Depending on air temperature, after 2–6 weeks, the larvae feed on roots of shrubs, trees, and grass growing in reforested areas. Larvae pupate in August and the new generation of beetles appears toward the end of August, in September, or at the beginning of October.

2.5. Other species of low economic importance

Table 1 lists other species of pest insects occasionally occurring in Poland on small areas of forest plantations and thickets. Buds and shoots of Scots pine trees younger than 15 years are mainly damaged by Lepidoptera larvae. At present, the European pine shoot moth *Rhyacionia buoliana* Schiff (Lepidoptera: Tortricidae) is the most common and important pest in Polish pine thickets. It finds suitable conditions for its development in sunny and weakened stands, which become reservoirs of this pest. Severe infestations of pine trees by the European pine shoot moth inhibit height growth, cause deformations of trees, and thereby lower the value of timber products.

Pine needles and buds are also infested by *Exoteleia dodecella* L., which appears in Poland in stands of all stages, but most rapidly and in largest numbers in plantations and thick-

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ets aged 6–30 years. For a number of years, considerable damage in pine thickets caused by *Thecodiplosis brachyntera* Schwaegr. and accompanied by *Contarinia baeri* Prell. (Diptera: Cecidomyiidae) has been reported. The larvae of these Diptera suck on needles and cause premature shedding and dropping. Similar damage to pine needles is also caused by the weevil *Brachonyx pineti* Payk. From the group of sucking insects, the pine bark bug *Aradus cinnamomeus* Panz. (Hemiptera: Aradidae) can be a serious pest in young pine stands. It occurs on dry and depleted soils and in areas affected by industrial pollution.

Insect species	Damaged species	Damaged parts of tree	Insect instar causing damage
Rhyacionia buoliana Denis and Schiff. *Rh. duplana* Hübner *Blastethia turionella* L. (Lepidoptera: Tortricidae)	*Pinus sylvestris*	Buds, shoots	Caterpillar
Aradus cinnamomeus Payk. (Hemiptera: Aradidae)	*Pinus sylvestris*	Stem	Larva, imago
Neodiprion sertifer Geoff. (Hymenoptera: Diprionidae)	*Pinus sylvestris*	Needles, shoots	Larva
Acantholyda hieroglyphica Christ (Hymenoptera: Pamphiliidae)	*Pinus sylvestris*	Needles	Larva
Barbitistes constrictus Brunner von Wattenwyl (Orthoptera: Tettigoniidae)	*Pinus sylvestris*	Buds, needles	Imago
Exoteleia dodecella L. (Lepidoptera: Gelechiidae)	*Larix decidua* *Pinus sylvestris*	Needles	Caterpillar
Dreyfusia nordmannianae Eckst. (Hemiptera, Adelgidae)	*Abies alba*	Needles, shoots	Larva
Cryptocephalus pini L. (Coleoptera: Chrysomelidae)	*Pinus sylvestris* *Picea abies* *Abies alba*	Needles	Imago
Brachonyx pineti Payk. (Coleoptera: Curculionidae)	*Pinus sylvestris*	Needles	Larva
Thecodiplosis brachyntera Schwägrichen (Diptera: Cecidomyiidae)	*Pinus* spp.	Needles	Larva
Contarinia baeri Prell (Diptera: Cecidomyiidae)	*Pinus sylvestris*	Needles	Larva
Hylastes spp. Erich. (Coleoptera: Curculionidae)	*Pinus, Picea, Abies* spp.	Stem	Imago
Magdalis spp. Germar (Coleoptera: Curculionidae)	*Pinus*, *Picea*, *Abies* spp.	Shoots	Larva, imago

Table 1. Insect pests of less economic importance in Polish young conifer stands.

3. Integrated management of weevils in reforested areas

3.1. Background

In Poland, contemporary forest protection against insect pests is based on the strategy of integrated pest management (IPM) (**Figure 1**). The plant is the main objective of all treatments, and its genetic specificity, response to the colonizing organisms, and the relationship with the environment are taken into account. Prevention based on prophylactic measures is a very important element of this strategy and followed by protection methods in which priority is given to biological and biotechnical methods covering the use of biological insecticides and also substances that affect insect behavior. Chemical treatments, as the last option, are used when other methods are not effective and in cases of high threats to crop sustainability.

In practice, prophylactic measures are aimed at strengthening stand resistance to attacks by pest insects and take into account the recommendations of forest silviculture, utilization, and

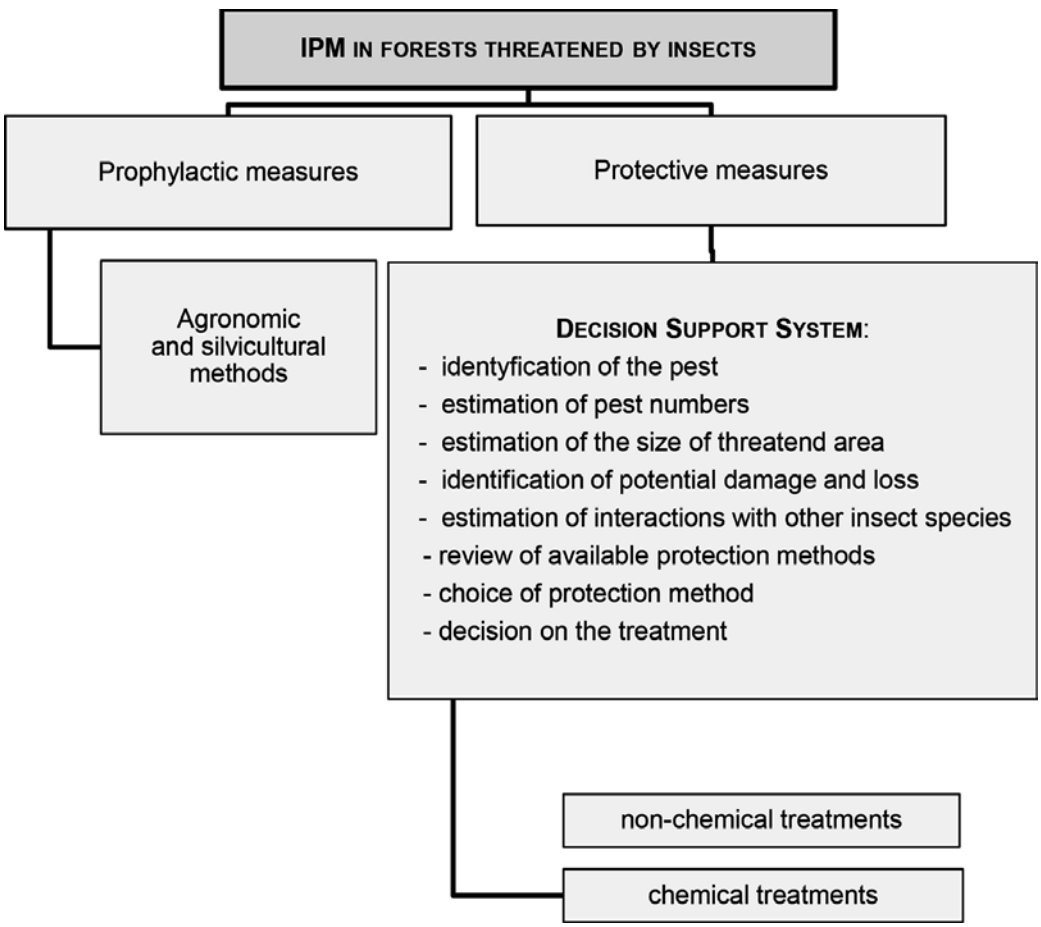

Figure 1. Integrated pest management to protect forests against pest insects.

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protection. The most suitable protection method is selected on the basis of a multi-step decision support system (DSS), which includes identification of the pest and determination of the amount of tree damage, estimating potential losses. It is also important to define potential interactions, e.g., coexistence with other species of pest insects. The final stage of DSS includes a review of available protection methods and selects the most appropriate method for the given situation.

Protective measures are mostly taken to reduce the abundance of *H. abietis*, in some cases also of *P. castaneus*. Treatments that protect crops against other species of insects are performed locally in small areas. The integration of different methods to reduce the damage caused by insects in forest plantations, particularly by *H. abietis*, is an example of the IPM strategy. It was developed based not only on research but also resulted from long-term observations of pest biology and ecology and scientific analysis of the causal sources of pest outbreaks. Integrated pest management strategies to protect reforestation stands against *H. abietis* were also introduce into the UK to replace the use of insecticides, with particular emphasis on the development of methods of risk assessment as well as biological control methods with the use of entomopathogenic nematodes (EPNs) [53, 54]. In Sweden, the IPM strategy, in addition to risk assessment, includes the use of different barriers on seedlings and silvicultural measures, such as soil scarification and leaving the shelter trees on site to reduce the damage [55–58].

3.2. Prophylactic measures

Clear-cutting is the method most frequently employed in Polish forests. Postcutting regeneration leads to the formation of evenly aged stands of poor species composition, mainly Scots pine and Norway spruce. This facilitates the concentration of pest insects associated with defined developmental phases of stands. The most important preventive measures include agronomic and silvicultural methods that improve seedling growth, making them more resistance to insect damage.

The establishment of forest plantations composed of a variety of trees species or the promotion of natural regeneration on sites with favorable regeneration conditions can increase resistance of the biocoenosis to pest insects. Results of Scandinavian studies showed that naturally regenerated plants were less susceptible to weevil attacks than planted ones. Water stress and some other physiological effects related to transplantation may be some of the reasons why planted trees are more susceptible to insect attacks.

According to Moore et al. [53], the within-season felling date is one of the most important factors affecting the development of *H. abietis* in stumps, its abundance, and damage to seedlings. In the second year after felling, they observed more weevils in the stumps created between May and early August than in those from late August to November. Similar results were obtained by Korczynski [59] who stated that in plantations established in areas where the stand was felled in winter, the number of *H. abietis* beetles was in all cases higher than in adjacent stands, whereas in plantations established on summer clear-cuts, the number of these insects was always smaller. Similarly, Sklodowski [60] stated that plantations established on clear-cuts from summer showed low susceptibility to the large

pine weevil. In contrast, Koehler and Kolk [61] considered that plantations established on clear-cuts established in May–June are increasingly threatened by insects than those established on clear-cuts from autumn or winter. In their opinion, *H. abietis* prefers to colonize stumps created during the summer period.

Delaying replanting for two to four years after clear-cutting can be another method to reduce *H. abietis* abundance in plantations. Damage is reduced because most of the weevils would have left the area before the beginning of reforestation activities [62]. Although this method is recommended for Poland, it can only be applied on 1–2-year-old areas, as intensive weed growth, resulting in high costs for weeding, renders this practice unsuitable [60]. In Poland, the planting takes place during early spring (March–April), frequently on fresh or 1-year-old clear-cuts, i.e., before the heaviest attack of *H. abietis* in May. Similar rules apply in Sweden, where Wallertz et al. [63] estimated the effect of planting time on *H. abietis* damage to *P. abies* seedlings. They found reduced damage to trees planted in August–September on clear-cuts established in January of the same year compared to late planting in November or May the following year.

From the start, the planted seedlings require optimal growing conditions. Proper site preparation by soil scarification and weeding, then careful handling, and planting are very important for the further development of trees and make them more resistant to weevil attacks [62, 64]. Örlander and Nordlander [65] found that fresh scarification significantly reduced *H. abietis* damage and increased seedling survival. These results were supported by Björklund et al. [66], who observed less damage to seedlings planted into pure mineral soil. They concluded that the presence of pure mineral soil around seedlings reduces the likelihood of damage caused by the large pine weevil. Similarly, Sklodowski [60] reported lower numbers of beetles collected by traps placed on the mineral soils. To effectively reduce impacts of *H. abietis*, soil scarification should be carried out in the first year after clear-cutting [62]; after two or four years, it has no effect on insect attacks. Adjustment of tree species composition and increasing the share of deciduous species, which are much less susceptible to these pest insects, can help to keep crops in good health condition and prevent mass occurrences of pest insects.

The size of the reforested area also has a significant effect on the number of weevils and the extent of the damage [64, 67]. Previous studies have found that larger areas are more threatened by pest insects than smaller ones. Korczynski [68] observed the correlation between the increase of damage to seedlings and the increase of distance from the plantation edge. In Poland, clear-cuttings usually do not exceed an area of 4 ha, and 1–2-year-old *P. sylvestris* seedlings are used for reforestations. Larger seedlings are more susceptible to damage than smaller ones, and this observation was supported by Korczynski [69], who found that higher seedlings ($16 \leq 35$ cm) were more frequently damaged by the large pine weevil than lower ones ($5 \leq 15$ cm).

Swedish studies showed reduced seedling damage on plantations with shelter trees. This may result from an extra supply of food, such as bark of branches and ground vegetation under the shelter trees [70–72].

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3.3. Estimation of population numbers and risk assessment

A number of studies have predicted and assessed *H. abietis* damage in forest plantations; however, so far, no successful methods to prevent such damage have been developed. The main reason for this might be the large number of factors influencing the dispersal of these insects. Leatcher et al. [11] listed four categories of risk factors related to large pine weevil biology—(1) suitability of breeding site, (2) weevil development rate, (3) planting site factors, and (4) weevil-seedling interactions—whereas Wilson et al. [73] indicated eight categories related to forest location, felling and planting, adjacent forest, soil, stumps, weevils, vegetation, and treatments.

An important part of these studies is the relationship between pest abundance and the extent of the damage. Some authors suggest that even in periods of high weevil abundance, seedling damage can be relatively small, while serious impacts can be recorded when pest abundance is low [7]. Results of Swedish and Polish studies showed that the numbers of beetles and impacted seedlings were only positively correlated in 1–2-year-old plantations. In Poland, the 1980s, a method of estimating the damage caused by *Hylobius* beetles was developed [7]. This method was based on the comparison of the damaged bark surface of 30 sections (20 cm long and 1 cm diameter) detached from fresh pine branches and placed in the investigated plantations. However, this method was never adopted in practice. In the UK, a method of risk assessment was developed and introduced to the strategy of Integrated Forest Management for *H. abietis*. It was based on the correlation between the time of clear-cutting and the period of oviposition and, subsequently, the extent of damage caused by the beetles [53, 54].

At present, assessment of weevil threats to plantations is based on the number of beetles captured in different kinds of traps baited with kairomones to attract weevils. Experiments with mass trapping systems were conducted in Sweden in the 1980s, where pitfall traps baited with resin derivative α-pinen and ethyl alcohol that act synergistically were evaluated [74]. Swedish traps with different modifications have been applied in several European countries in *H. abietis* control programs [13, 75–77]. In the UK, the emergency trap was developed to capture and monitor the population of *H. abietis* and its parasitoid *Bracon hylobii* Ratz. developing in the stumps [78]. The trap baited with turpentine and ethanol is formed by a tripod covered by a net and placed over a cut stump.

In Poland, to assess the risks for forest plantations, it is recommended to observe changes in pest abundance from April to September, based on the numbers of beetles captured in traps made from freshly cut *P. sylvestris* billets, slices of fresh bark (**Table 2** and **Photo 3**). It has been accepted that a single trapping of more than 10 *H. abietis* beetles provides a basis for taking protective methods. In the 1990s, IBL-4 pipe traps were developed and introduced into Polish forestry to monitor and control *H. abietis* populations (**Photo 4**). This trap consists of a pipe 60 cm in length and 10 cm in diameter, with two rows of inlet holes. This construction prevents the escape of beetles from the trap. The trap is baited with a mixture of α-pinen and ethanol and works as a food attractant. Contrary to pine billets, the use of IBL-4 traps

Insect species	Type of traps and their use
Hylobius abietis, H. pinastri *Cneorhinus plagiatus,* *Hylastes* spp.	– Pine billets; size, length of 1 m, diameter of 10–15 cm; slightly stripped on one side and this side placed on the ground – Fresh bark of pine or spruce; size, 30 × 30 cm; placed with phloem to the ground – Bundles of fresh coniferous brushwood; size, length of ±30 cm, diameter to 10 cm – Pine wood rings in a bark placed in the holes; the size of holes, 30 × 30 cm IBL-4 traps baited with an attractant Placing the traps from April to September Recommended trap density: – 5–10 traps/ha in risk assessment – To 50–100 traps/ha in protective measures Checking the traps: 1–3 times/week depending on the pest numbers Dry traps exchanged for new ones
Pissodes castaneus	– Sections of pine stems prepared from living trees: length of ±1.5 m; the diameter of 6–10 cm Placing the traps in early April: digging into a soil to a depth of 30 cm Recommended trap density, 10–20 traps/ha Checking the traps, 1–2 times/week Colonized traps are removed and destroyed
Rhyacionia buoliana	– Sticky trap (triangular or rhombic) with a dispenser containing a sex pheromone to collect the males of small butterflies Recommended trap density, >30 traps/ha Traps are hanging out before butterflies swarming—in the second half of June

Table 2. The use of traps for estimation of insect numbers and their control in forest plantations and thickets.

is much more effective and less time-consuming (**Photo 5**). Sklodowski and Gadzinski [79] compared the effectiveness of pine billets and IBL-4 pipe traps and found that pipe traps collected almost three times more beetles. The high effectiveness of IBL-4 traps was also confirmed by Kuzminski and Bilon [80], who estimated numbers of large pine weevils collected by different types of traps, including Scots pine billets and slices with or without addition of sawdust soaked with turpentine. The use of natural traps in forms of fresh pine bark or branches impregnated with a combination of α-pinene, turpentine, and ethanol was most effective; this method has also been carried out in Spain [81]. The results showed that most beetles could be caught using pine bark soaked with a mixture of these substances. There was no significant difference between the use of α-pinen and turpentine, and using pine bark with turpentine and ethanol was recommended as an effective and cost-efficient method to monitor *H. abietis* populations.

Natural Scots pine traps are also used to evaluate threats by other weevils, such as *C. plagiatus*, *Hylastes* spp., *Otiorhynchus* spp., and *Magdalis* spp. In order to successfully evaluate threats, plantations established on sandy soils and postfire areas should be subject to special control during the spring. Estimations of insect occurrence are performed on the basis of beetle numbers collected by traps and on the basis of needle damage.

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Photo 3. *Pinus sylvestris* billet used for protection of reforestations; under the trap there is a hole to collect *Hylobius abietis* beetles.

Evaluation of the number of *P. castaneus* and the level of damage to *P. sylvestris* plantations and thickets is performed on the basis of the number of trees colonized by the pest on areas of its occurrence in the previous years and in young forests weakened by biotic (fungi, insects, deer) and abiotic (drought, hail, fire) factors. The observations are performed every two to three weeks from mid-May to the end of September.

Susceptibility of *P. sylvestris* plantations to *B. incanus* is evaluated on the basis of beetle number per tree and percentage share of damaged needles of the highest whorl of branches [52, 82]. Observations should be made at the turn of April and May and in September. The number of beetles is determined every few days on 10 randomly selected trees by shaking them and counting the beetles dropping on sheets placed under the tree canopy. The degree of threat is then defined as the average number of beetles per tree calculated based on the results of 10 trees according to the following classification of threat:

– weak: five beetles/tree, damage to needles <30%
– medium: 6–30 beetles/tree, damage to needles 31–60%
– strong: >30 beetles/tree, damage to needles >60%

In the case of *Neodiprion sertifer*, evaluation of pest numbers in forest plantations and thickets is performed in early autumn on the basis of the number of eggs found in the trees. The level

Photo 4. IBL-4 trap used for collection of *Hylobius abietis* beetles.

of the threat depends on the age of the trees and is critical for 3–10-year-old forests, when the number of eggs reaches, respectively, 50–1,500 per tree. Evaluation of threats by *Tortricidea* spp. is based on the estimation of the number of pine buds or higher shoots damaged by larvae. It is generally carried out from May 15 to June 15 and consists of the observations of 30 trees growing on the edge and 30 trees growing in the center of the forest. Critical damage is defined as damage of at least 30% of buds or shoots. A complementary method of *Rh. buoliana* observation involves the counting of butterflies attracted by pheromone traps installed before the start of swarming in the second half of June (**Table 2**).

Assessment of the occurrence of *A. cinnamomeus* should be carried out in Scots pine plantations and thickets where cracking and pushing aside of bark scales as well as yellowing of needles are observed. In the threatened young stands, three pairs of control trees (one at the edge, two in the center of the stand) are evaluated. Subsequently, sticky bands (5 cm width) are placed on the control trees at a height of 20 cm in early spring, the period in which the insects leave their wintering places, or in autumn—the period in which the insects retreat to their wintering places in the forest litter. The sticky bands are checked every week; the stand is seriously threatened when 10 insects are found within the plantation and 50 insects on one tree.

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Photo 5. *Hylobius abietis* beetles collected by IBL-4 trap, visible dispenser in the form of tube filled with synthetic attractant.

3.4. Physical methods and baited traps

Different mechanical methods are integrated to effectively reduce damage caused by weevils. In Sweden, plastic collars and coated barriers of paper or plastic fibers were designed to surround and protect seedlings from damage caused by *H. abietis* weevils [55, 83, 84]. In 2009, Nordlander et al. [85] described a new method of physical protection which consists of covering the lower part of the seedling stem with flexible sand coating (Conniflex). The use of this kind of barrier resulted in increased survival rates of 97% of *P. sylvestris* and 86% of *P. abies* seedlings.

In Poland, at the turn of March and April, it is recommended to dig grooves with vertical walls (width and depth of 25–30 cm) along the border to older stands, where beetle invasion is expected (**Photo 6**). The grooves surrounding the plantations are commonly used to collect *H. abietis* weevils walking from adjacent stands into the plantations. Additionally, sections of fresh pine branches are placed in the grooves to collect and stop more beetles. To directly reduce the number of weevils (*H. abietis*, *C. plagiatus*, *Hylastes* spp.), freshly cut and split billets, pieces of fresh pine bark, or IBL-4 traps are used. For control measures, approximately 20–40 traps are set per 1 ha of plantation. Unfortunately, IBL-4 traps can also collect nontarget insects [79, 86], and only 92% of all caught insects were large pine weevils. The majority of

Photo 6. Plantation surrounded by groove with slice of pine wood to collect pest beetles.

captured nontarget insects belonged to the family Carabidae, which entered the traps accidentally or on the search for shelter. Beetles from the families Dermestidae, Geotrupidae, and Silphidae that feed on dead insects were probably attracted by the smell of decomposing insects inside the traps. Removal of stumps from the clear-cuts can reduce populations of

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the large pine weevil within reforestation areas [77], but in Poland, this method is time- and labor-consuming and not used in practice.

Damage caused by *P. castaneus* may be avoided by controlling the breeding of these insects in pine thickets. Potential breeding material such as windfalls, stems broken by wind, or trees damaged by fire is removed from the thickets. In areas with *P. castaneus*, trees showing signs of infestation are removed during the winter or before the end of April to destroy overwintering larvae. In areas with high density of pest populations, special "trap stems" may be prepared and placed before the middle of April (**Table 2**). They are examined at certain intervals, and when heavily infested by *P. castaneus*, they are peeled to destroy the larvae. Mechanical methods of *Rhyacionia bouliana* and *E. dodecella* control are not used in practice. The method of hand picking of infested buds, which has been suggested in some cases, is impractical for most situations. Also, mechanical control of *A. cinnamomeus* or weevils damaging pine needles is not feasible.

3.5. Biological methods

3.5.1. Pathogens

Wegensteiner et al. [87] reported for the first time the occurrence of the eugregarine *Gregarina hylobii* Fuchs, the neogregarine *Ophryocystis hylobii* Purrini and Ormières, and the microsporidium *Nosema hylobii* Purrini in populations of *H. abietis* and *H. pinastri* from a few locations in Austria and Poland.

Some species of entomopathogenic fungi may be important in regulating numbers of the large pine weevil. *Beauveria bassiana* (Bals.-Criv) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. belong to the most common species developing on *H. abietis*. Popowska-Nowak et al. [88] studied the species structures and densities of entomopathogenic fungi in soils of forest plantations in Poland. They isolated five species of entomopathogenic fungi: *B. bassiana*, *Isaria farinosa* (Holmsk.) Fr., *Isaria fumosorosea* Wize, *M. anisopliae*, and *Verticillium lecanii* (Zimm.), of which *I. fumosorosea* and *M. anisopliae* were found most frequently.

So far, there is little information on the potential use of entomopathogenic fungi in controlling *H. abietis*. Wegensteiner and Fuhrer [89] found mortality rates of up to 100% for large pine weevil beetles infected with conidia of *B. bassiana* under laboratory conditions. However, no fungal infections were noted in beetles feeding on bark treated with the fungus under field conditions. Similar results were obtained by Ansari and Butt [90], who observed 100% mortality of all growth stages of the large pine weevil infected by *B. bassiana* and two fungi of the genus *Metarhizium*: *Metarhizium robertsii* (Metschn.) Sorokin and *Metarhizium brunneum* Petch. under laboratory conditions. Williams et al. [91] carried out field experiments to control populations of the large pine weevil with *B. bassiana* and *M. anisopliae* applied together with entomopathogenic nematodes of the species *Steinernema carpocapsae* (Weiser) and *Heterorhabditis downesi* (Stock, Griffin, and Burnell). They observed a higher effectiveness of nematodes, which were responsible for 50% mortality of *H. abietis*, while fungi infected 20% of larvae and pupae of the pest. No synergy effect between the applied species of nematodes and fungi was found. The use of metabolites of fungi growing in the insect environment

could be another direction in plant protection against pests. Azzem et al. [92] isolated the fungus *Penicillium expansum* Link ex. Thom from feces and frass of *H. abietis* and described its metabolites (styrene and 3-methylanisole), which reduced the weevil's attraction to pine twigs in multi choice tests. These authors suggest that metabolites produced by microbes may be useful to reduce the damage caused by *H. abietis* and can be considered as alternatives to chemical insecticides.

A number of studies have evaluated the use of entomopathogenic viruses from the family Baculoviridae to control forest pest insects. In the case of insects occurring in young forests, especially in 5–15-year-old stands, the experiments were set up to evaluate the efficacy of the granulosis virus in the biological control of *Lepidoptera* larvae. Preliminary laboratory and field tests were established to use the granulosis virus of the codling moth *Laspeyresia pomonella* L. against *R. buoliana* [93]. The promising results of the first experiments indicated that granulosis virus might be suitable for microbial control of these pests. *N. sertifer* and its virosis belong to the most frequently reported example of biological control [94]. Research on the practical use of nuclear polyhedrosis virus of *N. sertifer* (NsNPV) causing epizootic has been conducted from the 1940s. Since then, NsNPV has been tested and practically applied in many countries, including Canada, the USA, Germany, the UK, Sweden, Finland, Norway, Russia, Austria, Poland, Balkan countries, and Italy. In Poland, due to the lack of registration and the low risk by this species, viral preparations are not currently used in practice.

3.5.2. Parasitoids

In natural environments, parasitoids from Hymenoptera (Braconidae) belong to the group of natural enemies regulating populations of the large pine weevil. This group includes *B. hylobii* (Ratzeburg, 1848), *Perilitus areolaris* (Gerdin & Hedqvist, 1985), and *Perilitus rutilus* (Nees, 1812). *B. hylobii* was described in many European countries (Hedqvist 1958). In the UK, it occurs wherever larvae of *H. abietis* are found and can cause mortality of up to 50% of *H. abietis* larvae developing in Sitka spruce (*Picea sitchensis* CARR.) stumps during the first three years after felling [95–97]. Henry and Day [96] studied the interactions between *B. hylobii* and *H. abietis* larvae and evaluated the possibility of the use of braconids to suppress large pine weevil populations.

Research on the use of natural enemies to limit numbers of *P. castaneus* has been concentrating mainly on the biology of parasitoids. So far, Alauzet [46, 98] and Kenis et al. [99, 100] provided most of the information on the parasitoids of *P. castaneus.* These authors listed species from Braconidae, such as *Eubazus semirugosus* (Nees), *Eubazus robustus* (Ratzeburg), *Eubazus crassigaster* (Provancher), and *Coeloides abdominalis* (Zetterstedt).

3.5.3. Competitive fungi

In Poland, a biological method to suppress *H. abietis* populations breeding in Scots pine stumps was developed in the 1990s. The experiments aimed at the use of *Phlebiopsis gigantea* (Fr.: Fr) Jülich—a fungus decomposing the stumps and disturbing the development of *H. abietis* in colonized stumps [23, 101]. The results indicated that *Ph. gigantea* grows rapidly on the cambium of stumps, making them unsuitable for pest development. It was also found that infection of

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stumps with mycelium of *Ph. gigantea* reduced the number of eggs on stumps and their roots. Subsequent field studies were conducted to evaluate the abundance of *H. abietis* beetles and the extent of seedling damage in 1–3-year-old plantations established on clear-cuts with pine stumps treated with *Ph. gigantea*. Evaluation of pest catches in traps in the second growing season following the treatment showed that pest abundance in plots treated with the fungus was 40% lower than in untreated plots, probably due to lower attractiveness of stumps colonized by *Ph. gigantea.* The reduction of weevil numbers could have also been caused by increased mortality of pest larvae in infected stumps. In addition, in the clear-cuts with infected stumps, less *P. sylvestris* seedlings were damaged by the large pine weevil. Based on these results, *Ph. gigantea* application was introduced into practice as a part of IPM.

3.5.4. Botanical antifeedants

Along with more information about the effectiveness of the insecticide azadirachtin, (a natural compound isolated from *Azadirachta indica* A. Juss). in plant protection, a number of experiments were undertaken to apply this compound against new groups of pest insects. There was described the antifeedant influence of azadirachtin on *H. abietis* under laboratory conditions, while field treatments of Norway spruce seedlings resulted in reduced damage to seedlings protected with azadirachtin [102, 103]. Other studies showed an insecticidal activity of azadirachtin only when this substance was used in high concentrations, which makes this method unviable from the economic point of view [104]. Despite promising results, azadirachtin was not registered for the protection of young forests and cannot be used against forest weevils.

In Poland, problems of the influence of extracts from plants of different species on *H. abietis* feeding were examined by Korczynski et al. [105, 106], who found antifeedant activity of common box (*Buxus sempervirens* L.), large-leaved lupine (*Lupinus polyphyllus* Ldl.), fern (*Dryopteris filix-mas* L.), and spurge (*Euphorbia peplus* L.). Kuzminski [107] described the repellent activity of extracts from anemone (*Anemone nemorosa L.*) against beetles. Unfortunately, the results of these studies have not found practical application.

Intensive research on the use of plant-derived antifeedants has been conducted for many years in Sweden, where extracts from the bark of 38 tree and shrub species were tested for antifeedant activity against *H. abietis* [108]. The study found that the bark of willow (*Salix caprea* L.), aspen (*Populus tremula* L.), yew (*Taxus baccata* L.), ash (*Fraxinus excelsior* L.), and especially lime (*Tilia cordata* Mill.) contains compounds which inhibit feeding activity of the large pine weevil. In further studies, carboxylic acid, limonene, carvone, and verbonen compounds, which demonstrated antifeedant activity against *H. abietis* in laboratory experiments, were isolated from extracts of *T. cordata* bark [109].

3.5.5. Nematodes

In northern Europe, studies to evaluate the possibility of using nematodes from two families, Steinernematidae (*S. carpocapsae*, *Steinernema feltiae* Filipjev, *Steinernema kraussei* Steiner) and Heterorhabditidae (*Heterorhabditis bacteriophora* Poinar, *Heterorhabditis megidis* Poinar,

Jackson & Klein and *H. downesi* Stock, Griffin & Burnell), have been conducted to reduce the populations of *H. abietis* larvae. Entomopathogenic nematodes (EPNs) have many attributes of an excellent biological control agent: they naturally occur in the soil environment; they are safe for mammals and other organisms, including humans; and they are characterized by long-term survival in the absence of host insects [110]. In addition, the potential of nematodes is not weakened by the simultaneous use of plant protection products. For these reasons, the use of preparations based on EPNs does not exclude the use of chemical pesticides [111]. In addition, EPNs for plant protection can also be produced on a large scale [112].

Treatments to reduce *H. abietis* populations consist of spraying of stumps and adjacent soil with suspensions of EPNs containing 3.5 millions of nematodes/stump. In northern European countries, the application of EPNs against the large pine weevil takes place in June, when pine weevil larvae that hatched from eggs laid between the end of May and the beginning of June are present in the stumps. The first attempts to reduce *H. abietis* using *Neoplectana carpocapsae* Weiser (= *Steinernema carpocapsae*) were performed in Sweden, where mortality rates of 50–60% were obtained [113, 114]. The use of different nematode species of the genera *Steinernema* and *Heterorhabditis* in Ireland resulted in 60–80% reduction of larvae [115–117]. Field studies carried out in Scotland resulted in a reduction of the number of pine weevil larvae of 60% [118, 119].

Similar EPN applications were conducted in Poland; however, treatments were applied at different times. Nematodes were not applied in the summer season, but in early autumn, when mainly overwintering *H. abietis*, larvae were present in the stumps. The choice of this treatment timing was based on results obtained after the application of EPNs in mid-June to reduce the newly emerged larvae of the first generation [120]. Only 5% mortality of *H. abietis* in treated stumps was observed, which did not differ from natural pest mortality in nontreated stumps. Most probably, these results were influenced by unfavorable weather conditions for nematode development during the study (high air and soil temperatures, lack of precipitation), which might have caused increased nematode mortality. On the other hand, applications conducted in early autumn—when weather conditions were considerably more beneficial for nematode development—indicated nematode parasitism in 80% of large pine weevil larvae overwintering in treated stumps. Subsequent studies aimed at evaluating the effectiveness of commercially produced biopreparations and consisted of the spraying of *P. sylvestris* stumps with *S. carpocapsae, S. feltiae, H. bacteriophora, H. downesi*, and *H. megidis.* All tested nematodes showed the ability to parasitize *H. abietis* larvae overwintering in *P. sylvestris* stumps. Highest mortality rates were observed in the groups of larvae parasitized by *S. carpocapsae* and *H. downesi* and lowest rates in larvae parasitized by *H. megidis* [121].

In summary, despite many attempts to use natural enemies to reduce *H. abietis* populations, the range of biological methods is very limited and potentially applies to entomopathogenic nematodes and saprotrophic fungi used to suppress *H. abietis* populations developing in stumps. Currently forest protection does not possess effective methods of biological control which can be used to suppress populations of other insect species affecting the youngest forests.

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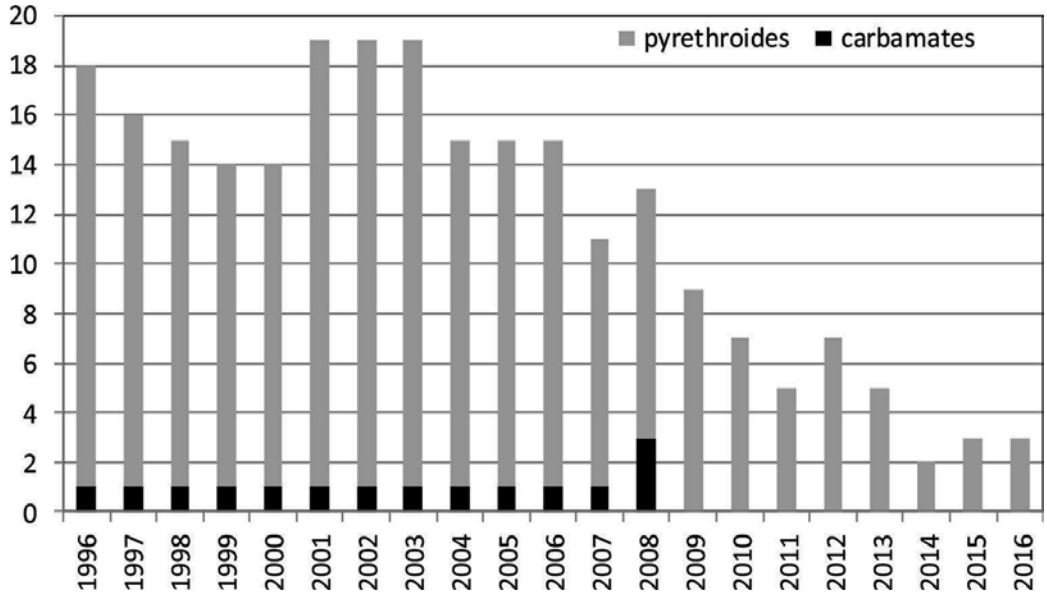

Figure 2. The use of insecticides in the protection of restock areas against weevils in Poland in years 1996–2016.

3.6. Chemical methods

Until recently, the use of insecticides was the most common method of protecting forest plantations against weevils, especially large pine weevils. However, limitation of pesticide use implemented by EU law and forest certification systems introduced by the Forest Stewardship Council (FSC) reduced the use of insecticides, particularly in young stands. The dynamics of changes in the numbers of pesticides registered for the protection of forest plantations showed an 86% reduction in insecticides that can be used against weevils (**Figure 2**). Pyrethroids are a group of insecticides most frequently used against weevils in the youngest forests. They particularly contain derivatives of cypermethrin, deltamethrin, esfenvalerate, lambda-cyhalotrin, and other compounds with *contact* and *stomach* action and repellent effects. Rose et al. [122] confirmed that *H. abietis* was able to detect the presence of lambda-cyhalotrin in multiple choice tests and feeding of food treated with this pyrethroid was significantly depressed and, in most cases, did not occur.

Carbamates were the second group of commonly used preparations to protect especially 1–2-year-old plantations. These preparations contained carbofuran and carbosulfan characterized by contact, stomach, and systemic actions. Granular formulations of carbamates applied to the soil through the roots of seedlings were particularly useful because the gradual release of active ingredients protected the tree up to two years after application [123]. These insecticides were absorbed by tree roots and showed a higher selectivity than pyrethroids. Due to toxic effects on nontarget insects (e.g., soil organisms), the use of carbamates was banned in EU countries.

The frequent use of pyrethroids can eliminate sensitive insects in the treated population. As more resistant insects are not affected, the development of insect resistance may be accelerated. Dobrowolski [124] found that *H. abietis* beetles from different populations significantly differed in their susceptibility to pyrethroids, and the author confirmed the importance of cytochrome P-450 monooxygenases in pest resistance to insecticides. To avoid the problem with resistance of *H. abietis* to pyrethroids, current research on chemical crop protection includes testing of other substances such as neonicotinoids. Rose et al. [122] observed the death of *H. abietis* weevils within three weeks after feeding on insecticide-treated Norway spruce. Similar results were obtained by Olenici et al. [125], who compared the activity of neonicotinoids and metaflumizone insecticides used against *H. abietis*. They found that beetles feeding on Scots pine twigs treated with neonicotinoids (acetamiprid, imidacloprid, thiacloprid) were either dying in three weeks or did not feed on metaflumizone-treated food.

Chemical protection of plantations against weevils includes preventive treatments consisting of dipping aboveground parts of the seedlings in the insecticides immediately before planting or the application of emergency postplanting sprays. Hereby, dipping seedlings is more effective than spraying them with the same concentration of insecticide [126, 127]. Thus, in Poland, in regions with high abundance of weevils, preplanting treatments are the most common way of plant protection.

As mentioned above, the number of insecticides registered for the protection of forests against weevils was significantly reduced because of:

- implementation of EU law (Directives of the European Parliament and of the Council 2009/128/EU and 1107/2009) for agricultural and forest practice aimed at the elimination of chemicals from the environment;
- the limited interest of chemical companies based on high costs of pesticide registrations for young forests which cover very small areas of the country compared to agricultural lands;
- the forest certification system by FSC.

As a result, in 2016, Polish foresters have the choice between three registered pyrethroids for the protection of plantations against *H. abietis* and other weevil species: Fastac Forest 15 SC with alpha-cypermethrin, Forester 100 EW, and Sherpa 100 EC, all based on cypermethrin. Currently, as threats by other species of insects have been relatively low for a number of years, chemical treatments are applied only to limit the numbers of the large pine weevil.

4. Conclusions

Curculionidae is the most important group of pest insects of forest plantations established at the clear-cut areas, which are most frequently used in Polish forests. Postcutting regeneration leads to the formation of even-age stands of poor species composition, attacked by pest insects associated with defined developmental phase of stands. Until recently chemical

plant protection was the most frequently used form of forest protection from insect pests and pathogens. Systematic decrease in number of plant protection products available in forestry as well as introduction in 2014 in the European Union of the principles of integrated plant protection calls for searching for plant protection methods using natural insect pest enemies such as pathogenic microorganisms, parasites, and predators. Therefore, contemporary forest protection requires advancement of integrated methods protecting forest plantations from insect pests through:

- studying the influence of climate warming on changes in biology of pest insects and changes in insect assemblages affecting reforestations;
- developing methods of monitoring and forecasting of forest dangers depending on site and stands characteristics;
- countering of threats caused by insect pests and pathogens within the large-scale disaster areas resulting from climate change;
- strengthening natural resistance of trees to insect pests and fungal pathogens;
- the use of natural enemies and agro-technical methods for regulation of population size of dangerous forest pests;
- evaluation of effectiveness of new plant protection products including studies intended for registration of pesticides for forestry;
- development of decision support systems as a tool facilitating introduction of integrated forest protection principles. Such support systems help to establish optimal terms for implementation of protection activities, which allows to increase their efficiency while limiting chemical pesticides to the absolute minimum.

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Chapter 8

Determination of Nucleopolyhedrovirus' Taxonomic Position

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Additional information is available at the end of the chapter

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Abstract

To date, over 78 genomes of nucleopolyhedroviruses (NPVs) have been sequenced and deposited in NCBI. How to define a new virus from the infected larvae in the field is usually the first question. Two NPV strains, which were isolated from casuarina moth (*L. xylina*) and golden birdwing larvae (*Troides aeacus*), respectively, displayed the same question. Due to the identity of polyhedrin (*polh*) sequences of these two isolates to that of *Lymantria dispar* MNPV and *Bombyx mori* NPV, they are named LdMNPV-like virus and TraeNPV, provisionally. To further clarify the relationships of LdMNPV-like virus and TraeNPV to closely related NPVs, Kimura 2-parameter (K-2-P) analysis was performed. Apparently, the results of K-2-P analysis that showed LdMNPV-like virus is an LdMNPV isolate, while TraeNPV had an ambiguous relationship to BmNPV. Otherwise, MaviNPV, which is a mini-AcMNPV, also exhibited a different story by K-2-P analysis. Since K-2-P analysis could not cover all species determination issues, therefore, TraeNPV needs to be sequenced for defining its taxonomic position. For this purpose, different genomic sequencing technologies and bioinformatic analysis approaches will be discussed. We anticipated that these applications will help to exam nucleotide information of unknown species and give an insight and facilitate to this issue.

Keywords: nucleopolyhedroviruses, Kimura-2-parameter analysis, next-generation sequencing, bioinformatic analysis

1. Introduction

Baculoviruses are insect-specific viruses which have a large circular double-stranded DNA genome packaged in enveloped, rod-shaped nucleocapsid and occluded within a paracrystalline protein occlusion body (OB) [1, 2]. The family *Baculoviridae* has four genera, including

Alphabaculovirus, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus*. Nucleopolyhedrovirus (NPV) is a member of *Alphabaculovirus* (lepidopteran-specific NPV) [3]; NPV replicates in the nucleus of the infected host cell and causes a disease of nuclear polyhedrosis. Epidemic outbreak of NPV may play a role in regulation of the host nature population [4]. Thereby, it is a potential agent for biological control with a number of eco-friendly benefits including high virulence and specificity against target insects, environmental safety and sustainable existence with target insects. Several baculoviruses showing promising results have been commercialized as biopesticides for the control of insect pests around the world [5]. For biotechnological applications, baculoviruses have been constructed as a eukaryotic protein expression vectors (baculovirus expression vector system (BEVS)) over the last 30 years and used to gene therapy trials. So far, many recombinant proteins have been expressed in insect cells by BEVS and contribute to human life [6].

To date, baculoviruses are known to infect more than 660 insect species; most of them are belonging to the order of Lepidoptera, Diptera and Hymenoptera [7, 8]. Baculoviruses exhibit genetic variations among species and its isolates [9]. Although a large number of baculoviruses in the nature, only a few have been well studied. To the best of our knowledge, a total of 78 fully sequenced genomes have been deposited in GenBank [10] and also several baculoviruses of whole genomes may soon be sequenced and deposited (**Table 1**). However, these published viral genomes represent only a small fraction and the genetic relationship among nucleopolyhedroviruses (NPVs) in the natural environment remains a puzzle.

Previously, Sanger sequencing was employed to sequence the viral genomic sequences cloned in plasmids. With the advances of sequencing technologies, next-generation sequencing (NGS) is becoming an important technology for large-scale viral genomic sequencing. The high cost of NGS and requirement of intensive bioinformatic analysis remain a hurdle for this application. In a word, NGS is an available tool to facilitate on the study of the genetic relationship of baculoviruses.

2. Identification of NPVs

Biochemical and biotechnology-based methods are the most common approaches employed to identify the NPVs. In most cases, more than one method is employed to compensate the pros and cons for each other. For example, restriction enzyme profiling of viral genomic DNA was used to reveal genetic variations among different isolates [97–99] and to distinguish one species from another between closely related viruses such as *Rachiplusia ou* (RoMNPV), AcMNPV, *Trichoplusia ni* (TnMNPV), *Galleria mellonella* (GmMNPV) [100, 101] and the MNPVs of *Spodoptera frugiperda* [102].

Polymerase chain reaction (PCR)-based methods were then established. These methods have been shown not only to be more sensitive and faster but also more reliable than restriction enzyme analysis for classifying baculoviral species [4, 103–105]. Multiple genetic markers (e.g., *egt*, *ac17*, *lef-2*, *polh*, *p35*, *pif-2*) could be used for the identification of baculoviruses [7, 106–109]. The *late expression factor 8* (*lef-8*), *late expression factor 9* (*lef-9*) and *polyhedrin*

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
Alphabaculovirus (Group I)	*Anticarsia gemmatalis* MNPV	AgMNPV-2D	NC_008520	132,239	36,623	29,338	29,513	36,765	44.5%	158	Sanger	PHRED/ ALIGNER	[11]
		AgMNPV-26	KR815455	131,678	36,411	29,288	29,405	36,574	44.6%	157	Roche 454 GS FLX	Geneious	[12]
		AgMNPV-27	KR815456	131,172	36,273	29,176	29,331	36,392	44.6%	157			
		AgMNPV-28	KR815457	130,745	36,185	29,018	29,242	36,300	44.6%	157			
		AgMNPV-29	KR815458	130,506	36,072	28,989	29,216	36,229	44.6%	157			
		AgMNPV-30	KR815459	130,741	36,195	29,011	29,173	36,362	44.5%	156			
		AgMNPV-31	KR815460	132,126	36,543	29,363	29,564	36,656	44.6%	158			
		AgMNPV-32	KR815461	131,494	36,341	29,234	29,384	36,535	44.6%	157			
		AgMNPV-33	KR815462	131,059	36,322	29,114	29,244	36,379	44.5%	157			
		AgMNPV-34	KR815463	131,543	36,435	29,233	29,383	36,492	44.6%	158			
		AgMNPV-35	KR815464	132,176	36,552	29,384	29,558	36,682	44.6%	159			
		AgMNPV-36	KR815465	131,216	36,293	29,127	29,270	36,526	44.5%	156			
		AgMNPV-37	KR815466	131,855	36,531	29,255	29,400	36,669	44.5%	156			
		AgMNPV-38	KR815467	130,740	36,194	29,012	29,172	36,362	44.5%	156			
		AgMNPV-39	KR815468	130,698	36,219	29,026	29,184	36,269	44.5%	157			
		AgMNPV-40	KR815469	132,180	36,542	29,409	29,583	36,646	44.6%	158			
		AgMNPV-42	KR815470	130,949	36,274	29,098	29,275	36,302	44.6%	157			
		AgMNPV-43	KR815471	132,077	36,539	29,369	29,528	36,641	44.6%	159			
	Antheraea pernyi NPV	AnpeNPV	NC_008035	126,629	29,513	34,041	33,664	29,406	53.5%	147	Sanger	ContigExpress9.1.0 + SeqMan5.0/ DNASTAR	[13]
	Autographa californica MNPV	AcMNPV	NC_001623	133,894	39,195	27,151	27,347	40,201	40.7%	156	Sanger	GCG package	[14]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Autographa californica MNPV-WP10	AcMNPV-WP10	KM609482	133,926	39,205	27,157	27,346	40,199	40.7%	151	Illumina HiSeq 2000	Newbler	[15]
	Bombyx mandarina NPV	BomaNPV	NC_012672	126,770	37,358	25,398	25,601	38,413	40.2%	141	Solexa GA	GENETYX-win Software + DNASTAR	[16]
	Bombyx mori NPV	BmNPV	NC_001962	128,413	37,747	25,828	26,056	38,782	40.4%	143	Sanger	DNASIS/PROSIS	[17]
	Catopsilia pomona NPV	CapoNPV	KU565883	128,058	38,938	25,348	25,444	38,328	39.7%	131	Roche 454 GS FLX+	GS *de novo* assembler	[10]
	Choristoneura fumiferana DEF MNPV	CfDEFMNPV	NC_005137	131,160	35,474	30,110	29,993	35,580	45.8%	149	Sanger	MacVector + Lasergene/ DNASTAR	[18]
	Choristoneura fumiferana MNPV	CfMNPV	NC_004778	129,593	32,224	32656	32,261	32,452	50.1%	146	Sanger	Gene Runner	[19]
	Choristoneura murinana NPV	ChmuNPV	NC_023177	124,688	31,408	30,986	31,370	30,924	50.0%	147	Roche 454	CLC Genomics Workbench	[20]
	Choristoneura occidentalis NPV	ChocNPV	NC_021925	128,446	32,108	31,905	32,481	31,952	50.1%	148	Roche 454 GS FLX	SeqMan Pro Lasergene/ DNASTAR	[21]
	Choristoneura rosaceana NPV	ChroNPV	NC_021924	129,052	33,309	31,261	31,425	33,057	48.6%	149			
	Condylorrhiza vestigialis MNPV	CoveMNPV	NC_026430	125,767	35,904	26,937	27,038	35,886	42.9%	138	Roche 454	Geneious + MIRA	[22]
	Dasychira pudibunda NPV	DapuNPV	KP747440	136,761	31,022	37,008	37,454	31,277	54.4%	161	Illumina MiSeq	Geneious	[23]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Ectropis obliqua NPV	EcobNPV	NC_008586	131,204	40,683	24,676	24,708	41,137	37.6%	126	Sanger	Genetyx-win	[24]
	Hyphantria cunea NPV	HycuNPV	NC_007767	132,959	36,031	30,039	30,465	36,424	45.5%	148	RISA-384	DNASIS	[25]
	Lonomia obliqua MNPV	LoobMNPV	KP763670	120,023	38,995	20,932	21,966	38,104	35.7%	134	Roche 454 GS FLX	Geneious	[26]
	Maruca vitrata MNPV	MaviMNPV	NC_008725	111,953	34,041	21,669	21,563	34,680	38.6%	126	Sanger	PHRED/PHRAP	[27]
	Orgyia pseudotsugata MNPV	OpMNPV	NC_001875	131,995	29,463	36,477	36,295	29,758	55.1%	152	Sanger	GCG package	[28]
	Philosamia cynthia ricini NPV	PhcyNPV	JX404026	125,376	28,966	33,461	33,809	29,140	53.7%	138	Sanger	N/A[1]	[29]
	Plutella xylostella MNPV	PlxyMNPV	NC_008349	134,417	39,437	27,303	27,396	40,281	40.7%	152	Sanger	Lasergene/ DNASTAR	[30]
	Rachiplusia ou MNPV	RoMNPV	NC_004323	131,526	39,674	25,630	25,793	40,429	39.1%	149	Sanger	Wisconsin package + Lasergene/ DNASTAR	[31]
	Thysanoplusia orichalcea NPV	ThorNPV	NC_019945	132,978	40,022	26,388	26,142	40,426	39.5%	145	Solexa GA	Edena	[32]
Alphabaculovirus (Group II)	*Adoxophyes honmai* NPV	AdhoNPV	NC_004690	113,220	36,505	20,025	20,328	36,362	35.6%	125	RISA-384	PHRED/PHRAP	[33]
	Adoxophyes orana NPV	AdorNPV	NC_011423	111,724	36,306	19,404	19,694	36,320	35.0%	121	Sanger	SeqMan II Lasergene/ DNASTAR	[34]
	Agrotis ipsilon MNPV	AgipMNPV	NC_011345	155,122	40,201	37,490	7,860	39,571	48.6%	163	Sanger	Lasergene/ DNASTAR	[35]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Agrotis segetum NPV	AgseNPV	NC_007921	147,544	40,237	33,200	34,247	39,860	45.7%	153	Sanger	Gap4	[36]
	Agrotis segetum NPV B	AgseNPV-B	NC_025960	148,981	40,490	33,698	4,371	40,422	45.7%	150	Roche 454	DNASTAR	[37]
	Apocheima cinerarium NPV	ApciNPV	NC_018504	123,876	41,223	20,865	20,449	41,332	33.4%	117	Sanger	SeqMan Pro Lasergene/ DNASTAR	unpublished
	Buzura suppressaria NPV	BusuNPV	NC_023442	120,420	37,568	22,152	22,142	38,558	36.8%	127	Roche 454 GS FLX	GS de novo assembler	[38]
	Chrysodeixis chalcites NPV	ChchNPV	NC_007151	149,622	45,151	29,304	29,060	46,107	39.0%	151	Sanger	Gap4	[39]
	Chrysodeixis chalcites SNPV	ChchSNPV-TF1-A	JX535500	149,684	45,090	29,324	29,133	46,137	39.1%	150	Roche 454	Newbler	[40]
		ChchSNPV-TF1-C	JX560539	150,079	45,146	29,384	29,096	46,447	39.0%	150			
		ChchSNPV-TF1-B	JX560540	149,080	44,989	29,152	28,987	45,952	39.0%	150			
		ChchSNPV-TF1-G	JX560541	149,039	45,075	29,136	28,869	45,958	38.9%	151			
		ChchSNPV-TF1-H	JX560542	149,624	45,162	29,285	29,034	46,143	39.0%	150			
	Clanis bilineata NPV	ClbiNPV	NC_008293	135,454	41,557	25,560	25,558	42,779	37.7%	129	Sanger	N/A	[41]
	Epiphyas postvittana NPV	EppoNPV	NC_003083	118,584	35,221	24,287	23,956	35,120	40.7%	136	Sanger	DNASTAR	[42]
	Euproctis pseudoconspersa NPV	EupsNPV	NC_012639	141,291	41,736	28,455	28,549	42,551	40.3%	139	Sanger	Wisconsin package + GENETYX-win	[43]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Helicoverpa armigera SNPV AC53	HaSNPV-AC53	NC_024688	130,442	39,121	25,389	25,606	40,326	39.1%	138	Ion Torrent PGM	CLC Genomics Workbench	[44]
	Helicoverpa armigera MNPV	HearMNPV	NC_011615	154,196	46,371	30,731	31,060	46,031	40.1%	162	Sanger	SeqMan 5.0/ DNASTAR	[45]
	Helicoverpa armigera NPV	HearNPV	NC_003094	130,759	39,345	25,340	25,552	40,522	38.9%	137	Sanger	Wisconsin package + Lasergene/ DNASTAR	[46, 47]
	Helicoverpa armigera NPV G4	HearNPV-G4	NC_002654	131,405	39,529	25,530	25,738	40,608	39.0%	135	Sanger	PHRED/PHRAP	[48]
	Helicoverpa armigera NPV NNg1	HearNPV-NNg1	NC_011354	132,425	39,754	25,791	26,054	40,826	39.2%	143	RISA-384	DNASIS	[49]
	Helicoverpa zea SNPV	HzSNPV	NC_003349	130,869	39,273	25,471	25,675	40,450	39.1%	139	Sanger	Wisconsin package + Lasergene/ DNASTAR	[50]
	Hemileuca sp. NPV	HespNPV	NC_021923	140,633	42,827	26,977	26,595	44,234	38.1%	137	Sanger	Wisconsin package + Lasergene/ DNASTAR	[51]
	Lambdina fiscellaria NPV	LafiNPV	NC_026922	157,977	45,363	34,616	34,350	43,648	43.7%	137	Roche 454	CLC Genomics Workbench	[52]
	Leucania separata NPV	LeseNPV	NC_008348	168,041	42,546	40,683	40,927	43,885	48.6%	169	MegaBACE 1000	DNASTAR	[53]
	Lymantria dispar MNPV	LdMNPV	NC_001973	161,046	34,229	46,226	46,331	34,260	57.5%	164	Sanger	GCG package	[54]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Lymantria dispar MNPV-27	LdMNPV-27	KP027546	164,158	35,020	47,133	47,118	34,887	57.4%	162	Illumina ZMiSeq	CLC Genomics Workbench	[55]
	Lymantria dispar MNPV-BNP	LdMNPV-BNP	KU377538	157,270	38,788	39,579	39,567	39,336	50.3%	154	Illumina MiSeq	Geneious	[56]
	Lymantria dispar MNPV-2161	LdMNPV-2161	KF695050	163,138	34,855	46,648	46,812	34,823	57.3%	174	Roche 454 GS Junior	SeqMan NGEN Lasergene/ DNASTAR	[9]
	Lymantria dispar MNPV-3029	LdMNPV-3029	KM386655	161,712	34,321	46,434	46,457	34,500	57.4%	163	Roche 454	Lasergene/ DNASTAR	[57]
	Lymantria dispar MNPV-45	LdMNPV-45	KU862282	161,006	34,234	46,192	46,314	34,264	57.5%	155	Illumina	CLC Genomics Workbench	[58]
	Lymantria dispar MNPV-3054	LdMNPV-3054	KT626570	164,478	35,151	47,119	47,140	35,068	57.3%	174	Roche 454 GS Junior	LaserGene/ DNASTAR	[59]
	Lymantria dispar MNPV-3041	LdMNPV-3041	KT626571	162,658	34,715	46,478	46,647	34,818	57.3%	178			
	Lymantria dispar MNPV-Ab-a624	LdMNPV-Ab-a624	KT626572	161,321	34,282	46,302	46,405	34,332	57.5%	176			
	Lymantria xylina MNPV	LyxyMNPV	NC_013953	156,344	36,207	41,674	41,933	36,530	53.5%	157	Sanger	PHRED/PHRAP	[60]
	Mamestra brassicae MNPV	MabrMNPV	NC_023681	152,710	46,042	30,311	30,604	45,753	39.9%	159	Roche 454	GS de novo assembler	[61]
	Mamestra configurata NPV-A	MacoNPV-A	NC_003529	155,060	45,336	32,160	32,463	45,101	41.7%	169	Sanger	Wisconsin package + Lasergene/ DNASTAR	[62]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Mamestra configurata NPV-B	MacoNPV-B	NC_004117	158,482	47,831	31,504	31,953	47,194	40.0%	168	Sanger	Sequencher 4.0	[63]
	Orgyia leucostigma NPV	OrleNPV	NC_010276	156,179	46,420	31,270	31,020	47,469	39.9%	135	Sanger	Agencourt BioScience	[64]
	Peridroma NPV	PespNPV	NC_024625	151,109	35,060	40,593	39,822	35,633	53.2%	139	Roche 454	CLC Genomics Workbench	[65]
	Perigonia lusca single NPV	PeluNPV	NC_027923	132,831	39,968	26,167	26,362	40,256	39.6%	145	Roche 454	Geneious	unpublished
	Pseudoplusia includens SNPV	PsinNPV	NC_026268	139,132	41,843	27,452	27,210	42,609	39.3%	141	Roche 454 GS FLX	MIRA	[66]
	Spodoptera exigua MNPV	SeMNPV	NC_002169	135,611	38,445	29,486	29,929	37,751	43.8%	139	Sanger	Wisconsin package + Lasergene/ DNASTAR	[67]
	Spodoptera frugiperda MNPV virus	SfMNPV	NC_009011	131,331	39,417	26,346	26,507	39,061	40.2%	143	Sanger	Lasergene/ DNASTAR	[68]
	Spodoptera litura MNPV	SpliMNPV-AN1956	JX454574	137,998	37,469	30,803	30,846	38,880	44.7%	132	Roche 454 GS Junior	LaserGene/ DNASTAR	[69]
	Spodoptera litura NPV	SpltNPV	NC_003102	139,342	39,180	29,691	29904	40,567	42.8%	141	MegaBACE1000	DNASIS + DNASTAR	[70]
	Spodoptera litura NPV II	SpltNPV-II	NC_011616	148,634	40,998	33,210	33,671	40,755	45.0%	147	n/a	N/A	unpublished
	Sucra jujuba NPV	SujuNPV	KJ676450	135,952	41,395	26,157	26,399	42,001	38.7%	131	Roche 454	GS de novo assembler	[71]
	Trichoplusia ni SNPV	TnSNPV	NC_007383	134,394	40,601	6,256	26,117	41,384	39.0%	145	Sanger	PHRED/PHRAP	[72]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
Betabaculovirus	*Adoxophyes orana granulovirus*	AdorGV	NC_005038	99,657	33,077	17,098	17,275	32,207	34.5%	119	Sanger	SeqMan II Lasergene/ DNASTAR	[73]
	Agrotis segetum granulovirus	AgseGV	NC_005839	131,680	41,892	25,179	23,953	40,656	37.3%	132	n/a	n/a	unpublished
	Clostera anastomosis GV isolate Henan	ClasGV-A	NC_022646	101,818	27,115	23,832	23,739	27,132	46.7%	122	Illumina GA	SOAPdenovo	[74]
	Clostera anastomosis granulovirus-B	ClasGV-B	KR091910	107,439	33,648	19,904	20,673	33,214	37.8%	123	Roche 454 GS FLX	Newbler	[75]
	Cnaphalocrocis medinalis GV	CnmeGV	NC_029304	111,246	36,021	19,756	19,385	36,084	35.2%	118	Roche 454 GS FLX	GS de novo assembler	[76]
	Cnaphalocrocis medinalis granulovirus	CnmeGV	KP658210	112,060	36,295	19,904	19,529	36,332	35.2%	133	PacBio RS II	HGAP2.2.0	[77]
	Choristoneura occidentalis GV	ChocGV	NC_008168	104,710	36,132	17,268	16,938	34,372	32.7%	116	Sanger	PHRED/PHRAP	[78]
	Clostera anachoreta granulovirus	ClanGV	NC_015398	101,487	28,188	22,554	22,523	28,222	44.4%	123	Illumina GA	SOAPdenovo	[79]
	Cydia pomonella granulovirus	CpGV	NC_002816	123,500	34,029	27,722	28,183	33,566	45.3%	143	Sanger	Wisconsin package + Lasergene/ DNASTAR	[80]
	Cryptophlebia leucotreta granulovirus	CrleGV	NC_005068	110,907	38,095	18,090	17,890	36,832	32.4%	128	Sanger	Lasergene/ DNASTAR	[81]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Diatraea saccharalis granulovirus	DisaGV	NC_028491	98,392	32,133	17,032	17,337	31,880	34.9%	125	Roche 454	Geneious	[82]
	Epinotia aporema granulovirus	EpapGV	NC_018875	119,082	35,524	24,984	24,403	34,171	41.5%	132	Roche 454 GS FLX	Newbler	[83]
	Erinnyis ello granulovirus	ErelGV	NC_025257	102,759	31,707	19,440	20,324	31,288	38.7%	130	Roche 454 GS FLX	Geneious	[84]
	Helicoverpa armigera granulovirus	HearGV	NC_010240	169,794	50,336	34,518	34,810	50,130	40.8%	179	Sanger	SeqMan Lasergene/ DNASTAR	[85]
	Plodia interpunctella granulovirus	PiGV	KX151395[2]	112,536	n/a	n/a	n/a	n/a	n/a	123	Roche 454 GS Junior	SeqMan NGEN Lasergene/ DNASTAR	[86]
	Phthorimaea operculella granulovirus	PhopGV	NC_004062	119,217	38,306	21,127	21,431	38,353	35.7%	130	Sanger	N/A	[87]
	Plutella xylostella granulovirus	PlxyGV	NC_002593	100,999	30,252	20,546	20,546	29,655	40.7%	120	DSQ-1000 L	GENETYX-win	[88]
	Pieris rapae granulovirus	PrGV	NC_013797	108,592	36,619	17,863	18,168	35,942	33.2%	120	Sanger	NN/A	[89]
	Pseudaletia unipuncta granulovirus	PsunGV	NC_013772	176,677	53,572	34,993	35,311	52,799	39.8%	183	n/a	N/A	unpublished
	Spodoptera frugiperda GV isolateVG008	SpfrGV	NC_026511	140,913	38,131	32,852	32,288	37,642	46.2%	146	Roche 454 GS FLX	Newbler	[90]

Genus	Virus	Virus Abbreviation	GenBank accession	Genome size (bp)	A	C	G	T	GC content	ORFs	Sequencing	Assembler	Reference
	Spodoptera litura granulovirus	SpliGV	NC_009503	124,121	38,360	23,813	24,377	37,571	38.8%	136	Sanger	N/A	[91]
	Xestia c-nigrum granulovirus	XcGV	NC_002331	178,733	53,166	36,079	36,627	52,861	40.7%	181	Sanger	DNASIS/PROSIS	[92]
Gammabaculovirus	*Neodiprion abietis* NPV	NeabNPV	NC_008252	84,264	28,292	13,948	14,177	27,847	33.4%	93	Sanger	PHRED/PHRAP	[93]
	Neodiprion lecontei NPV	NeleNPV	NC_005906	81,755	27,741	13,596	13,640	26,616	33.4%	89	Sanger	SeqMan Lasergene/ DNASTAR	[94]
	Neodiprion sertifer NPV	NeseNPV	NC_005905	86,462	29,158	14,444	14,745	28,115	33.8%	90	Sanger	Sequencher 4.1	[95]
Deltabaculovirus	*Culex nigripalpus* NPV	CuniNPV	NC_003084	108,252	26,623	27,228	27,839	26,562	50.9%	109	Sanger	CAP3	[96]

N/A: no information is available either in the paper or GenBank file.
The GenBank file with accession number KX1513952 is not available in GenBank website.

Table 1. List of sequenced baculoviruses genomes.

(*polh*) were found in a highly conserved genes among baculoviruses [110], therefore, used as targets for degenerating PCR to characterize lepidopteran NPVs through the amplification of the conserved regions from a variety range of baculoviruses [111–113]. The Kimura 2-parameter (K-2-P) distances between the aligned *polh*/*gran*, *lef-8* and *lef-9* nucleotide sequences were described by Jehle et al. for baculoviruses identification and species classification [3]. The K-2-P nucleotide substitution model from aligned nucleotide sequences were determined by using the pairwise distance calculation of MEGA version 3.0 applying the Kimura 2-parameter model [114].

Due to the higher cost of NGS for viral genome sequencing, it is frequently required to combine various approaches to cut down the cost but still ensure precision, e.g., PCR-based K-2-P analysis and NGS approach for identifying the potential new NPV species. Two NPVs were isolated from casuarina moth (*Lymantria xylina*) and golden birdwing larvae (*Troides aeacus*) collected from the fields, respectively, will be as representative cases for explanation in the following sections. We will focus on the characterization of these two potential new NPVs first and then the use of the sequences of three genes, *lef-8*, *lef-9* and *polyhedrin* of two NPV candidates was used to examine their taxonomic position by K-2-P analysis. Finally, we will focus on the genome sequencing technology and bioinformatic analysis on NPVs.

3. The identification of ambiguous NPVs

In this section, the discussion of molecular identification of NPV species based on K-2-P distance [3] is presented. Two new NPVs were used as examples in this study to reveal different issues regarding the classification of NPVs.

3.1. LdMNPV-like virus

The K-2-P distances, based on the sequences of three genes, between different viruses could mostly evaluate the ambiguous relationship among the NPVs. It was defined that distances less than 0.015 indicates that the two isolates are the same baculovirus species. On the other hand, the difference between two viruses is more than 0.05 should be considered as different virus species. For the distances between 0.015 and 0.05, complementary information is needed to determine whether these two viruses are of the same or different species [3, 9, 115].

A new multiple nucleopolyhedrovirus strain was isolated from casuarina moth, *L. xylina* Swinhoe, (Lepidoptera: Lymantriidae) in Taiwan. Since the *polyhedrin* sequence of this virus had high identity to *L. dispar* MNPV (98%), it was named LdMNPV-like virus [116]. To precisely clarify the relationship of three Lymantriidae-derived NPVs (LdMNPV-like virus, LdMNPV and LyxyMNPV [60]), the K-2-P of *polh*, *lef-8* and *-9* was performed. The distances between LdMNPV-like virus and LyxyMNPV exceeded 0.05 for each gene, *polh*, *lef-8*, or *lef-9* and also for concatenated *polh*/*lef-8*/*lef-9* (**Figure 1**). For LdMNPV-like virus and LdMNPV, not only the single *lef-8* and *lef-9* sequences but also concatenated *polh*/*lef-8*/*lef-9*, the distances were generally lower than 0.015, but only the *polh* sequence distance (0.016) exceeded slightly

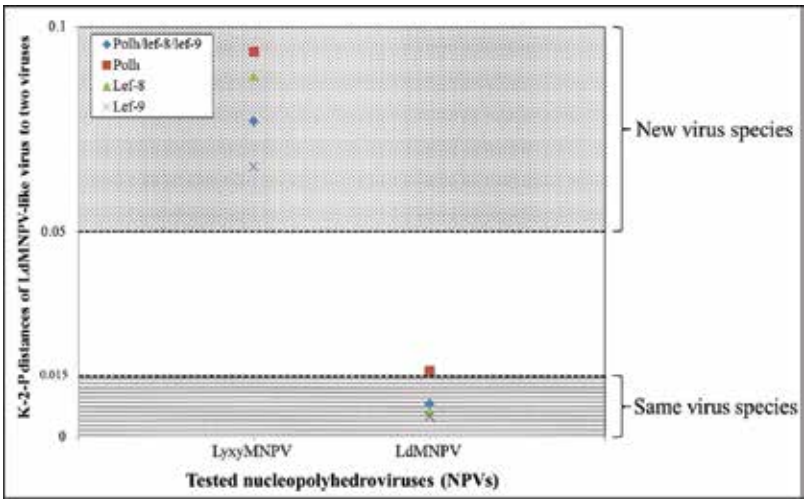

Figure 1. Pairwise K-2-P distances of the nucleotide sequences of *polh*, *lef-8* and *lef-9* and concatenated *polh*/*lef-8*/*lef-9* fragments of LdMNPV-like virus, LyxyMNPV and LdMNPV. Modified data reproduced with permission of the Elsevier [116].

0.015 (**Figure 1**). These results strongly suggested that LdMNPV-like virus is an isolate of LdMNPV. However, as indicated by our previous report, the genome of LdMNPV-like virus is approximately 139 Kb, due to large deletions compared to that of LdMNPV [116]. To further investigate the LdMNPV-like virus, a *Hin*dIII-*Pst*I fragment (7,054 nucleotides) was cloned, sequenced and compared to the corresponding region of LdMNPV. Nine putative ORFs (including seven with full lengths and two with partial lengths) and two homologous regions (hrs) were identified in this fragment (**Figure 2**) and those genes, in order from the 5′ to 3′ end, encoded part of *rr1*, *ctl-1*, *Ange-bro-c*, LdOrf151, LdOrf-152-like peptides, *Ld-bro-n*, two *Ld-bro-o* and part of LdOrf155-like peptides (**Table 2**). The physical map of *Hin*dIII-*Pst*I fragment of LdMNPV-like virus showed that the gene organization was highly conserved compared to the corresponding region of LdMNPV, although several restriction enzyme recognition sites were different. Additionally, the *ld-bro-o* gene in the LdMNPV-like virus was split into two ORF7 and ORF8, due to a point deletion in the downstream (+669) of ORF7 and this deletion causes a frameshift that results in the formation of a stop codon (TGA) after 73 bp. Afterward, ORF8 was overlapped with the last four base pairs (ATGA) in ORF7. The nucleotide identities of these genes were 96–100% homologous to those of LdMNPV, except ORF3 which was 68% homologous to *Ange-bro-c* and ORF7 and ORF8 showing low identities to *Ld-bro-o* (73% and 26%, respectively). The deduced amino acid sequences of these genes were similar to those of LdMNPV, with identities of 81–100%, except the similarity of ORF3 to *Ange-bro-c* was 70% and ORF7 and ORF8 also showed low similarity to *Ld-bro-o* (67% and 26%, respectively). These results imply that the LdMNPV-like and LdMNPV viruses are closely related but not totally identical.

Based on these results, LdMNPV-like virus has a genomic size significantly smaller than that of LdMNPV and LyxyMNPV and appears to be an NPV isolate distinct from LdMNPV or LyxyMNPV. Moreover, a gene, *ange-bro-c* of LdMNPV-like virus, was truncated into two ORF7

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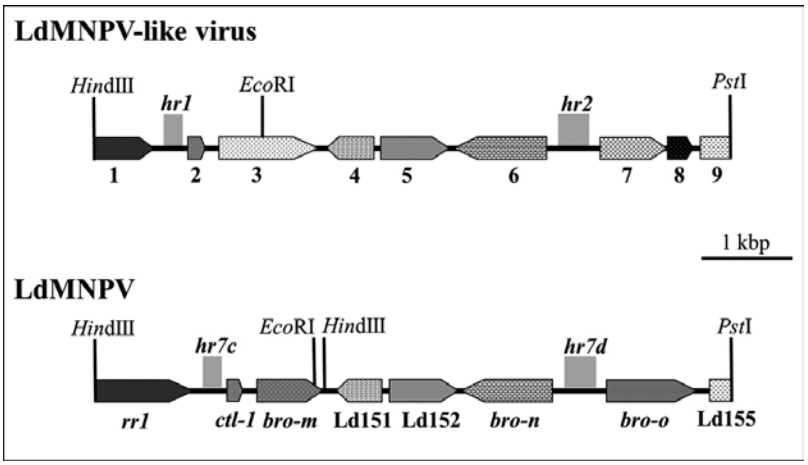

Figure 2. Comparison of relative restriction sites and gene locations in the LdMNPV-like virus *Hind*III-*Pst*I fragment with those of the corresponding LdMNPV fragment. Arrows denote ORFs and their direction of transcription. Gray boxes represent the homologous repeat regions (hrs). ORF homologues in the corresponding regions are drawn with the same patterns. Numbers below the arrows indicate the nine putative ORFs listed in **Table 2**.

and ORF8 and the sequence showed relatively low identity to that of LdMNPV (**Table 2**). Taken together, these results indicate that LdMNPV-like virus is a distinct LdMNPV strain with several novel features. Otherwise, LdMNPV-like virus and LdMNPV have distinct geographical locations (from subtropical and cold temperate zones, respectively) and are

No*	LdMNPV-like virus			LdMNPV§		
	Position†	Length		Name	Identity (%)	
		nt	aa		nt	aa
1	1 → 654	654	217	rr1	96	81
2	1063 → 1224	162	53	Ctl-1	100	100
3	1397 → 2473	1077	358	Ange-bro-c	68	70
4	2590 → 3596	504	168	LdOrf-151	99	98
5	3200 → 3952	753	251	LdOrf-152	99	99
6	4019 → 5026	1005	335	Ld-bro-n	93	91
7	5645 → 6391	744	248	Ld-bro-o	73	67
8	6388 → 6654	264	88	Ld-bro-o	26	26
9	6758 → 7054	297	99	LdOrf-155	100	100

†The directions of the transcripts are indicated by arrows.

§Reference from the genome of LdMNPV (Kuzio et al. [63])

*The nine potentially expressed ORFs are numbered in the order in which they occur in the LdMNPV-like virus genomic fragment from the 5′ to 3′ end. Two ORFs extend past this cloning site are printed in bold; only the N-terminus which contains 217 amino acids (654 nucleotides) and 99 amino acids (297 nucleotides) was examined.

Table 2. Comparison of the nucleotide (nt) and deduced amino acid (aa) sequences for putative ORFs in LdMNPV-like virus genomic fragment and their corresponding LdMNPV homologues.

distinct in genotypic and phenotypic characteristics and it also showed broad genetic variation among LdMNPV isolates [9].

3.2. An NPV isolate from *T. aeacus* larvae

A nucleopolyhedrosis disease of the rearing of the golden birdwing butterfly (*T. aeacus*) larvae was found and the polyhedral inclusion bodies (PIBs) were observed under light microscopy (**Figure 3**). PCR was performed to amply the *polh* gene by 35/36 primer set (**Figure 3**) to further confirm NPV infection [117, 118]. Therefore, this NPV was named provisionally TraeNPV. The three genes, *polh*, *lef-8* and *lef-9* of TraeNPV, were cloned and sequenced and then the K-2-P distances between the aligned single and concatenated *polh*, *lef-8* and *lef-9* nucleotide sequences were analyzed. The results indicated that TraeNPV belonged to the group I baculoviruses and closely related to BmNPV group. **Figure 4** showed that most of the distances between TraeNPV and other NPVs were between 0.015 and 0.050, whereas the distances for *polh* between TraeNPV, PlxyNPV, RoNPV and AcMNPV group exceeded 0.05. It should be noted that for all the concatenated *polh/lef-8/lef-9* sequences, the distances were apparently much more than 0.015 and even to 0.05. These results left an ambiguous situation of this NPV isolate; so far, we could conclude that TraeNPV neither belongs to BmNPV group nor AcMNPV group. More complementary information is needed to determine the viral species of TraeNPV.

In summary, K-2-P distances were employed to further clarify the relationship between closely related NPVs. We discussed two different cases analyzed by K-2-P. From the sequence data

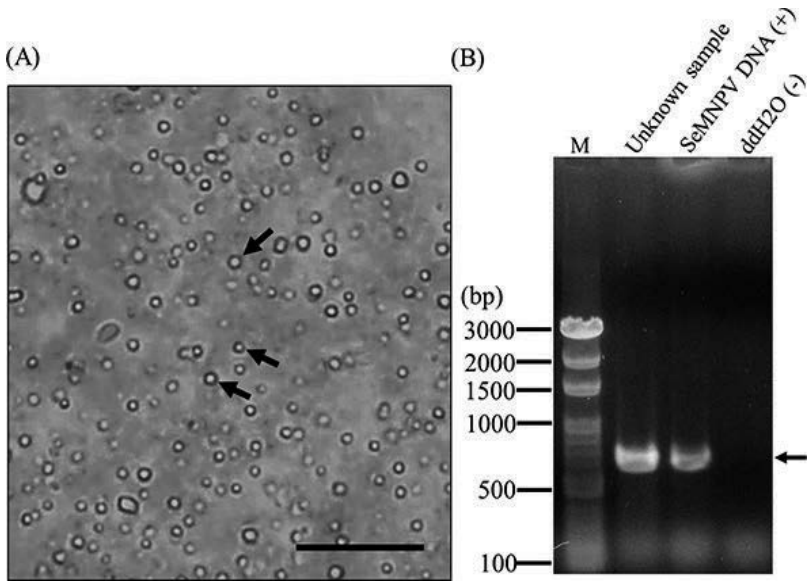

Figure 3. Identification of unknown NPV. (A) Light microscopy observation of liquefaction from the cadavers of *T. aeacus* larvae, scale bar = 20 μm. Black arrows indicated the polyhedral inclusion bodies (PIBs). (B) PCR detection of partial *polyhedrin* gene, M = 100 bp marker, (+) = positive control and (−) = negative control.

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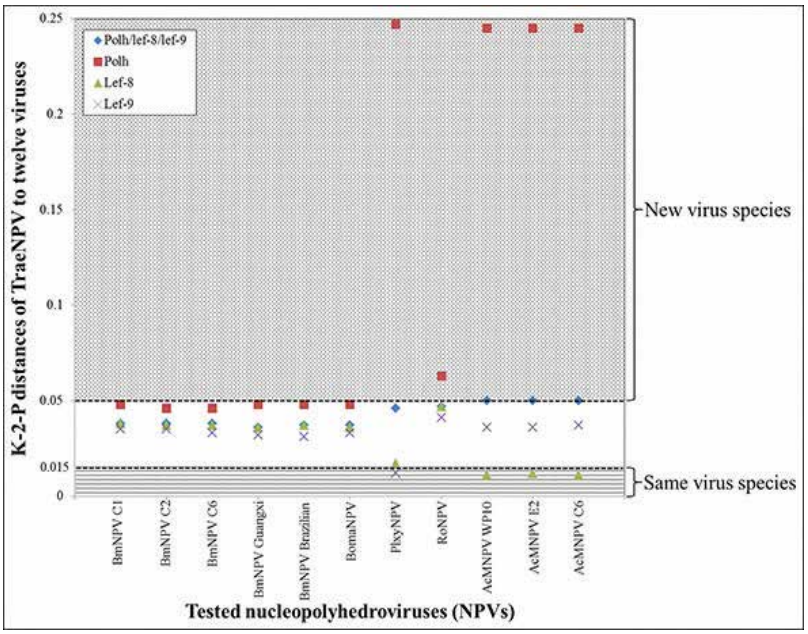

Figure 4. Pairwise Kimura-2-parameter distances of the nucleotide sequences of *lef-8*, *lef-9* and *polh* and concatenated *polh*/*lef-8*/*lef-9* fragments of TraeNPV and 12 viruses.

of LdMNPV-like virus, results strongly supported that LdMNPV-like virus is an isolate of LdMNPV. Since the RFLP profiles of the LdMNPV-like virus showed the genome of this isolate was deleted tremendously, this deletion also showed coordinately in our partial sequences of genomic DNA fragments and the results of K-2-P. The K-2-P distances between TraeNPV and BmNPV or AcMNPV were among 0.05 and 0.015. Anyway, we cannot define that this virus is a new species with the evidences of RFLP, part gene sequences and K-2-P results; therefore, it is necessary to get more data, especially the whole genome sequence of TraeNPV.

4. The importance of whole genome sequencing on baculoviruses

The rapidly growing mass of genomic data shifts the taxonomic approaches from traditional to genomically based issues. The K-2-P distance supported LyxyMNPV as a different viral species (K-2-P values = 0.067–0.088), even though they were still a closely relative species phylogenetically. But, "how different did LyxyMNPV and LdMNPV?" become another question. Thus, the whole genome sequence could provide deep information of this virus. For example, as the genomic data revealed, the most part of the ORF (151 ORFs) between LyxyMNPV and LdMNPV was quite similar while still have several different ORF exhibits or absent in LyxyMNPV, e.g., two ORFs were homologous to other baculoviruses and four unique ORFs were identified in the LyxyMNPV genome and LdMNPV contains 23 ORFs that are absent in LyxyMNPV [60]. Besides, there is a huge genomic inversion in LyxyMNPV compared to LdMNPV [60]. Another example is *Maruca vitrata* NPV (MaviNPV). All of the K-2-P distance-

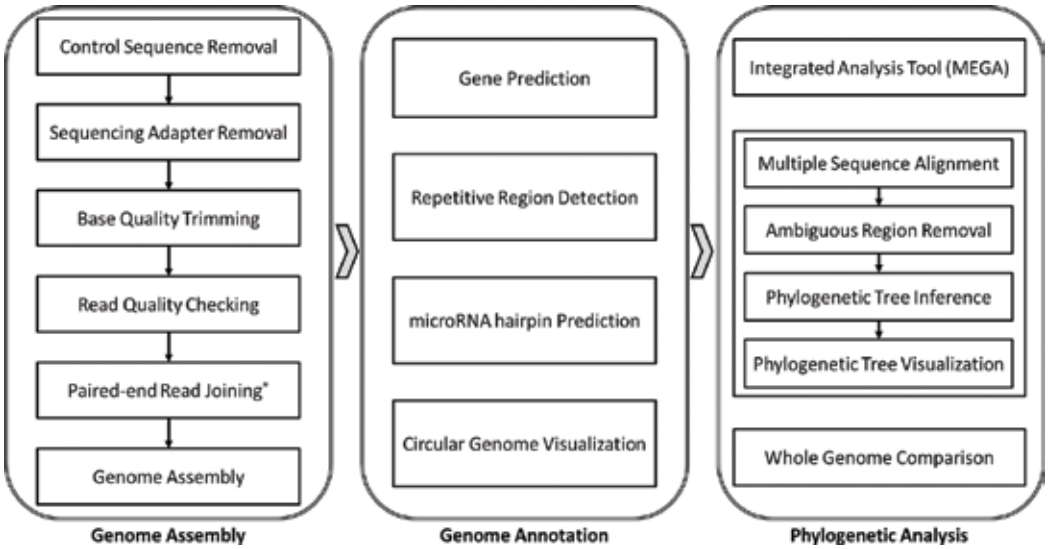

Figure 5. Pairwise Kimura-2-parameter distances of the nucleotide sequences of lef-8, lef-9 and polh and concatenated polh/lef-8/lef-9 fragments of MaviNPV and 12 viruses.

supported MaviNPV is quite different from other NPVs (K-2-P values = 0.092–0.237) (**Figure 6**). While the gene content and gene order of MaviNPV were highly similar to that of AcMNPV and BmNPV, through the genomic sequencing, it showed the 100% collinear to AcMNPV [27] and MaviNPV shared 125 ORFs with AcMNPV and 123 with BmNPV. The detailed information could only be captured after whole genome sequencing rather than partial gene

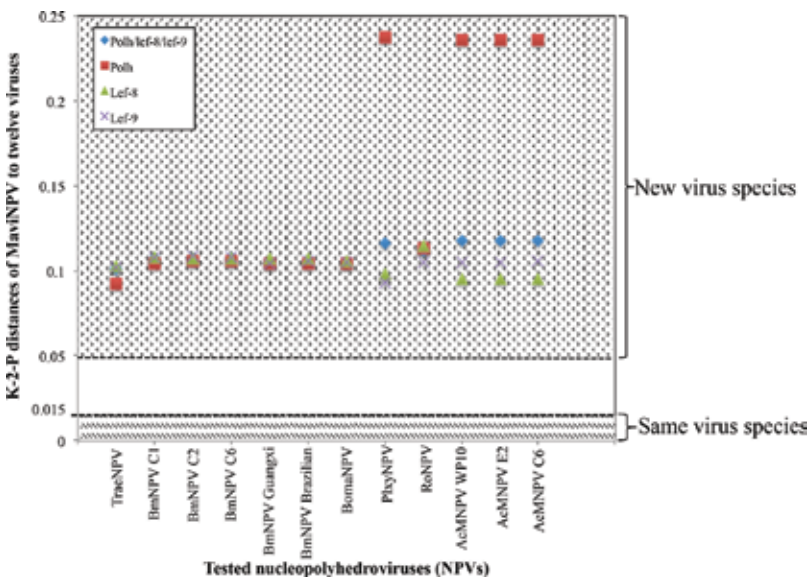

Figure 6. Common bioinformatic workflow for genome assembly and analysis.

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sequences or other phylogenetic analyses. Sometimes, usage of K-2-P data may raise other problems, which we mentioned above; it seems LdMNPV-like virus and LdMNPV were the same viral species. While through the restriction enzyme profile and partial genomic data, we could identify that there are some deletion fragments and different gene contents within the LdMNPV-like virus genome. For the TraeNPV, most of the K-2-P values were ranged from 0.015 to 0.05; thus, whole genome sequencing could be one of the best ways to figure out this ambiguous state. The more detailed information we can get, the more deep aspect we can evaluate, e.g., the taxonomic problems and further evolutionary studies.

5. Genomic sequences of NPVs

5.1. Genome sequencing technology

Previous NPV genome sequencing employed three types of approaches: plasmid clone (or template) enrichment, NGS, or a combination of the two methods. Initially, the most common approach used restriction enzymes to fragmentize the viral genome into smaller pieces. Plasmid-based clone amplification was then employed to enrich templates for sequencing. Later, conventional Sanger sequencing and/or next-generation sequencing was employed for genome assembly. In addition, purely high-throughput sequencing-based approach from isolated viral genome was also employed [9, 15]. To date, next-generation sequencing technology plays an increasingly important role on viral genome assembly. Previous researches showed that Illumina HiSeq has superior performance in yield than 454 FLX [119–121]. Baculoviruses usually contain a novel homologous region (hr) feature, which comprises a palindrome that is usually flanked by short direct repeats located elsewhere in the genome [122]. Thereby, the shorter single-read length of Illumina sequencers might lead the difficulty during genome assembly. Further application of paired-end read sequencing method could certainly provide alternative for sequencing overlap the hrs in baculoviral genomes.

5.2. Bioinformatic analysis

Construction of a complete genome map is essential for future genomic investigations. Besides sequencing, bioinformatic approaches are also required for determining the order and content of the nucleotide sequence information for the viral genome of interest. In general, bioinformatic approaches can be separated into three consecutive steps: genome assembly, genome annotation and phylogenetic relationship inference (**Figure 5**).

5.2.1. Genome assembly

Sequence reads are the building blocks for genome sequencing and assembly. Thus, quality control of sequence reads plays a key role in determining the fidelity of a genome assembly. The procedure of read quality checking includes, but not limited to, the removal of unrelated sequences such as control sequences, adaptors, vectors, potential contaminants, etc., trimming of low-quality bases and selection of high-quality reads. The control sequences (e.g., PhiX control reads in Illumina sequencers, control DNA beads in Roche 454 sequencer) are routinely

used by sequencer manufacturers to evaluate the quality of each sequencing run. There are software applications made available to be utilized to identify and remove control sequences and low-quality bases. For NGS, sequencing adapters could be identified in reads if the fragment size is shorter than read length. Cutadapt [123] was implemented to trim the adapter sequences. Ambiguous bases or bases with lower-quality values can be removed by PRINSEQ [124] from either 5′ or 3′ end. NGS QC Toolkit [125] has programmed module to select high-quality reads. If paired-end technology was applied, paired-end reads could be joined by PANDAseq [126], PEAR [127], FLASH [128] and COPE [129], if a fragment size is shorter than read length.

Genome can be assembled from quality paired-end or single-end reads with de novo or reference-guided approaches. There are two standard methods known as the de Bruijn graph (DBG) approach and the overlap/layout/consensus (OLC) approach for de novo genome assembly. The idea of de Bruijn graph is to decompose a read into kmer-sized fragments with sliding window screening. Each kmer-sized fragment will be used to construct graph for longer path (e.g., contigs). Then, long-range paired reads can be utilized to build scaffolds from contigs with given insert size and read orientation. SOAPdenovo [130] is one of the DBG assembler that has an extreme speed by utilizing threads parallelization [131]. The OLC assembler starts by identifying all pairs of reads with higher overlap region to construct an overlap graph. The contig candidates are identified by pruning nodes to simplify the overlap graph. The final contigs are then output based on consensus regions. Additionally, Newbler [132] is a widely used OLC assembler distributed by 454 Life Sciences.

Reference-guided genome assembly is another solution for genome assembly if the genome of a closely related species is already available. For viral genome assembly, closely related species can be identified by mapping quality reads against sequenced viral genomes deposited in GenBank (http://www.ncbi.nlm.nih.gov/genome/viruses/) and select top-ranked species as the reference genome(s) to facilitate the assembly of the genome of interest. Reference-guided assembler is also called mapping assembler that the complete genome is generated by mapping quality reads with variant (single nucleotide polymorphism (SNP), insertion and deletion) identification. For example, MIRA (a computer program) [133] can create a reference-based assembly by detecting the difference between references.

During the assembly process, gap filling (or gap elimination) is conducted to resolve the undetermined bases either by bioinformatics or other approaches such as PCR and additional sequencing. Bioinformatic approaches normally use paired-end reads to eliminate gaps. PCR coupled with Sanger sequencing is a common approach to finalize the undetermined regions [134]. In addition, Sanger sequencing can also be used for genome validation and homologous region (hr) checking.

5.2.2. Genome annotation

Annotation determines the locations of protein-coding and noncoding genes as well as the functional elements in the genome. Glimmer [135], N-SCAN [136], NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), GeneMark [137] and VIGOR [138] are gene prediction tools for identifying protein-codivng genes in the genome. Repetitive sequence regions were detected by

RepeatMasker (http://www.repeatmasker.org/). Viral microRNA candidate hairpins can be predicted by Vir-Mir [139]. The circular map of the viral genome was generated by CGView [140].

5.2.3. Phylogenetic analysis

Phylogenetic relationship inference reveals the evolutionary distances of various, especially closely related, species. MEGA [141] was the most widely used software suite that provides the sophisticated and integrated user interface for studying DNA and protein sequence data from species and populations. Alternatively, phylogenetic relationships among species based on the complete viral genomes or functional regions could also be estimated with Clustal Omega [142]. Clustal Omega was employed for multiple sequence alignment on the complete genomes and DNA fragments, respectively. ClustalW [143] was employed to do file format conversion of multiple sequence alignment. Ambiguously aligned positions were removed by using Gblocks version 0.91b [144, 145] under default settings. Phylogenetic tree inference could be constructed by hierarchical Bayesian method (e.g., MrBayes [146]) or maximum likelihood method (e.g., RAxML [147]) to estimate phylogeny [148]. Tree was depicted with FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The divergence times of different species were estimated using BEAST version 1.8 or version 2.3.2 [149]. In addition, pairwise sequence identity was determined by BLASTN (NCBI BLAST Package) [150] to analyze sequence-level variation. Also, whole genome pairwise alignment can be done by LAGAN [151]. CGView comparison tool (CCT) [152] was used to represent the block similarity among different species. Mauve [153], one of the multiple genome alignment tools, can help us to visualize the consensus sequence blocks among distant-related species.

Up to 78 baculoviruses have been reported; most of baculoviruses have a narrow host range, only infect their homogenous hosts, such as BmNPV, SpltNPV, SpeiNPV, MaviNPV and so on; LyxyNPV can infect LD and LY cell lines, while AcMNPV has a wide host range; at least 40 hosts in vitro have be found. Therefore, a new baculovirus isolate needs to define its taxonomic position and to analyze its phylogenetic relationship with a known baculovirus member.

6. Conclusion

With the accomplishment of the sequencing technologies, more NPV genomes were sequenced. So far, more than 78 baculoviruses have been fully sequenced and based on the sequencing methods, we can divide into two parts, one is sequencing by Sanger method and another is sequencing by NGS method (**Table 1**). Among these sequenced genomes, 35 genomes were sequenced by Sanger method and 43 genomes were sequenced by NGS methods. It could be expected that whole genome sequencing by NGS method would get much common in this field; however, the upcoming metagenomic era is imperative that one remains aware of and careful about the shortcomings of the information presented about the organisms that are being sequenced and that these databases can oversee neither the correctness of the organismal identifications nor of the sequences entered into the databases.

The natural environment harbors a large number of baculoviruses. However, only a few of them have been sequenced and studied. A lot more information related to the genetic relationship of NPVs in the natural environment is needed to facilitate our understanding of these creatures. Though NGS technology has become an important technology for viral genomic sequencing, high cost of NGS for whole viral genome sequencing remains a barrier. To reduce the cost, it is necessary to evaluate whether the newly collected NPVs are suitable for whole genome sequencing or not. Alternatively, biochemical approaches and biological tools, such as PCR-based K-2-P analysis, can be good options to facilitate the process. As expected, all these applications are anticipated to help us reveal the genetic information of unknown species, so that more detailed insights of their genetic makeup and functional composition can be obtained to help us better understand the nature of these viruses. By using the powerful sequencing technique, the metagenomic progress (e.g., transcriptome analysis of insect host), new pathogen species in the natural environment would be easier to be found in the future. With the increase of new baculoviral genomic data, improvement of bioinformatic analysis methods and further validation of biological information would generate a group of genes, which connect to the viral host range and solve the contradiction situation in the baculoviral genomics.

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Chapter 9

Detection of *Yersinia pseudotuberculosis* in Apollo Butterfly (*Parnassius apollo*, Lepidoptera: Papilionidae) Individuals from a Small, Isolated, Mountain Population

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Additional information is available at the end of the chapter

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Abstract

Yersinia pseudotuberculosis is a bacterium pathogenic to humans and other mammals; however, its insecticidal activity has also been documented in laboratory studies. A small population of Apollo butterfly (*Parnassius apollo*), reconstituted from less than 30 individuals in 1990s, occurs in Pieniny National Park (Poland). In this report, we demonstrate that a DNA fragment specific to *Y. pseudotuberculosis* could be detected in 40% of biological samples isolated from insects belonging to the Apollo butterfly population. Although *Y. pseudotuberculosis* DNA occurred in both normal and malformed insects, the difference between the fractions of infected individuals was statistically significant ($p = 0.044$ in the Fisher's exact test). No such DNA could be detected in analogous samples from other butterflies (*Pieris napi*, *Pieris rapae, and Zerynthia polyxena*) occurring in separate habitats (either a meadow near the city of Cracow, Poland, or in a mountain region of Greece). It is suggested that infection with *Y. pseudotuberculosis* might weaken the general condition of the *P. apollo* population from Pieniny and contribute to the appearance of developmental abnormalities of the butterflies. Thus, it appears that *Y. pseudotuberculosis* infections of insects may be of biological significance in natural environment.

Keywords: Apollo butterfly, deformed wings, reduced wings, *Yersinia pseudotuberculosis*, isolated butterfly population

1. Introduction

Yersinia pseudotuberculosis is a bacterium pathogenic to humans and other mammals. However, it also reveals insecticidal activity due to the production of specific toxins [1, 2]. Infections with this bacterium cause a serious, often deadly, disease of various insects, including species belonging to Lepidoptera [3, 4]. Nevertheless, reports indicating occurrence of *Y. pseudotuberculosis* in insects and its pathogenicity to these hosts are based on laboratory, rather than environmental, studies.

Parnassius apollo (Lepidoptera: Papilionidae), known as Apollo butterfly, is a rare, seriously endangered species. It is often considered as near threatened [5], despite the fact that its population in Europe was relatively large for some 100 years ago [6]. While reason(s) for *P. apollo* extinction are debatable, and only partially explained [7], various programs for saving and reconstitution of this butterfly have been established. In Pieniny National Park (Poland), the population of Apollo butterfly declined to less than 30 individuals at the beginning of the last decade of twentieth century [8]. Nevertheless, a specific program allowed to enlarge this population significantly [9]. On the other hand, surprisingly frequent appearance of malformed butterflies has been noted [10]. Such insects occurred in the natural environment of Pieniny National Park, but this phenomenon was more pronounced in the reared population, kept in seminatural conditions in order to increase the number of *P. apollo* individuals (most probably, malformed insect died and/or were eaten in the natural environment). The most striking malformed phenotypes include deformation and reduction of wings [10]. Examples of malformed individuals, in comparison with the normal one, are depicted in **Figure 1**.

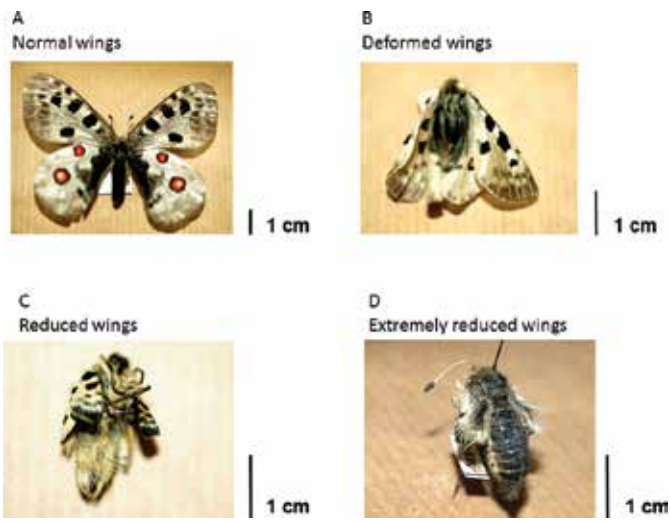

Figure 1. Examples of *P. apollo* individuals with different patterns of wings: normal (A, wings characteristic for healthy butterflies), deformed (B, wings of the size similar to normal, but with changed shape and arrangement), reduced (C, wings smaller than normal, sometimes with different morphology), and extremely reduced (D, very small wings, resembling buds rather than mature organs, sometimes almost invisible). Photographs made by the authors.

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Until recently, the cause of the malformations in *P. apollo* from Pieniny was unknown. However, when genetic materials from normal and malformed insects were compared, some significant differences could be identified. In butterflies with deformed or reduced wings, mutations in the *wingless* gene, coding for a protein involved in wing development, were found to be common [11]. Deficiency in laccases, enzymes which are involved in detoxification of some compounds found in normal diet of caterpillars, was significantly more frequent in malformed than in healthy butterflies [12]. Moreover, many individuals with deformed or reduced wings did not contain *Wolbachia*, a prokaryotic symbiont that can modulate some important physiological processes in insects [13]. These results indicate that there are genetic, biochemical, and microbiological reasons for malformations of wings in the isolated population of *P. apollo*. On the other hand, statistical analyses indicated that none of the mentioned reasons can be considered a sole cause of the developmental changes [11–13]. Therefore, further studies on this phenomenon appear to be warranted. In this report, we present evidence that a considerable fraction of the population of Apollo butterfly from Pieniny is infected with *Yersinia pseudotuberculosis*.

2. Materials and methods

2.1. Insects

Insects used in this work were either withdrawn from a meadow near the city of Cracow, Poland (individuals of *P. napi*, *P. rapae*), taken from a mountain region in Greece and obtained from a private collection of butterflies (individuals of *Z. polyxena*) or obtained from the collection of dried insects of Pieniny National Park (individuals of *P. apollo*). The permission for the use of this material has been obtained from the Director of Pieniny National Park (permission no. PB-5232-24/07, topic ID: p0748). For DNA isolation, a material from 3 specimens of *P. napi*, 4 of *P. rapae*, and 2 of *Z. polyxena,* and 15 of *P. apollo* was used. Among *P. apollo* individuals, 12 had normal wings and 3 had malformed wings.

2.2. DNA isolation and amplification

A material extracted from legs of investigated insects was used for DNA studies. This material was subjected to wash using deionized water before the procedure to avoid environmental contamination. The procedure was conducted by employing the Sherlock AX Purification Kit (A&A Biotechnology), according to the manufacturer's instruction. Following PCR-mediated amplification of specific DNA fragments (using primers listed in **Table 1**), they were separated by agarose gel electrophoresis and analyzed as described previously [14].

2.3. DNA cloning and sequencing

Selected products of DNA amplification were cloned into a plasmid vector by using the TOPO TA Cloning Kit Dual Promoter (with pCR II-TOPO vector) with One Shot TOPO10F′ Chemically Competent *Escherichia coli* (Invitrogen). DNA sequencing was conducted com-

mercially in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Warsaw, Poland).

Gene	Primers (forward and reverse)	References
dpp	5′ AGA GAA CGT GGC GAG ACA CTG	[24]
	5′ GAG GAA AGT TGC GTA GGA ACG	
hh	5′ AAG GAA AAA CTG AAT ACG CTG GC	[24]
	5′ CGA GAC GCC CCA ACT TTC C	
ptc	5′ CTC CGA AGA AGG TCT GCC GCA AG	[24]
	5′ AAT TCG TGC TCG TCG TAT TTT C	
inv	5′ TAA GGG TAC TAT CGC GGC GGA	[15]
	5′ CGT GAA ATT AAC CGT CAC ACT	

Table 1. Specific primers used in PCR.

2.4. Statistical analysis

Since only a low number of samples could be analyzed (due to restrictions caused by regulations of *P. apollo* protection and protective rules of the collection of Pieniny National Park), the statistical analysis was performed by using the Fisher's exact test. Statistical significance was assumed when $p < 0.05$.

3. Results

In the course of our studies on the reasons of deformation and reduction of wings in the population of *P. apollo* from Pieniny National Park, we tested various genes involved in the development of differentiation of various insect organs. Since Apollo butterfly genome has not been sequenced yet, in order to amplify some genes, primers were designed on the basis of DNA sequences from other insects. Although this strategy was often successful [11, 12], specific DNA fragments were unambiguously identified (e.g., those amplified with primers for *dpp*, *hh*, and *ptc* genes, listed in **Table 1**; this was also a positive control for the quality of DNA samples) in some cases and no amplification products of desired genes could be obtained. Instead, in a few cases, PCR-derived DNA fragments of unexpected lengths appeared and were particularly abundant. An example was ~160-bp PCR product, amplified with the use of primers (5′-TCG GAA AAA TTG TGG ATC GAG G and 5′-AAA TCC GAA GCC GAT GTT GTC) initially devoted for amplification of the *wg* gene fragment (with expected length of 220 bp, assuming a sequence homology of the *wg* gene from *P. apollo* to that from other insects). This ~160-bp DNA fragment was cloned in a plasmid vector and sequenced (the actual length of the insert was 158 bp). The BLASTx-mediated search indicated a homology to two proteins of *Y. pseudotuberculosis*, an RND family efflux transporter and hemolysin secretion protein D.

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These results suggested a possibility of the presence of this bacterium in a biological material withdrawn from bodies of investigated insects. Therefore, we aimed to test this hypothesis.

Using primers specifically designed to identify *Y. pseudotuberculosis* (reported previously [15]), it was possible to detect the presence of this bacterium in samples from normal and malformed *P. apollo* individuals. Among 15 samples tested, the *Y. pseudotuberculosis*-specific PCR product was detected in 6 (**Table 2**). Three of them were from normal individuals, and three of them were from insects with deformed or reduced wings. Statistical analysis indicated that the malformed butterflies were significantly more often infected than normal individuals ($p = 0.044$ in the Fisher's exact test). In control experiments, no *Y. pseudotuberculosis*-specific DNA could be detected in samples from *P. napi, P. rapae,* and *Z. polyxena* (**Table 2**). These control samples came from insects withdrawn from habitats located outside of Pieniny National Park, that is, either a meadow near the city of Cracow (Poland) or a mountain region in Greece.

Species and characteristics	**Number of individuals used for DNA isolation**		
	All tested	**With *inv* specific PCR product**	**Without *inv* specific PCR product**
P. napi (normal)	3	0	3
P. rapae (normal)	4	0	4
Z. polyxena (normal)	2	0	2
P. apollo (normal)[a]	12	3	9
P. apollo (with malformed wings)[a]	3	3	0

[a]The *p* value, in the Fisher's exact test, for normal individuals vs. malformed insects was 0.044.

Table 2. Results of PCR-mediated DNA amplification with the use of indicated templates and primers specific to the *inv* gene of *Y. pseudotuberculosis*.

4. Discussion

Pathogenicity of *Y. pseudotuberculosis* to insects was demonstrated previously under laboratory conditions [3, 4]. Its detection in samples from *P. apollo* individuals coming from Pieniny National Park indicates that this bacterium can infect butterflies in natural habitats and may suggest that the investigated Apollo butterfly population is endangered by insecticidal activity. Although the extinction of this population due to *Y. pseudotuberculosis* infection is rather unlikely, the presence of this pathogen may significantly weaken the insects. One might suggest that *Y. pseudotuberculosis* infections could contribute to developmental changes observed in these insects. Although statistically significant difference was found between the frequency of infected normal and malformed Apollo butterflies, detection of *Y. pseudotuberculosis* in samples from healthy individuals demonstrated that the infection occurs in the entire population. Perhaps, butterflies weakened by other factors, such as deficiency of laccase or the

absence of *Wolbachia*, might be more susceptible and more sensitive to *Y. pseudotuberculosis* infection.

The presence of *Y. pseudotuberculosis* in butterflies from Pieniny National Park, and its absence in samples from other butterflies withdrawn from other habitats (either in Poland or in Greece), might seem surprising. However, this bacterium has also been described as a pathogen of sheep around the world [16–19]. There is a broad area of a sheep pasture ecosystem in Pieniny, where sheep grazing is particularly extensive [20]. Importantly, it occurs even at upper mountain parts. Therefore, sheep can be considered as a source of *Y. pseudotuberculosis* in this region. Bacteria may be excreted with feces of sheep, causing contamination of local plants [21, 22], and then, they can be spread through various animals, becoming potential infectious agents for insects in Pieniny National Park. One might suppose that infections of *P. apollo* by *Y. pseudotuberculosis* could contribute to developmental abnormalities of butterflies, due to weakening of the insects and causing physiological disturbance, especially in combination with genetic, biochemical, and symbiosis problems which the population in Pieniny suffers from (and which were described previously [11–13]). Interestingly, insecticidal activity of cell extracts from *Yersinia enterocolitica*, a species closely related to *Y. pseudotuberculosis* and producing the same kinds of toxins, was demonstrated to be present only when bacteria were cultured at low temperature (10°C), in contrast to higher temperature (30°C) [23]. Because the population of *P. apollo* in Pieniny exists in the mountain region, where temperatures are commonly around 10°C from late spring to early fall, a deleterious effect of *Y. pseudotuberculosis* infection on this population seems likely.

The question appears what might be effects of infections of Apollo butterflies with *Y. pseudotuberculosis*? In fact, in our work, focused on the biological material from a collection, we could only detect the presence of this bacterium in samples of insect bodies. To determine how severe such infections could be, laboratory studies, with experimental administration of bacteria to insects' bodies would be necessary. Then, symptoms of the infection might be observed and investigated, with assessment of their severity. Moreover, it would be particularly interesting to test whether *Y. pseudotuberculosis* infection affects the development of Apollo butterfly. Again, experimental studies with the use of *P. apollo,* including larvae and imago forms, would be necessary. The problem is that Apollo butterfly is a rare species (particularly subspecies *frankenbergeri,* occurring in Pieniny), protected by law. Thus, no individuals can be withdrawn from their natural habitat to conduct biological experiments. The only possibility would be to use insects from a culture; however, to our knowledge, no such culture is currently available.

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Section 2

Insects as Disease Vectors

Chapter 10

Culicoides spp. (Diptera: Ceratopogonidae) in Tunisia

Darine Slama, Hamouda Babba and Emna Chaker

Additional information is available at the end of the chapter

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Abstract

Culicoides is a genus of biting midges in the family Ceratopogonidae. The female midges require blood meals for egg production. There are over 1000 species in the genus, which is divided into many subgenera. Several species are known to be vector of many diseases and parasites, which can affect animals. As vectors of viruses, *Culicoides* species are of the higher veterinary importance. More than 75 arboviruses, belonging to Bunyaviridae, Reoviridae and Rabdoviridae families, were isolated from different *Culicoides* species. In Mediterranean region, the principal vector of Bluetongue virus is represented by *Culicoides imicola*, and also other European *Culicoides* biting midges are implicated in virus transmission. Despite the virulence of these species and his colonisation in Tunisia, they are still considered as neglected area due to the rarity or the absence of programmes to control these biting midges. Thus, the available data on species composition, dominant species, breeding sites and host preferences are urgently needed to better understand these biting midges and to develop reliable tools to prevent the spread of other diseases that threaten human and animal life.

Keywords: *Culicoides*, biting midges, Tunisia, species, geographical distribution

1. Introduction

Culicoides biting midges are important vectors of a number of arboviruses causing disease in domesticated livestock such as bluetongue (BT) and African horse sickness. These midges are smaller (1–3 mm) than mosquitoes and can be much more abundant [1] (**Figure 1**).

The development cycle of *Culicoides* consists of egg, four larval instars, pupa and adult. Almost, 1400 extant and extinct species of *Culicoides* have been described from a highly diverse range of ecosystems, and the genus is present in all major land masses with the exception of Antarctica and New Zealand and at altitudes of up to nearly 4000 masl. The first description

Figure 1. Female of *Culicoides* sp.

to these biting midges is by reverend W. Derham who described their life history and biting biotops in 1731. The primary studies on sub-Saharan *Culicoides* date to 1908 when two species were described from Namibia [2].

The bites of females species of *Culicoides* cause skin lesion and comprise dermatitis in livestock [3, 4], affecting the general health status of domestic animals and wildlife [4, 5]. Regardless of transmitted disease, *Culicoides* midges play an important role in human health. In fact, they can transmit Oropouche virus, leading in severe cases to febrile illness Oropouche fever, between humans beings [1–7]. Oropouche virus is currently restricted to the Neotropics and infects humans, causing major outbreaks of febrile illness. After Bluetongue (BT) appeared, entomological studies were implemented to establish which species of *Culicoides* had acted as vectors.

The aim of this chapter is to review epidemiological features of *Culicoides* species in Tunisia.

2. Life cycle of *Culicoides*

Almost all *Culicoides* exige moisture-rich habitats for development of egg, larval and pupal forms and the availability of these environments is a key determinant for their distribution, abundance and seasonal occurrence [1]. All *Culicoides* species present a complete metamorphosis life cycle. The female midges require blood meals for the completion of the gonotrophic cycle, but those of a few species are autogenous and therefore may produce an initial batch of eggs without feeding using reserves stored from the larval period (**Figure 2**).

2.1. Eggs

The eggs are usually about 400–500 μm in length. They are laid in wet soil in boggy flushes, mires and in the transition zone at the edge of bogs. The eggs have an elongate, curved and

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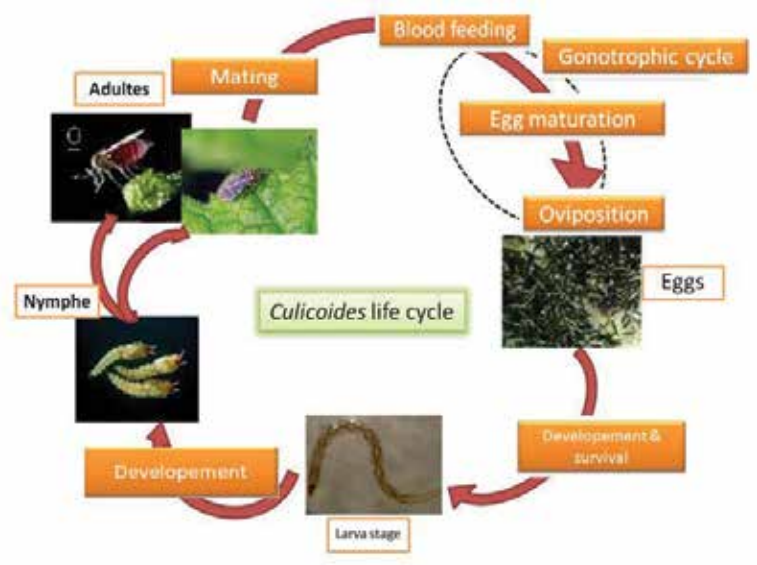

Figure 2. Life cycle of *Culicoides* vectors.

pointed form at each end. Concerning the number of eggs produced, this later varies among species and also size of blood meal. It seems agreed that a blood meal is important in the egg laying in *Culicoides*.

Species	Breeding sites	References
Culicoides imicola	Cow dung rich in organic material, grassed margins of streams, muddy habitats	[11–15]
Culicoides sonorensis	Edges of waste water, irrigation run off in pasture, puddles, trough spillover	[16–18]
Culicoides brevitarsis	Cattle dung	[19, 20]
Culicoides oxystoma	Paddy fields, stream edges, pond margins	[20, 21]
Culicoides obsoletus	Cattle dung, cowshed, dried dung on the walls of the building, leaf compost, tree holes	[22–26]
Culicoides scoticus		
Culicoides dewulfi	high soil moisture, cattle dung breeders	[27]
Culicoides chiopterus		
Culicoides cataneii	Ponds and river margins, rich organic matter, soils poor in organic matter, unpolluted sites, grass covered pool	[12, 15, 28, 29, 30, 31]
Culicoides sahariensis	Mud in drainage channels rich in organic matter, mud fringing a salt lake, unvegetated pond, shorelines of the unvegetated pond and the grass covered pool moist	[12, 15, 29, 30]
Culicoides circumscriptus	Puddles of water contaminated withy animal excreta, inundated soils	[15, 28]
Culicoides newsteadi	Breeding in shallow, brackish pools, lined with decaying vegetable materialx	[31]

Species	Breeding sites	References
Culicoides jumineri	Mud near irrigation channel	[32]
Culicoides nubeculosus	Mud rich in dying near the water reservoirs and in mud from swap, organic matter	[33]
Culicoides puncticollis	Sites rich in organic matter, mud rich of dung near water reservoirs and mud from swamps and less in mud from reed sites areas	[11, 33]
Culicoides gejgelensis	Mud with poor organic matter alongside streams, mud from around dams, mud from reed sites	[33]
Culicoides riethi	Rich organic matter, mud swamps contaminated by feces of poultry animals, mud rich in dung near water reservoirs	[33, 34]

Table 1. Some example of breeding sites for certain *Culicoides* species.

Figure 3. Breeding sites of some *Culicoides* species. Photograph: LP3M: Laboratory of Medical and Molecular Parasitology-Mycology, University of Monastir Tunisia.

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2.2. Larva

The larvae are vermiform, usually pale. They have a distinct head capsule with toothed mandibles and eyespots. There are three thoracic and nine abdominal segments. The larvae are narrow and worm-like, and they live in the soil. Neverthless, the larvae of some species are omnivorous, and their diet includes small animals such as Nematodes, other insect larvae, fungi and parts of plants. They grow slowly when compared to some other species in the genus, due to the nutrient-poor soil [8]. According to *Culicoides* species, the breeding sites of *Culicoides* larva were very variable, usually defined as humid rich and enriched in animal or vegetal organic matter. Many larval biotopes are defined worldwide including damp or wet decomposing vegetation, wet leaf packs, manure, many different types of tree holes, swamps, ponds, lakes, streams and river margins, bogs and salt marshes [9] (**Table 1**), (**Figure 3**). Such great variety of habitats of many species of biting midges makes it difficult to find out the immature stages [10]. Nevertheless, it was state that the last stage larvae overwinter and pupate the following spring to early summer.

2.3. Pupa

The pupal stage is formed in the same site as the last larval stage. Pupal colour can be pale yellow to light brown. They are 2–5 mm in length with an unsegmented cephalothorax that has a pair of respiratory horns that may bear spines or wrinkles. The pupae of most *Culicoides* species are aquatic and have the ability to float.

3. Disease transmission and distribution of *Culicoides* sp.

3.1. Disease transmission

Biting midges of the genus, *Culicoides* play a big threat role, and this when several species serve as biological vectors of pathogens of medical and veterinary importance. Almost, over 50 arboviruses have been isolated from species of *Culicoides* [1, 35, 36]. In addition, only few *Culicoides* species have a significant deleterious impact on human existence. Opportunistic feeding of *Culicoides* species on humans can have impact on tourism, forestry and agricultural industries [1]. Actually, the major economic impact of *Culicoides* resides in their ability to transmit bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV). These arboviruses are of greatest importance in ruminants and equines. The biting midges have recently been identified as the vector of the Orthobunyavirus, Schmallenberg virus [37].

In the context of pathogen transmission to or between humans, *Culicoides* include a range of filarial nematodes transmitted between humans, especially *Mansonella ozzardi, M. perstans* and *M. streptocerca* [38].

It is noteworthy that biologically transmitted *Culicoides* species have the ability to transmit Oropouche virus (OROV), the aetiological agent of the febrile illness Oropouche fever, between human beings [1, 38]. Indeed, the symptoms of Oropouche fever include headache and also lead to generalised arthralgia, anorexia and in rare cases meningitis [1]. **Table 2** summarises major disease transmitted by *Culicoides* species in worldwide.

		Viruses							Filarial Nematodes						Parasites					
		BTV	AHSV	EHDV	EEV	OROV	Vesicular stomatitis Indiana	West Nile	Mansonella ozzardi	M. perstans	M. streptocerca	Onchocerca cervicalis	Onchocerca gibsoni	Onchocerca reticulata	Haemoprote-us	Plasmodium	Leucocytozoon	Hepatocystis	Leishmania	References
Subgenera	**Vectors**																			
Amossovia	*C. arboricola*							x												[39]
Avaritia	*C. bolitinos*		x																	[1]
	C. brevitarsis	x																		[40]
	C. dewulfi	x																		[42]
	C. fulvus	x																		[42]
	C. grahami									x	x									[43]
	C. imicola[*]	x	x		x														x	[1, 40, 42, 43]
	C. obsoletus	x																		[40]
	C. orientalis												x							[41]
	C. pungens												x							[41]
	C. wadai	x																		[40]
Beltranmyia	*C. circumscriptus*[*]														x	x			x	[42, 44]
Haematomyidium	*C. debilipalpis*								x											[41]
	C. paraensis								x											[41, 45]
	C. lahillei								x											[41]
Hoffmania	*C. guttatus*								x											[41]
	C. insignis	x							x											[41]

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		Viruses							Filarial Nematodes							Parasites				References
		BTV	AHSV	EHDV	EEV	OROV	Vesicular stomatitis Indiana		West Nile	Mansonella ozzardi	M. perstans	M. streptocerca	Onchocerca cervicalis	Onchocerca gibsoni	Onchocerca reticulata	Haemoprote-us	Plasmodium	Leucocytozoon	Hepatocystis	Leishmania	
Macfiella	*C. phlebotomus*									x										[41]
Monoculicoides	*C. varriipennis var sonorensis*	x			x															[40]
	C. nubeculosus												x		x					x [41, 43]
	C. sonorensis	x					x													[40, 46]
Meijerehelea	*C. arakawei*																	x		[47]
	C. distinctipennis										x									[41]
Miscellaneous	*C. adersi*																		x	[47]
Oecacta	*C. furens*									x			x							[41]
	C. stellifer								x											[39]
Remmia	*C. oxystoma*													x						[41]
Silvaticulicoides	*C. biguttatus*								x											[39]
Trithecoides	*C. fulvithorax*										x									[41]
Unplaced	*C. austeni*											x								[41]
	C. bwambanus										x									[41]
	C. inornatipennis										x									[41]

(*detected in Culicoides spp. In Tunisia)

Table 2. Pathogen associated with the genus *Culicoides* worldwide.

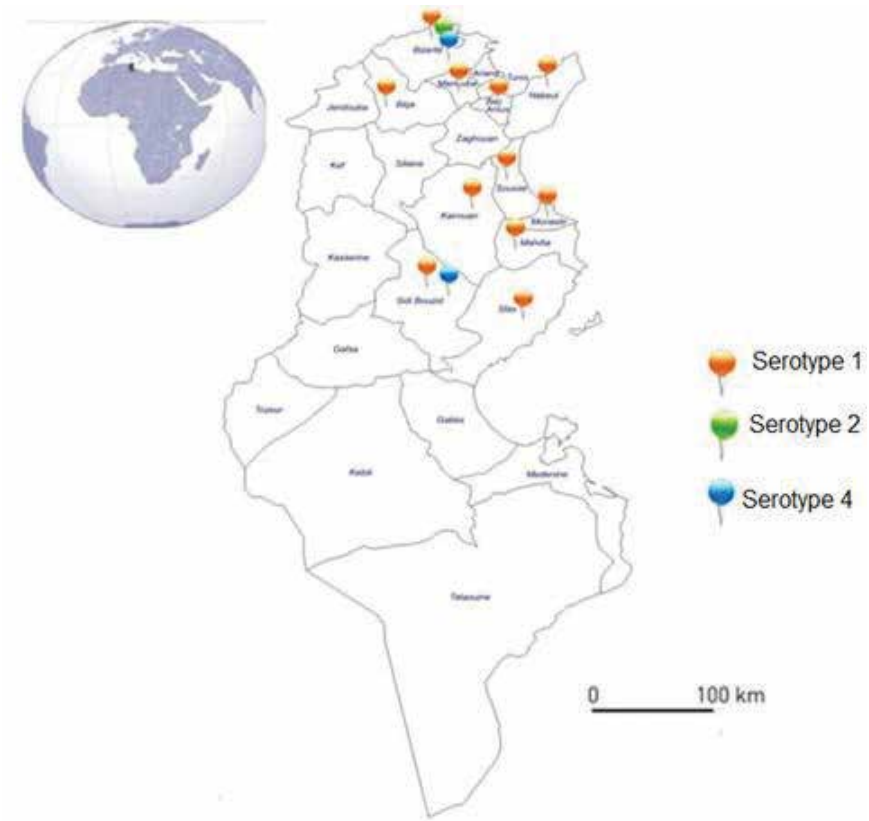

Figure 4. BTV serotype distribution in Tunisia.

4. Distribution of *Culicoides* spp.

Culicoides midges are found on all large landmasses ranging from the tropics to the tundra, with the exception of Antarctica and New Zealand.

In Tunisia, first incursion of BTV dates from 1999 (serotype 2), where the autumn was characterised by high temperatures and heavy rain. This weather created favourable conditions for BTV vector activity. It is noteworthy that the optimum conditions for activity of these biting midges are temperatures of 18–29°C and high humidity [48]. During this first incursion, severe clinical signs were observed in affected sheep: high temperature (41–42°C), nasal discharge, salivation, oedema and congestion of the head and the mucous membranes. Affected sheep flocks were located in the eastern part of Tunisia along the cost. The overall morbidity and mortality rates were 8, 35% and 5, 5%, respectively. In 2000, 72 outbreaks of BT were reported during the period extending from June to October. Indeed, 6120 clinical cases were diagnosed in sheep, of which 1318 died. Moreover, outbreaks were reported in 10 districts with most cases appeared in the eastern and central parts of the country [49].

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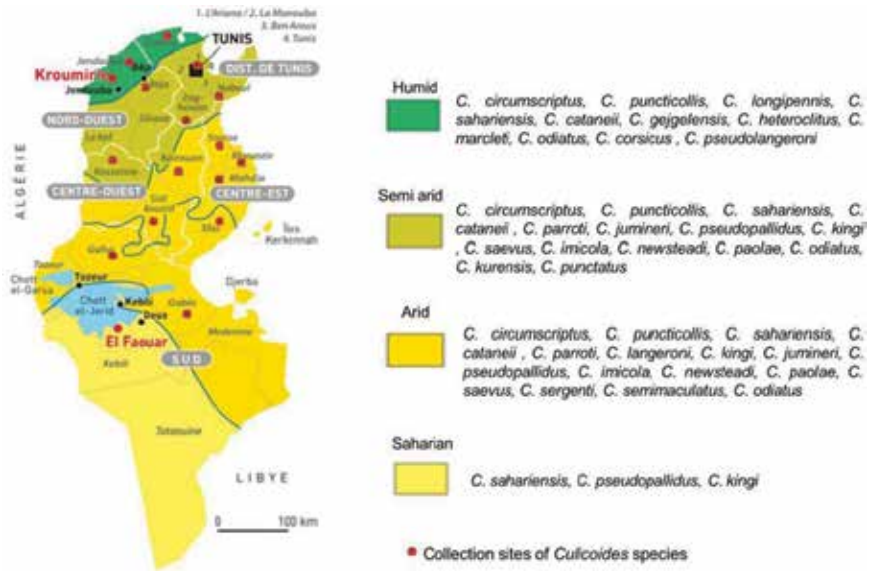

Figure 5. Distribution of *Culicoides* species in Tunisia. Source of the map of Tunisia from [54].

In total, three serotypes of BTV were reported in Tunisia: serotype 1, 2 and 4. **Figure 4** shows the distribution of BTV serotype in Tunisia.

Since the epizootic of vector-borne disease (AHS in 1966 and BT in 1999) in Tunisia, the veterinary authorities of the region have implemented surveillance programmes to detect and identify the presence and the distribution of the known vectors of disease, notwithstanding that fewer studies have been made in comparison with other Mediterranean countries. Indeed, in 1981, a study of [29] reported that the presence of 22 *Culicoides* species, with the most abundant species, was *C. circumscriptus, C. sahariensis, C. longipennis* and *C. puncticollis*. However, no *C. imicola* was detected. It is only to be expected that the presence of *C. imicola* in Tunisia was reported only in 2005 in the Monastir governate [50]. Evenly, Hammami et al. [51] reported 14 species with one new for the fauna *C. punctatus*. Since 2009, the national veterinary authorities of Tunisia have implemented an epidemiovigilance programme. Entomological studies have been conducted aimed to the detections of any new competent vectors. Thereby, Sghaier et al. [52] have identified 25 species of which seven were identified for the first time: *C. obsoletus, C. submaritimus, C. santonicus, C. univittatus, C. fascipennis, C. subfasciipenis* and *C. indistinctus*. However, this study was conducted in different regions: eastern and northern part of the country. Since this date, no studies were made to update knowledge on the *Culicoides* fauna present in Tunisia. But in 2016, Slama et al. [53] identified 22 species in Central Tunisia (Monastir, Kairouan and Sidi Bouzid). This study reported the presence of two new species: *C. semimaculatus* and *C. sergenti*. Indeed, the numbers of the *Culicoides* species recorded from Tunisia were increased to 35 species. **Figure 5** represents the distribution of *Culicoides* species in different regions of Tunisia.

5. Tools for *Culicoides* species identification

The most common method of *Culicoides* identification relies in the use of taxonomic keys. The observation of wing patterns allows the classification of the insects into vector relevant groups of *Culicoides* spp. Moreover, certain species can be identified based on wing pattern, while others need microscopic analysis of slide-mounted parts of bodies [55, 56]. Morphological identification can be a time-consuming procedure and laborious process that requires intensive training and most importantly that the biting midges be undamaged. If for any reason the *Culicoides* specimen of interest is damaged, morphological identification may not be possible. Withal, some species can only be identified by differences in the male genitalia, making it difficult or impossible to identify adult females, or may even be morphologically indistinguishable [57]. Many PCR-based tests have been used for identification of *Culicoides* spp., targeting the mitochondrial cytochrome oxidase I gene (mt COI) and the ribosomal RNA genes internal transcribed spacer 1 or 2 (ITS1, ITS2). Moreover, the fused carbamoylphosphate synthetase, aspartate transcarbamylase and dihydroorotase (CAD) nuclear marker have also developed for its utility in differentiating species [58]. **Table 3** summarises the molecular markers used for molecular analysis within *Culicoides*.

Another molecular technique (matrix-assissted laser desorption/ionisation time of flight mass spectrometry, MALDI-TOF MS) has proven its benefits for rapid, simple and cost-effective characterisation and identification of biting midges [59].

Genomic region	Molecular marker	References
Mitochondrial	COI	[57, 60–67, 78]
	COII	[68–70]
	28S	[71]
	18S rRNA	[72]
	16S rRNA	[73, 74]
	Cytb	[66]
Ribosomal	ITS1	[75–78]
	ITS2	[79]
Nuclear	CAD	[58]

Table 3. Molecular markers used for *Culicoides* species identification.

6. Conclusion

Despite the fact that the epidemiological studies realised till now, *Culicoides* species in Tunisia are yet neglected vectors. Their geographical propagation is increasing because of the environmental changes. For this reason, more epidemiological studies and many surveillance and control systems are required to be created.

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Chapter 11

Transmission of Major Arboviruses in Brazil: The Role of *Aedes aegypti* and *Aedes albopictus* Vectors

Thaís Chouin-Carneiro and
Flavia Barreto dos Santos

Additional information is available at the end of the chapter

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Abstract

Arthropod-borne viruses (arboviruses) are transmitted to a mammalian host by an infected arthropod vector. More than 130 types of arboviruses are known to cause disease in susceptible vertebrate hosts and are responsible for some of the most explosive epidemics of emerging infectious diseases in recent decades. The transmission cycle requires three essential components: virus, vector and vertebrate. Understanding the role of the vector in the arboviruses transmission is critical to improve emerging arbovirus disease control strategies. Since 2015, Brazil is faced with the challenge of three co-circulating arboviruses of major public health importance. Dengue virus (DENV) infection has been a public health for 30 years, which has suffered several epidemics caused by all four serotypes. The emergence of Chikungunya virus (CHIKV) and Zika virus (ZIKV) in Brazil poses new challenges to clinicians and public health authorities. In urban and suburban areas, those arboviruses are transmitted between people by *Aedes* mosquitoes in the subgenus *Stegomyia*, especially *Ae. aegypti* (the main vector) and potentially *Ae. albopictus*. Factors relating to the environment and the vector-virus interactions can influence the dynamics of arboviruses transmission. This chapter describes the main biology aspects of the *Ae. aegypti* and *Ae. albopictus* that can influence the success of the transmission of main arboviruses in Brazil and provide information to understand the role of those factors in this dynamic relations

Keywords: *Aedes aegypti*, *Aedes albopitcus*, Arboviruses, virus-vector interactions, transmission

1. Introduction

A critical premise of epidemiology is that disease and other health events do not occur randomly in a population but are more likely to occur in some members of the population than others because of risk factors that may not be distributed randomly in the population.

As noted earlier, one important use of epidemiology is to identify the factors that place some members at greater risk than others. Agent, host and environmental factors interrelate in a variety of complex ways to produce disease. Different diseases require different balances and interactions of those components. In the case of many communicable diseases, such as Dengue, Chikungunya and Zika, the agent can only reach the host via a third party, the vector. Infectious diseases transmitted by insects have long been associated with significant human illness and death. Vector-borne diseases account for more than 17% of all infectious diseases, causing more than 1 million deaths annually [1].

Development of appropriate, practical and effective public health measures to control or prevent vector-borne diseases usually requires assessment of all components and their interactions, and much remains to be elucidated, in particular about the complex biological and ecological relationships that exist among pathogens, vectors, hosts and their environments, **Figure 1**.

Arbovirus or arthropod-borne virus is the ecological term used to define viruses maintained in nature by biological transmission between a susceptible vertebrate host and a hematophagous arthropod, such as mosquitoes, the best known disease vector [1]. More than 130 types of arboviruses are known to cause disease in susceptible vertebrate hosts, being responsible for some of the most explosive epidemics of emerging infectious diseases in recent decades. Moreover, the global expansion of these arboviruses was preceded by the global spread of their vectors [2].

1.1. Major arboviruses currently affecting Brazil: Dengue, Zika and Chikungunya

Descriptions of a dengue-like disease were reported in China during the Chin Dynasty (265–420 A.D); however, the first well-documented cases believed to be dengue occurred in 1779–1780 on Asia, Africa and North America [3], and the first viruses were isolated by the Japanese [4] and American investigators [5] during World War II [6].

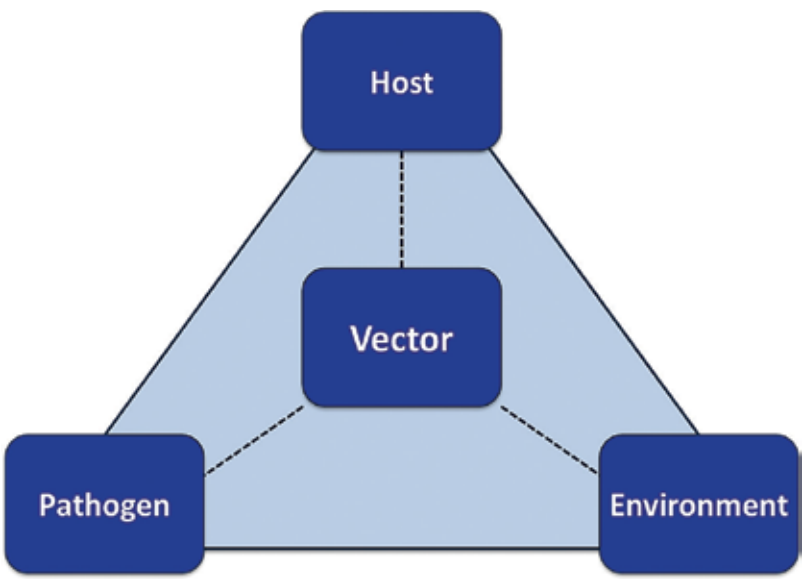

Figure 1. Epidemiological triad of vector-borne diseases.

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Dengue viruses (DENV) exist in either sylvatic or human transmission cycles, most prevalently in tropical and subtropical areas in the world, and due to its impact poses relevant social and economical effect related to the increased geographic extension, number of cases and disease severity [7]. The four serotypes (DENV-1 to DENV-4) that belong to the family *Flaviviridae* and the genus *Flavivirus* show only 62–67% homology based on amino acid sequences [8], and despite they could have been classified as separate viral groups, the four serotypes are classified as belonging to a single group. Within each serotype, distinct genotypes are characterized based on a nucleotide divergence ≥6% for a given region of the genome [9, 10].

In the last 50 years, the disease has gradually reached the status of a pandemic, hospitalizing more than 5 million children and resulting in more than 70,000 deaths [11]. In Brazil, DENV has become a major public health problem of significant social and economic impact after DENV-1 introduction in 1986 [12]. In 1990, DENV-2 was also introduced in Rio de Janeiro and led to the first severe cases and increase in the number of hospitalizations [13]. DENV-3 was first detected in December of 2000, again in Rio de Janeiro, and caused one of the most severe epidemics in 2002. In 2007–2008, an epidemic caused by the reemergence of DENV-2 led to severe cases and deaths on children 15 years-old and under. DENV-1 reemergence in 2009–2010 caused explosive epidemics throughout the country, and severe cases on patients with comorbidities were reported. Despite its detection in 1982 in Boa Vista, Roraima, North of Brazil, DENV-4 emerged and caused epidemics, after its introduction in 2010. Currently, the four DENV serotypes are circulating in the country in a hyperendemic scenario, with increased number of cases occurring year after year. Only in the first semester of 2016, a total of 1,399,480 probable dengue cases were reported in Brazil [14].

Chikungunya virus (CHIKV), *Togaviridae* family, genus *Alphavirus*, was first isolated from human serum during a febrile illness outbreak in Tanzania in 1953 [15]. It is an Old World alphavirus belonging to the Semliki Forest antigenic complex, which also includes Bebaru virus, Mayaro virus, O'nyong nyong virus, Ross River virus, Getah virus, Semliki Forest virus, and Una vírus. It has four genetically distinct genotypes characterized as West African, East-Central-South African (ECSA), Asian and Indian Ocean [16].

As another emerging arbovirus, CHIKV represents nowadays a global risk. Since the 60s, chikungunya outbreaks were reported in Southeast Asian countries. After years of its isolation, the virus caused epidemics in Congo in 1999–2000 [17] and Indonesia from 2001 to 2003 [18]. Until then, chikungunya cases were restricted to Asia and Africa; however, in 2005–2006, epidemics were reported in several Indian Ocean Islands [19]. In October of 2013, the CHIKV Asian genotype was first reported in the island of Saint Martin in the Caribbean, and the increased occurrence of cases in the Caribbean and its spread to other Latin American countries led to the introduction of this arbovirus also in Brazil. First autochthonous CHIKV infections in the country were reported in Oiapoque, Amapá, bordering French Guiana in North region and Feira de Santana, Bahia [20, 21], and viral genome sequencing characterized the Asian genotype circulating in the North of the country and the ECSA genotype in Bahia, suggesting this genotype introduction in the Americas for the first time. Despite the susceptible population, CHIKV infections were restricted to four Brazilian states (Bahia, Amapá, Mato Grosso do Sul and Roraima) and Distrito Federal in 2014. In 2015 and 2016, the virus

spreads to other Brazilian states, and in the first half of 2016, Brazil reported 170,000 cases, 10 times the number reported in the same period of 2015 and the country accounts for 94% of confirmed cases in the Americas [22].

Zika virus (ZIKV), member of the *Flaviviridae* family, genus *Flavivirus*, also related to Ilheus virus, Rocio virus, St. Louis Encephalitis viruses, Yellow Fever virus and DENV, was first isolated in 1947 from a rhesus monkey in the Zika forest in Uganda; however, the first human case was reported in Uganda in 1964. Since then, sporadic human cases were reported in countries of Asia and Africa. The first reported large outbreak of ZIKV human infection occurred at the Federated States of Micronesia in 2007 [23], when 73% of the local population became infected [24]. The first laboratory testings performed at the time suggested that patients were infected by DENV, what proved to be untrue after ZIKV was later confirmed as the causative agent of the epidemic [23]. More recently, epidemics due to ZIKV were reported in French Polynesia, New Caledonia, Easter Island and the Cook Islands and imported cases to Australia and Germany [25–27]. The potential emergence and spread of ZIKV outside Africa, such as to the Pacific Islands and Americas, were stressed previously [28].

ZIKV was previously believed to cause only a mild and self-limiting illness; however, it has emerged as a new public health threat since the outbreak in French Polynesia in 2013–2014 and the explosive epidemic in Brazil in 2015. In Brazil, the virus was introduced in Bahia and Rio Grande do Norte, in March [29, 30], and an increase in severe congenital malformations (microcephaly) and neurological complications, mainly Guillain-Barré Syndrome (GBS), was reported in the country. Moreover, ZIKV has been associated with fetal microcephay and other birth defects in both humans [31–35] and mice [36–38]. By December 2015, all regions of the country had already reported autochthonous transmission, and estimates were that zika suspected cases ranged from 440,000 to 1,300,000 [39]. A recent study reports that the introduction and rapid spread of ZIKV in the Americas resemble that of CHIKV, after its introduction and spread by and from the Caribbean. Furthermore, it was estimated that it took approximately 5–6 months for the virus to spread from the northeastern coast to the southeastern coast and western border of Brazil [40]. In 2016, a total of 174,003 probable cases of zika were reported in Brazil [14]. Following its spread to other American countries, the World Health Organization declared the zika epidemic, a Public Health Emergency of International Concern, on February 1, 2016.

2. *Aedes aegypti* and *Aedes albopictus* vectors

Aedes (Ae.) aegypti and *Ae. albopictus* are the most important vectors for arboviruses transmission to humans. Both are exotic species and took advantage of trade developments to spread throughout the tropics from their native area: *Ae. aegypti* from Africa and *Ae. albopictus* from Southeast Asia. With the presence of the two species in the American Continent, the transmission of arboviruses among humans occurs, but factors relating to the environment and the vector-virus interactions can influence the dynamics of that transmission.

Ae. aegypti most likely originated in Africa; since then, the mosquitoes spread globally and adapted easily in tropical and subtropical areas, and parts of the temperate world. Their

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distribution is associated to urban areas, specifically to human dwellings, feeding preferentially on human blood. *Ae. albopictus* originated in Asia and is considered one of the most important invasive species worldwide. Its colonization of temperate regions such as North America and Europe as well as tropical and subtropical regions such as South America and Africa was facilitated by the species' strong biological and behavioral plasticity. Currently, both *Ae. aegypti* and *Ae. albopictus* are present in most Asian cities and large parts of the Americas [41].

Ae. aegypti adults are relatively small and range in size from 4 to 7 mm and could be mistaken with *Ae. albopictus*. However, lyre-shaped white scales on the dorsal surface of the thorax are a marked characteristic. White basal bands that appear as stripes are present on each tarsal segment of the hind legs. Its abdomens are generally dark brown and may also present white scales [42]. Females are larger than males and are distinguished by minute palps with silver or white scales present on their tips. Females also differ from males by short, sparse hairs whereas males have plumose antennae.

2.1. *Aedes* mosquitoes' life cycle

The mosquitoes' species have a complex life cycle with dramatic changes in shape, function and habitat. They have four distinct stages during their life cycle: egg, larva (L1, L2, L3 and L4), pupa and the adult insect (**Figure 2**). Both male and females mosquitoes are nectar feeders, but females are adapted for blood feeding, and sucking blood of vertebrate animals to mature her eggs. Generally, about 3–4 days after the blood meal, the females produce on average 100–200 eggs per batch.

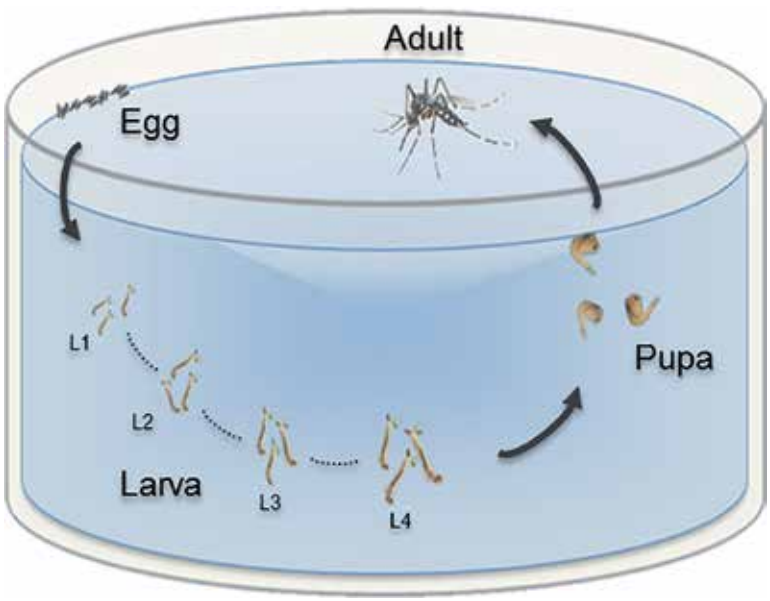

Figure 2. The Aedes mosquitoes' four life stages: egg, larva, pupa and adult.

Both species lays their eggs in internal and damp surfaces of containers that, permanently or intermittently, contain water. When first laid, eggs appear white but within minutes turn a shiny black. The embryogenesis is complete in 2–3 days after layer but can be variable depending on ambient temperature. In warm climates, eggs may develop in 2–3 days, whereas in cooler temperate climates, this time can extend and the development can take up to a week [43]. Laid eggs can survive for very long periods in a dry state, often for more than a year. However, the proportion of eggs hatched from the same batch varies according to the ambient temperature, relative humidity and the time of exposure to desiccation [44]. This desiccation peculiarity enables the eggs are transported over long distances in dry containers, allowing the dispersion of the vector.

When in contact with the water, triggering the process of hatching of the larvae and the cycle of larval development begins [45]. The larval phase is the period of feeding and growth of immature stage. The larvae feed on suspended particulate matter (i.e., detritus, bacteria, diatoms, algae and other microorganisms) by filtering water with modified mouth parts often referred to as "mouth brushes," shedding their skins three times to be able to grow from first to fourth instars. When the larva has acquired enough energy and size and is in the fourth instar, metamorphosis is triggered, changing the larva into a pupa. Strongly dependent on food availability and water temperature, the larvae can develop from first instar to pupae within several days to a few weeks [46]. In optimal conditions, the period between hatching of the egg and the formation of the pupa may not exceed 5 days, or in most adverse conditions such as low temperatures, insufficient nutrients and high larval density, it can extend for several weeks [47]. In fact, males develop faster than females, so males generally pupate earlier, and consequently, they are the first ones to appear on refuges.

The pupa is the last immature stage. Mosquitoes' pupae are mobile, do not feed and expend almost all the time breathing near the surface. They just change in form until the body of the adult, flying mosquito is formed. This stage lasts for 2–3 days, depending mainly on the temperature. Unlike the larval stage, which is influenced by many other environmental conditions, the mortality rate of the pupae is practically null. For this reason, it is suggested that the number of pupae found in one location corresponds directly to the number of adults that will emerge and occupied the houses in brief [48].

The adult emerges slowly through the longitudinal opening in the pupal case and remains at rest for a few minutes about water, due to the surface tension. The terrestrial phase has an essential function to reproduction and dispersion.

2.2. *Aedes* mosquitoes′ reproduction and feed behavior

The males are attracted to the females due to the sound that is made by their wing beat 2.5 h after emergence [49, 50]. The attracted male clasps the tip of the female abdomen with his genitalia and inserts his aedeagus into the female genital chamber. The duration of the copulation is brief and lasts less than a minute [49]. Older and larger males as well as larger females have greatest mating success. Density and ambient environmental conditions are influential factors of mating biology of mosquitoes [51].

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During copulation, sperm and seminal fluid are transferred from the male into the female's bursa copulatrix [52], and the males' seminal fluid of male contains a large number of proteins that are transferred to females during mating, possibly affecting the female biology and behavior [53]. The seminal fluid is thought to be responsible for female refractoriness to mating in both species. However, there are indications, especially in *Ae. albopictus*, that multiple inseminations occur in the field species and also that the fertilization of eggs could be done by the sperm issued from several males [54], and in *Ae. aegypti*, there are evidence that polyandrous behavior depends on the postmating interval [55]. Mating errors between biologically incompatible species may result in varying degrees of reproductive loss that decreases fitness [56].

The feeding behavior of females includes the intake of blood to provide energy for the maturation of eggs at every cycle of ovarian development, called gonotrophic cycle. Many females blood-sucking insects will develop and lay a batch of eggs each time a sufficient blood meal is taken gonotrophic concordance. However, *Ae. albopictus* and *Ae. aegypti* often take multiple blood meals in different individuals or not, in each gonotrophic cycle, a phenomenon that has high epidemiological importance, once maximize the chances of viral transmission.

Apparently, in nature, sugar intake by *Ae. albopictus* occurs more often than by *Ae. aegypti*. Moreover, *Ae. aegypti* and *Ae. albopictus* females can also feed in other animals; however, the *Ae. aegypti* feeds preferentially on humans, which increases its fitness, and synthesis of energy reserves, therefore, an effective disseminator of human pathogens. *Ae. albopictus* has also been shown to exhibit strongly anthropophilic behavior similar to *Ae. aegypti* in specific contexts [57, 58].

The females of both mosquitoes may bite at any time of the day, although the biting peak periods are early in the morning and before dark in the evening [59]. Host finding by mosquitoes is largely driven by olfactory cues that are given off by individual hosts. Mosquitoes use the wind direction and odors, such as carbon dioxide, emitted by the hosts in order to locate a host to bite [60].The bites can occur in any part of the body but are more frequent in feet and lower parts of the legs, where normally concentrate CO_2 molecules and sweat components that are attractive to anthropophilic species, and variation in sweat composition may cause differential attractiveness within and between individuals and also between humans and other mammals [61].

The *Culicidae* family females may enhance the development and survival of their immature forms by obeying some specific preferences, determined by physical and chemical characteristics of the water, by the degree of exposure to sunlight or shade, the location and the size of the site, among other factors [62]. In general, female mosquitoes laid her eggs at once in a single focus of reproduction. However, the skip oviposition behavior is clearly observed in *Ae. aegypti* females and is also observed in *Ae. albopitcus* ones. The females choose to oviposit a few eggs in several different sites. "Skip oviposition" behavior may ensure the greater distribution of progeny from an individual female which, in turn, tends to increase the genetic diversity of populations and reduces sibling competition, that may maximize the survival of their offspring, and even if one site are destroyed, some of their eggs have the possibility to become adult mosquitoes in another site, neglected by the control [63, 64]. It is possible that

the transovarial transmission represents a way of maintenance of the virus in nature, because the virus can persist until the seventh generation in the mosquitoes' tissues [65–67]. Even at this low rate, transovarial transmission may allow virus survival in unhatched eggs during dry or cool periods [68, 69].

2.3. *Aedes* mosquitoes′ breeding sites

For mosquitoes, location of suitable sites for oviposition requires a set of visual, tactile and olfactory cues that influence females before laying their eggs. The ability of gravid females to distinguish among potential oviposition sites that will or will not support the growth, development and survival of their progeny is critical [70].

Ae. aegypti and *Ae. albopictus* are sympatric species that tend to breed in similar sites, most commonly in artificial containers [71]. Interspecific competition between these species has been documented. Both prefer breeding sites that contain stagnant, clean and unpolluted water. The containers with dark background and kept in locations shaded are the breeding sites hotspots.

Ae. aegypti is highly endophilic and anthropophilic, therefore frequently found in urban and suburban environments, with high concentrations of humans and houses. The immature stages are found in water-filled habitats, mostly in artificial containers or objects for domestic use, closely associated with human dwellings and often indoors. Water storage containers, such as wells, tanks, cisterns, barrels, jars, buckets, should be kept clean and sealed so mosquitoes cannot use them as aquatic habitats. Such breeding sites are, as a rule, which ensures the development of immature forms with adequate space and less competition inter- and intraspecific and must be strategically inspected and mitigated [46]. In addition to these, plant pots and dishes, plastic pools, tires, damaged appliances, animal drinking pans whose water is not changed periodically, gutters clogged and traps of drains are also frequently used as breeding sites for this species.

The urban landscape has several implication characteristics for the life parameters of *Ae. aegypti* females. The organization and structure of the modified environment, the lack of infrastructure, sewage and drainage systems, as well as the cultural habits of human populations pose direct influence on the presence and density of the *Ae. aegypti* [72, 73]. *Ae. albopictus*, on the other hand, inhabits at the edges of forests and breeds in natural habitats (e.g., tree holes, bamboo stumps and bromeliads), and it was previously considered a rural vector [74]. However, this species has adapted well to urban environments with larvae also breeding in artificial containers. In some opportunities, it has become the most important and sometimes sole vector in urban areas [75, 76]. Due to its considerable ecological valence, easily adapting to the rural, urban and periurban environments, it is presumed that *Ae. albopictus* may serve as a bridge between the urban and sylvatic cycles.

Both species showed seasonal variation in their larval densities. The rainfall and the ambient temperature have direct influence in the adults' population density. Overall, larval densities are greater during the wet seasons. However, in tropical climate, its proliferation is continuous, even though during that period and lower precipitation and lower temperatures, the population density tends to decrease significantly. The temperature increases above 20°C in temperate

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areas, or 22–24°C in tropical areas in South America, is strongly associated with the increase in the *Ae. aegypti* density and, consequently, the risk of transmission of arboviruses [48].

2.4. Vector-virus interactions

In the arboviruses transmission cycle, the arthropod is exposed to and becomes infected when ingesting blood from the viremic host. The arbovirus cycle requires replication in the cellular environment of the arthropod vector. The extrinsic incubation period (EIP) comprises the time between the ingestion of an infectious blood meal by susceptible mosquito and the presence of infective viral particles in the salivary secretion. After this period, the insect becomes able to transmit the virus to a new vertebrate host [77–79]. This period in the vector is required for viral replication and dissemination and is conditioned by the kinetics and tropisms of virus replication in the vector. The EIP is an important epidemiological factor, as it is a temporal process. The life span of a mosquito is intimately tied to this period, and thus, potential transmission of those viruses cause transmission is only permitted when the longevity of the vector exceeds the EIP. To be transmitted by a susceptible vector, the viral particles must adhere to cell receptors on target cells in the midgut epithelium of the insect for establishing infection [80]. Virions need to enter epithelial cells through the microvilli before the blood meal is surrounded by the peritrophic matrix, which will prevent the virus to infect the midgut. The pore size of the peritrophic matrix is smaller (20–30 nm) than all arboviruses [81]. In the *Ae. aegypti*, the peritrophic matrix becomes evident at 4–8 h after blood feeding and attains mature thickness and texture by 12 h [82]. Infection patterns of midgut epithelial cells vary according to virus-mosquito species combinations. In order for productive infection of a mosquito, enough virus must be ingested to infect the midgut, and thus, only vertebrate hosts that manifest sufficient titers can contribute to the transmission cycle. The blood meals containing high concentrations of DENV enhance the probability of disseminating the virus for secondary tissues, increasing the chances of virus being found in the salivary gland of *Ae. aegypti* and the prevalence of infectious mosquitoes after the blood feeding [83, 84].

After the penetration into the midgut epithelial cells, the virus begins the replication process. The virions need to pass through the basal lamina of the midgut epithelium to enter the hemocoel. The hemocoel is the mosquito's body cavity, which contains the organs and muscles and is an open circulatory system that contains hemolymph fluid. Following escape from the midgut into the hemocoel, arboviruses typically disseminate to secondary tissues and organs such as fat body, ovaries, hemocytes and nerve tissue, occurring the viral dissemination in the body of the insect. In non-susceptible mosquitoes, the dissemination does not occur, and the infection is confined to the midgut, in general, in low titers [85].

Finally, it is necessary to establish the infection in salivary glands. Mosquito salivary glands are laterally paired organs located in the thorax. Each gland consists of three lobes or acini, two lateral lobes and one medial (shorter median lobe), connected to a main salivary duct [86]. The lateral lobes can be divided into proximal and distal regions. The glands are made of a single layer of epithelial cells, which are surrounded by a basal lamina and different regions of the glands excrete different proteins. Arboviruses' infection of salivary glands typically begins in the distal lateral lobes [85, 87]. DENV-2 and CHIKV, for instance, infect the proximal

lateral and median lobes of *Ae. aegypti*. The distal lateral lobes of salivary glands in *Aedes* mosquitoes are speculated the site containing receptors to enable endocytosis of arboviruses [85].

Following replication, the virus is released into salivary ducts for horizontal transmission to an uninfected vertebrate host [88] (**Figure 3**). Once the salivary glands of the mosquito become infected, the mosquito transmits the virus throughout his life [89]. The arthropods' saliva is known to facilitate transmission and modulate host responses to virus replication by injecting a variety of substances, which contains complex protein peptide mixtures such as glycosidases, antimicrobials, antihemostatics, proteins with angiogenic or anti-inflammatory properties, and immune modulators [90].

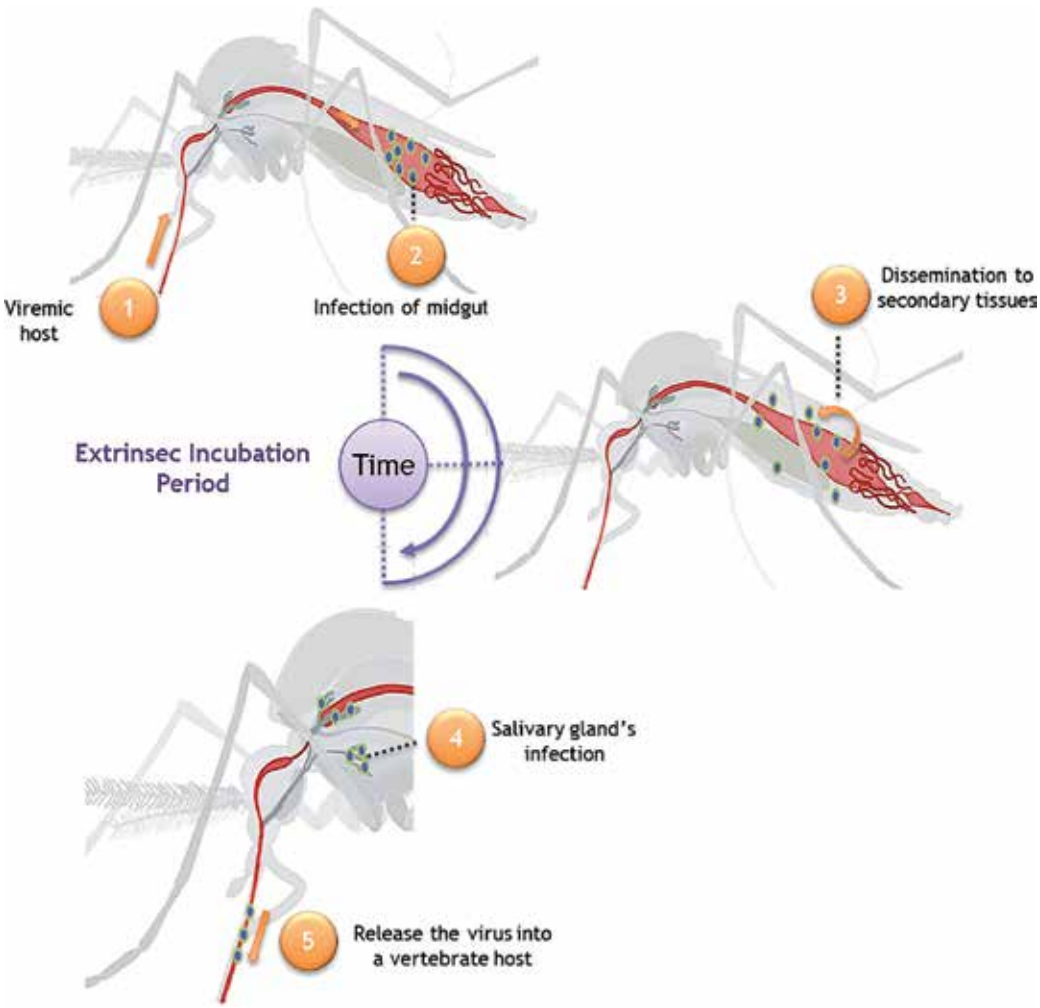

Figure 3. The main steps for an arbovirus infection in the vector: (1) the arthropod is exposed to and becomes infected when ingesting blood from the viremic host; (2) epithelial cells' infection of the midgut by the ingested viral particles, thus occurs viral replication and spread within the midgut epithelium; (3) viral dissemination and amplification from the midgut to secondary tissues; (4) infection of salivary glands; and (5) release of the virus into salivary ducts for horizontal transmission, which can lead to inoculation into a uninfected vertebrate host upon refeeding.

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The extrinsic incubation period is dependent on the genetic characteristics of the virus, the viral titer and the amount of blood that the insect feeds [89]. In addition, environmental factors, mainly temperature, humidity and intrinsic factors of the vector competence and the viral genotype involved, may influence the spread of the virus to the salivary glands of the mosquito, affecting the EIP [85, 91–93].

The biological transmission of an arbovirus by a mosquito vector implies overcoming a series of physical and physiological barriers to allow the virus to be transmitted in a new blood meal along with the saliva and a subsequent gonotrophic cycle. Barriers to the insect able to prevent the virus to replicate and spread to the salivary glands, such as the innate immunity, the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection barrier (SGIB) and salivary gland escape barrier (SGEB), can significantly affect the vector competence.

It is known that a close combination between genotypes of the mosquitoes and viral genotypes is imperative in determining these phenotypes [83, 94]. Intraspecific genetic variations in populations of the mosquito vector influence the various systems of barriers mentioned above, preventing or allowing the infection of various cells and tissues and the spread of the virus. These barriers are genetically controlled and can be expressed in various proportions in a population of mosquitoes, affecting the arboviruses epidemiology [77].

Genetic variation among mosquito's populations contributes significantly to the transmission potential and length of EIP, affecting the nature of human outbreaks [95]. Different samples of the DENV serotypes can replicate with different intensities and spread with distinct efficiencies, until the salivary gland in a same population exhibiting a wide variation in vector competence to transmit dengue [96, 97]. After feeding on a viremic individual, the mosquito Ae. aegypti becomes infected, and then a reported EIP of 7–14 days is required before the mosquitoes can transmit the virus to a new host [85]. Coinfections with different DENV serotypes in a single mosquito demonstrate competition between serotypes leading to a different transmission potential [98, 99].

In addition to vector competence, several other entomological parameters contribute to vector capacity, which reflects the overall contribution of the vector population to pathogen transmission [100], that is, the vector biting rate, vector density and vector survival.

Experimental infection and transmission of DENV in *Ae. aegypti* and *Ae. albopictus* have been extensively performed since the 1970s. Currently, the *Ae. aegypti* is the main vector for all four DENV serotypes, although *Ae. albopictus* has been incriminated in small-scale dengue epidemics and it is considered a minor vector compared to *Ae. aegypti* [101]. The potential role of *Ae. albopictus* as a dengue vector has become a major concern in dengue-free temperate regions where this mosquito has been established in the absence of *Ae. aegypti.*

In Brazil, the presence of *Ae. aegypti* is found in all regions and federal units of the country [102]. *Ae. albopictus* was detected for the first time in 1986, and by 2014, the mosquito was identified in 25 out of the 27 Brazilian states [103, 104]. It inhabits suburban and rural vegetated areas in Brazil whereas *Ae. aegypti,* more urban areas. Under resource-limited conditions, *Ae. albopictus* demonstrated to be a superior competitor than *Ae. aegypti* [105]. Coexistence of both

species in vegetated areas in Brazil is likely affected by seasonal environmental differences, such as detrital resource levels or egg desiccation [106].

Some ecological aspects of the interaction between DENV and *Ae. aegypti* have been explored. It was observed a negative impact on mosquito fecundity, since infected females laid fewer eggs per clutch than uninfected controls in the third and subsequent oviposition cycles [107]. Moreover, it was observed that infected mosquitoes spent more time ingesting blood [108], and *Ae. aegypti* females infected intrathoracically with DENV-2 had an increase of up to 50% in their locomotor activity when compared to uninfected control [109]. In DENV-infected mosquitoes, increased locomotor activity could potentially increase the chances to find a host [110]. However, a recent study shown that, vertical or horizontal viral transmission has no reproductive cost on *Ae. aegypti* females, suggesting why both types of transmission are sustained evolutionary [111]. Despite the existence of DENV vertical transmission was recently report that asymptomatic infections in human host and infected individuals' movement are more important determinants of DENV's persistence [112].

CHIKV is also transmitted by *Ae. aegypti* and *Ae. albopictus*, and occasional coinfection has been reported [113]. The extrinsic incubation period (EIP) ranges from 2 to 9 days, with an average of 3 days [114]. A number of studies have focused on identifying particular viral genetic determinants that could be driving successful infection of mosquitoes as hosts.

In the CHIKV outbreak occurred in La Reunion island in 2005–2006, a single viral mutation at the position 226 on the E1 glycoprotein in ECSA genotype (E1-A226V) was associated with an enhanced ability of the *Ae. albopictus* significantly infect and disseminate the virus [115]. This viral variant was selected after passing through the midgut barrier, the first step in mosquito infection [116].

American populations of *Ae. aegypti* and *Ae. albopictus* are responsible and highly efficient in transmitting the Asian and ECSA CHIKV genotypes (with and without the E1-A226V mutation [117]. Interestingly, several positions in the CHIKV genome were later discovered to exert strong epistatic effects on the E1-A226V substitution [118, 119]. Recently, a double mutant virus containing E1:K211E and E2:V264A mutations in background of E1:226A revealed remarkably higher fitness for *Ae. aegypti*, as indicated by significant increase in virus infectivity, dissemination and transmission compared to parental E1:226A virus [120]. Therefore, CHIKV represents a threat to the public health in infested areas or in the process of infestation by both *Ae. aegypti* and *Ae. albopictus*. In Brazil, the CHIKV ECSA genotype was detected in 2014; however, the isolates did not contain the A226V mutation on the viral genome [20, 121].

Currently, only *Ae. aegypti* has been implicated in CHIKV transmission in the Americas and Brazil [122, 123]; however, experimental infection of *Ae. albopictus* by Asian strains of CHIKV has been reported [117]. In fact, the current chikungunya epidemic in the Americas could potentially spread on regions infested by both vectors, but with low risk to regions in Europe infested by *Ae. albopictus* [124]. Actually, it has been shown that CHIKV potential transmission by *Ae. albopictus* strong relied on the combination of the mosquito population, virus strain and temperature [125].

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The ZIKV emerged in the Pacific Ocean and subsequently caused a dramatic Pan-American epidemic after its first appearance in Brazil in 2015 [24, 30, 126, 127]. By October 2016, 60 American countries or territories have already reported active ZIKV transmission [128]. Although the virus can be transmitted between humans, it is believed that the most common mode of biological transmission in epidemic and endemic zones is by vector transmission [129, 130].

Although the virus has been discovered in Uganda for almost 70 years, little is known about natural ZIKV vectors. *Aedes* mosquitoes are considered the primary vectors of ZIKV in Africa with reported viral isolations from several species, especially from *Ae*. *africanus* [130, 131]. More recently, natural infections screened by molecular methods in sylvatic African mosquitoes were again predominantly found in *Aedes*, but also in other species [132, 133]. Nevertheless, ZIKV transmission in the wild has remained poorly understood. In laboratory assays, only two sylvatic species (*Ae*. *vittatus* and *Ae*. *luteocephalus*) proved to be able to transmit ZIKV [134].

The domestic mosquito *Ae*. *aegypti* was early shown to be competent to experimentally transmit ZIKV [135]. Due to its high anthropophilic and domestic behaviors and virus detection in field caught specimens [136, 137], this mosquito has been incriminated as the urban and periurban vector in Africa and Asia [130].

ZIKV has only recently emerged outside of its natural distribution in Africa and Asia and has caused a series of epidemics in urban and periurban sites on Pacific islands [24, 138, 139] before reaching the Americas, probably in 2013 [140]. The spreading virus belonged to the Asian genotype. Despite multiple efforts, the mosquito vectors involved in the ZIKV outbreaks across the Pacific Ocean from 2007 to 2015 were not identified. Experience with ZIKV in the Pacific confirmed that the virus may be transmitted by different vectors during outbreaks, that is, by *Ae. hensilii* in Yap State, *Ae. aegypti* in New Caledonia and *Ae. aegypti* and/or *Ae. polynesiensis* in French Polynesia. In Gabon, *Ae. albopictus* introduced into an environment where the *Ae. aegypti* level was low was the vector for ZIKV [141]. Further experimental studies supported the role for Asian populations of *Ae. albopictus* as vectors of ZIKV transmission concomitantly with *Ae. aegypti* [142, 143].

The global number of zika cases, either suspected or confirmed in the Americas, reached levels never seen before [144, 145]. The virus proved to have a high potential for geographic expansion in regions where *Ae*. *aegypti* mosquitoes are present and concomitantly with DENV 1–4 and CHIKV, as it has occurred in Brazil and other American tropical and subtropical countries [128, 145].

It has been shown that American *Ae*. *aegypti* and *Ae*. *albopictus* populations showed to be competent to transmit the ZIKV belonging to the circulating genotype but displayed heterogeneous infection, dissemination and transmission rates in laboratory assays [146]. Currently, our knowledge of the ZIKV vectors in all reported studies from Africa, Asia, the Pacific region and the Americas is pointing the *Aedes* mosquitoes as the main vectors [147]. Furthermore, the identification of those potential vectors has important implications for the disease outbreak control, especially with the rapid disease spread in the world.

3. Conclusion

Anthropogenic environmental modifications, climate change, global transport network expansion, disordered urban growth are some factors that influence the emergence or reemergence and transmission of vector-borne diseases. The Brazilian population is exposed to infections caused by arboviruses previously described and transmitted by mosquito vectors with anthropophilic habits, widely distributed on the national territory.

The characterization of behavioral patterns allows a better understanding of the transmission dynamics and the design of more effective vector control strategies. No vaccine or specific treatments are available to most arboviruses diseases; therefore, the emergences and epidemics rely mostly on vector control and personal protection. Furthermore, the cocirculation of distinct arboviruses in a same region leads to a complicated clinical and laboratorial diagnosis, as signs and symptoms are similar, and much diagnostic tests are difficult due to cross-reactions.

The transmission' cycles are dynamic with ecological and molecular interactions, between the vector and the pathogen. Many of the steps of those interactions are now seen as of potential use in the control of endemic diseases, through strategies that have targeted the vector, the pathogen transmitted or the transmission' mechanism. In that scenario, understanding the mechanisms of viral-vectors' interactions, as well behavioral characteristics contributing to their competence in transmitting the viruses, is still in need.

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Chapter 12

Major Disease Vectors in Tanzania: Distribution, Control and Challenges

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Additional information is available at the end of the chapter

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Abstract

Disease vectors remain a major public health challenge in spite of efforts done to control across Tanzania. Different disease vectors have been controlled and efforts are on to eradicate them but challenges are still emerging and managed. In spite of all these success, different disease vectors have been observed to have developed resistance to all classes of insecticides used in public health practices in Tanzania.Resistance reports to main different vectors have been coming throughout Tanzania. The resistance of vectors to insecticides has been of different mechanisms depending on species, insecticides and mechanisms of action of the pesticides. Social economic factors and housing style still a major factor for the distribution and foci of vector abundance. The impact of public health intervention has been observed but still disease vector existence is noticed. Careful monitoring of the public health priorities for disease vectors control should be rethought to keep the elimination track live. Different tools such as insecticides use, understanding control measures, vector distribution and human lifestyle can lead to reduced burden caused by disease vectors. This chapter has described mosquitoes, tsetse flies, soft ticks, blackflies, and houseflies in terms of distribution, abundance, control and challenges of eradication in Tanzania.

Keywords: vectors, disease, insecticides, control, Tanzania

1. Introduction

In Tanzania, like any other developing countries, disease vectors are distributed throughout different ecological zones. Vector abundance and distribution depend on the host availability, climate and breeding sites availability [1–3]. In different regions of Tanzania, disease vector

abundance depends on the use of intervention tools, human activities, social economic status and knowledge on disease vector control (such as traditional practices) [4, 5].

Disease vectors in Tanzania play different roles in transmitting disease from man to man or from animal to man and vice versa. Mosquitoes from two families of Anophelines (all Anopheles mosquitoes) and Culicines (Aedes, Mansonia and Culex) are potential vectors across the country for malaria, filariasis, dengue, chikungunya and of recent Zika viruses. Tsetse flies of different species are principal vectors of African trypanosomiasis, which is caused by *Trypanosome brucei*, most foci are found around national parks and savannah areas. Soft ticks (Ornithodorous sp) are important vectors of *Borrelia duttonii*, the causative agent of tick-borne relapsing fever in Tanzania. In black flies, the most important genus of medical importance is Simulium that has most of the vector groups such as *Simulium damnosum* complex and *S. naivae* group, which transmit *Onchocerca volvulus* that causes human blindness. House flies are common vectors of human diseases such as pathogens (such as *Vibrio cholerae* and Shigella), viruses of polio, Coxsackie and protozoan such as Entamoeba, cryptosporidium, enterobius and giardia.

The government of Tanzania has been putting much effort to ensure reduction and subsequently control of disease vectors [6–9]. The most important targeted disease vectors include mosquitoes, tsetse flies, soft ticks, black flies and house flies. All of these mentioned disease vectors are distributed in different ecological area with the varying abundance and infectivity [10, 11].

In this chapter, all five disease vectors are discussed with focus in vectors distribution, control and challenges in Tanzania.

1.1. Mosquito (Culicidae)

In Tanzania, the main mosquito vectors for diseases are *Anopheles gambiae* s.l. (including *An. gambiae* s.s., *An. arabiensis* and *An. merus*), *Anopheles funestus*, *Culex quinquefasciatus* and *Aedes aegypti*. In Tanzania, among members of *An. gambiae* s.l. found to be vectors of malaria and filariasis are *An. gambiae* s.s., *An. arabiensis* and *An. Merus* [10–14]. In the past, *An. gambiae* s.s. population dominated the areas with high humidity from Coast of the India Ocean and decreased toward mainland Tanzania where *An. arabiensis* dominated [15, 16]. Currently due to different factors including land use changes, climate change and intervention to uses, the vectors species composition in coastal area have changed with *An. arabiensis* taking an upper hand against *An. arabiensis* [12, 17, 18]. The distribution of *An. arabiensis* and *An. gambiae* s.s. has been observed to occur in different proportions in other regions of mainland Tanzania [19–22]. *An. merus* is still restricted in the coast of mainland Tanzania and Zanzibar [12, 21–25].

Anopheles funestus in Tanzania is distributed throughout the country [10, 13, 26–30]. Among the Sibling species of *An. funestus* complex, *An. funestus* s.s., *An. leesoni*, *A. rivulorum* and *An. parensis* are the most abundant throughout the country [10, 27, 30]. They have been found to vector malaria and filariasis in Tanzania [13, 31].

The *Aedes aegypti* distribution in Tanzania is countrywide but with more focus on urban areas and areas with high altitude [32–35]. These vectors are found mainly in human settlement

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with regard to its anthropophilic behavior [36]. These vectors feed during the daytime and breed within the human settlements water holding containers and tanks [35, 37–39] and also in vegetation leave axis [40]. These vectors feed indoors and outdoors [41]. These are the mainly vectors: dengue, chikungunya, yellow fever in Tanzania [35, 42] and ZIKA virus outside Africa [43].

An. gambiae s.s. and *An. arabiensis* breed in clean water exposed to sunlit [44]. The breeding habitat varies in size and type from footprint, abandoned goldmines and drainage ditches, cultivated swamps [45] and Paddy farms [46, 47]. The populations of *An. gambiae s.s.* and *An. arabiensis* decrease with an altitude increase and temperature decrease [48]. Climate change and deforestations in highlands of Tanzania have led to colonization of these vectors in those areas such as in Usambara Mountains [49–51] and Hai district [48]. Farming in highlands has led to productive habitats and subsequently vectors colonization in highlands [45].

An. gambiae s.s. feed indoor and rest indoor due to their behavior of being anthropophilic and endophilic [52]. Due to high bed nets and indoor residual spray coverage, the indoor surfaces with insecticides repel mosquitoes or kill them hence they have forced to be outdoor feeders [53].

An. arabiensis are zoophilic and exophilic [48]. They feed outdoors in bovines and only on unprotected human when the bovines are not available [54, 55]. The use of LLINs and IRS programs affects the indoor feeding behavior of *An. arabiensis* (endophagic) for increasing irritation, knockdown and exophily to mosquitoes [53]. These vectors have developed avoidance behavior for the treated indoor surfaces [53]. Due to genetic feeding behavior of being anthropophilic and zoophilic for *An. gambiae* s.s. and *An. arabiensis*, respectively, the population of *An. gambiae* s.s. has decreased drastically due to high coverage IRS and LLINs, hence shift in population from *An. gambiae* s.s. to *An. arabiensis* in most areas [12, 17, 18]. Both *An. arabiensis* and *An. gambiae* are potential malaria and filarial vectors in spite of control efforts [12].

An. merus breeds in the salt water along the shore of the Indian Ocean [56]. These vectors breed in salt water exposed to sunlit. The distribution of these vectors is limited to Indian Ocean coast. These vectors are anthropophilic and rest indoor. It disease transmission efficiency is restricted to small-scale because of their breeding sites preferences.

An. funestus sibling species that are abundantly found in Tanzania breed in shaded habitats with high vegetation cover [57]. The feeding and resting behavior of *An. funestus* sibling species differs between them depending on the host preference. *An. funestus* s.s. is anthropophilic, endophagic and endophilic. The vector population of *An. funestus* sibling species distribution has been affected with wide range of LLINs and IRS coverage [13, 29, 57].

Culex quinquefasciatus are vectors associated with the urbanization breeding mostly in the polluted habitats such as sewage system, pit latrines and septic tanks [58, 59]. These vectors are distributed throughout the country [60]. In spite of being distributed throughout the country, they are nuisance vectors in the mainland Tanzania, while they are suggested to be vectors of filariasis in the coastal Tanzania [61, 62]. In coastal Tanzania, they transmit filariasis because of high humidity and presence of microfilaria in human population. In mainland Tanzania, *Culex quinquefasciatus* has been considered as one of the potential vectors of harbovirus

diseases (Rift Valley fever) vector [32, 35]. *Culex quinquefasciatus* feeds and rests indoors (been endophilic and endophagic) [63, 64].

1.1.1. Mosquitoes control

1.1.1.1. Indoor residual spray

In Tanzania, the classes of insecticides currently used for IRS are pyrethroids; carbamate and organophosphate IRS have been effective by increasing mortality knockdown and exophily of mosquitoes. To some classes of insecticides such as pyrethroids, high resistance has been reported throughout the country, which reduces its efficiency against pyrethroids resistant vectors [19, 20, 65–67].

1.1.1.2. LLINs indoor residual spray

LLINs play two major roles in mosquitoes control. First, it plays a physical barrier role [68] and second, it plays a chemical role for irritating, knocking down and increasing exophily for mosquitoes [68]. Mosquito nets are treated with pyrethroids alone. Of the recent, incorporation of PBO on the LLNs has shown to increase the efficacy of the LLINs against resistant wild populations of mosquitoes [69, 70]. Currently, multiple resistance interested in pyrethroid throughout the country against different vector species threatens the use of LLINs to remain as physical barrier only [68, 70]. LLINs have shown to be more efficient when combined with other tools such as IRS [28, 71] and larval source reduction [72, 73].

1.1.1.3. Larval source management (LSM)

LSM has been applied in small scale and mostly in urban areas. The most areas in Tanzania covered by LSM practice are in the city of Dar es Salaam through urban malaria control [6, 7]. This method has been found to be effective when larval sources are few and manageable (reference). The advantage of this method it utilized well is that the immature stage of mosquito is nonmotile [64]. LSM has shown effectiveness in vectors and disease transmission when done well alone [74–76] in combination with other methods such as LLINs and IRS [72].

1.1.1.4. Use of repellents

Repellents are the compounds used to keep mosquitoes away of the host when applied properly. Among the number of brands of repellents that are available in the market, DEET has been considered as the best reference repellent [77]. Other plant-based repellents include Citronella oil, Lemon grain oil, MRO8, Maskitaa and Ocimum brands have been considered as effective repellents [78–82].

Repellents are used as supplementary tools for LLINs and IRS to prevent bites before retiring to bed or for those getting out of the bed early in the morning in active biting cycle of mosquito. These are effective for all mosquito species.

1.1.1.5. Use of coils

Mosquito coils have irritancy and knockdown effect against mosquitoes. The coil protection time has been found to be 6–8 h [83]. Mosquito coils are burnt inside the house in a room where protected population is expected to have asleep. Burning mosquito coils protects those who are not under bed nets by repelling and forced exophily. The use of coils in areas with LLINs and IRs coverage might strengthen the protection against infective bites.

1.1.1.6. House modification

In Tanzania, in last two decades, there has been much in house structure improvement for better settlements. In traditional houses, more than 70% of mosquitoes entering the house were through caves (the space between a wall and a roof) [84, 85]. The rest of 30% or less was entering through unscreened doors and windows [84, 85]. With the public health education given to community, house improvement in different regions in Tanzania has shown that sealing the caves, screening the doors and windows reduced house entry of mosquitoes [85, 86]. The risk of disease incidences is directly proportional to the house modifications [86–88]. House modifications mostly play a major role in reduction and control of indoor vector dusty and disease transmission risks. House improvements have vividly shown to be effective in different ecological setting in reducing indoor vector density [86–88].

1.1.2. Challenges in mosquito control

Despite of the successful efforts invested in mosquito control in Tanzania, which have led to reduced mosquito-borne disease outcomes, there are still some emerging challenges in control. These challenges are:

1.1.2.1. Insecticides resistance

In Tanzania, the intensive use of insecticides for public health and Agricultural pests control has been the best sources of mosquitoes insecticide resistance [19, 65, 67]. Insecticide resistance has been found against pyrethroids, organochlorines, organophosphates and of recent in carbomates insecticides [89–91].

Insecticide resistances have different mechanisms involved. These mechanisms have enhanced the reduction of toxicity efficiency of insecticides, hence survival of vectors.

In current time, several mechanisms have been realized in Tanzania such as metabolic resistance [91]. This mechanism deals with elevating enzymes efficiency in detoxification of insecticides. The other mechanism is knockdown resistance (kdr), which has been found in both caring genes for Western African *kdr* (*kdr* West) and East African *kdr* (*kdr* East) [19].

1.1.2.2. Behavioral changes

One decade ago after intensive LLINs distribution and scaling up, vectors have changed feeding and resting behavior [53, 92]. Other factors such as house modification of installing

window mesh, door mesh and sealing the eaves have caused vectors to feed and rest outdoors [53, 85, 86]. Most vectors such as *An. gambiae* s.s. have changed the natural ecological feeding and resting behavior from feeding and resting indoors [93] to feeding and resting outdoors [53]. This has caused vectors to avoid LLINs contact and indoor sprayed surfaces for biting and resting outdoors. Odor-baited traps with insecticides can be an alternative to be deployed outdoor for controlling outdoor malaria transmission, which cannot be targeted by neither LLINs nor IRS.

1.1.2.3. Urbanization and poor planning

Most urban areas are growing fast with more people migrating from rural for better jobs and opportunities in urban. The settlement demand has caused the emerging growth of unplanned settlement, which subsequently has led to poor land use planning and drainage systems in which mosquitoes have capitalized as potential breeding sites [76]. The increased population in urban has led to demand for more agriculture produce, which have created potential breeding sites that are difficult to be attended at a point of time, hence leading to adult vector productivity in urban [75, 76]. The quality of houses in unplanned urban areas is poor and cannot protect occupants against disease vector, which have house entry behavior such as mosquitoes.

1.1.2.4. Social economic status

The low social economic status mostly in rural and in populated urban areas has caused the impairment of the efficiency of disease control incidences and cases [94]. The low income has caused the communities to fail to improve healthy living status for not meeting the costs of vector control such as house improvements, LLINs and IRS programs when they are not provided for free. In rural setting, the improvement of livelihood, health seeking behavior and use of protective tool such as LLINs have been found to correlate with the income of the family [95–97].

1.2. Tsetse flies (Glossinidae)

1.2.1. Tsetse distribution and occurrences in Tanzania

The tsetse flies (*Diptera: Glossinidae*), referred to by Nash 1996 as "Africa's bane," are small insects that resemble a house fly. It ranges in size from 8 to 17 mm. These insects are characterized by a distinct proboscis, antenna with branched arista hairs and by wings that fold at rest and have a characteristic "hatchet" cell. There are 31 living tsetse species belonging to the *Glossina* genus and recent genetic studies have identified new markers meaning that the list may be expanded in the future [98, 99]. However, out of the 31 known species of tsetse flies, only 8–10 species are considered of veterinary and public health importance. Tsetse flies occur in 38 African countries, infesting a total area of 10 million km^2 in sub-Saharan Africa [99]. The *Glossina* fly is solely responsible for the cyclical transmission of the protozoa *Trypanosoma brucei*, which causes human African trypanosomiasis (HAT), also called sleeping sickness and African animal trypanosomiasis (AAT), also known as nagana [100–102].

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Humans and livestock who live in tsetse-infested areas are continually exposed to the risk of these infections [102–105].

The genus *Glossina* is divided into three taxonomically distinct groups based on morphological characteristics, habitat requirements and preferred hosts [106]. (i) Subgenus *Morsitans* are mainly found in Savannah areas, including open areas and thickets. The most widely distributed species in Tanzania is *G. morsitans,* followed by *G. pallidipes.* Others in this group are *G. morsitans centralis*, *G. swynnertoni* and *G. austeni.* (ii) Subgenus *Palpalis* inhabit riverine and lakeshore habitats. These include *G. fuscipes fuscipes and G. fuscipes martinii*. (iii) Subgenus *Fusca* can be found in forest areas and near riverbanks e.g., *G. fusca*, *G. longipennis*, *G. brevipalis* and *G. fuscipleuris* [99, 107]. According to a distribution map produced by Ford and Katondo in 1977, two thirds of Tanzania was infested by 10 species and subspecies of tsetse fly. The infestation was distributed between four separate fly belts [108] and is predominantly of the *Morsitans* subgenus, but also includes species from the *Fusca* and *Palpalis* groups, which were restricted toward Lake Victoria and Tanganyika, respectively.

1.2.2. Current situation on tsetse distribution in Tanzania

Since Ford's tsetse distribution map of 1977 for Tanzania, there has been no clear updated distribution map available showing tsetse distribution across the country. However, a number of surveys were undertaken countrywide from 2003 to 2012 to better understand the current distribution. The current updates show that 43% of the country has high to low risk tsetse infestation and 57.4% has no risk. This estimated percentage was surveyed across 16 infested regions in the country [109]. According to a study conducted from 2005 to 2007 [110], tsetse distribution has been altered due to changes in land cover and usage, which is driven by population growth, expansion of human settlements and associated agricultural and infrastructure development activities and land reform policies. These have significantly contributed to the destruction of tsetse habitat ecology, causing a new tsetse distribution limit with fragmented pockets of tsetse flies [111].

1.2.3. Life cycle and reproduction

The life cycle of tsetse is unusual since they do not lay eggs. Instead, after mating, a female tsetse fly develops the egg and young larva within her uterus. A full grown larva is produced every 10 days and quickly deposited in a shady area. Larvae burrow into the soil and pupate virtually immediately, in contrast with other insects. The adults emerge 20–45 days later, depending on temperature. Pupal development does not succeed below 17°C or above 32°C. Thus, each female can produce only one offspring at a time and can produce up to 12 offspring during her typical adult lifespan of 2–3 months. Thus, the tsetse population growth tends to be low. Both male and female adult tsetse take blood meals from a variety of vertebrate hosts every few days and in so doing may cyclically transmit the pathogenic trypanosomes and cause HAT or AT [112, 113].

From precipitin tests, it is concluded that the principal hosts of G. *swynnertoni* from one locality were: (1) a large bovid, possibly roan or kudu, (2) giraffe, (3) wart-hog or bush pig and (4) primate [114]. The absence of abundant antelope species such as impala and duiker from

the list of probable hosts and the exclusion of man and baboons from the list of primates suggest that these tests should be treated with a degree of reserve.

1.2.4. Population ecology and dynamics

Tsetse distribution is mainly influenced by density independent (abiotic) factors such as temperature and humidity, which in turn influence vegetation cover. In contrast, fly density is determined by the availability of suitable habitats and hosts, which is influenced by human activity such as expansion of settlement and agriculture, deforestation, livestock movements and habitat fragmentation. These alterations in tsetse distribution and density may have an influence on the transmission of infectious diseases [112].

Generally, tsetse flies are unable to fly for long periods but instead fly in short bursts, with a relatively low capacity for active dispersal. The average total distance flown per day varies between 4.5 and 9 km. They can also be passively dispersed by vehicles, floating vegetation and animals. It is also reported that movements of tsetse flies within a uniform habitat are fairly constant in length and can be related to host-seeking behavior for a blood meal. This behavior is modulated by exogenous and endogenous stimuli. Exogenous stimuli include temperature, vapor, pressure deficit, visual and olfactory stimuli, while endogenous stimuli include levels of starvation, age, sex, pregnancy status and circardian rhythm of activity [115]. Tsetse flies locate their hosts by a combination of olfactory and visual cues. The ability for the tsetse fly spread over a long distance even though in short bursts still causes risk of transmission to new areas or reintroduction to areas/places that have been under control [106].

1.2.5. Tsetse control practices in Tanzania methods (past and current experiences)

Tanzania is among the African countries, which is highly infested by tsetse flies, thus they continue to pose a risk for both humans and domestic animals, despite considerable investments toward control of tsetse over many decades [116]. Attempts to control testes files in Africa including Tanzania were initiated during the colonial era and soon after independence [110]. In the mid 1950s to 1980s, large-scale control programs were implemented, including aerial spraying, clearing vegetation and destruction of hosts to eliminate tsetse and disease eradication seemed a possibility at that time [110, 117].

1.2.5.1. Clearing of vegetation

The former method of clearing vegetation was either by total removal or by removal of only vegetation that was important to support tsetse flies through bush burning. This method was not environmentally beneficial and had left some areas with permanent effects, exacerbated by drought episodes. This control strategy is no longer used due to the environmental degradations it caused [110, 111].

1.2.5.2. Destruction of hosts

Since tsetse flies are hematophagous and feed on wild animals, wild animals have become reservoirs for the trypanosome infections that then spread to domestic animal and humans.

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Widespread mass killing of wild animals led to the decline of tsetse fly infestations and ultimately to reduction in trypanosomiasis cases. Despite being effective at the time, the method is no longer acceptable due to its association with environmental destruction [118, 119].

1.2.5.3. Insecticides

After independence, aerial spraying of insecticides on the ground was in use. This method was extensively used in Northern Tanzania, mainly in Arusha, in the early days of control against vectors of sleeping sickness in areas of Babati. The insecticides used were either residual such as DDT, Endosulfan or nonresidual such as synthetic pyrethroid compounds. However, aerial spraying had challenges including how to minimize product loss due to spray drift and ensure maximal deposition on the targeted ground. Despite the method being widely used and considered successful, there was still a challenge with reinvasion of tsetse flies and also insecticide resistance [120]. The methods caused a significant reduction of fly infestations and ultimate control of trypanosomiasis. This method was also considered as being environmentally hazardous and is no longer applied [118, 121].

The use of ineffective methods, the emergence of resistance and environmental concerns motivated the engagement of better strategies; these include the use of chemicals (insecticides) on cattle, traps and targets, bait technology and biological controls such as sterile insect technique.

1.2.5.4. Traps and targets

Traps are black and blue insecticide-impregnated fabric screens that attract the flies by the blue segment, which then land on the black segment and quickly succumb to the insecticides [122]. Apart from the control of tsetse flies, the traps can also be used for entomological surveying, as they attract the flies and trap them upwards. Targets are simpler to traps; both are impregnated with biodegradable pyrethroid like deltamethrin. The efficiency of traps and targets is enhanced by the use of odor attractants such as acetone and cow urine. This technique is important for monitoring and estimating the control and prevention of trypanosomiasis epidemics; they are also important as a suppression tool before other technique was being applied such as SIT [123].

Efficiency of traps and targets varies depending on type of tsetse flies species surveyed, location (habitat) and type of traps/targets used for tsetse collection or survey. For instance, NGU, Epsilon and F3 are markedly superior to the biconical and pyramidal traps for *G. pallidipes* and *G. brevipalpis* [124]. Also, some targets are found to be more efficient when limited to a certain size [125].

The technique is credited as the most ecologically friendly technique [126]. In addition, traps and targets can be used with the combination of live baits to speed up suppression of the vectors before elimination is achieved. In recent years, modifications of traps have been achieved to increase their efficiency in catching specific *Glossina* spp. in specific environments [119, 125]. Despite the method being cost-effective and environmentally friendly, a widespread implementation of this technique in Tanzania has failed, due to lack of proper

infrastructures to manage and sustain the traps/targets over large areas and its failure to eradicate residual tsetse populations [127]. The insecticide-impregnated targets have some drawbacks in terms of insecticide efficiency reduction in the targets due to rain, sunlight, wind and dust.

This control method has been present and applied in a sporadic manner. However, Tanzania National Parks Authority (TANAPA) has extensively utilized the technique to control tsetse flies across several national parks and communities around national parks [128]. The tsetse control strategy played a significant role in the control of previous HAT outbreaks to tourists, park staff and surrounding communities in northern parks. This is one of the major achievements by parks control efforts. However, there are still challenges with tsetse flies in national parks, which might be attributed by the fact that there is a possibility of reinvasion from areas in which tsetse flies have not been controlled. Also, the environment and habitats in the parks support the thriving of tsetse, due to the availability of stable vegetation for resting and breeding, abundant supply of blood meal from wild animals and with vegetation attracting large density of tsetse flies [128].

1.2.5.5. Bait technology

Another useful method is bait technology; this can be used in live animals or moving objects such as sprayed vehicles. The technique involves treating cattle with appropriate insecticide formulation, usually by means of cattle dips, or as pour-on, spot-on, or spray-on treatments. The formulations are highly effective against tsetse flies as well as ticks. The method has been used in Mkwaja, Mzeri and Kagera ranches in Tanzania, since the early 1990s [110, 129]. To date, the method is still commonly practiced and easily adopted by farmers. However, there are challenges associated with the effective use of insecticides due to farmer's diluting stock solutions incorrectly, which may led to insecticide resistance [124]. In national parks, vehicle spraying has been used in recent tsetse control programs, whereby vehicles travelling into the Parks are sprayed with insecticides to serve as moving targets. The method attracts flies to the moving object and hence serves as a control of tsetse flies in the national parks. The lower half of a vehicle is sprayed with approximately 16 L of Glossinex® diuted to 0.25 ml/L [128].

1.2.5.6. Biological control

Sterile insect technique (SIT) was used as a biological control method that involves three main steps: (1) production of large numbers of target insects, (2) sterilization of male flies and (3) sustained and systematic release of sterile males over the targeted area with large numbers of flies (March 2013). The method has significantly proved its potential against riverine and savannah tsetse species. A first full-scale project was implemented in Zanzibar Island, Tanzania and the project was successful in eradicating tsetse flies (*G. morsitans morsitans*) in Zanzibar Island (1994–1997), also in Burkina Faso (1980s) and Nigeria (1979–1988). The technique was enhanced by integrating the release of sterile males with the use of targets [99]. The mating of sterile males with females lead to female infertility for the rest of its life span; however, recently this has been shown not to occur in species like *G. fuscipes*. Theoretical models clearly

demonstrated that, the method is efficient and cost-effective, as the natural population declines with the increase of the sterile male population [99]. Despite of the method being advantageous, there are challenges associated with this technique such as the quality of the released insects and require a low target population density. The method has failed in some areas, where population targets have been high or when there are other technical and logistic difficulties involved [130]. Other countries have used SIT as part of an area-wide integrated pest management approach in combination with other control tactics to eradicate, suppress, or contain pest population of Diptera, Coleoptera and Ledioptera (Screwworm fly) in the USA, Mexico, Central America and Libya [99].

Unintentional causes of tsetse control also occur when anthropogenic landscape modifications involve the destruction of tsetse habitat. This is influenced by demographic pressure including expansion of human's settlement and increases in agricultural development. This control is very effective and less expensive and has been increasing in recent times; however, it is not a feasible approach to tsetse control [131].

1.2.6. Challenges for tsetse control in Tanzania

Current endeavors in the control of tsetse flies across Tanzania have been hampered by a lack of funds or different priorities and subsequent improper policies set by the government. These challenges began during larger economic structural adjustments about three decades ago and since that time, there has been no embracing of tsetse fly control. Withdrawal of donor support and a reduced role of central government in veterinary services have caused discontinuity of the existing control programs. This has impaired research and capacity building both in terms of infrastructure and manpower. A number of government research institutes are no longer active, nor is there a tailored course for junior tsetse experts. As for livestock keepers, control of parasites is within their means, with high variation to standard procedures, hence increasing the risk of insufficient preparation usage of insecticides and irregular treatment of chemotherapy, which may lead to drug/acaricides resistance potentials.

Another challenge facing tsetse control in Tanzania associates with the identified hot spots for tsetse breeding. These spots are currently confined in protected areas in the form of game reserves and reserved forests. Hence, the effective control can only be achieved by joint efforts between authorities responsible for protected areas like Tanzania National Parks (TANAPA), the Wildlife Division in the Ministry of Natural resources and Local governments. The use of GIS and GPS for recording and updating distribution patterns of tsetse flies and trypanosomiasis can also be useful for control in these pocket locations. It is only through jointly coordinated efforts against tsetse that the vector will be eliminated from the county.

1.2.7. New opportunities in control

The existing collaborations in tsetse control activities through various organizations including TANAPA, Ministry of Health and Social welfare, Ministry of Agriculture, livestock and fisheries development, the Wildlife Division in the Ministry of Natural resources and Local governments must be strengthened and honored continuously for sustainable tangible impacts. Furthermore, the involvement of interested private sectors would strengthen the fight against

tsetse flies and trypanosomiasis across the country. The surrounding communities living in hot spot areas must be well involved in tsetse control activities to ensure sustainability of the control tsetse and trypanosomes efforts.

More research projects must be prioritized as a way forward to increase the efficiency of existing control methods. Updated data are crucial for efficient control of diseases. There is a significant work undertaken in reporting HAT cases, but this has not been the case for AAT. Researches into improved techniques are also needed, such as methods to maximize the lifespan and durability of tsetse fly targets and traps.

1.3. Houseflies (Muscidae)

In Tanzania, houseflies are distributed throughout the country colonizing both rural and urban areas [132, 133]. These disease vectors have been found to consume and survive well in household water throughout the country. Houseflies feed on several types of substances, almost all food materials for human, carcasses, rotting material, excreta and other inorganic materials. In feeding, the physical state of food material causes different feeding modes. For thin fluids, such as milk and tea, the labella are placed in contact with food, which is then sucked through the pseudotrachea. When the feed is in semisolid state such as fecal material, food leftovers and sputum, the labella are completely everted and food staff is suckled up directly into food channel. When feeding on a complete solid material such as sugar, cooked meat and dry blood, the labella and prestomal teeth which surround the food channel scrape the solid food. Then, a fly moistens small food particles. In Tanzania, the abundance of houseflies have been associated with poor hygiene and lack of sufficient amount of water supply in populated areas. The main houseflies species of medical importance belongs to genus *musca*. The important species in Tanzania are *Musca domestica* and *Musca sorbens* [132, 133]. These are the main species distributed and have the impact on public health for transmitting microorganisms for mostly trachoma, diarrhea and cholera [132, 134, 135].

1.3.1. Medical importance of housefly

1.3.1.1. Disease vector

Houseflies are the main vector transmitting microorganisms for mostly trachoma, diarrhea and cholera

1.3.1.2. Nuisance

The landing of houseflies on face frequently is disturbing and making a person uncomfortable. High occurrence of the houseflies makes house occupants uncomfortable which is regarded as nuisance.

1.3.2. Control

1.3.2.1. Treated curtains

Curtains are treated with insecticides with low mammalian toxicity such as pyrethroids.

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1.3.2.2. Spray

Use of spray aerosols in walls and roofs.

1.3.2.3. Treated cords

Insecticide treated cords are hanged indoor for flies to rest on them and pick up lethal dose of insecticide, hence increase mortality of flies.

1.3.2.4. Screened windows and doors

Screened windows and doors are physical barriers with for houseflies to enter the house.

1.3.3. Challenges

Due to poor planed urban and rural waste management, the perfect control of the house flies has been a task to be tackled in cross cutting manner.

1.4. Bedbugs (Cimicidae)

Bedbugs are distributed in all urban and rural areas of Tanzania [136–139]. Bedbugs distribution has been mostly associated with the human being movements worldwide [140]. This might be the case in Tanzania as well. Mostly, the bedbugs infestation is associated with poor hygiene and poor housing. In houses, bedbugs have been breeding in furnitures, bed and house wall cracks. They are nocturnal, but when they are hungry, they feed at any time on availability of host.

1.4.1. Control

1.4.1.1. Indoor residual spray

Different classes of Insecticides approved for use against pests of public health importance. The spray is targeted in furnitures, wall cracks and beds.

1.4.1.2. Use of LLINs

The wide coverage of LLINs increases the exposure of bedbugs to insecticides and increases mortality.

1.4.1.3. Hygiene

Household hygiene prevents and limits the distribution and survival of bedbugs.

1.4.1.4. House style improvement

House structure improvement from traditional to modern houses has led to increased hygiene and reduced the possible breeding sites for the bedbugs.

1.4.2. Medical importance

1.4.2.1. Annoyance

The highly bedbugs infested houses per room cause disturbance to occupant who are not been able to sleep. This causes uncomfortability for the room occupants.

1.4.2.2. Anemia for children

In highly infested family houses, the children and infants suffer from anemia due to high blood loss.

1.4.2.3. Sleeping stress

This is caused with high biting rates per night, which reduced the interrupted sleeping time.

1.4.3. Challenges

1.4.3.1. Insecticide resistance

Due to wide coverage of LLINs and IRS programs across Tanzania, bedbugs resistances have been reported from all areas with intensive coverage of LLLINs and IRS, due to extended exposure of insecticides as reported from Tanga, Zanzibar and Bagamoyo [136, 138, 139].

1.4.3.2. House improvements

House structure improvement progresses still in low rate from rural to urban areas, hence handicapping the efforts of bedbug control.

1.4.3.3. Human movements (student to school and travelers)

Human movements from infested to uninfested areas cause the spread of bugs and human movements cannot be restricted.

1.5. Black flies (Simuliidae)

Black flies are major Africa vectors of human onchocerciasis (river blindness), caused by filarial nematode *Onchocerca volvulus*. In Tanzania, foci are in southern central and northern east, which include Ruvuma focus and the Kilosa, Uluguru, Tukuyu and Mahenge and Amani forest where parasite transmission is mediated by *S. damnosum* s.l. Members of *S. neavei* group are the principal or sole vectors in two or three foci; they are associated with freshwater crabs and also known to attack human population [141–143]. All these foci are either located along the river valley or clustered along the Arc chain of mountains.

1.5.1. Medical importance

1.5.1.1. Annoyance

Black flies cause serious biting problems, although the severity of the reaction to bites differs in different individual, localized swelling and inflammation, which might be accompanied by irritations for several days.

1.5.1.2. Onchocerciasis

This is a nonfatal disease, called river blindness, which is caused by the filarial parasite *Onchocerca volvulus*. There are no annual hosts, the disease is not zoonosis. Black flies are the only vector of human onchocerciasis. Their feeding habit of tearing skin and feeding makes it possible for parasite to penetrate the human skin.

1.5.2. Control

1.5.2.1. Use of repellents

The reduction of human—black flies contract can be achieved by using repellents such as DEET or wearing pyrethroid-impregnated or sprayed clothing.

1.5.2.2. Use of insecticides (larviciding)

The water rivers found to be habitats are sprayed with larvicidal such temephos or *Bacillus thuringiensis var. Israelensis* (Bti). In areas with high infestations, applications should be repeated in 1–2 weeks interval, throughout the year to prevent recolonization. Due to the nature of habitat, the ground application of insecticides is more difficult, hence the aerial application is recommended.

1.5.3. Challenges

It has been difficult to reaching all the active breeding sites throughout the year.

1.6. Soft ticks (Argasidae)

Soft ticks have worldwide distribution. There are 193 species, which belongs to four genera. The most medical important genus is Ornithodoros. The most important species is *Ornithodoros moubata,* which is a vector-borne (endemic) relapsing fever (*Borrelia duttonii*). In Tanzania, soft ticks are distributed across the country. The regions mostly infested are Dodoma, Iringa, Mara, Dodoma, Mwanza, Tabora, Morogoro, Shinyanga, Manyara and Arusha and Zanzibar prisons [144–146]. The distribution of eggs, larvae, nymphs and adults of soft ticks is usually restricted to the infested structures occupied by any host [145, 147]. The most identification feature in soft tick is the absence of scutum (shield).

1.6.1. Medical importance

1.6.1.1. Tick-borne relapsing fever

Ornithodoros moubata transmits *Borrelia duttonii,* which is ingested during blood feeding and multiplies in the midgut ready for being transmitted to the host during next feeding.

1.6.1.2. Q-fever

Argasidae ticks can be vector but the most serious vectors are ixodid ticks.

1.6.1.3. Tick-bite allergies and tick paralysis

Ticks cause allergies such as itching, skin rashes and fevers.

1.6.2. Control

1.6.2.1. Use of repellents

The standard approved repellents for the use against soft ticks are DEET, Picaridin-based products or indalone.

1.6.2.2. Use of insecticide treated clothes

Clothes are treated with recommended dosage of pyrethroid like permethrin.

1.6.2.3. Indoor residual spray

Infested houses are sprayed with insecticides such as organophosphates, carbamates, propoxur and pyrethroids targeting cracks on walls and floors, furnitures and all possible sites where ticks can be hiding.

1.6.2.4. Frequent house maintenance

Plastering of house walls and floors can play a major role in reduction of hiding and breeding sites of soft ticks.

1.6.3. Challenges

1. Insecticides resistance among soft ticks population.
2. Poor house structure and quality in infested rural areas.
3. Culture and belief of some tribes of staying with animals in the same shelter.

2. Conclusion

Main diseases vector (mosquitoes, tsetse flies, black flies, sand flies and soft ticks) control in Tanzania has taken a new direction with great success in population decline. Community awareness has been done for long with aid of government and donor project funds for vector control. Community involvement during the campaign for vector control is an asset, which needs to be natured for maintaining the attained progress and go beyond. Community-based vector control programs should be institutionalized and operationalized by community for maintenance and sustainability.

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Chapter 13

The African *Chrysops*

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Additional information is available at the end of the chapter

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Abstract

African *Chrysops* are less studied than their European and American counterparts. The bionomics of only *Chrysops silacea* and *Chrysops dimidiata* is frequently reported. These two species feed on mammals in general but humans remain their main host. From the resting place in the canopy of the natural and secondary forest, they locate their hosts as they move but smoke of wood is a much better attractant than the movement. Other species live either in the rain forest or in the wooden savannah feeding on mammals and reptiles. *Chrysops* are biological and mechanical vectors of diseases in human and livestock. They also cause painful bites often resulting in open wounds, which can serve as open door for bacterial infections. In the past, control relied on the use of insecticides and clearing of vegetation around the habitations. Nowadays, recourse to repellents, trappings and destruction of the canopy around houses is recommended. The detailed geographical distribution of African *Chrysops* is still to be elucidated, as well as any genetic variability within and among species. The aims of the chapter are to provide the reader with the state-of-the-art knowledge on African *Chrysops*, and to present the gap in knowledge of this genus species.

Keywords: Africa, *Chrysops*, biting density, fly range, vector

1. Introduction

The genus *Chrysops* known as deer flies is under studied in Africa. Most of the knowledge on this genus date back to works performed in the years fifties to sixties. Interest was again raised on this genus in the years nineties when loiasis, a filarial disease transmitted by *Chrysops* species, stood as an obstacle to onchorcerciasis elimination programme. The literature on the topic is not

only old but also very limited and scarce, probably because *Chrysops* species are mistakenly considered as of little economic importance and also because few species are anthropophilic while the majority is mainly zoophilic. Although a number of *Chrysops* species are transmitters of many pathogens to livestock, what is currently known on the genus *Chrysops* is mostly derived from studies on *Chrysops silacea* and *Chrysops dimidiata*, the main vectors of *Loa loa* to humans. Nowadays, *Chrysops* species in Africa are neglected, under studied flies in comparison to other flies of veterinary and medical importance such as ticks, tsetse flies, mosquitoes, sand flies or black flies. They are only mentioned in various studies pertaining to loiasis or sporadically in mere fauna studies. Apart from its role in the transmission of loiasis, its vectorial role in transmitting both human and livestock diseases evoked decades ago has not been fully investigated. In addition, their detailed geographical distribution is still to be elucidated, talkless of the investigation of any genetic variability within and among species that may impact their distribution and their disease transmission potential. The aims of the chapter are to provide the reader with the state-of-the-art knowledge on African *Chrysops*, to present the gap in knowledge and to develop interest for further studies of this genus.

2. Importance of African *Chrysops*

Chrysops species are of medical and veterinary importance. They do not only transmit loiasis to human and animals but also cause harm to their hosts. The losses incurred by *Chrysops* transmitted diseases or bites have not been estimated but coma, encephalopathy and death are some of the outcomes of loiasis in patients treated with diethylcarbamazine or ivermectine [1, 2]. Other clinical signs observed in patients with loiasis include generalized arthralgia, headache and painful oedema [2]. All these signs lead the patients to inactivity, resulting in economic unproductiveness. From the veterinary point of view, *Chrysops* are harmful to livestock, as they have been reported to feed on cattle, camels and dogs [3–5]. *Chrysops* are large bloodsucking insects. Therefore, heavy infestations of the animal may lead to anemia. They may also act as mechanical vectors of diseases to livestock [4].

3. Hosts and species

3.1. Hosts

There is very little detailed information on the host range of *Chrysops*. *Chrysops* species are known to feed on mammals in general. These include humans, wild animals and laboratory animals such as guinea pigs [2, 6]. Some species seem to feed preferably on some hosts referred to as "normal hosts" and accessorily on other hosts when their normal host is not available. Regarding the host preferences, Gouteux and Noireau [7] showed no significant difference between *C. silacea* and *C. dimidiata* in their preference for humans, with about 90% of blood meals taken on man. The other source of blood meal (10%) was shown to be taken from a range of nonhuman hosts which was of great variety and included hippopotamuses, rodents, reptiles, wild pigs and wild ruminants (antelopes, principally *Tragelaphus scriptus* and buffalos). The difference between the two *Chrysops* species regarding the preference for hippopotamuses

or wild ruminants was not significant nor was that for rodents or reptiles. The authors also found that domestic animals (sheep, goat, cats and dogs) though available were not attractive to *C. silacea* and *C. dimidiata*. Nevertheless, this latter observation disagreed with a previous finding indicating that *C. silacea* feeds well on cattle and dogs under natural conditions and on rabbits while in the laboratory [3]. Detailed knowledge of the full host range of zoophilic species is not known. *Chrysops streptobalia*, a wild species, has recently been captured in an area harboring cattle, small ruminants (sheep, goats) and Equidae [4].

3.2. Species

Data on *Chrysops* species and on their exact number in Africa are scarce. Whatever their number, two of them *C. silacea* and *C. dimidiata* are the mostly recorded species. According to Fain [8], about thirty *Chrysops* species are known to occur in sub-Saharan Africa. Of these species, only a few are recorded to feed on human and livestock and to transmit diseases. The list of some of the species recorded in Africa, with their geographic location is presented below (**Table 1**). There is a huge gap in the knowledge of species and also in the genetic variability that may occur within and among species. Whether sibling species or subspecies occur in *Chrysops* species is not known. The genetic variability may affect both the ecological spread of the species and also its vectorial transmission potential. Thus, as for other species such as mosquitoes or black flies, molecular tools need to be developed for the accurate identification and genotyping of *Chrysops* in Africa.

***Chrysops* species**	**Geographic location**	**Reference**
C. silacea, Austen, 1907	Cameroon, Nigeria, Equatorial Guinea, Democratic Republic of Congo, Rwanda	[8–11]
C. dimidiata Wulp, 1885	Cameroon, Equatorial Guinea, Democratic Republic of Congo, Rwanda	[8–11]
C. flavipes Meigen, 1804	Egypt	[12]
C. streptobalia Speiser, 1912	Ethiopia	[4]
C. centurionis Austen, 1911	Nigeria	[8, 13]
C. distinctipennis Austen, 1906	Nigeria, Democratic Republic of Congo	[8, 13]
C. longicornis Macquart, 1838	Nigeria, Democratic Republic of Congo	[8, 13]
C. langi, Bequaert, 1930	Democratic Republic of Congo	[8]
C. laniger Loew, 1860	Democratic Republic of Congo	[8]
C. distinctipennis, Austen, 1906	Democratic Republic of Congo, Rwanda	[8]
C. obliquefasciata Macquart, 1838		[8]
C. funebris Austen, 1907	Democratic Republic of Congo, Rwanda	[8]
C. brucei Austen, 1907	Democratic Republic of Congo, Rwanda	[8]
C. griseicollis Bequaert, 1930	Democratic Republic of Congo	[8]
C. neavei Austen, 1911	Democratic Republic of Congo	[8]

Table 1. List of some *Chrysops* species recorded in Africa.

4. Classification

The taxonomy of *Chrysops* species is well known. The genus *Chrysops* is one of the three genera of veterinary and medical importance of the Tabanid family. There is no controversy on the taxonomic classification of *Chrysops* species.

Chrysops are members of the Arthropod (Arthropoda) phylum. Arthropods consist of invertebrates species whose major characteristic are the division of the body into clusters of segments notably the head, thorax and abdomen; the presence of a hard chitinous exoskeleton and jointed limbs. Each set of segments is known as tagma, with each tagma (head, thorax and abdomen) having specialized functions. Segmentation has almost disappeared in some species (mites) but still remains in the embryo.

They belong to the insect (Insecta) class with three pairs of legs in adults, a single pair of antennae and a broad tagmatisation (division into tagma) of the body into three distinct sections: the head, thorax and abdomen.

They are part of the dipterous (Diptera) order or true flies. True flies are characterized by a thorax bearing a single pair of functional wings. Other winged insects have two pairs of wings but in dipteran flies, the second pair of wings, the hind pair, is reduced to small knob-like sensory organs called halters which help the insect to maintain a balanced flight. The larvae are different in behaviour and structure to the adults so that the fly can parasitize the tissues of the host either as larvae or as adults but not in both states. Some are also mechanical or biological vectors of diseases.

Chrysops are brachyceran flies (suborder Brachycera) whose short antennae are usually composed of different sized segments. These antennae project in front of the fly. The Brachycera face is bulbous and there is no arista on the antennae.

Chrysops belong to the Tabanidae family. This family gathers the large robust flies known as horse flies (*Tabannus*), deer flies (*Chrysops*) and clegs (*Haematopoda*). These are flies with antennae made up of three sections, the third one being enlarged and composed of four to eight segments; they have two-jointed palps with the second segment enlarged and feet with three pads.

The genus *Chrysops* is made up of flies having wings with a simple pattern of a dark band across the width. Their antennae are long with five segments and the proboscis is shorter than the head.

5. Geographic distribution and ecological zones

Studies on the geographic distribution of *Chrysops* species have so far aroused little interest. The only detailed study showing the confinement area of the fly at country level dates back to many decades ago [8]. Zouré et al. [14] provided a comprehensive distribution of loiasis

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(**Figure 1**) in Africa, which nearly corresponds to the distribution of *Chrysops* vectors [2, 8] because the transmission of loiasis is correlated to the distribution of its vectors. According to these authors, two main zones of highly endemic loiasis can be distinguished: a western zone that comprises part of the Equatorial Guinea, Gabon, Cameroon, Republic of Congo, Central African Republic and Chad, Democratic Republic of Congo (DRC) and Angola; the second hyper-endemic zone is mainly made up of the North-Eastern part of the DRC. Areas of low endemicity include most parts of DRC, north Cameroon and large sections of Angola, Nigeria, Chad and Sudan. Because some *Chrysops* are essentially zoophilic, the geographic distribution likely expands beyond the previously described area. For instance, *Chrysops* species have been described in Egypt [12] and in Rwanda [8], countries not mentioned in the previously described area of distribution.

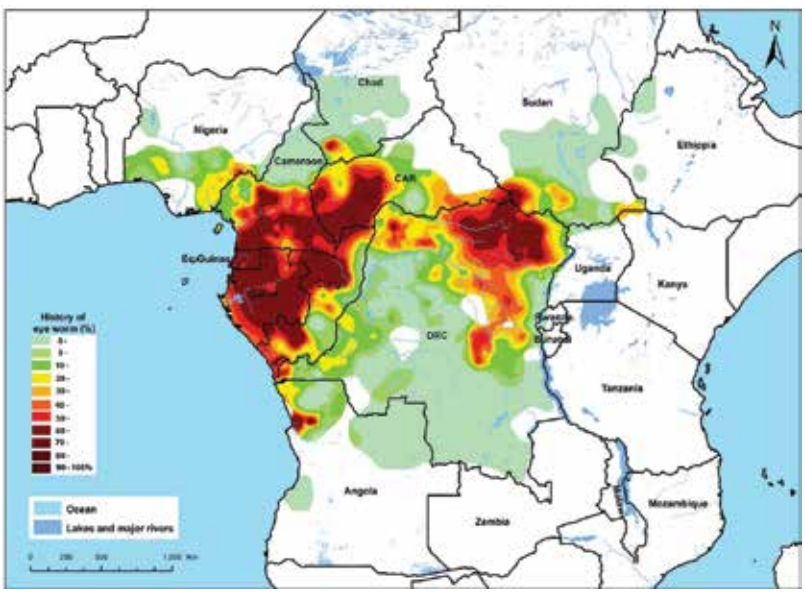

Figure 1. Geographic distribution of loiasis, a *Chrysops*-transmitted disease (reproduced from Zouré et al. [14]).

Within a given area, *Chrysops* are found in some particular ecological zones. In general, most species dwell in forested areas. *C. silacea* was found to be predominant in the cleared forest, particularly in the villages and in their immediate vicinity, whereas *C. dimidiata* prefers natural vegetation, particularly in the forest [15]. Whether the natural or artificial vegetation, presence of the canopy seems to be the most important criteria for *Chrysops* to settle, as this offers a resting place from where the host is spotted [2]. The artificial vegetation found as suitable ecological area include cacao farms, crop fields, mixed crop fields and inhabited areas [9, 16], regenerated forest [17] and commercial plantations such as rubber tree plantations [2]. Some but few species also live in savanna areas, as is the case for *Chrysops flavipes* collected in the Sinai in Egypt [12].

6. Morphology

Species of the genus *Chrysops* are relatively large biting flies with size varying from 5 to 30 mm in length. The head is large, presents bulky eyes with brightly coloured marks and a prominent proboscis (**Figure 2**). The short, stout, anteriorly projecting antennae have no arista and consist of three markedly different segment. The three segments are expanded and the third is marked by five annulations that make the genus antenna look as though it consists of more than three units. The wings have a dark band across the width and when the fly is at rest, they are held apart over the abdomen (**Figure 3**). The wing venation is characteristic, especially the branching of the fourth longitudinal vein.

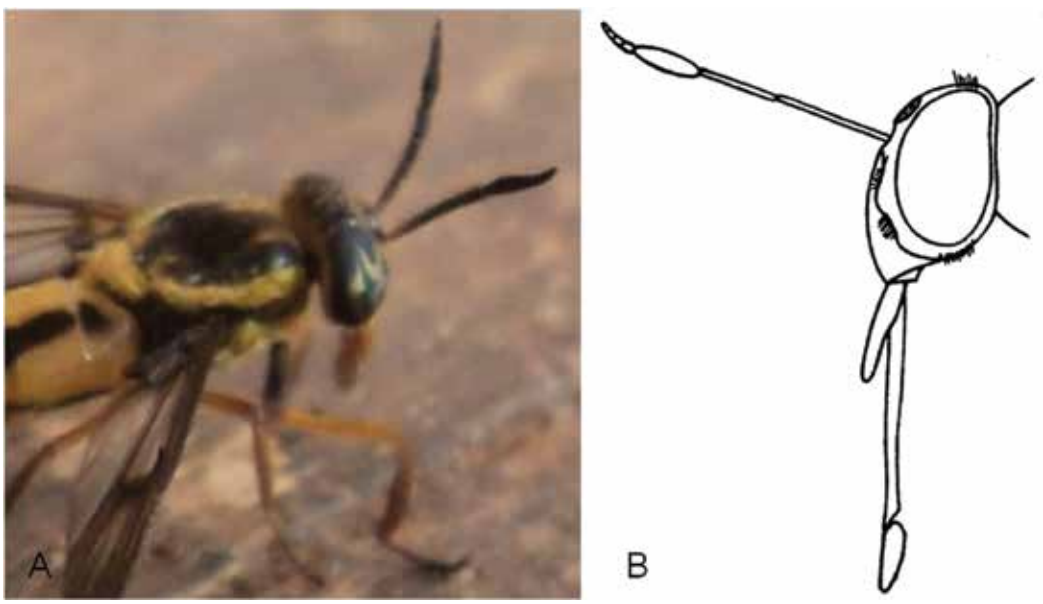

Figure 2. Morphology of *Chysops dimidiata's* head (A) (photograph by Dr Marc Kouam) and diagrammatic representation of *C. silacea's* head (B) showing the three-segmented antenna (reproduced from Gordon and Crewe [6].

Figure 3. Female *Chrysops silacea,* dorsal (A) and ventral view (B) and female *Chrysops dimidiata,* dorsal view(C) (photographs by Dr Marc Kouam).

C. silacea and *C. dimidiata*, the two mains vectors of diseases among African *Chrysops* species are presented below. In *C. silacea*, the two longitudinal stripes on the yellow abdomen never extend beyond the third abdominal segment and may be so attenuated or interrupted as to be almost absent. In *C. dimidiata*, the abdominal stripes are broader and reach the fourth segment, where they usually become merged into the darker brown colour of the terminal segments. The wing markings of the two species are similar.

7. Life cycle

It should also be noticed that recent information on the life history of *Chrysops* is untraceable and no published work on this topic on African *Chrysops* species, apart for *C. silacea* [3] is available. Adult females of the Tabanidae are known to live on a mixed diet, feeding on sugar and blood, whereas adult males feed exclusively on carbohydrates. In the Tabanidae, development of the ovaries is dependent on the taking of a previous blood meal and after the ovoposition, a further blood meal is required before a further batch of eggs will develop.

After mating, the female *C. silacea* seeks a blood meal until sufficient blood has been ingested and then retires for egg maturation. During the "gestation period", the fly feeds on carbohydrates obtained from fruits or flowers to keep alive or active. The eggs are then laid after development and a new cycle is resumed after which other eggs are laid. The complete development of the ovaries and subsequent ovoposition take not less than about 6 days after blood meal. The normal development of the ovaries was shown to occur when the blood meal was larger than 8 mg and if the blood meal happened to be insufficient, the fly must return to attack another host to feed and retire to gestate, only when the blood amount is sufficient to initiate ovaries development [3]. The ovoposition sites of various species of *Chrysops* have been described to be over water. Female *Chrysops* laid the eggs in the mud along the rivers and lakes, on various objects (vegetation, stones) in the water near the shore, in permanent swamps and in small swampy patches formed throughout the rain forest during the wet season [18–21]. The eggs laid in batch of 100–800 units are 1.1 mm long and 0.2 mm wide at the broadest part and tapering more towards the apex than towards the base.

The eggs hatch between 5 and 9 days after ovoposition and all the larvae from one egg mass hatch almost simultaneously. After hatching, the larvae leave the substratum to sink in the mud that is covered with very shallow slowly running water. Larvae are saprophageous, whitish in colour, vermiform and hemicephalous. Larval development is very slow, consisting of 7–10 instars according to environmental conditions [19]. In the rainy season, the duration of a larval live history is estimated at 27 days for *C. dimidiata* and 15 days for *C. silacea* [19]. During the dry season, the duration of the larval stage is longer for all *Chrysops* species. For instance, the normal life history of *C. silacea* appears to occupy 1 year but the eighth or ninth instar fail to pupate in any year before the onset of unfavourable conditions, then the fly can apparently survive in the larval stage for a considerable period; this would account for the small number of pupae and adult flies which are found even during the driest and therefore unfavourable season [3]. Before pupation, the mature larvae of *C. silacea* moves to the edge of the stream or swam in which it is living and takes up a position in the mud just beyond and

above the water's edge. Pupation takes place at the edge of water and the pupa, first pale yellow in colour becomes brown or yellow brown as it ages, showing a size varying from 10 to 13mm. The time slot between pupa and imago is 4 to 7 days, but the commonest period is 5 or 6 days. When the pupa is about to emerge to imago, it moves upwards to the surface of the mud until the thorax completely get out. The adult fly gradually works its way out from the puparium, taking several minutes to emerge completely and then rests on the mud for up to 1 hour until the body and winds are sufficiently hardened for it to fly away for the resting site in the canopy.

The time slot between pupa and imago is 4 to 7 days, but the commonest period is 5 or 6 days. When the pupa is about to emerge to imago, it moves upwards to the surface of the mud until the thorax completely get out.

8. Pathology

8.1. Cutaneous effect

African *Chrysops* not only cause deep painful bites but also cause irritation that result in painful wounds in some people (**Figure 4**). These wounds are potential entry doors for many pathogens.

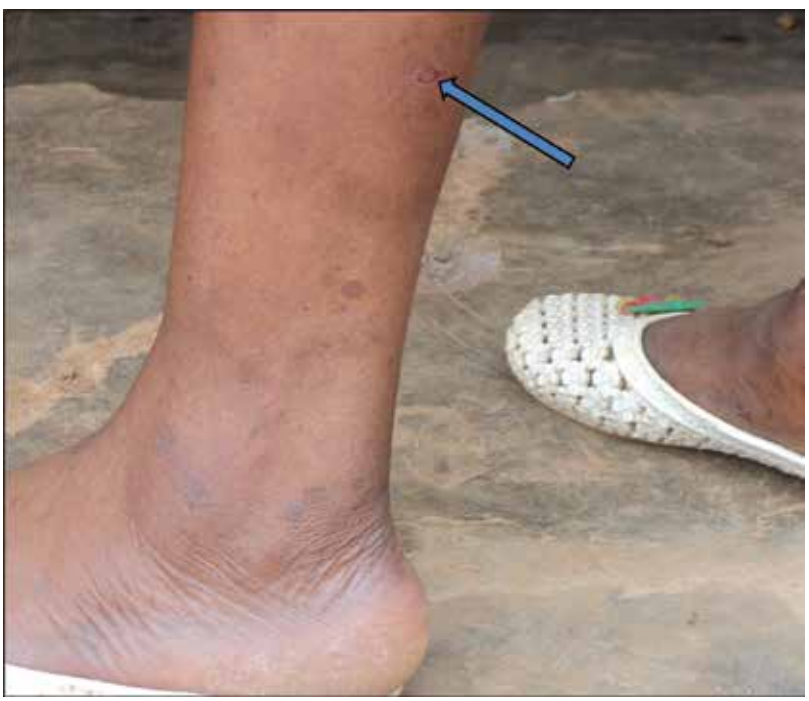

Figure 4. Photograph of a healing wound (see arrow) following a *Chrysops* bite on a woman leg in Kokodo, Central Cameroon (photograph by Dr Marc Kouam).

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8.2. General effect (vector of diseases)

African *Chrysops* are vectors of *L. loa* in human and *Loa papionis* in monkeys [2, 9, 22]. Due to the biting habit of *Chrysops* whereby several hosts are often necessary to feed to repletion, they are responsible for the mechanical transmission of diseases [23]. They are vectors of many pathogens to livestock; this includes bacteria, viruses, protozoa, *Trypanosoma evansi* in equines, dogs and camels, *Trypanosoma equinum* in equine, *Trypanosoma simiae* in pigs *Trypanosoma vivax* and *Trypanosoma brucei* in equine, cattle, sheep and other ungulates [4, 5, 24]; other *Trypanosoma* transmitted by *Chrysops* are *Trypanosoma gambiense* and *Trypanosoma rhodesiense*, the causative agent of human African trypanosomiasis [24].

9. Epidemiology

Chrysops species are either nocturnal or diurnal biters. The feeding time is correlated with the time when the host is active and available. Thus, *C. silacea* and *C. dimidiata* which feed on humans are diurnal feeders [9, 17]. In contrast, *Chrysops langi* and *Chrysops centurionis* having monkeys as hosts are crepuscular biters. Environmental conditions probably influence the biting habits of *Chrysops*. Temperature, humidity and light intensity are some interdependent factors influencing the biting activity of *Chrysops*. Brilliant sunshine and very dull days were reported to reduce the biting activity of *Chrysops* [3], whereas the daily biting cycle of *C. silacea* was showed to present two peaks of activities, 9–11 a.m. and 2–4 p.m. [17]. The *Chrysops* biting density has been shown to vary according to ecological zones, being higher in forested than in clear areas and habitations [15, 16]. In the Lekie Division in central Cameroon, Demanou et al. [16] reported a *Chrysops* biting density of 568 and 4696 bites/man/year in inhabited and forest areas, respectively. In general, *Chrysops* species bites all over the year but *Chrysops* biting density is the highest in the rainy season (the favourable breeding season) for some species such as *C. silacea* and *C. dimidiata* [16, 17], whereas some species (*Chrysops neavi*) have been collected solely in the dry season [8]. Meanwhile, other species (*Chrysops brucei*, *Chrysops distinctipennis*) have been reported solely in the rainy season [8]. To feed on human, African *Chrysops* usually fly up to the habitations to attack the host at the veranda and even inside well lighted houses [9, 16]. They are persistent and furtive, mostly attacking the legs or walk on the clothing probably in search of a biting site. They can be observed flying around the human host but the usual sign of their presence is the great pain of the bite, since they are pool feeders. In the forest, African *Chrysops* have also been reported to follow a moving vehicle like other Tabanids and to pursue a human being on foot for at least half a mile [3]. The population density of adults *Chrysops* is fairly low (~1000/km^2) and their flight range usually not great (theoretical range: <6000 m and maximum distance: 4500 m) in the secondary forest [25]. Adult female *Chrysops* spot their hosts using visual or olfactory means. Females are attracted by the movement of people or animals who are directly visible from the canopy [3]. Smoke of wood fire is extremely attractive to *C. silacea* [26, 27]; this therefore increases the opportunity of contact between human and the flies. The attraction to fire may be related to the diffusion of odorous molecules other than CO_2, contained in the smoke in the canopy [2, 27]. Catches carried out around a wood fire is

multiple fold higher than in catches without wood fire [2, 28–30]. As reported by Duke [31], this visual attraction to humans appears to be less than that of a wood fire.

10. Laboratory diagnosis

The coloration of the wing and the three-segmented antennae is used in differentiating the three major genera of the family Tabanidae (**Figure 5**). In *Chrysops* species, the three antennal segments are expanded and the third is marked by five annulations that make the genus *antenna* look as though it consists of more than three units. The wings have dark bands across the width and when the fly is at rest, they are held apart over the abdomen. *Tabanus* species have transparent wings and the first two antennal segments are small and the terminal segment has a tooth-like projection on its basal part and four annulations. *Haematopota* species have characteristically mottled wings that are held divergent when at rest; its first antennal segment is large, the second is narrower and the third presents three annulations.

Figure 5. Specimen of the genus *Chrysops* (A), *Haematopota* (B) and *Tabanus* (C). A=*C. dimidiata* (photograph by Dr Marc Kouam); B=*Haematopota pluvialis* (reproduced from De grote, http://www.eaaci.net/site/content.php?l1=17&sel=400); C=*Tabanus* (*Tabanus*) *gertrudae* (reproduced from Maity et al. [32]).

11. Control

Attempts to large scale control of *Chrysops* population in the past relied on the use of insecticides. Dieldrin, DDT and Gamma-BHC have been used in Kumba in the southwest region of Cameroon against *C. silacea* and *C. dimidiata* larvae and pupae. The treatment was successful, leading to a drop in the fly density of 30%, 2 years after dieldrine spreading [21]. Although the result was promising, the method was not recommended due to difficulties to access to breeding sites in densely vegetated areas, the high cost of the treatment and the risk of environmental pollution and contamination of food and water [2]. A 60% solution of dimethylphtalate has also proven to be a good repellent of *Chrysops* in Kumba. Another promising method attempted was the creation of anthropic savanna hostile for *Chrysops* development around habitations [33]. Nowadays, it is well established that adults fly dwell in the canopy where they locate their host, that the fly range and density are limited (less than 6000 m and 785–3682 flies/km^2) and that the smoke of wood is attractive to *Chrysops*. So, based on these current knowledge on the biology and ecology of *Chrysops*, control measures against

these pests may encompass: the clearance of large area of bush around habitations in order to destroy the habitats (canopy) of the adult flies, use of repellents for crop or forest workers and livestock, trapping with attractant to reduce the fly density. In this respect, Morlais [30] showed that smoke of wood increased the Loapi trap performance to 14 fold. Also, the Harris trap has been reported to be efficient on wooded savanna–dwelling *Chrysops* and need to be tested on forest-dwelling *Chrysops* [2]. If traps have been developed and largely used for other disease vectors in Africa (tsetse flies, mosquitoes), little has been done as regards African *Chrysops*. Yet, traps have the advantages of being cheap, harmless to the environment and can be used by a common man. For a rapid and large scale control of *Chrysops*, modern chemicals without a permanent effect on the environment need to be developed.

12. Conclusion

The gap in the knowledge of African *Chrysops* is huge. The *Chrysops* fauna in Africa still needs to be elucidated, as well as the role of each species, subspecies or genotype in the transmission of diseases to human and livestock. But before this is done, molecular tools need to be developed for epidemiological studies to clarify whether currently known species vary genetically across geographic areas. Research works are also to be focused on repellents, attractants, traps and environment-friendly insecticides that can be used for an efficient control of *Chrysops.* With the present knowledge on the biology, ecology and behavior of *Chrysops*, different control measures could be combined at small and large scale level. At small scale level, insecticide-treated traps or Harris-type traps could significantly reduce the fly density in rural areas if they are set next to firewood smoke (attractant). At large scale level, aerial spraying of insecticides at the resting sites (canopy) could be done but the most efficient technique would involve limiting or preventing female from breeding by using its natural enemies or the "sterile male" technique. This technique consists of introducing barren males in the population to compete with wild males for mating, as is the case in tsetse fly control. In sum, there is still a lot to know on African *Chrysops* and a long way to go before their successful control or eradication.

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Chapter 14

Functional Anatomy of the External and Internal Reproductive Structures in Insect Vectors of Chagas Disease with Particular Reference to *Rhodnius prolixus*

Ralem Gary Chiang and Jennifer Ann Chiang

Additional information is available at the end of the chapter

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Abstract

The insect vector of Chagas disease, *Rhodnius prolixus*, has become a very popular model organism for exploring, among other things, the physiology of insects. Its ability to remain in a state of stasis until after engorging a blood meal has focussed most studies on those physiological and developmental processes triggered by the blood meal leaving the details of its sexual physiology vague. This chapter summarizes the relationship between the male and female by describing their respective reproductive systems and genitalia, and how they function during and after copulation. A number of novel processes are noted, such as the transfer of male secretions without the formation of a spermatophore, pump/valve mechanism in the male aedeagus, sensory and a chemical means by which copulation may be facilitated, and the possible mechanism by which adhesive protein is applied to an egg during ovipositioning. Combined with knowledge of its genome, further studies into the functional anatomy of reproduction in this insect have the potential to increase our understanding of sexual reproduction in Reduviidae bugs, and to suggest new ways to control their population growth and the spread of Chagas disease.

Keywords: *Rhodnius prolixus*, sexual physiology, male genitalia, female genitalia, copulation, Chagas disease, Reduviidae, Triatominae, aedeagus, spermatophore, accessory reproductive glands

1. Introduction

This chapter describes the anatomy and physiology of internal and external reproductive structures in Reduviidae bugs, the blood-feeding insect vectors of Chagas disease. Chagas disease is endemic to Central and South America, and is also known as American trypanosomiasis [1]. The

disease is caused by a protozoan parasite, *Trypanosoma cruzi*, which completes one part of its life cycle in the digestive system of the Reduviidae bug, and the other part in the tissues of warm-blooded animals. Animal tissues are infected by the amastigote stage of this flagellate, which multiplies by binary fission, and transforms into trypomastigotes. These trypomastigotes burst from the cell, and enter the blood stream to infect other cells within the host. The trypomastigote is also the stage ingested by the insect during a blood meal. Once in the insect midgut, the trypomastigote transforms into the epimastigote and proliferates. The epimastigotes then enter the hindgut and transform into the metacyclic trypomastigote. When the infected insect takes its next blood meal it also defecates leaving the metacyclic trypomastigote, once in its hindgut, on the host. The metacyclic trypomastigote can enter the blood stream directly through the bite or through mucous membranes to find tissues to infect, and the cycle starts over again. Additionally, the mode of host infection may occur by ingesting food contaminated by the infected faeces of these bugs [2].

The causative agent of Chagas disease and its mode of transmission by Reduviidae bugs was discovered by Carlos Chagas in 1909 [3]. Although transmitted by several species of Reduviidae, one species, *Rhodnius prolixus*, has become the single most significant insect for advancing our understanding of insect physiology. Indeed, outside the endemic regions of the world, *R. prolixus* is known for being an ideal insect model for studying insect physiology rather than for its role in spreading Chagas disease. The events leading to this status include the discovery by Vincent B. Wigglesworth (1899–1994) that *R. prolixus* is able to survive considerable experimental manipulation, and will remain in a state of physiological stasis until it ingests a blood meal of sufficient size [4, 5]. These factors combined with the ease of raising them in the laboratory, has made *R. prolixus* a popular insect model in teaching and research.

In his studies on insect physiology, for which he was knighted in 1964, Wigglesworth concentrated on growth, development and metamorphosis in *R. prolixus* [6–8]. Other researchers have examined physiological processes associated with reproduction [9, 10], feeding [11–14], digestion [15, 16] and behaviour [17]. A wealth of knowledge has been gained in close to 100 years of research on this species and with the recent completion of the *Rhodnius* genome project [18], research on this bug should continue long into the future. To help set the stage on which to explore more fully the functional anatomy of both the external and internal features of the reproductive system, this chapter summarizes and clarifies our current understanding of the mechanics of egg-laying, copulation and the formation of the spermatophore. This work is specific to *R. prolixus*, but as noted with a few comparisons, it appears to be applicable to the Reduviidae as a whole.

2. Overall design of the adult abdomen

The adult abdomen in *R. prolixus* is flattened dorso-ventrally and in both the male and female, it is divided into seven full-sized segments on the dorsal side. On the ventral side, the female has six recognizable full size segments and the male has five. This number differs because of the way the first three ventral segments merge. Although there are a different number of

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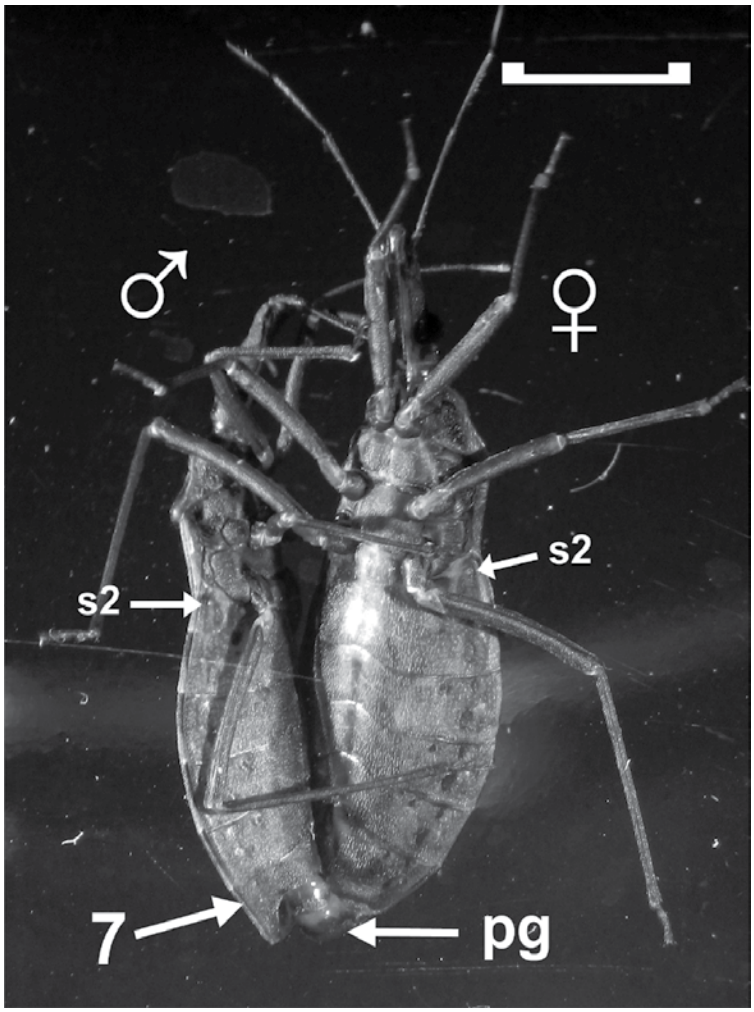

Figure 1. Ventral view of a copulating pair of *Rhodnius prolixus* seen through the side of a glass jar. Male is to left in picture holding onto the female and the female is standing on the glass surface. s2, second abdominal spiracle; 7, seventh full-sized abdominal segment; pg, posterior genital segment of male. The aedeagus, the male penis, extends from the pg into the female during copulation. Genitalia in both sexes are attached to the seventh full-sized abdominal segment. Scale bar: 5 mm.

full-sized dorsal and ventral segments, the corresponding tergal and sternal plates can be recognized in the lateral view and identified by the spiracle number they are associated with (see **Figure 1**). There are at least eight sets of abdominal spiracles and the first and the eighth pairs are not visible along the ventral lateral side of the abdomen. In both sexes, the first abdominal spiracle appears laterally on a sliver of cuticle on the dorsal side of the abdomen close to the thorax, and is partially covered by the first full-sized dorsal abdominal segment, whereas the eighth pair of spiracles is located on the ventral genital segment and becomes exposed when these segments are extended. By numbering the abdominal segments according to the spiracle they are associated with, the first full-sized abdominal dorsal segment is two

and the last is seven (refer to **Figure 1**). The genitalia in both sexes are specializations at the end of the abdomen and are attached to full-sized abdominal segment seven. In morphological studies that compare different species of insects, the segments of the genitalia are numbered according to their relationship to other very diverse species (for example, see Ref. [19]). For this chapter, which focuses on the functional anatomy in one particular species, the genital segments are referred to according to their position within the genitalia.

3. The female reproductive system

3.1. The dorsal and ventral genital segments of the female

The genitalia of the female are attached to abdominal segment seven, and are equipped with a single dorsal sclerite and a pair of ventral sclerites (see **Figure 2**). When pulled in towards the rear of the animal, these genital sclerites cover the sclerites that surround the opening to the genital chamber. The genital chamber in *R. prolixus* can be referred to as either the bursa copulatrix [20, 21] or the vagina [22, 23]. In keeping with its functional role, this chapter refers to the genital chamber as the vagina.

The dorsal genital sclerite is hinged on the posterior edge of full-size abdominal segment seven (**Figure 2A**), and narrows towards its posterior tip to take on a triangular shape. In its retracted position, it sits under the animal extending ventro-anteriorly (**Figure 2B**). It has symmetrically arranged lateral flaps (**Figure 2A**) to which the male can attach his parameres when this genital sclerite is extended during copulation. Its posterior tip has a prominent medial ridge that overlaps sclerites ventral to it when the vulva, the external opening to the vagina, is closed. When the genital segments are relaxed as a result of decapitation of the female, the third valvula becomes visible (**Figure 2C**). A slender branch of cuticle connects the lateral edge of the dorsal genital sclerite to the base of the second valvula which is one of the three pairs of sclerites associated with the vulva.

On its interior side, the dorsal genital sclerite has a pair of apodemes with each member of the pair located between the midline and the right or left side of the sclerite. Anchored to these apodemes are a pair of bilaterally symmetrical muscle bundles which fan out a short distance anteriorly to attach to the posterior lining of the vagina. Contractions of these muscles pull the dorsal genital sclerite anteriorly onto the underside of the animal to close off the vulva and the anus. Relaxation of these muscles allows the dorsal genital sclerite to extend exposing the anus during defaecation, or the vulva during copulation, egg-laying or the expulsion of the male secretions after copulation.

While the dorsal genital sclerite covers the dorsal to lateral sides of the rear of the abdomen, the ventral to lateral sides are covered by a pair of ventral genital sclerites. The relationship of these ventral sclerites to the ventral side of abdominal segment 7 is governed by the shape of the abdomen in cross section. Whereas the dorsal abdominal surface is flat, the ventral portion forms a deep trough. The anterior part of each pair of ventral genital sclerites sits in this trough so that they lay over part of the interior side of abdominal segment 7. The eighth abdominal spiracle is located on the ventral genital sclerite, but not on its outer nor inner surface. Instead,

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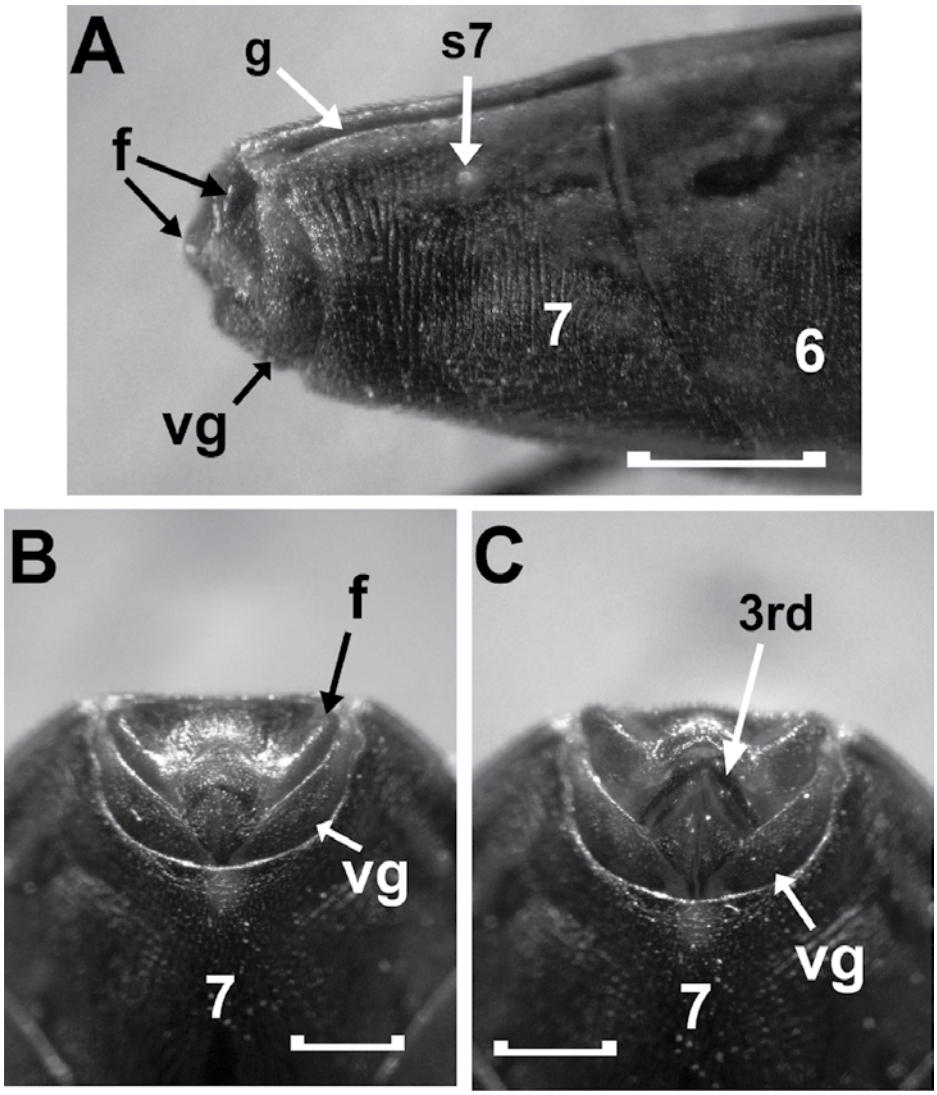

Figure 2. External female genitalia of an adult *Rhodnius prolixus*. (A) Lateral view showing the genitalia attached to the full-size abdominal segment seven (7) identified by the seventh spiracle (s7). 6, full-sized abdominal segment 6; f, the lateral flaps on the single dorsal genital segment; vg, the ventral genital segment consisting of a pair of bilaterally symmetrical sclerites; g, the lateral abdominal groove characteristic of adult *Rhodnius*. Scale bar: 2.5 mm. (B) Dorsal and ventral genital segments held close to the body closing off the anus and entry to the genital chamber. Scale bar: 1.0 mm. (C) Dorsal and ventral genital segments are partially relaxed after decapitating the animal, and the third valvula (3rd) becomes visible. Scale bar: 1.0 mm.

it sits approximately midway along its lateral edge (see **Figure 3**), and this edge becomes exposed to the outside when the ventral genital segments are extended out of abdominal segment 7 to open the vagina. The ventral genital segment is attached to the inside of the ventral side of abdominal segment 7 by at least four sets of skeletal muscles. These muscles, which have yet to be fully documented, provide the female with considerable control of the

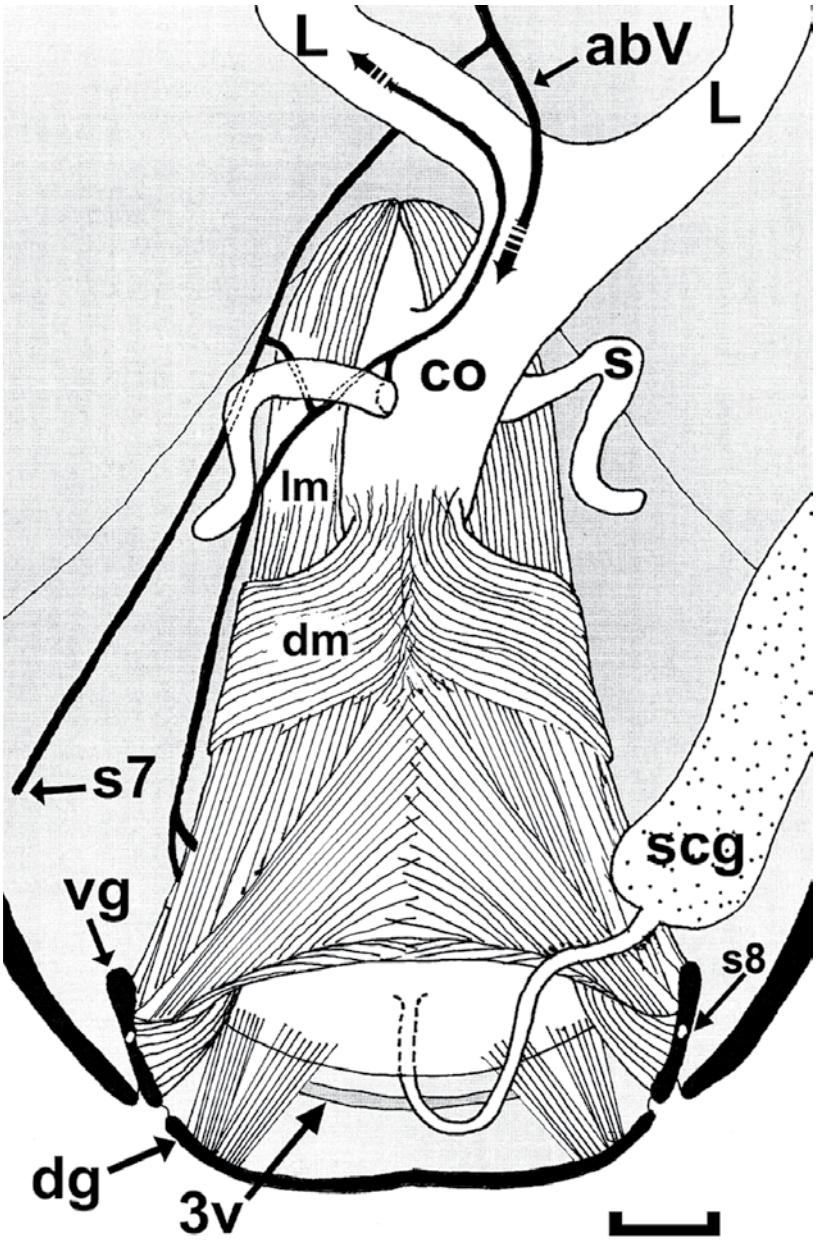

Figure 3. Line diagram of a dorsal view of the lower reproductive system in a female adult of *Rhodnius prolixus* as revealed by methylene blue staining and electrophysiology. abV, left branch of abdominal nerve 5 which supplies the genitalia. Hashed arrows denote the course of the nerves not included in this diagram. s7, is the seventh abdominal spiracle which is innervated by a nerve which branches off abV and travels under the lateral oviduct (L); s8, the eighth spiracle located on the edge of the ventral genital segment (vg); co, common oviduct to which the spermathecae (s) are attached; lm, longitudinal vagina muscles; dm, dorsal vagina muscles; scg, secretory portion of the cement gland whose excretory duct is attached to the inside of the vagina on the dorsal side of the vulva; dg, dorsal genital segment; 3v, the base of the third valvula which is attached to the lining of the vagina on the dorsal side of the vulva. Scale bar: 0.5 mm. (Adapted with permission from Ref. [22]).

sclerites of the ventral genital segment, a control that would be exercised during copulation and ovipositioning.

On the side facing the vagina, the ventral genital sclerites are directly attached to the bulk of the muscles that overlie the vagina (**Figure 3**). The muscle bundles fan out in three different directions and become intertwined as they proceed over the vagina. The most anteriorly attached muscle bundles extend anteriorly along the ipsilateral side of the vagina, past the common oviduct, to attach to the posterior medial edge of abdominal segment 7 where the muscle bundles associated with the dorsal genital sclerite also attach. The muscle bundles attached to the mid anterior region of the ventral genital sclerite form a distinct twisting pattern, and extend directly across the body over the posterior end of the vagina to the contralateral ventral genital sclerite. The more posteriorly attached muscle bundle extends anteriorly and contralaterally travelling across the top of the vagina around the contralateral side of the common oviduct to attach to the medial posterior edge of abdominal segment 7. The interwoven nature of the muscle fibres and the diagonal pattern assumed by many of them would help to ensure that pressure generated during their contractions would be evenly spread over an exiting egg.

3.2. The vulva

The vagina opens to the outside through the vulva, which is surrounded by three sets of sclerites (see **Figure 4**). These consist of a single dorsal sclerite, a pair of lateral sclerites which are attached to the dorsal genital segment by the previously mentioned slender branch of cuticle and a pair of ventral sclerites. The base of each of these sclerites is attached to the soft articulating cuticle that lines the vulva and is continuous with the soft cuticle lining the vagina. When using the scanning electron microscope to compare the external female genitalia in fourteen species of *Rhodnius*, da Rosa et al. [24] refer to the dorsal sclerite as the gonapophyse 9, and the other two sets as the gonapophyse 8. For this chapter, we have adopted the designation which is specific to *R. prolixus*, and refer to the sclerites that surround the vulva as the valvulae (see Text-**Figure 1** in Ref. [25]). The ventral pair of sclerites represents the first valvula, the lateral pair, the second valvula and the single dorsal sclerite is the third valvula (**Figure 4B**). The valvulae are more than simple pieces of cuticle that guide the material through the vulva and out of the vagina. Each set has an anatomical specialization to suggest that they play more than a passive role in sexual physiology.

As is the case for the dorsal genital segment above it, the third valvula has an overall triangular shape, but is smaller and displays a medial line that separates the sclerite into two distinct halves (**Figure 5**). The two halves are joined only from the anterior base of the sclerite to approximately 1/3 their length, beyond which they are completely separated. The lateral and distal margins of each half forms a thick rounded edge which possesses several long fine hairs. The similarity of these hairs to tactile sensors on the insect cuticle suggests that they have a sensory function, and the manner by which they line the edge of the third valvula suggests that this structure serves as a sensory organ.

The second valvula consists of a pair of sclerites that line the lateral edges of the vulva. They are bilaterally symmetrical and elongated or lacinate in shape. They are widest at their base

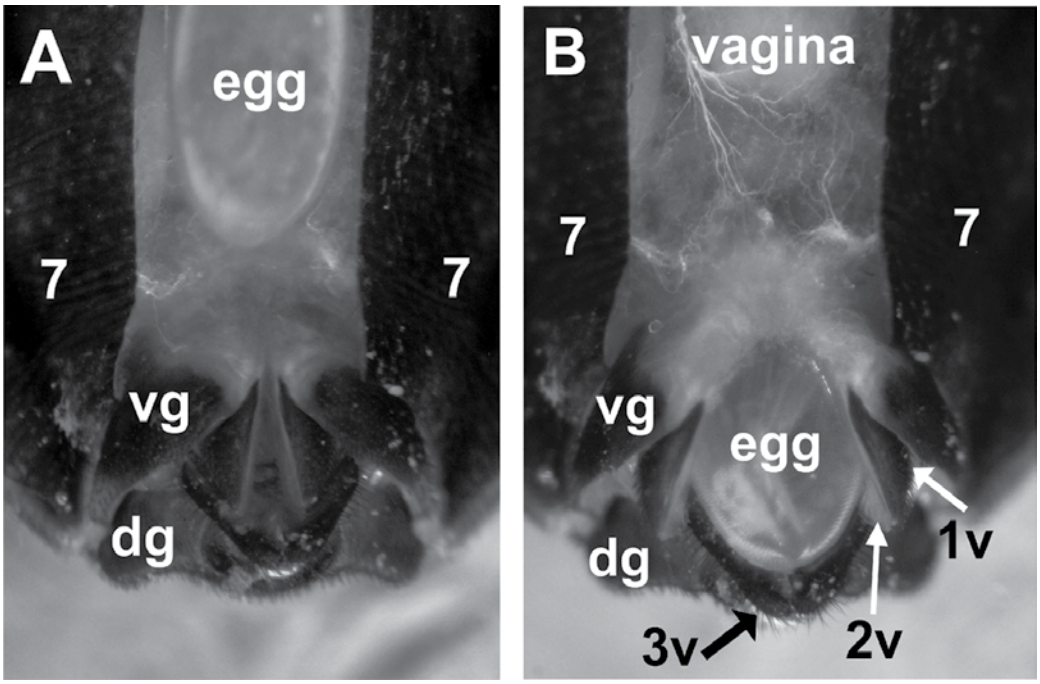

Figure 4. Ventral view of the female genital segments with the ventral cuticle of abdominal segment seven (7) partially removed to show the vagina. (A) An already laid egg with a developing embryo is laid over the vagina. vg, ventral genital segment; dg, dorsal genital segment. (B) Egg inserted into the vagina to show that the expansion of vulva occurs mainly from separating the two halves of the vg, the first valvula (1v) and the second valvula (2v). The halves of the third valvula (3v) remain together. The egg is approximately 1.0 mm in width.

where they attach to the soft cuticle lining of the vulva. They also curve along their long axis at their base to form a short tube-like structure, and they narrow posteriorly to a pointed end. The ventral edge of the second valvula forms a ridge along its margin, and this ridge fits into a groove that runs along the dorsal edge of the sclerite in the first valvula. As noted previously, the second valvula is attached at its base to the arm of cuticle that connects to the mid-lateral region of the dorsal genital segment.

The first valvula consists of a bilaterally symmetrical pair of sclerites that are more triangular in shape than the lacinate lateral sclerites of the second valvula (**Figure 4B**). The dorsal edge of the sclerites of the first valvula forms the groove in which the ventral ridge on the sclerites of the second valvula slides. This ridge and groove mechanism allows the second valvula to extend beyond the posterior end of the first valvula while keeping these two sets of sclerites firmly attached. This intricate structural relationship between the first and second valvulae may be an adaptation to serve a physiological role as the egg is passing out of the vagina. For instance, the excretory pore of the cement gland is situated on the dorsal side of the vulva near the tubular bases of the second valvulae (see **Figure 3**), yet on exiting the body, the cement gland secretions appear as dabs of secretions on the ventral, not dorsal, side of the egg [26]. In combination with the tubular nature of the base of the second valvula, and the ridge and

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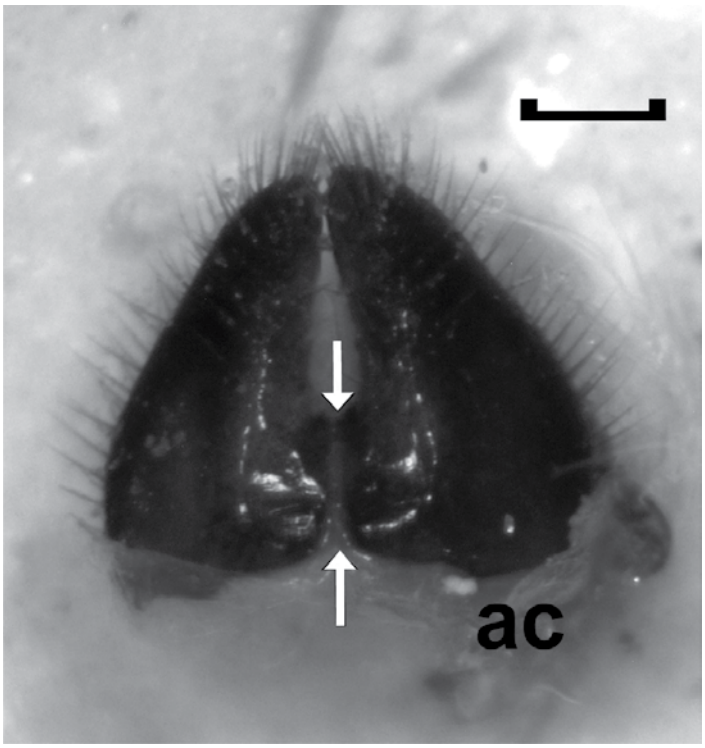

Figure 5. Dorsal view of the third valvula removed from the genitalia of the female of *Rhodnius prolixus*. The base of this pair of sclerites is attached to the articulating cuticle (ac) of the vagina on the dorsal side of the vulva. They are firmly joined along the midline between the arrows, and are covered with fine hairs typical for insect tactile receptors. Scale bar: 0.25 mm.

groove mechanism, these valvulae may function to direct cement gland secretions onto the ventral surface of an egg as it passes through the vulva.

The contralateral sclerites of the first valvula are not directly attached to one other, but are connected to each other by the soft cuticle that lines the entrance of the vulva (**Figure 4**). Thus, unlike the fused halves of the third valvula (**Figure 5**), these sclerites can separate, and stretch apart passively, as would be expected when an egg courses through the vulva. They can also be pulled back into the body to close off the vulva when the muscles associated with the vagina contract to eject an egg. Inserting a previously laid egg through the vulva demonstrates how readily the paired sclerites of the third valvula separate to allow the egg to pass (**Figure 4B**).

3.3. Female reproductive organs

The structure and function of the internal organs of the adult female reproductive system have been well documented for *R. prolixus* [27, 28]. At its anterior end, this system consists of a pair of ovaries each containing seven ovarioles. Since the developing oocytes in the ovarioles are connected by nutritive cords to the nutritive cells at the anterior end of the ovariole, these ovaries

are classified as telotrophic [29]. At their base, the ovarioles are connected to a calyx which, in turn, is connected to the anterior end of the ipsilateral lateral oviduct. Posteriorly, the lateral oviducts fuse to form a short common oviduct which enters the vagina and terminates in a muscular bulbous structure referred to as the vestibulum [20, 27]. The vestibulum undergoes spontaneous twitch-like contractions which may serve, after copulation, to propel the spermatozoa from the vagina into the common oviduct, then to the spermathacae, the sperm storage organs [20].

The spermathecae are one of the two accessory reproductive glands associated with the female reproductive system of *R. prolixus* (see **Figure 3**). They are a bilaterally symmetrical pair of slender blind-ended tubes arising from the sides of the common oviduct [10]. In *R. prolixus*, they take on a curved to twisted orientation, and when stretched, they can extend from 1/4 to 1/3 down the length of the vagina. After ingesting a blood meal of similar size, mated females make significantly more eggs than unmated females, and this difference can be correlated with the presence of spermatozoa in the lumen of the spermathecae [27]. This observation suggests that the cells lining the spermathecae are triggered by the male secretions into producing an endocrine secretion that increases the ability of the female to convert her blood meal into eggs.

The cement gland, the other accessory reproductive gland in *R. prolixus*, synthesises and releases an adhesive protein used to attach the egg to the substrate [28]. It consists of a cuticle-lined excretory duct which empties the secretions through an excretory pore located in the lining of the vagina on the dorsal side of the vulva. The duct receives secretions from the relatively large secretory portion which is folded over itself or the vagina to accommodate a length that is more than twice that of the vagina. As noted above, a laid egg has a thin layer of this adhesive material on its ventral surface [28] suggesting that the first and second valvulae may serve to position the adhesive protein onto the ventral side of the egg as it is being oviposited.

A comparative work on Reduviidae bugs shows that these blood-feeding insect vectors of Chagas disease can vary with respect to the presence of a cement gland and the morphology of their spermathecae [29]. All Reduviidae examined possess spermathecae that are paired blind ended tubes attached to the side of the common oviduct. However, the shape and location of the distal ends of the spermathecae differ depending on the genus. In *Rhodnius*, the spermathecae extend out from the common oviduct and are free to twist, whereas in *Triatoma*, *Nesotriatoma* and *Panstrongylus*, the ends of the spermathecae are held in place ventral to where the lateral oviducts attach to the common oviduct. In addition, the distal ends of the spermathecae take on the shape of flattened disks in *Triatoma klugi*, *Triatoma sordida*, and *Panstrongylus,* while in *R. prolixus* there appears to be no distal specializations. With respect to the cement gland, of the species examined, *Triatoma dimidiata* has a relatively small cement gland while this structure is absent in *T. klugi*, *T. sordida* and *Nesotriatoma bruneri*. All *Rhodnius* species examined (*R. prolixus*, *Rhodnius brethesi*, *Rhodnius nasutus*, *Rhodnius pictipes*) possess a prominent cement gland. This variability may be related to the ovipositioning behaviour since *Triatoma* tend to scatter their eggs loosely over a substrate whereas *Rhodnius* adheres its eggs to the substrate.

3.4. Physiology of muscles associated with the vagina and valvulae

The physiology of the muscles associated with the vagina and valvulae in *R. prolixus* has been studied by attaching a force transducer to a small metal hook inserted through the dorsal side

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of this chamber, then raising the hook with the force transducer to apply tension to the muscles [22, 30]. In all preparations set-up in this fashion, there is a slow gradual drop in baseline tension until a steady baseline is reached around the 5-minute mark. Many of the preparations show spontaneous contractions at the onset of recording, and these contractions could either disappear after a few minutes or become synchronised into 10–30 second bursts that occur regularly over the recording period. The ovaries, lateral and common oviducts and spermathecae are also capable of spontaneous contractions [31].

The overall pattern of innervation in the abdomen of *R. prolixus* is bilaterally symmetrical, and evoked contractions of the vagina muscles are elicited by stimulating either one of the paired abdominal nerve V supplying the genital segments. The response is dependent on the rate and duration of stimulation (see **Figure 6**). Any spontaneous contractions that may be present are eliminated with stimulations of 2 Hz or below showing that this system has a prominent inhibitory motor input, whereas slow prolonged contractions, typical of visceral muscle, are elicited at stimulation rates greater than 5–10 Hz. Continual stimulation at 10, 20 or 30 Hz does not maintain the tension suggesting that the excitatory input is not able to completely eliminate the inhibitory input. These physiological studies, combined with methylene blue staining, show that the ipsilateral regions of the vagina are innervated by motor neurons that travel from the thorax to the genitalia through the ipsilateral abdominal nerve V, then along the segmental nerve branch that serves spiracle 7, turning at the level of the common oviduct to travel to the side of the vagina (see **Figure 3**). At the vagina, the nerve divides to course anteriorly serving the ipsilateral spermatheca and lateral oviduct, and posteriorly to serve the vagina muscles. Near the dorsal posterior region of the vagina, the nerve appears to terminate within a nerve network and not on individual muscle fibres. Stimulation of the nerve elicits a relatively large negatively recorded potential at this point indicating the presence of a unique relationship between motor stimulation and smooth muscle contractions, a relationship that warrants further investigation.

Methylene blue also stains a network of fibres that extend over the base of the common oviduct and dorsal anterior region of the vagina [22]. This network resembles the nerves stained with an antibody for proctolin [32]. Application of various concentrations of proctolin to the preparation shows that increasing concentrations of proctolin have the same effect on tension generation as increases in electrical stimulation of the motor nerves [30]. Thus, proctolin plays a significant role in regulating contractions of the vagina muscles in *R. prolixus* and may serve as the primary excitatory transmitter. Such motor control over the muscles associated with ovipositioning provides the physiological mechanism enabling these insects to lay their eggs during a specific time of day [33], and to correlate the number of eggs laid with the substrate on which they are laid [34].

3.5. Egg laying

According to the structure and function of the genitalia in *R. prolixus*, ovipositioning includes the following steps:

1. A mature chorionated egg is released from the base of the ovariole in the ovary and enters the lateral oviduct;

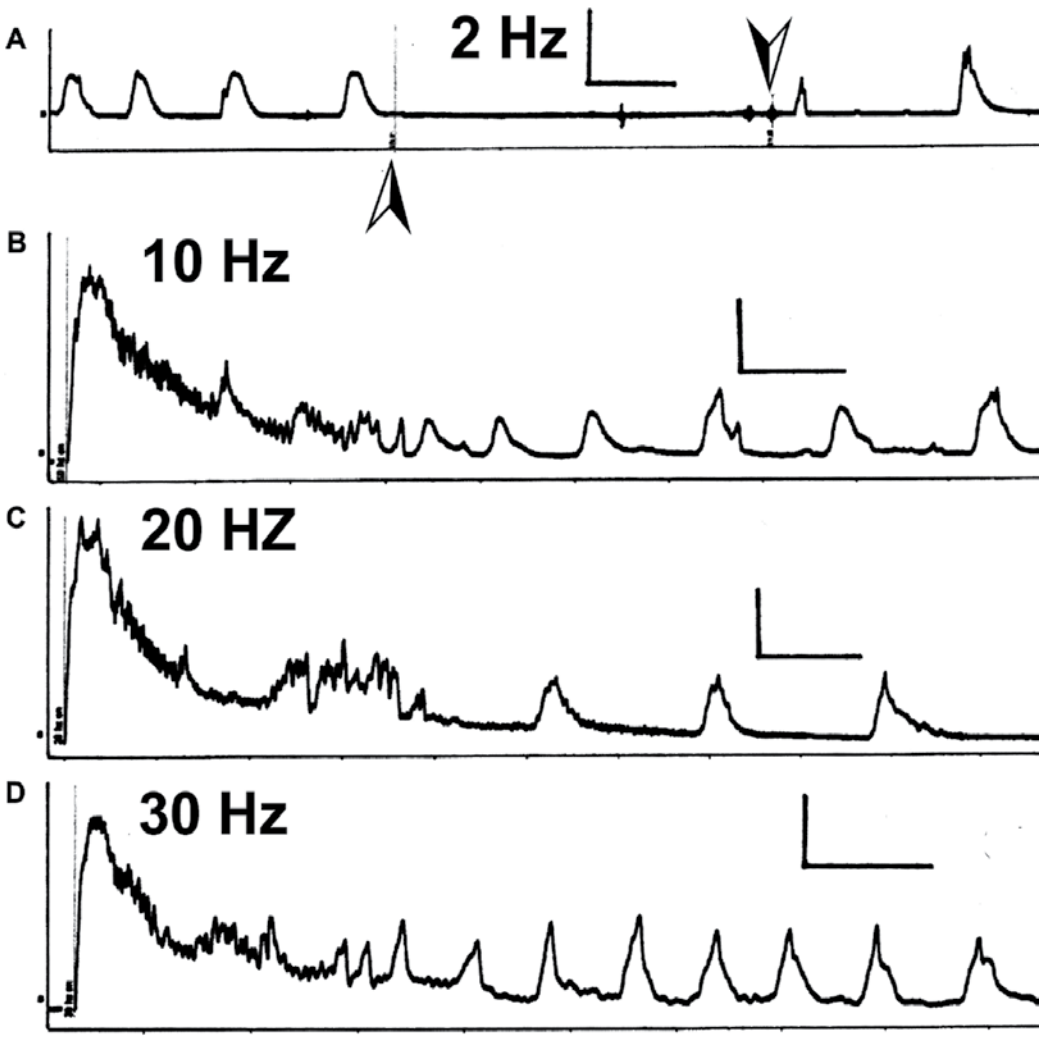

Figure 6. Tension versus time graphs generated by vagina muscles of *Rhodnius prolixus*. (A) Stimulating the motor neurons in abdominal nerve V (up arrowhead) eliminated the spontaneous contractions. Turning off the stimulus (down arrowhead) allowed the spontaneous contractions to return indicating the presence of an inhibitory motor input. (B), (C) and (D) Continual stimulation at 10, 20 and 30 Hz caused an immediate rise in tension at the beginning of the traces with tension gradually dropping which may be as a result of the inhibitory motor input combined with fatigue of the excitatory motor input. Scale bars: vertical, 300 mg; horizontal, 1 min. (Adapted with permission from Ref. [22]).

2. Peristaltic contractions of the lateral oviduct propels the egg into the common oviduct. These contractions may be spontaneous or evoked by a motor input;

3. The egg squeezes through the muscular vestibulum at the end of the common oviduct, and as it stretches the walls of the common oviduct, it stretches the opening of the attached spermathecae. This action allows for the release of some of the stored

spermatozoa onto the egg. Release of spermatozoa may also be enhanced by motor stimulation of the spermathecae;

4. The egg stretches the vagina muscles and the nerve plexus attached to the vagina, and this stretching elicits a contraction of the vagina muscles causing them to shorten and pull the valvulae anteriorly, at which point, the valvulae stretch apart in response to the presence of the egg allowing the egg to start its descent out of the vagina. This step probably involves a stretch reflex which causes contractions of the vagina muscles since eggs are often seen in the lateral oviducts, but seldom lodged within the vagina [10];

5. As the egg exits the vulva, secretions from the cement gland are delivered to the dorsal side of the egg, and the first and second valvulae relocate the cement gland secretion to the ventral side of the egg.

6. As the egg leaves through the vulva, inhibitory input can relax the vagina muscles allowing the valvulae to close off the vulva. This action, in conjunction with active retraction of the dorsal and ventral genital segments, squeezes the egg out of the vagina and onto the substrate. As the dorsal genital segment retracts, it may place pressure on the dorsal surface of the passing egg, and such pressure would ensure that the egg contacts the substrate. Two observations suggest this final action of the dorsal genital segment. First, a mature egg in the reproductive system shows no asymmetry but is equally rounded on all sides, whereas an egg which is laid has a distinct indentation on its dorsal surface as would be expected if pressure were placed on this location during its passage to the substrate. Second, this indentation appears to be directly related to the egg passing through the vulva, and not due to structural changes after being laid. In a SEM image of an egg passing out of the vulva, the exiting egg already shows a distinct indentation under the dorsal genital segment [26].

The role of the female reproductive system is also important in copulation and the ejection of male secretions after copulation, and these events will be considered following a description of the male reproductive system.

4. The male reproductive system

As in the case for the female genitalia this chapter simplifies the nomenclature by identifying the male genital segments according to their structure and function observed in the adult.

4.1. The male genitalia

The genitalia of the adult male are positioned on the underside of full-sized abdominal segment 7, and consist of two segments which move together (see **Figures 7** and **8**). The anterior segment is smaller with no cuticle specializations, and serves to attach the larger posterior genital segment to abdominal segment 7. The skeletal muscles extending between the anterior genital segment and abdominal segment 7 move the genitalia enabling the male to

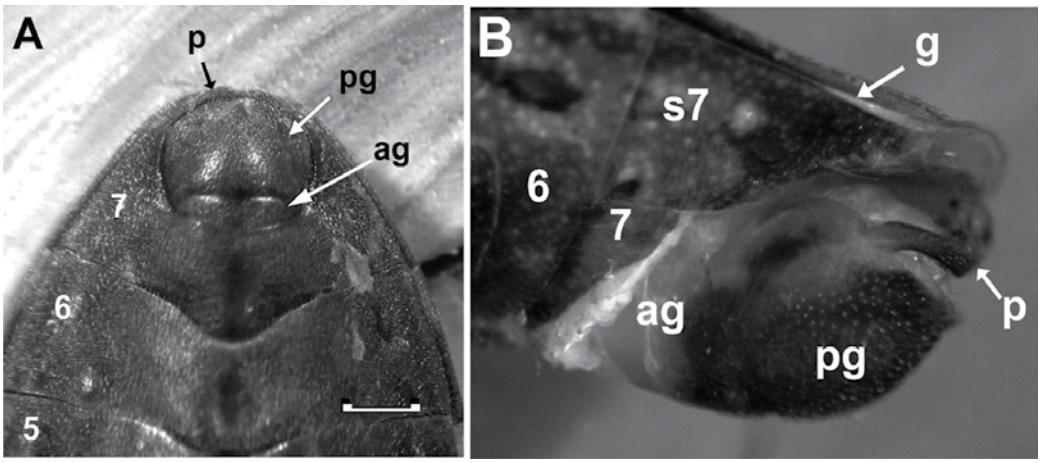

Figure 7. External view of the male genital segments in the blood-feeding insect, *Rhodnius prolixus*. (A) Ventral view showing abdominal segments 5, 6 and 7 (5,6,7). The genitals consist of an anterior genital segment (ag) which is attached to abdominal segment 7, and a posterior genital segment (pg) attached to the ag. p, the distal end of the right paramere sitting in a groove along the posterior dorsal edge of the pg. Scale bar: 1.0 mm. (B) Lateral view of the genital segment with the posterior edge of abdominal segment 7 removed to reveal the anterior region of the ag. g, lateral groove in adult abdominal cuticle; s7, seventh abdominal spiracle.

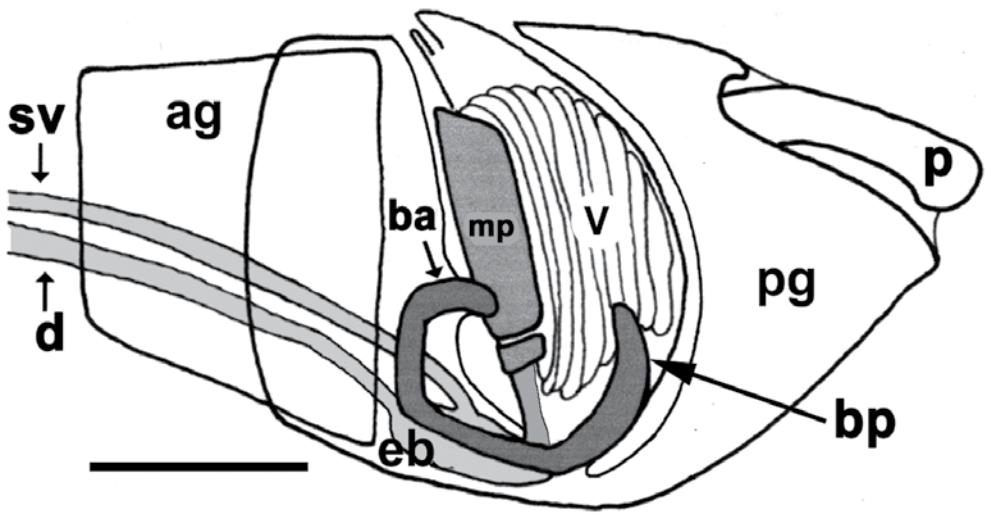

Figure 8. Line diagram summarizing the functional anatomy of the male genitalia in the blood-feeding insect, *Rhodnius prolixus*. The aedeagus sits in a pocket in the posterior genital segment (pg) and contains a convoluted bag-like structure which may serve as a valve (v). The aedeagus is anchored to the softer cuticle lining the pocket by the posterior (bp) and anterior (ba) arms of the basiphallus. mp, medial plate under which secretions from the ejaculatory bulb (eb) are delivered. sv, duct from seminal vesicle; d, duct from accessory reproductive glands; ag, anterior genital segment; p, paramere. Scale bar: 0.5 mm.

extend his genitals away from his body and to turn them laterally to face the corresponding female genitalia. It is this anatomical arrangement that determines the side-by-side position copulating pairs assume (**Figure 1**).

The posterior genital segment is twice the size of the anterior genital segment, and is rounded at its posterior end taking on a bulbous shape (**Figure 7**). On this posterior-rounded side, there are two bilaterally symmetrical arms of cuticle, the parameres, which are attached to the posterior lateral edges of the posterior genital segment (p in **Figures 7** and **8**). On their distal ends, the parameres possess fine hairs characteristic of sensory hairs associated with tactile stimulation in insects [35], and when not extended, they fit into a groove on the posterior dorsal edge of the posterior genital segment with their slightly flattened hook-like ends facing one another (**Figure 7A**). During copulation, the male extends his parameres to make contact with the female genitalia, and during insemination, the curved blunt tips of the parameres wrap around the lateral flaps on the dorsal genital segment of the female. These parameres have been considered homologous to claspers in other insect species [36], but they do not appear to firmly latch on to the female [37]. Preliminary results suggest that the parameres serve a sensory function aiding the male to determine the position of the female genitalia before and during copulation. Their position and sensory function suggest that they are homologous sensory organs to the third valvula in the female genitalia.

The posterior genital segment houses the aedeagus (see **Figure 8**). The aedeagus sits in a pocket lined with soft articulating cuticle and opened to the dorsal side of the posterior genital segment. During copulation, the aedeagus extends out of this pocket into the vagina. Viewed laterally, the aedeagus assumes a half-moon shape (**Figure 9C**). The curved portion of the aedeagus contains an elaborate bag-like structure formed from an invagination of soft cuticle with several overlying folds (**Figure 9B**). These folds allow the bag-like structure to be extended or compressed perpendicular to the flow of secretion from the male reproductive organs. In dissections where the vital dye, methylene blue, is added to the exposed abdomen, this dye is picked up by the reproductive glands and carried in their ducts to the aedeagus where it ends up in the space between the bag-like structure and the medial plate of the aedeagus. Methylene blue does not enter the bag-like structure suggesting that this structure is not designed to receive secretions from the male reproductive organs [37].

The male secretions reach the aedeagus through the ejaculatory duct which is the fused portion of the left and right ejaculatory bulbs. This duct is anchored to a ring of cuticle in the basiphallus which serves as the supporting base for the aedeagus. A pliable delicate duct extends from this ring into the aedeagus (**Figure 9A**), and carries secretions from the ejaculatory bulb into the aedeagus when the aedeagus is extended into the vagina. Since the secretions can be deposited in the space between the bag-like structure and the medial plate on the straight side of the aedeagus, the bag-like structure could serve as part of a pumping mechanism that forces secretions out of the aedeagus and into the vagina during the power stroke, but prevents back flow during the recovery phase of the pumping cycle.

4.2. The male reproductive organs

The male reproductive system anterior to the ejaculatory duct is bilaterally symmetrical and each side consists of two reproductive organs—the testis and the seminal vesicle, and two types of accessory reproductive organs—the three lobes of the transparent accessory reproductive gland (tag) and the one lobe of the opaque accessory reproductive gland (oag)

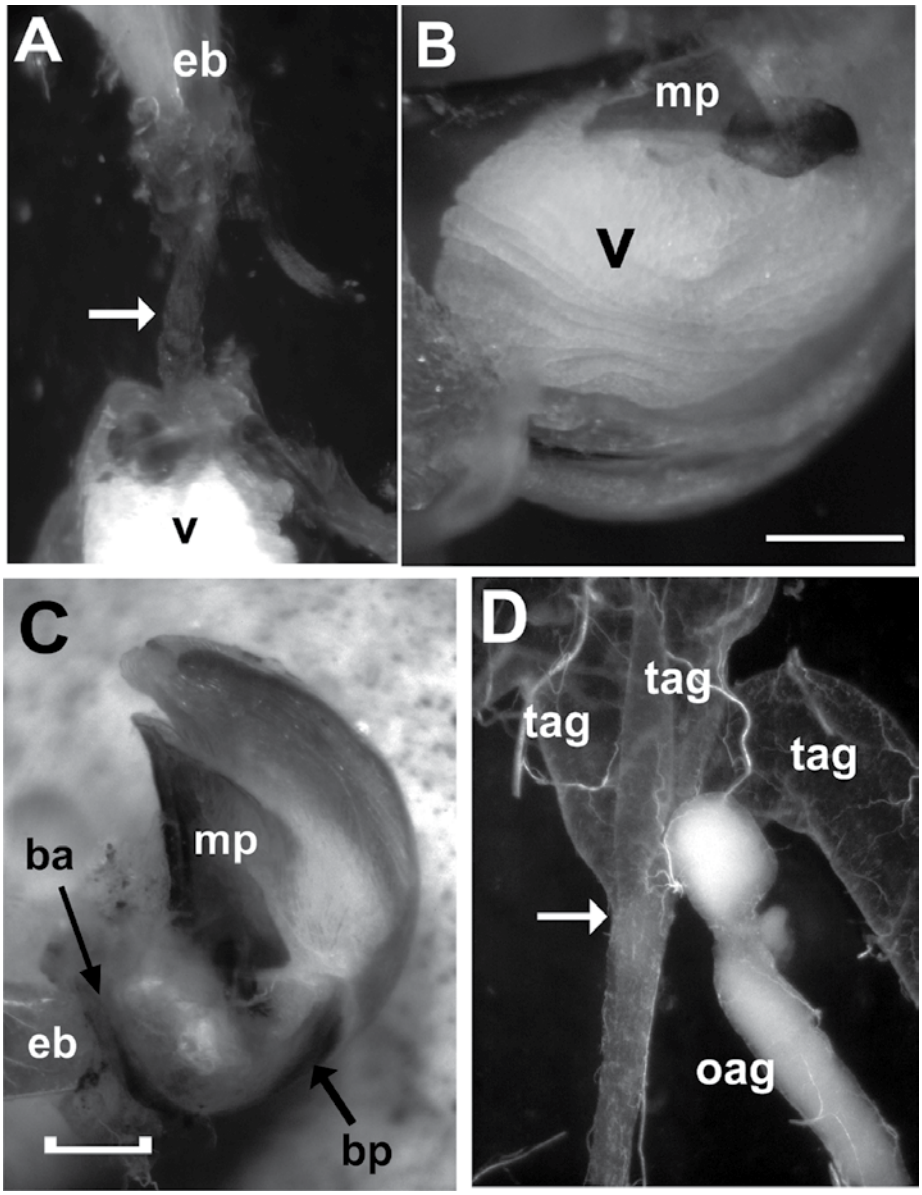

Figure 9. Structures in the male reproductive system of *Rhodnius prolixus* which provide a clue to the manner by which the male secretions are delivered to the vagina during copulation. (A) A small duct (arrow) from the base of the ejaculatory duct (eb) directs secretions into the aedeagus in the space above the valve (v). (B) Picture of the valve (v) showing that it consists of folds of soft articulating cuticle. Secretions enter the space between the medial plate (mp) and this valve. Scale bar: 0.6 mm. (C) A dissected aedeagus removed from the posterior genital segment. The whitish valve is just visible through the articulating cuticle between the medial plate and the curved back of the aedaegus. ba, anterior arm of the basiphallus; bp, posterior arm of the basiphallus. Scale bar: 0.5 mm. (D) The relationship between the ducts of the three transparent reproductive accessory glands (tag) and the opaque reproductive accessory gland (oag). They converge at the head of the common duct (arrow), travel down the duct separately merging into a single tube that empties into the ejaculatory bulb.

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(see **Figure 10**). The testes are located laterally near the mid-region of the abdomen. They are present in the penultimate larval stage (L5), and become connected to the seminal vesicle during metamorphosis. The testis consists of seven follicles folded onto each other and wrapped with a thin membrane. Two of the seven have a larger girth and a longer length than the other five, even in the L5 stage. As the testes increase in size during the adult stage, the growth is mainly attributed to the two larger follicles which increase considerably in length and girth [10], which has also been observed in other species of Reduviidae bugs [38].

Each testis is connected to the seminal vesicle by the vas deferens which extends a short distance from the testis, where the bases of the follicles are attached, to the tip of the anterior lobe of the tag. From this point, the vas deferens remains closely associated with the tag and courses along its side to the level of the lobe's base where the vas deferens connects to the seminal vesicle. Between the testis and the tip of the tag, the contents of the vas deferens tend to be transparent. From the tip of the lobe to the seminal vesicle, the contents are distinctly yellowish white and have a clump-like appearance. The seminal vesicle is a semi-rigid elongated sack which can increase considerably in girth as the adult matures. Its length is approximately the same length as the individual lobes of the tag (**Figure 10**), and its duct connects to the ejaculatory bulb posterior to the duct from the accessory reproductive glands.

Of the two types of accessory reproductive glands, the larger tag consists of three large tube-like lobes, and the smaller oag is a single elongated structure (**Figure 10**). The tag contains a clear proteinaceous material, and the oag contains a whitish milky substance, both of which are delivered to the female during copulation. Rather than sequestering from the haemolymph molecules made from another organ or tissue, the tag may make the secretions themselves [39], with their activity being under endocrine control [40, 41]. They also produce a polypeptide that is secreted into the haemolymph [42]. The tag possesses a relatively tough muscular lining that is supplied by motor axons which, when electrically stimulated, will cause each of the three lobes of the tag to constrict their girth and lengthen (personal observations). The tag secretions are viscous and pour slowly out of the lobe when it is cut. In contrast, the oag has a delicate lining, is easily damaged during dissection and its whitish secretions readily flow out of the lobe. It is widest at its anterior base, tapers towards its posterior end, and does not respond to electrical stimulation of the abdominal nerves. Early studies report that placing the contents of the oag onto an adult vagina can elicit strong twitch-like contractions of the vestibulum suggesting that this male secretion may aid delivery of the transferred spermatozoa to the spermathecae [20]. Because the response is described as capricious rather than consistent, this role is speculative.

Each lobe of each accessory reproductive gland empties through its own duct, and these ducts enter a tube which makes up the proximal end of the common accessory reproductive gland duct (**Figure 9D**). As these ducts enter this tube, they do not merge into a single duct at this point, but extend down the tube to become a single lumen before emptying into the ejaculatory bulb. This tube has a muscular sheath, and displays spontaneous contractions that tend to shorten the tube pulling it posteriorly towards the ejaculatory bulb.

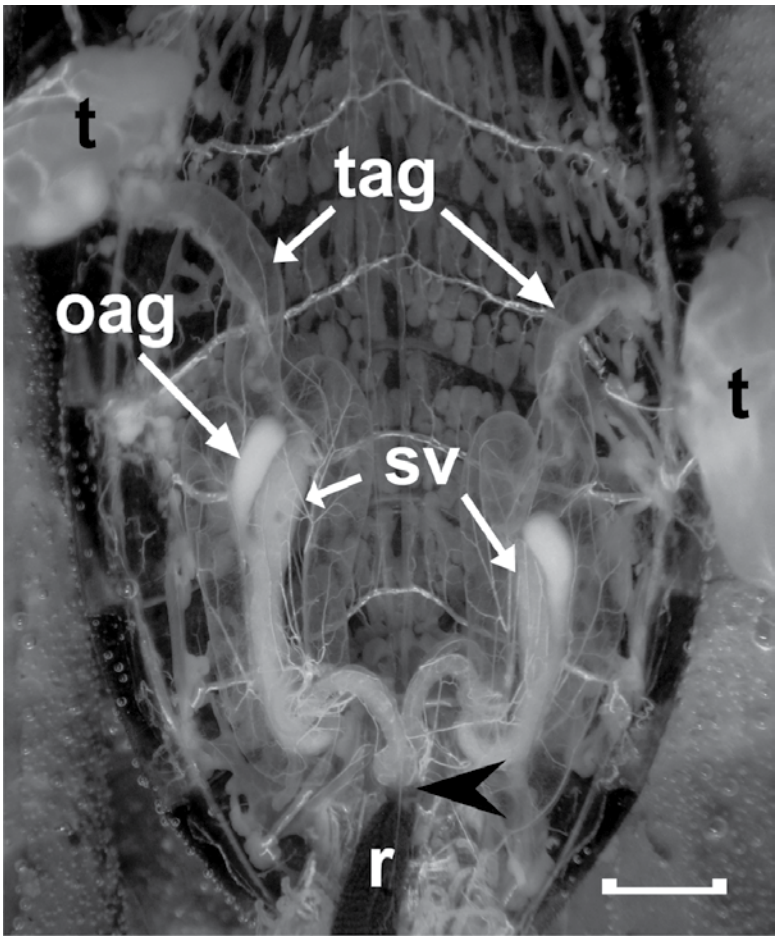

Figure 10. Live preparation of the abdomen of a male *Rhodnius prolixus* exposed to show the reproductive organs. t, right and left testes; tag, the anterior lobe of the transparent accessory reproductive gland; oag, left opaque accessory reproductive gland; sv, seminal vesicle; r, rectum. The ducts of the seminal vesicles and accessory reproductive glands, and the anus at the end of the rectum, come together to enter the anterior genital segment (arrowhead). Scale bar, 2 mm.

4.3. Delivery of male secretions to the vagina

The manner by which the male secretions are delivered to the female reproductive system in insects varies between two extremes. At the one extreme, the female has two genital openings, one to the bursa copulatrix, and the other to the egg pore. The male produces a distinct spermatophore which is a proteinaceous package containing spermatozoa and this package is deposited into the bursa copulatrix. There, the spermatophore is broken open allowing the spermatozoa to migrate along the sperm duct to the spermatheca [43]. At the other extreme, the female has a single opening to her reproductive system. The male inserts a long intromittent organ through the vagina, into an insemination duct which leads to an elaborate spermatheca. At the end of the insemination duct, the male extends his intromittent organ

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through a valve and pumps his secretions directly into the spermatheca. No spermatophore is needed [44].

The first description of sperm transfer in *R. prolixus* assumed that this species produced a distinct spermatophore. Khalifa [45] described the spermatophore in *R. prolixus* as a pear-shaped sac containing spermatozoa enclosed in a proteinaceous mass, and from his study of fixed tissue, Davey [36] proposed that before it is delivered to the female, this spermatophore is formed within the male spermatophore sac that everts from the end of the aedeagus into the female during copulation. While examining spermatophore formation in *Triatoma infestans* and *Rhodnius neglectus*, Pereira-Lourenço et al. [23] made observations which differ to that reported for *R. prolixus*. In these bugs, which are closely related to *R. prolixus*, there is no spermatophore sac and the spermatophores are transferred as a viscous or mucous substance directly to the vagina where they solidify. Chiang and Chiang [37] resolved this discrepancy by observing that the fixed tissue of *R. prolixus* observed by Davey [36] does not behave like a living tissue. As noted above in the description of the male reproductive system, the structure previously thought to be a spermatophore sac in fixed tissue of *R. prolixus* stays within the aedeagus, has no opening, and does not receive any secretions from the male reproductive system.

Considering these more recent findings, it is likely that all Reduviidae bugs lack spermatophore sacs, and the structure thought to be a sac is part of a pumping mechanism which enables the aedeagus to fill the vagina with the male secretions. Rather than resembling those arthropods which make encapsulated spermatophores that harden before they are inserted into the bursa copulatrix of the female [46], the Reduviidae are more closely related to insect species which lack spermatophores and deliver the semen by using a long intromittent organ that the male inserts through an insemination duct to the spermatheca [44]. In *R. prolixus*, the spermathecae are attached directly to the common oviduct so that no insemination tube is required since this location is very close to where the spermatozoa are delivered. Male Reduviidae bugs may still be described as spermatophore producers, but unlike the traditional description of a spermatophore, their spermatophores are naked, are formed directly in the female and harden after they have been delivered to the female.

As the male secretions are delivered to the female, they assume the pear-shape of the inside of the vagina with the narrower anterior end resulting from the male secretions being pushed up against the narrow base of the common oviduct (**Figure 11**). In a recently inseminated female, the secretions from the seminal vesicle appear as a clump of yellowish material at the base of the common oviduct whereas the rest of the vagina is filled with a slightly cloudy secretion from the accessory reproductive glands. Since the spermatozoa are positioned anteriorly, the seminal vesicle secretions are delivered first, followed by secretions of the accessory reproductive glands. With separate ducts to the ejaculatory bulb, differential motor activity from the central nervous system likely stimulates the seminal vesicles to deliver their secretions prior to transfer of the accessory reproductive gland secretions. In addition, the clump from the seminal vesicle is approximately the same size as the aedeagus suggesting that only one or two pulses from the pump in the aedeagus are needed to deliver the spermatozoa.

The remainder of the spermatophore consists of a large amount of secretion from the tag and oag. While in the body of the male, the secretions of the tag are transparent, but in the

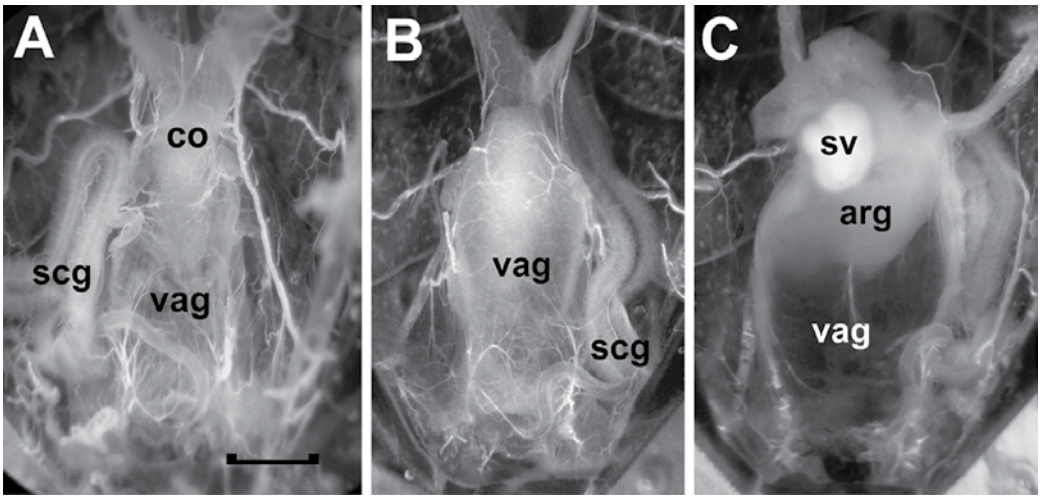

Figure 11. A freshly dissected female of *Rhodnius prolixus* showing in (A) an empty vagina (vag), the collapsed common oviduct (co), and the secretory portion of the cement gland (scg), in (B) a vagina expanded to accommodate the male secretions shortly after the completion of copulation and in (C) the same vagina in B which has been cut open on its dorsal side and the contents pulled slightly out of the vagina. sv, secretions from the seminal vesicles; arg, secretions from the accessory reproductive glands. The clear area in the vagina is the region from which the male secretions were pulled away. Scale bar: 0.5 mm.

spermatophore in the female, they take on a slightly cloudy appearance (**Figure 11C**). This change likely results from a small amount of oag material mixing with a large amount of tag material which is possible due to the relationship between the ducts from the three lobes of the tag, and the single duct from the oag. All four ducts enter the distal end of the tube of the common accessory reproductive gland duct, and this tube is able to produce bursts of contractions that rhythmically constrict and shorten the tube. In addition, the lobes of the tag can contract due to motor stimulation thus forcing the material into their ducts, whereas material from the oag enters passively. This anatomical arrangement could allow the peristaltic-like contractions of the common duct to 'milk' the ducts of the four lobes of accessory reproductive glands at the same time resulting in a large amount of tag material being mixed with a small amount of oag material before they are delivered to the aedeagus and the vagina. This scenario, which is supported by the anatomy and physiology, suggests that the oag secretions are affecting the tag secretions rather than eliciting contractions of the vestibulum. Davey [36] postulated that the secretions from the cells lining the ejaculatory bulb mix with the tag secretions to lower the pH, causing the tag secretions to harden. However, the oag secretions may also serve in hardening the tag secretions. Determining the relationship between the secretions from the tag and oag promises to be a fruitful area of study.

4.4. Facilitating copulation

In *R. prolixus*, the male completes insemination in about 50 minutes (52 + 14 minutes, $n = 26$, as reported in Ref. [10]), and has at least two physiological means to help maintain copulation for this length of time. One is sensory; the other is chemical.

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If the sensory hairs on the ventral lateral region of the abdomen are gently stroked with a probe, the heartbeat is inhibited [47]. Such a reflex could be part of a general thigmotactic response in which the insect becomes less responsive to external stimuli when it wedges itself into a confined space (see p. 313 in Ref. [35]). This response could be elicited as these sensory hairs touch the surface of the enclosed area, and the stoppage of the heart beat may be part of the general calming of the whole body. The ventral region of the abdomen linked to this tactile inhibition of the heartbeat is the same region where the male places his abdomen during copulation, which, in turn, could generate a thigmotactic response to help calm the female.

This sensory thigmotactic response could be enhanced chemically by rhodtestolin, a cardio-inhibitor first discovered in testes extracts of *R. prolixus* [48]. When a test saline containing rhodtestolin is applied to the isolated heart, the heart becomes flaccid and all beating immediately ceases. Rhodtestolin is a small, heat stable protein, and its dramatic cardio-inhibitory effect is dose dependent and reversible [10]. It has yet to be determined how rhodtestolin concentrated in the testes makes its way to the female, but it is delivered during copulation since extracts of spermatophores removed from the female shortly after the completion of copulation show this cardio-inhibitory effect [10]. Being delivered to the female during copulation could enhance the sensory thigmotactic response, but if rhodtestolin has a general inhibitory effect on insect visceral muscle, its major role might be to relax the vagina muscles and to prevent the female from expelling the spermatophore prematurely. In preparations in which the tension generated by the vagina muscles is monitored by a force transducer, stretching these muscles generates strong spontaneous contractions that would expel any vagina contents, whether it is an egg or a spermatophore [22]. Therefore, rhodtestolin may be a visceral muscle relaxant which reduces the excitability of the vagina muscles so they do not contract in response to being stretched by the male secretions. This role still needs to be substantiated but it would increase the changes of the spermatozoa delivered to the vagina reaching the spermathecae before the male secretions are ejected from the vagina.

5. Summary

Our knowledge of the details of sexual reproduction in *R. prolixus* has lagged behind other aspects of its physiology since studies using this bug have tended to take advantage of the fact that a blood meal triggers the onset of physiological processes related to feeding. To help address this shortfall, the present chapter details the functional anatomy of male and female genitalia, and highlights a number of significant points which may apply to insect vectors of Chagas disease in general. First, the female genital chamber is best referred to as the vagina since the male of this species does not form a distinct spermatophore. Second, the male inserts his aedeagus directly into the vagina and delivers his secretions with the aid of a novel pumping mechanism. Third, the design of the accessory gland ducts provides a mechanism whereby the male secretions from the tag and oag can be mixed before they enter the vagina, suggesting that these secretions have a chemical relationship that warrants further investigation. Fourth, the male parameres and the third valvula of the female appear to be homologous sensory structures which may have a function in both sexes during copulation, or in the female

during egg-laying. Fifth, the sclerites of the first and second valvulae in the female, and their proximity to the excretory pore of the cement gland, suggests that they are involved in placing the cement gland secretions onto the ventral side of the egg. Finally, the sensory and chemical aids for copulation, which still need to be further explored, provide yet another intriguing aspect of insect reproduction first to be observed in *R. prolixus*.

This knowledge gained by detailing the mechanics of copulation and egg-laying in this well-studied insect sets the groundwork from which further investigation of this important physiological process in this bug can be carried out. Along with the completion of the *Rhodnius* genome project, which provides a resource to investigate the genes and proteins associated with reproduction, *R. prolixus* is well equipped to maintain its status as a popular insect model for teaching and research.

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Chapter 15

Developing the Arsenal Against Pest and Vector Dipterans: Inputs of Transgenic and Paratransgenic Biotechnologies

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Additional information is available at the end of the chapter

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Abstract

Insects are the most numerous of all animals and are found in almost every inhabitable place on earth. The order Diptera (true flies) contains many members that are notorious agricultural pests, nuisance or vectors of diseases. The list is long: mosquitoes, tsetse flies, screw worms, fruit flies, sand flies, blow flies, house flies, gall and biting midges, black flies, leaf miners, horse flies, and so on. Efforts to combat some of these pests and vectors have resulted in control measures such as the chemical, physical, and cultural control methods. These methods, though largely beneficial, have disadvantages and limitations, which sometimes seem to outweigh the problems initially sought to be controlled. The chemical method, for example, is not environment-friendly since it negatively affects many nontarget organisms and disrupts ecosystem balance. Development of insecticide resistance by pests/vectors is another concern. Molecular biotechnology has introduced vast arrays of novel ways to tackle pests and disease vectors, as well as improve the potency of existing control methods. This chapter looks at transgenic and paratransgenic biotechnologies and how they have been applied so far to develop and expand the arsenal against dipteran pests and disease vectors. Further, we discuss the advantages, disadvantages, and limitations of these technologies.

Keywords: insects, dipterans, crop pests, disease vectors, transgenesis, paratransgenesis

1. Introduction

Insects are highly abundant and are the most numerous classes of all described living animals. They account for about half or more of all living animals and are found in almost every inhabitable place on earth [1, 2]. Their success and abundance could be attributed

to their ability to adapt and colonize diverse habitats. Among insects, the order Diptera is one of the largest orders with an estimated 120 families and 250,000 described species [1]. They are generally regarded as the two-winged insects or true flies [2, 3]. The main characteristics for members of this order include larvae that lack legs (apodous maggots), pupae enclosed in a thick larval cuticle (puparium), and adults that possess a pair of membranous forewings, vestigial hindwing modified into halters, as well as a tubular sucking or sponging mouthparts [2]. Dipterans are longtime foes and arguably considered the insect archenemy of man. This stems from the fact that many members of this order constitute pests of cultivated crops, are major causes of annoyance or are highly notorious as vectors of human diseases either in their larval or adult stages. Examples of pest, annoyance causing and vector dipterans are given in **Table 1** [4–8].

Efforts by man in the fight against dipteran pest and vector insects have resulted in the generation of an arsenal with several weapons. These range from chemical method which involves the use of insecticides to cultural methods such as sanitation, physical interference or destruction of breeding sites, and cropping methods. However, many of these methods have major disadvantages and (or) limitations that sometime seem to outweigh their benefits. For example, the chemical method is very widely used, but has the disadvantages of environmental consequences such as pollution, health challenges on man and livestock, killing of nontarget insect species, as well as the challenge of the targeted insect species developing resistance to the insecticides applied [9, 10]. Most cultural methods applied against dipteran pest or vector control are labor-intensive and can only be most suitably applied on a small scale.

Biologically-based approaches are generally friendlier to the environment, more sustainable and cost-effective than many other methods used for dipteran control. Here, control methods such as the use of natural enemies like predators and parasitoids are environment-friendly with varying levels of success, but the major limitation is the fact that it is unpredictable as chances are usually low on finding a suitable parasitoid or predator that can survive the weather and conditions wherever the pest or vector dipteran is and continue to effectively eat or parasitize the host [10]. The time it takes to find a good parasitoid may be so long that farmers or entomologists concerned may opt for other control methods, in addition to the fact that the process of actual control by a parasitoid or predator itself is slow. The biological method of using pathogens (microbial or biopesticides) has been quite promising, but recently there has been concerns of insect resistance as is the case with *Bacillus thuringiensis*, and also the disadvantage that the applied pathogen may infect other nontarget insects, livestock, or man himself. Major limitations of biopesticides are usually that one may need to find an efficient way to get the pathogens to their host and that the pathogens may be negatively affected by environmental conditions such as weather.

Another biologically-oriented and environment-friendly method for controlling dipterans is the use of pheromones or suitable attractants. However, the scale of its application and area that it covers is also limited, while the potency of the attractants does reduce gradually with time or could easily be influenced by environmental factors such as rainfall or masked by other chemicals within the vicinity.

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Family	Genus/species involved	Problematic or damage-causing stage; problem caused
Agromyzidae (leaf miners)	*Phytomyza angelicastri; Melani agromyza*	Larva; damage to leaves of crops
Anthomyiidae	*Anthérigona* spp; *Delia radicum*	Larva; damage to stems of crops like cauliflower and sorghum causing disease like "dead heart"
Calliphoridae (blow flies)	*Callitroga* spp.; *Cordylobia anthropophaga; Lucilia* spp; *Chrysomya bezziana*	Larva; myiasis or flesh infesting damage to man and livestock
Cecidomyiidae (gall midges)	*Contarinia sorghicola*	Larva; damage to leaves of crops such as rice, pear, sorghum, etc.
Ceratopogonidae (biting midges)		Adult; blood sucker from man and livestock
Chloropidae (chloropid flies)		Larva; damage to leaves of crops like rice and cereals
Culicidae (mosquitoes)	*Anopheles* spp.; *Aedes* spp.; *Culex* spp.; *Mansonia* spp.; *Psorophora* spp.; *Stegomyia* spp	Adult; blood sucker from man and livestock transmitting parasites that cause various diseases like malaria, dengue fever, West Nile fever, yellow fever, encephalitis, O'nyong nyong fever, Bancroftian filariasis, chikungunya, Igbo-Ora, Zika, etc.; nuisance, major cause of disturbance and annoyance to man at night
Drosophilidae	*Drosophila suzukii*	Larva; damage to fruits
Glossinidae (tsetse flies)	*Glossina* spp.	Adult; blood sucker from man and livestock transmitting the causative agent of Trypanosomiasis (sleeping sickness)
Muscidae (house flies)	*Musca domestica*	Adult; transmits microorganisms that cause cholera and amoebic dysentery; nuisance, major cause of annoyance to man during the day
Oestridae (warble or bot flies)	*Oestrus ovis; Gasterophilus* spp.; *Hypoderma bovis; Dermatobia hominis*	Larva; myiasis or flesh infesting damage to man and livestock
Psychodidae (sand flies and moth flies)	*Phlebotomus* spp.	Adult; blood sucker from man transmitting the parasite causing disease leishmaniasis
Sarcophagidae (flesh flies or screw worms)	*Cochliomyia hominivorax; Sarcophaga* spp; *Wohlfahrtia* spp.	Larva; myiasis or flesh infesting damage to man and livestock
Simuliidae (black flies)	*Simulium* spp.	Adult; blood sucker from man transmitting the causative agent of the disease onchocerciasis (river blindness)
Tabanidae (horse flies)	*Tabanus* spp.; *Haematopota* spp.; *Chrysops* spp.	Adult; blood sucker from man and livestock transmitting the causative agent of diseases like trypanosomiasis (sleeping sickness) and loasis
Tephritidae (fruit flies)	*Anastrepha* spp.; *Bactrocera* spp.; *Ceratitis* spp.; *Dacus* spp.; *Rhagoletis* spp.; *Tephritis* spp.	Larva; serious damage to fruits and vegetables

Table 1. Dipteran crop pests, nuisance or vectors of diseases [4–8].

Genetic methods such as the sterile insect technique (SIT) majorly use radiation to sterilize male insects and thus reduce the fertility of females that mate with them [11, 12]. This method works well with sexually reproducing insects and has so far has great success among many dipterans [13]. Its major problem is the fact that the gamma radiation used for sterilization also reduces the fitness of the males and makes them less competitive than the wild males.

Obviously, no present method of pest control is devoid of disadvantages or limitations. As such, an integration of different control methods that are compatible is the recent paradigm. Integrated pest management (IPM) has offered a way to augment control methods to achieve a more efficient and sustainable management of pests and vectors.

The new millennium has witnessed advanced progress in genetic biotechnology which in turn has greatly influenced insect control. Biotechnology approaches have been used and are continually been pursued as a means to develop novel ways or improve some of the methods used to fight pest and vector dipterans. For example, new strains of reproductively sterile insects or strains exhibiting other desired traits could be engineered to control a population or designed to fit into control methods like SIT, entomopathogens or biopesticides that are adversely affected by weather conditions where a pest or vector is located or have environmental concerns regarding nontarget insects could be encapsulated in materials that will release the pathogens only in a desired condition, while nonharmful microorganisms could be engineered to deliver therapeutic or antiparasitic molecules to pathogens in their environment. Many of these new biotechnology approaches could also be used as a part of IPM programs which is suitable for other methods. In this chapter, we focus on how transgenic and paratransgenic biotechnologies have been applied to expand the array of weapons in man's arsenal against dipteran crop pests and vectors of diseases.

2. Transgenic biotechnology

Transgenesis aims at the transformation of an organism by altering its genetic composition and the final outcome is the generation of a transgenic or a genetically modified organism (GMO). Basically, desired genes or genes-of-interest from a different organism(s) are inserted into the genome of a wild type organism majorly with the aid of "jumping genes" called transposable elements or transposons and the transgenic organism generated carry these desired genes (transgenes), while exhibiting characters or traits encoded by the transgenes as well (**Figure 1**). For insects, germline transformation is sought and microinjections are performed to achieve it, allowing the genome modification to be passed on from generation to generation [14]. To enable detection of successfully transformed organisms, fluorescent proteins such as the green fluorescent protein (GFP), the red fluorescent protein (RFP), and fluorescent proteins of other colors are used as markers [15–18]. Consequently, GM dipterans harboring a transgene that incorporates a fluorescent protein gene cassette as marker would express the fluorescent protein and can be visualized under a fluorescent microscope (**Figure 2**).

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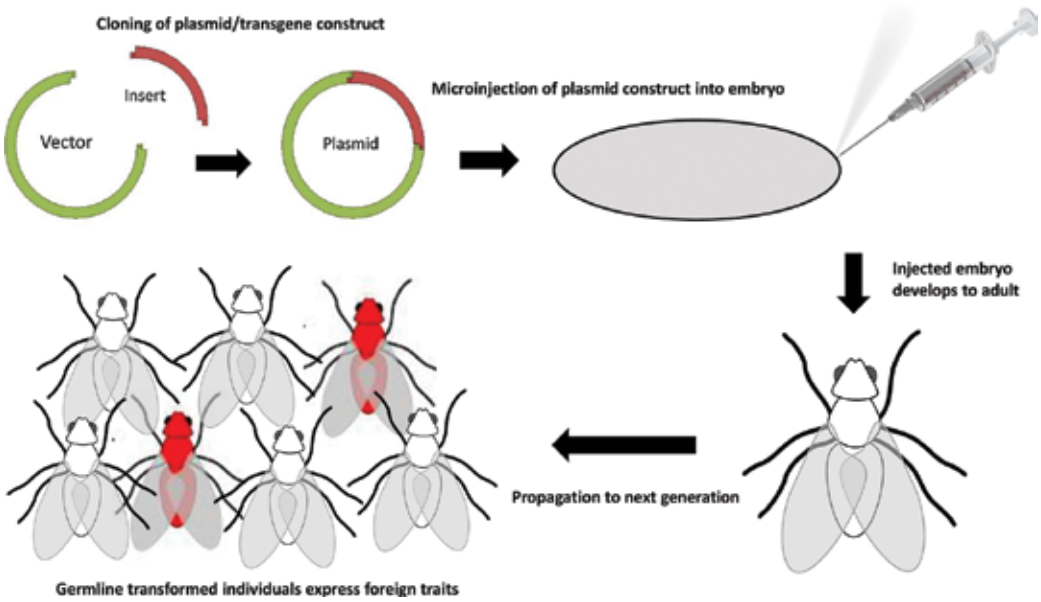

Figure 1. Schematic representation of transgenesis: desired gene-of-interest is cloned into a vector to generate a plasmid or transgene construct which is then microinjected into embryos. Adults developing from the injected embryos are outcrossed to non-injected ones and their progeny are screened. Progeny that are stably germline transformed express traits encoded by the genes in the plasmid construct injected, for example a red fluorescent protein, and as such are distinguished from untransformed ones.

2.1. Transgenic strategies against agricultural crop pest dipterans

2.1.1. Drosophilids

The family Drosophilidae consists of many members including the well-known model fly *Drosophila melanogaster*, but only the spotted wing drosophila, *Drosophila suzukii*, is considered a major pest of cultivated crops [19]. However, *D. melanogaster* has been immensely beneficial in genetic studies and many proofs-of-principle of transgenic strategies against dipteran population control, or even for other insect orders, have been developed in this model insect.

A proof-of-principle transgene-based, embryo-specific lethality system for insect control was developed by Horn and Wimmer [20]. The system used embryo stage-specific promoters such as serendipity alpha (sryα) to regulate the expression of a hidAla5 lethal effector placed under the control of a tetracycline-response element [20]. Such a strain would effectively achieve reproductive sterility in insect populations because the offspring die during the embryo stage and could replace radiation sterilization of insects as is the case for conventional SIT.

Two proofs-of-principle for transgenic sex-specific lethality systems for insect population control were developed: (i) using female-specific enhancers of yolk protein 1 (yp1) gene to drive expression of a hid effector under control of tetracycline-responsive element [21]. (ii) using a female-specific yolk protein and fat-body enhancer Yp3 to regulate the expression of a Ras64BV12 effector under control of tetracycline-responsive element, as well as using a

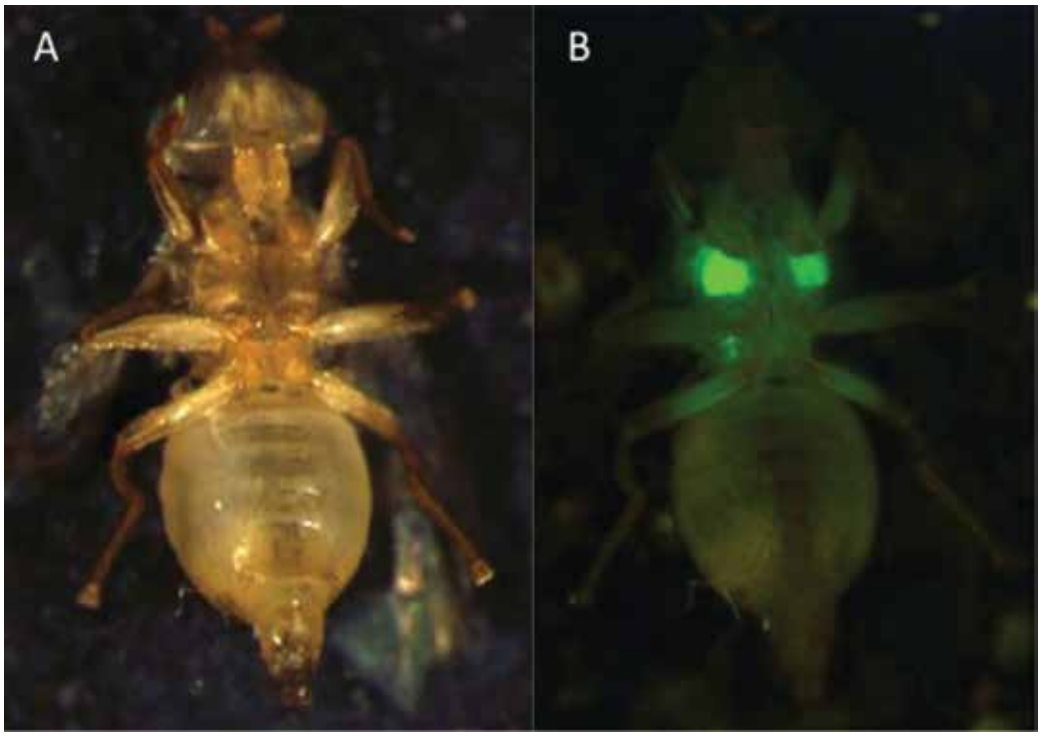

Figure 2. A transgenic strain of the Mediterranean fruit fly Ceratitis capitata: (A) visualized under cold light, (B) visualized under fluorescent light, the same transgenic fly shows a pattern of expression of green fluorescent protein GFP in its thorax and legs.

Hsp26 promoter to regulate expression of a dose-compensation gene, mutant male-specific lethal 2 (msl-2NOPU), under the control of a tetracycline-response element [22]. These kinds of systems limit lethality or death of offspring to only female individuals and could be used for efficient sex separation of dipterans prior to field release during area-wide dipteran pest control programs such as SIT.

Besides these afore-mentioned transgenic lethality systems which were all based on the tetracycline-repressible binary expression system (**Figure 3**) [23], a gene-driven system capable of driving population replacement was also developed in Drosophila [24]. Basically, a gene-driven system such as a maternal-effect dominant embryonic arrest (Medea) system use a combination of two genes that encode for a toxin and its antidote, respectively, to create a condition whereby a heterozygote female would express only the maternal toxin in half of her oocytes without the antidote resulting in death of those offspring. The Medea strain which was developed by Chen et al. in Drosophila used microRNA-mediated silencing of a maternally expressed embryonic development gene, my88, as its toxin and early zygotic expression of a rescuing transgene as the antidote. A more complex Medea system employing additional mechanisms such as targeting signaling pathways like the Notch pathway has since been also demonstrated in *D. melanogaster* [25].

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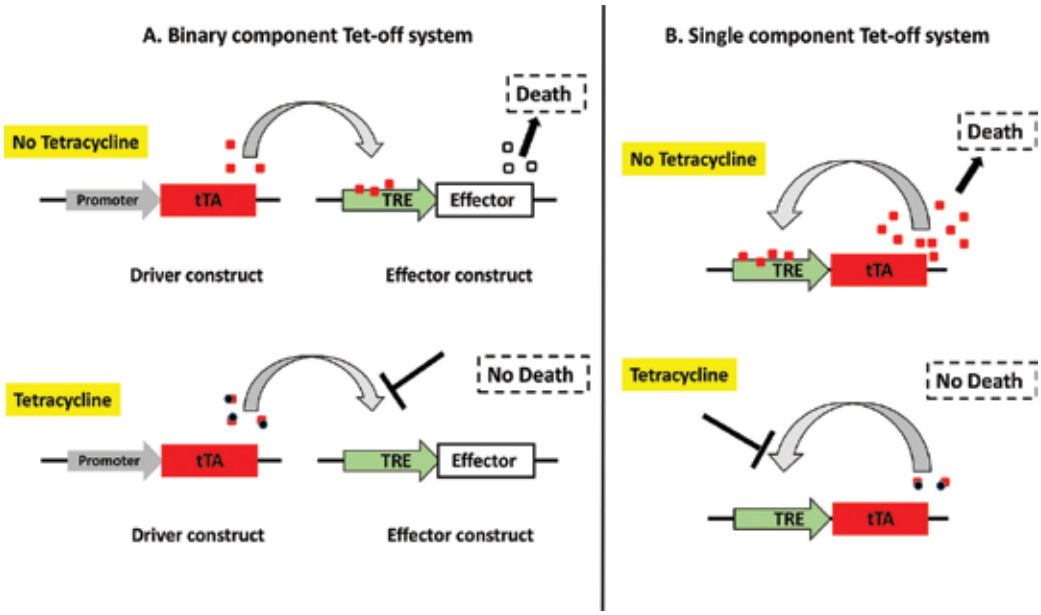

Figure 3. Diagrams showing different versions of tetracycline-repressible expression system: (A) binary component tet-off system, (B) single component tet-off system [23, 29]. In the absence of tetracycline, tetracycline-represssible transactivator (tTA) is produced and goes on to bind to the tetracycline-response element (TRE) to activate expression of a downstream gene. Both systems are turned off in the presence of tetracycline which bind to the tTA and stops expression of a downstream gene. The binary system needs an effector for lethality, while the single component uses tTA which is toxic at high concentration.

For the crop pest Drosophilid, the spotted wing drosophila *D. suzukii*, germline transformation has recently been performed and transgenic strains for control of this strain may soon be generated [26].

2.1.2. Tephritid fruit flies

Tephritids are very important pests of fruits and vegetables and majority of transgenic strategies for crop pests have been developed against members of this group. Lethality systems that their proofs-of-principle have earlier been developed in Drosophila have also been successfully transferred to many Tephritids. Among these are the conditional embryonic lethality strains transferred from *D. melanogaster* to both the Mediterranean fruit fly, *Ceratitis capitata* and the Caribbean fruit fly, *Anastrepha suspensa*, and using the tetracycline-regulated binary expression system, embryonic promoters/enhancers and proapoptotic hid effector [27, 28]. In addition, the lethality strains not previously established in Drosophila was developed for *C. capitata* using a simplification of the tetracycline-regulated binary expression system to a single expression component that relies on auto feedback-driven overexpression of a version of the tetracycline-repressible transactivator (tTA) for its lethality (**Figure 3**) [29].

For sex separation of Tephritids, transgenic sexing strains were developed for different fruit fly genera: (i) an RNA interference (RNAi) system developed for *C. capitata* based on knockdown

of transcripts of the sex determination gene transformer (tra) [30], (ii) lethality systems relying on a simplified single component tetracycline expression system and developed for *C. capitata* and the olive fruit fly, *Bactrocera oleae* [31, 32], (iii) lethality systems relying on a tetracycline-regulated binary expression system [23], embryonic promoters/enhancers and proapoptotic hid effector, and developed for *C. capitata, A. suspensa*, and the Mexican fruit fly, *Anastrepha ludens* [33–35]. Unlike the two proof-of-principle transgenic sexing systems based on lethality earlier developed in Drosophila, all the afore-mentioned transgenic sexing systems based on lethality in Tephritids employed the sex-specifically spliced intron of the gene transformer (tra) to confer lethality only to the female individuals. However, only those systems employing the tetracycline-regulated binary expression system and embryonic promoters or enhancers achieved female-specific lethality in the embryo stage [33–35]. Another type of transgenic sex-specific lethality system has recently been developed for the Oriental fruit fly, *Bactrocera dorsalis* [36]. This system combined the mechanism of alternative splicing of the double sex (dsx) gene and the toxicity of expressed ricin to ensure female-specific lethality and kill off the female progeny in *B. dorsalis* [36].

Since area-wide dipteran pest control strategies like SIT involve release of sterile males, a way to monitor the released males is also as important as the sterilization and sex separation of the males. Scolari et al. developed a transgenic strain that would facilitate such monitoring in C. capitata by using the promoter of a sperm-specifically expressed gene β2-tubulin (β2t) to regulate the expression of RFP and GFP to only male testis. Males of this sperm-marked fly strain were shown to still have brightly glowing fluorescent testis for several months after they had died [37]. As such, the released males could easily be monitored if caught in traps or found dead in the field in the case they were used in any SIT control program.

2.2. Transgenic strategies against dipterans of medical and veterinary importance

2.2.1. Mosquitoes

The battle against any other dipteran insects has perhaps never been as intense as it is for mosquitoes due to the wide range of diseases they vector and transmit. Almost every kind of approach that is imaginable is under development or has been developed in the effort to win the battle against mosquitoes. Ever since the first germline transformation of an Anopheles mosquito [38], several transgenic strategies have been constructed including gene drive systems, lethality, flightless, sperm-monitoring, as well as spermless systems, and mosquito strains that have been impaired in their ability to transmit a parasite.

Among the gene-driven systems include a maternally-regulated transposition system in the yellow fever mosquito, *Aedes aegypti*, which utilized regulatory elements of a maternal gene Nanos to control events in mosquito embryos [39]. A synthetic gene drive system developed in the human malaria mosquito, *Anopheles gambiae*, exploited I-SceI which is a selfish genetic element known as a homing endonuclease gene (HEG) to drive rapid invasion of mosquito population genomes by the engineered gene of interest [40]. The recently developed clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas), better known as the CRISPR-Cas9 system that enables flexible genome editing in both prokaryotic and eukaryotic cells [41–43] using a guide-RNA to direct the nuclease to its

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target has also been exploited to develop a gene drive system with high transmission rate to progeny of up to 99.6% in *An. gambiae* [44].

Strains exhibiting dominant lethality or major incapacitation in the form of a flightless phenotype have also been generated. A transgenic strain based on the expression of dominant lethality in *Ae. aegypti* was constructed using similar components (a single expression component that relies on auto feedback-driven overexpression of a version of tTA) as was used for the Mediterranean fruit fly *C. capitata* [29, 45]. Fu et al. also generated a flightless strain of *Ae. aegypti* for dengue fever control by using its Actin4 (Act4) gene promoter in the single component tet-off expression system (**Figure 3**) to regulate the expression of tTA [46]. Act4 is female-specifically expressed in the indirect flight muscles [47], and as such, the tTA-mediated lethality regulated by its promoter is obtained predominantly in the female indirect flight muscles rendering them flightless and providing a way to genetically separate the sexes or enable possible male-only mosquito release in an SIT program [46].

For transgenic sexing and sperm monitoring, Catteruccia et al. established a strain that exhibited fluorescent sperms in the Asian malaria mosquito *Anopheles stephensi*, employing the promoter/enhancer elements of the β2 tubulin gene to control and ensure expression of enhanced green fluorescent protein (EGFP) in male testis [48]. This has been followed by further sperm manipulation whereby to study possible roles of sperms in regulation of postmating female responses in the major malaria mosquito *An. gambiae*, a spermless strain was engineered by RNAi-mediated silencing of a developmental gene required for early germ cell differentiation, zero population growth (zpg) [49]. Mosquito control programs may benefit from the interesting report that female mosquitoes mated to the spermless males become refractory to further mating [49]. Moreover, such a spermless mosquito strain also possesses reproductive sterility and could also find application in SIT programs for mosquito population control. A very recent sex distorting system developed in An. gambiae employs the CRISPR/Cas9 endonuclease to shred the X chromosome and lead to male bias in progeny without significantly reducing the adult's fertility [50].

Another strategy that has also been pursued is to transgenically impair the ability of mosquitoes to transmit malaria Plasmodium parasites. To this end, transgenic Anophelines were developed that were unable to vector Plasmodium parasites as they express an antiparasitic peptide, the salivary gland and midgut peptide 1 (SM1) in their midgut epithelia under regulation by a carboxypeptidase promoter [51]. In the wake of insecticide and drug resistance by both vector and parasite, respectively, this approach offered an avenue to curtail transmission while not removing the vector and could easily be spread to wild mosquito population using some of the gene drive systems developed. Several other researchers have followed this strategy and developed transgenic mosquitoes that cannot transmit their parasites. Bee venom phospholipases, synthetic antimalaria proteins like vida3, single chain antibodies (scFv) targeting malaria parasites, as well as an antimicrobial peptide cecropin A have been used as effectors and mosquitoes engineered to express them lack the ability to effectively transmit parasites [52–56]. RNAi-based resistance to dengue virus has also been engineered in *Ae. aegypti* mosquitoes by using inverted-repeat RNA (IR-RNA) from the premembrane protein coding region of the DENV-2 RNA genome whose expression

was regulated by a carboxypeptidase promoter to suppressed viral replication in the midgut [57].

2.2.2. Blow flies

Veterinary pests such as Blow flies that inflict enormous damage to sheep and other livestock have also received attention lately. Transgenic sexing strains that allow male-only production for control of the Australian sheep blow fly Lucilia cuprina were developed using both the single and binary component tetracycline-repressible expression system. An initial single component female-specific lethality system showing lethality in pupa used a heat shock promoter Hsp70 and the transformer (tra) intron from *Cochliomyia hominivorax* to limit lethality to only females [58, 59]. A later strain which used the binary component of the tetracycline-repressible expression system and showed lethality in embryos utilized promoters of cellularization genes to drive expression of tTA and the transformer (tra) intron from *C. hominivorax* placed inside a hidAla2 effector gene to confer the lethality to only female individuals [60].

2.2.3. Screwworms

Though the very successful strategy of SIT had originally been developed against the New World Screwworm *C. hominivorax* [11], this has not translated into success in development of transgenic strains of this insect. Transformation of *C. hominivorax* is much more challenging than other dipterans and efforts have resulted in few transgenic strains that allow genetic marking with fluorescent proteins for management and control of screwworms [61, 62]. It is expected that similar transgenic sterile male strains, sexing strains, sperm marked, and organismal lethal strains will be developed for screwworms in the near future as had already been done for other dipterans [62].

3. Paratransgenic biotechnology

Similar to transgenesis, paratransgenesis also involves the genetic transformation of organisms. However, paratransgenesis targets to achieve the genetic transformation or transgenesis of the symbionts that live inside an insect instead of the insect itself and cause the symbionts to express or secrete substances that act against parasites and pathogens that are transmitted by the insect (**Figure 4**). Consequently, paratransgenesis is suitably applied against disease vectors. Originally developed against the triatomine bug vector of Chagas disease, Rhodinus prolixus using its symbiont Rhodococcus rhodnii and the antimicrobial peptide cecropin A as an effector [63], this strategy has been adopted for many dipterans that vector diseases of humans and livestock.

3.1. Paratransgenic strategies against medical and veterinary important dipterans

3.1.1. Mosquitoes

Since mosquitoes transmit several disease-causing pathogens, many paratransgenic studies have been conducted on it. Inhibition of vectorial competence in mosquitoes via bacterial

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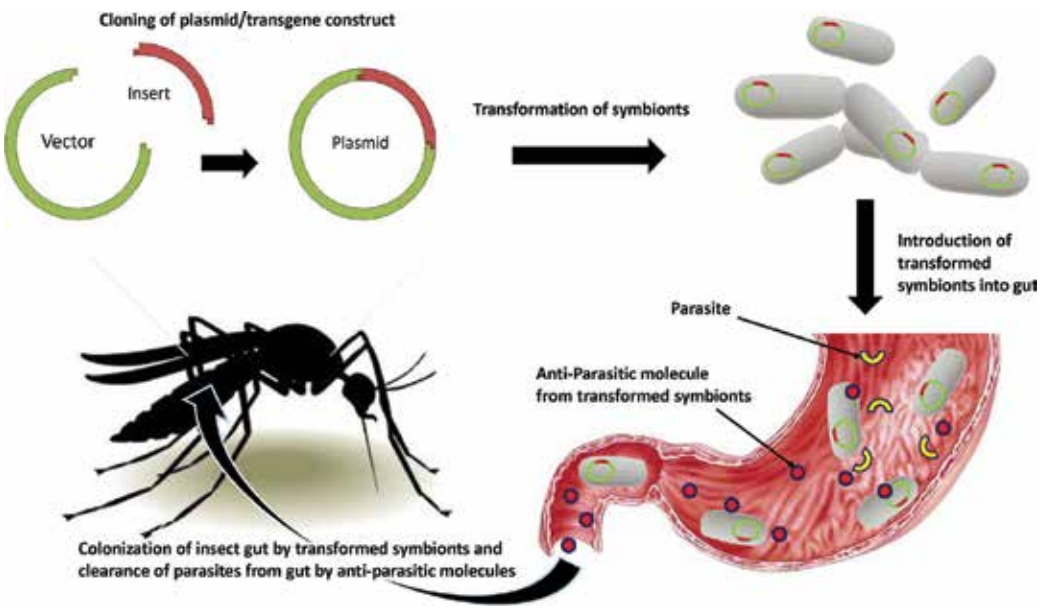

Figure 4. Schematic representation of paratransgenesis: transgenes encoding anti-parasitic molecules are cloned into a plasmid which is used to transform suitable symbionts of a vector insect. When the transformed symbiont is introduced into the gut of the vector, they colonize the gut whereas the anti-parasitic molecules they produce act against the parasites and clear them off.

symbiont paratransgenesis were demonstrated in the malaria mosquito *An. stephensi* using genetically modified strains of the popular gram negative bacteria Escherichia coli to express scFvs that block development of the parasite *Plasmodium berghei* [64] or an anti-Plasmodium molecule such as SM1 [65]. Other bacteria such as Asaia spp and Pantoea agglomerans (formerly Enterobacter agglomerans) have also been used. Favia et al. showed that the Asaia associates stably with *An. stephensi* and that transgenic strain of this bacteria expressing GFP are able to colonize the gut and salivary gland of females of this mosquito [66]. In another study using Asaia spp, paternal transmission of recombinant strains expressing the green fluorescent protein GFP or the red fluorescent protein DsRed to progeny through mating of paratransgenic males with wild females was obtained in *An. stephensi* showing that it is possible to utilize nonbiting male mosquitoes in malaria transmission [67]. Working with *Pantoea agglomerans*, Wang et al. were able to express several anti-Plasmodium molecules such as SM1 peptide, scFv, mutated phospholipase (mPLA2), Plasmodium enolase-plasminogen interaction peptide (EPIP), synthetic antiparasitic lytic peptide Shiva1, etc., in both *An. gambiae* and *An. stephensi* and successfully suppressed transmission of *Plasmodium falciparum* and *P. berghei*, respectively [68].

Besides bacteria, fungi and viruses have also been utilized in mosquito paratransgenesis. The entomopathogenic fungi Metarhizium anisopliae was engineered by Fang et al. to express the anti-Plasmodium molecules SM1, a sporozoite-agglutinating scFv, as well as an antimicrobial toxin scorpine in An. gambiae [69]. Using the densonucleosis virus (DNV) in a proof-of-concept viral paratransgenesis work in *An. gambiae*, the potential of virions in paratransgenesis

was demonstrated by ability of transgenic DNV expressing GFP to infect larvae, persist to the adult stage and disperse to vital tissues such as fat bodies, ovaries and midgut, and be transmitted subsequently to other generations [70]. This shows that viruses such as DNV could be used to express antiparasitic molecules not only in one but several mosquito developmental stages and subsequent generations and could effectively mitigate and eliminate malaria transmission. Another virus, the Sindbis virus, has also been exploited in paratransgenesis and used to express scFv that acts against *Plasmodium gallinaceum* sporozoites in *Ae. aegypti* [71]. As it appears, the Sindbis virus has great potentials for control of various viruses transmitted by Aedes mosquitoes [72–74].

3.1.2. Sandflies

Efforts on control of sandfly vectors that transmit Leishmania parasites which cause the disease Leishmaniasis has been done mainly with chemical insecticides. To develop a more environment-friendly strategy, Hurwitz et al. recently demonstrated the feasibility of paratransgenesis for sandflies in a proof-of-principle work in *Phlebotomus argentipes* in which they used recombinant *Bacillus subtilis* fed to larvae to express GFP in the gut lumen of emerging adults (**Figure 5**) [75]. This proof-of-principle study has paved the way for future development of strains that will express anti-Leishmania molecules and block transmission of the parasites by the sandfly vector.

3.1.3. Tsetse flies

Although several control strategies including SIT have been applied against tsetse flies, continual effort is made to develop other methods that would not have limitations of the existing methods, be more sustainable, more cost-effective or suitable for use in IPM control. To investigate the possibility of paratransgenesis in tsetse flies, transgenic *Sodalis glossinidius* were introduced into adult females where they were able to express GFP and interestingly passed on subsequently to the progeny of those females [76, 77]. Actual utilization of antitrypanosomal molecules to block parasite development and transmission by tsetse flies could be achieved in the near future.

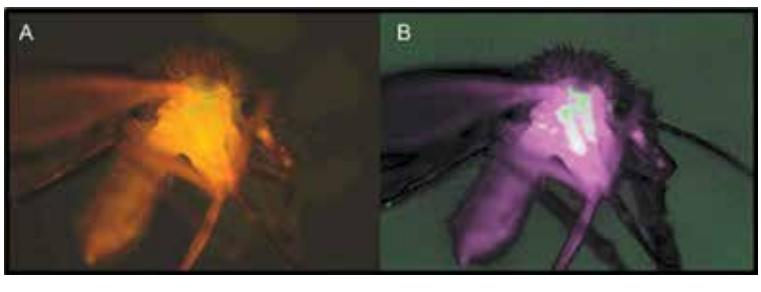

Figure 5. Paratransgenic sandfly Phlebotomus argentipes: (A) auto fluorescence of the outer carapace of the sand fly is seen amidst the presence of GFP expressed by the symbiont, (B) subtilis in the sand fly's midgut B. visualization of GFP specifically localized in the sand fly's midgut chamber upon uncoupling of the GFP signal from the background [75].

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4. Advantages, disadvantages and limitations of transgenic and paratransgenic technologies

4.1. Transgenic technology

4.1.1. Advantages of transgenic technology

The main advantage of transgenic biotechnology is its ability to generate strains that possess traits that are unique and special, and accurately designed or tailored to be specific as desired. Also, the flexibility of transgenic technology allows generation of such desired strains in many species which would have been very difficult or impossible to achieve by other means. Transgenic strains are usually generated after one generation (**Figure 1**), and hence take less time to generate compared to other methods like classical genetics. Moreover, generation of strains possessing desired traits in one species can almost always be reproduced and transferred to related species with relative ease [78]. Quite unlike earlier genetic methods such as SIT where the use of radiation generates unknown and uncharacterized genetic mutations, transgenic technologies generate known and characterizable genetic modifications. Transgenes could easily be thoroughly characterized, and same goes for genomic positions in the dipteran insect where desired transgenes had got inserted. Also, most transgenic strategies are environment-friendly, sustainable and target-specific. For example, while chemicals developed against fruit flies may kill pollinator insects, transgenic strains developed for control of fruit flies are not likely to have any negative effect on pollinators that their wild counterparts do not already exhibit. Also, the development of resistance against control agents is less likely to occur when using transgenics.

In terms of costs, transgenic technologies as well as many other control strategies are not so cheap to develop. But it is difficult to say with all certainty whether transgenic (and paratransgenic technology) is cheaper than many other methods as there have not been any such economic studies to the best of our knowledge. Nevertheless, transgenic (and paratransgenic) approaches are considered less expensive with regards to the farmer or public beneficiaries as they are usually area-wide-oriented and implemented by big organizations at overall little or no cost to the individual farmers or the public.

4.1.2. Disadvantages of transgenic technology

Probably due to the fact that transgenic technology is just beginning to move from laboratory to the field [79, 80], there are yet no scientifically proven disadvantages. Despite this, many public negative concerns already exist on the use of transgenics [78], mostly environmental and social, as well as safety and ethical issues. These are mainly due to speculations and the uncertainty as to what might happen in nature following field use of transgenics, and whether unintentional and unforeseen mutations could lead to harmful consequences (though these can potentially occur also in nontransgenics). There are also thoughts on how field use of transgenics could interfere with diversity and evolution due to possible loss of genetic material of original insects and the associated future downstream events. Potential horizontal transfer of transgenes could also be a potential disadvantage that could be associated with the use of transgenic technology. However, a ”self-limiting” transgenic approach such as use of

transgenic lethality dipteran strains should not present some of the afore-mentioned environmental problems since they are most likely to remove themselves from the environment with time unlike "self-sustaining" strains [81]. Though not always the case, there may be fitness costs that might arise in dipterans and other insects due to the various loads of transgenes they carry [82, 83]. When well assessed, the fitness costs could be determined and measures taken to eliminate them if necessary or avoid using strains that suffer such lack of fitness. New transgenic strains that may not have the observed fitness cost could also be developed and utilized instead. Proper assessment should be done to determine the associated risks and benefits before any GMO can be utilized [84].

4.1.3. Limitations of transgenic technology

Transposable elements or "jumping genes" have been the main tool relied on to achieve germline transformation and generate transgenic dipterans. However, most of the transposons used in dipterans (and other insects too) are insect-derived [85] and a major concern is that a transposon could potentially be remobilized from its integrated genomic position in the insect if transposases required for its activity is encountered in the field. The consequence of such transgene-transposase exposure could be the remobilization of a transgene to another genomic location or total loss of a transgene from an insect's genome. Measures to avoid potential transgene remobilization in engineered dipterans such as postintegrational transgene modification to alter the transposon and achieve nonmobilization or stability has been demonstrated in *D. melanogaster* and *C. capitata* [86–88]. Other strategies that offer transgene stability are becoming available. The recently developed genome editing tool, CRISPR/Cas9, which allows RNA-guided modification of target DNA locations [41] has been utilized to achieve stable germline transformation in *D. melanogaster* [43]. Unlike transposon-mediated germline transformation, CRISR/Cas9-mediated germline transformation is seamless and should not be prone to subsequent remobilization.

Transgenesis is not yet possible in all dipterans as not all members are amenable to it. Since the development of a transgenic insect strain involves germline transformation (**Figure 1**), it is therefore important that the biology of a target insect must be in such a way that allows the necessary manipulations to achieve genetic transformation. Tsetse flies are yet to be genetically transformed due to their viviparity which makes it difficult to obtain embryos needed for microinjections and subsequent germline transformation [77].

4.2. Paratransgenic technology

4.2.1. Advantages of paratransgenic technology

While similar to transgenic approach in terms of its ability to generate within a short time strains that possess unique and special traits designed specifically as desired, paratransgenesis also has an additional advantage of leaving the insect itself genetically unmodified and rather targets the parasites transmitted. This gives paratransgenic approaches a major plus in the sense that it has a more positive public perception than transgenic approaches as many of the disadvantages with use of transgenics would not be present [78, 89]. In addition, this technology has a high potential to be transferred between different species [78]. Moreover,

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paratransgenic biotechnology mostly employs microorganisms that live within the target dipterans (symbionts) and as a result also has a high likelihood of field success. Another advantage of paratransgenics is the absence of fitness cost of genetic manipulation compared to transgenics or other control strategies [90].

4.2.2. Disadvantages of paratransgenic technology

Field application of paratransgenic strategies is yet to be actualized and any potential disadvantage of this technology is still to be proven scientifically. Nevertheless, safety concerns and risk assessments have become necessary requirements that need to be addressed to ensure that the benefits outweigh the risks of utilized genetically modified organisms [84]. One concern for paratransgenics is the potential exposure of engineered symbionts to the environment and likely consequences such as horizontal gene transfer. Measures such as symbiont encapsulation to ensure regulated release are being taken to address some of these regulatory concerns [91].

4.2.3. Limitations of paratransgenic technology

Despite the known advantages of the paratransgenic approach, a major limitation is that it is not suitable for most dipteran crop pests and has been developed mostly for those dipterans (and other insects) that transmit disease pathogens. Symbiont choice and utilization in a paratransgenic expression approach depend not only on availability of symbionts that can be isolated, cultured, reintroduced, and survive well in the targeted host, but also on the ability of the symbiont to be genetically transformed and to possibly express antiparasitic molecules [77]. The lack of some of these requirements would render several good symbionts unusable for paratransgenic control. The bacteria symbiont Wolbachia is one such microorganism that is promising for paratransgenic application, but the lack of success in genetically transforming it has hindered its further utilization for expression of antiparasitic molecules.

5. Future of transgenic and paratransgenic technologies

In the near future, transgenic and paratransgenic pest/vector control strategies may become common place and more widely applied than it is now. Some of the novel approaches of these technologies are promising and offer great hopes for control of several human diseases and could be implemented in the near future if regulatory and ethical issues are satisfied [92, 93]. This could usher in a new era where cases major dipteran-vectored diseases of man such as malaria and dengue, as well as agricultural pest like Tephritid fruit flies become much reduced or even eradicated.

The arrays of weapons in man's arsenal against his dipteran enemies are also expected to continue to expand. Continuous improvement will be made to existing control strategies, while new and better strategies are expected to be developed in the future as more advances are made in genetics and molecular biology. The RNA-guided genome editing tool, the CRISPR/Cas9 endonuclease recently developed from bacteria such as *Streptococcus pyogenes*

and *Neisseria meningitidis* [41, 94] has equally enabled genome modification and generation of transgenic control strategies in dipterans [43, 44, 50, 95]. More recently, a DNA-guided genome editing which makes use of an argonaute from the bacteria *Natronobacterium gregoryi* [96] has also been developed and it is expected that this new tool, as well as others that may soon be developed, will definitely lead to the generation of new transgenic or paratransgenic approaches to better control pest, nuisance or vector dipterans.

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This book provides recent contributions of current strategies to control insect pests written by experts in their respective fields. Topics include semiochemicals based insect management techniques, assessment of lethal dose/concentrations, strategies for efficient biological control practices, bioinsecticidal formulations and mechanisms of action involving RNAi technology, light-trap collection of insects, the use of sex pheromonal components and attractants for pest insect capture, measures to increase plant resistance in forest plantations, the use of various baculoviruses as biopesticides, and effect of a pathogenic bacterium against an endangered butterfly species. There are several other chapters that focus on insect vectors, including biting midges as livestock vectors in Tunisia, mosquitoes as vectors in Brazil, human disease vectors in Tanzania, pathogenic livestock and human vectors in Africa, insect vectors of Chagas disease, and transgenic and paratransgenic biotechnologies against dipteran pests and vectors. This book targets general biologists, entomologists, ecologists, zoologists, virologists, and epidemiologists, including both teachers and students.

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