2009

BIOLOGY AND MANAGEMENT OF THE BED BUG, Cimex lectularius L. (HETEROPTERA: CIMICIDAE)

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ABSTRACT OF DISSERTATION

Alvaro Romero

The Graduate School
University of Kentucky
2009
BIOLOGY AND MANAGEMENT OF THE BED BUG, *Cimex lectularius* L.  
(HETEROPTERA: CIMICIDAE)

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Alvaro Romero

Lexington, Kentucky

Director: Dr. Kenneth F. Haynes, Professor of Entomology

Lexington, Kentucky

2009

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ABSTRACT OF DISSERTATION

BIOLOGY AND MANAGEMENT OF THE BED BUG, Cimex lectularius L. (HETEROPTERA: CIMICIDAE)

The near absence of bed bugs from human dwellings for fifty or more years has left us with limited knowledge of its biology and few answers to eliminate populations. I explored a diverse set of objectives to answer key questions concerning bed bug biology and control. Major objectives were studies of circadian rhythmicity, pyrethroid resistance, sublethal effects of insecticides, synergism of pyrethroids, and evaluation of a pyrrole insecticides, chlorfenapyr. Additional studies included persistence of Borrelia in bed bugs after ingestion, and aggregation factors from feces.

In the absence of host stimuli, insects were much more active in the dark than in the light. Nocturnal activity was periodical under continuous light conditions, which indicates that locomotion is endogenously generated by a circadian clock. Circadian rhythm was entrained to reverse dark-light regimes. Short–term starved adults moved more frequently than long-starved adults. These results suggest that starved bugs reduce locomotor activity as a strategy to conserve metabolic reserves.

Pyrethroid resistance in C. lectularius was documented for the first time. Extremely high levels of resistance to deltamethrin and λ-cyhalothrin, was detected in populations collected in Kentucky and Ohio. The resistance ratios reported are among the highest documented in any arthropod. Evaluations of more than 20 populations from across the United States indicate that resistance to pyrethroid insecticides is widespread.

Bed bugs avoided resting on surfaces treated with deltamethrin but not with chlorfenapyr. Video recordings of bed bugs showed that insects increased their activity when they contacted sublethal doses of deltamethrin. However, harborages treated with a deltamethrin remained attractive. A nearby heat source overcame avoidance to deltamethrin.

The P450 inhibitor piperonyl butoxide (PBO) enhanced toxicity of deltamethrin to resistant bed bugs. However, the residual resistance after PBO treatment indicated that other resistance mechanisms are involved. The effectiveness of combining PBO with
pyrethroids varied among populations, which indicates that this synergist is not a comprehensive solution to pyrethroid resistance. Chlorfenapyr was effective against pyrethroid resistant strains. While it does not cause quick knockdown, long residual activity and no avoidance behavior to dry residues appears to make this insecticide a useful tool for bed bug control.

KEYWORDS: Locomotor activity, Insecticides, Resistance, Sublethal Effect, Pest Management

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Alvaro Romero

December 15 2009
BIOLOGY AND MANAGEMENT OF THE BED BUG, *Cimex lectularius* L.  
(HETEROPTERA: CIMICIDAE)

By

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DISSENTATION

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Lexington, Kentucky

Director: Dr. Kenneth F. Haynes, Professor of Entomology

Lexington, Kentucky

2009

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To my parents Cecilia Nasayo and Alvaro Romero R.
for their support, encouragement, and constant love throughout my life.
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It was a privilege to have pursued my PhD in the Department of Entomology of the University of Kentucky, working on a topic for which I feel passionately. Because of the international momentum in bed bug research in recent years, I feel really lucky to have had the chance to conduct research on this pest with an excellent research group.

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Chapter One: Introduction

Brief history and resurgence of bed bugs

Bed bugs (*Cimex lectularius* L.: Heteroptera: Cimicidae) have been associated with humans since ancient times (Panagiotakopulu and Buckland 1999). Usinger (1966) suggested that the ancestors of *C. lectularius* fed on cave–dwelling bats. According to this hypothesis, our ancestors became an alternative host when they shared caves with bats. Subsequently, bed bugs evolved parallel to the social evolution of the human species (Usinger 1966). Historic records also indicate that bed bugs spread throughout the world with the increase of urbanization and improvement of housing conditions (Panagiotakopulu and Buckland 1999, Potter 2008a). Bed bugs were reported in England in 1593 and it is believed they were brought to America by European colonizers (Potter 2008a). An escalation of bed bug infestations occurred because of the increasing use of central heating in some cities in Europe and the United States during the beginning of the 20th century (Usinger 1966). Infestations of bed bugs in society, particularly in poorer and overcrowded places, were a common issue during the 1930s and 40s and numerous control methods were used to eliminate infestations with little success.

A turning point in the history of bed bug control occurred after the advent of synthetic insecticides such as DDT in the 1940’s, and subsequently organophosphates and carbamates. Since then bed bugs nearly disappeared, particularly in developed countries, with only sporadic infestations occurring in dwellings with high occupant turnover and questionable sanitation conditions (Krueger 2000). Over the last ten years, a resurgence of bed bugs has been reported in North America, Australia and Europe (Krueger 2000, Boase 2001, Doggett et al. 2004, Hwang et al. 2005). In the United
States, the first indication of bed bug resurgence was provided by Krueger (2000) who reported an increase of incidence of infestations in large cities between 1998 and 2000. Since then, bed bug reports have increased dramatically across the United States with an intense media coverage reflecting the public reaction to this pest (Anderson and Leffler 2008). Bed bug infestations have been reported in all 50 U.S. states, occurring in a variety of places including single-family dwellings, apartments, hotels, health care facilities, laundries, theaters, college dormitories, and means of transportation (Hwang et al. 2005, Potter 2008a). While bed bug infestations occur across all social and economic classes, the most severe infestations occur among the socially disadvantaged, including low-income minority communities (Pinto et al. 2007). Bed bug control is an increasing part of services offered by many pest control companies in the United States (Gangloff-Kaufmann et al. 2006). An online survey among the pest management industry indicated that 91% of responders had serviced bed bug jobs during a two-year period (2006-2007) (Potter 2008b).

Many explanations have been proposed for the resurgence of bed bugs. Social and human issues such as decline of public awareness of the pest (Reinhardt et al. 2008), increased domestic and international travel, and increased turnover of second hand items are believed to have contributed to the resurgence of this pest (Potter 2005, Pinto et al. 2007, Reinhardt and Siva-Jothy 2007). Other reasons explaining the resurgence are related to insecticides, including changes in the pattern of use, withdrawal of effective insecticides such as DDT, and development of resistance of bed bugs to insecticides (Doggett 2005, Potter 2005, Pinto et al. 2007, Romero et al. 2007a, b). Research to determine the origin of the resurgence is just beginning. For example, researchers are
using diverse molecular tools to gain insights about the source of current bed bug populations in the United States and to determine biogeographical patterns of spread (Szalanski et al. 2008). Analysis of mitochondrial DNA (mDNA) of more than a hundred samples, including bed bugs collected in poultry facilities, suggested multiple introductions of bed bugs into the United States. This population variability may also indicate the presence of isolated bed bug populations parasitizing non-human hosts. Because some mDNA haplotypes of bed bugs collected in poultry facilities were common in human structures, the authors suggested that these facilities may be playing a role in bed bug dispersal.

**Biology**

The human bed bug is a flightless blood-sucking insect that usually feeds at night on warm-blooded hosts including humans, chickens, bats, and occasionally, domesticated animals (Usinger 1966). Adult bed bugs are 3-5 mm long, oval insects that are flattened and brown when unfed, but become swollen and elongated after feeding, turning reddish-brown (Fig. 1.1, C and D). Adult females and males ingest nearly 7 and 4 µl of blood at one feeding (respectively), increasing their body weight > 250% (A. R. unpublished data). Frequency of feeding depends on temperature, host availability and life stage. When bed bugs are reared in a laboratory setting (27°C at 50-60 % RH), adults will take a blood meal from a membrane-based system as soon as 3 d after the last meal. Nymphs feed only after molting, their feeding interval is longer than adults, and also varies according to temperature. When feeding, the bugs fully engorge in 10-15 min and may probe the skin several times before actual feeding begins. Itchy welts appearing where bed bugs have bitten the host are the result of immune system reactions to proteins of
insect saliva. These welts usually appear in groups. Once feeding is terminated, bed bugs return to their refuges.

Adult females can be differentiated from males because the tip of their abdomen is rounded while in males it is pointed (Fig. 1.1, C and D). This characteristic is less evident in recently fed adults. Eggs are white and about 1 mm long. There are five nymphal instars, and each stage requires a blood meal before molting proceeds. The first instar is as small as a pinhead (about 1 mm) (Fig. 1.1, A), while fifth-instar nymphs are slightly smaller than an adult (Fig. 1.1 B). When nymphal stages are unfed they are a yellowish transparent color, turning dark red after ingesting a blood meal. Increase in weight of nymphs after a blood meal is about 4-7 times their unfed weight, depending on the instar. The small size and flatness of bed bugs allows them to fit in crevices or cracks present in their environment and it is in these harborages where they mate, lay eggs, and aggregate. Vertebrate blood is the only source of nutrition for all stages of bed bugs.

Feeding triggers mating behavior and rate of copulation is high within 36 h post-feeding. A male copulates with a female by introducing an intromittent organ into a specialized external groove of the female’s abdominal wall. Because copulation involves puncture of the cuticle, it is known as traumatic insemination (Usinger 1966, Stutt and Siva-Jothy 2001). Adult females require a blood meal to initiate oviposition. Under laboratory conditions at 27°C, oviposition begins four days post-feeding and lasts seven days with a total production of about 9-10 eggs (A. R. unpublished data). Females require a new blood meal to produce subsequent batches of eggs. An adult female can produce up to 541 eggs in her lifetime (Usinger 1966), but egg production might be reduced under laboratory conditions (A. R. unpublished data). Eggs hatch within six days at 27°C and
50-60 RH (A. R. unpublished data). Adults can survive up to a year without feeding and longevity depends on feeding status as well as temperature and humidity conditions (Usinger 1966). Laboratory-reared bed bugs, deprived of blood may live between three and six months (A. R. unpublished data). The total period of development from egg to adult at 27°C and 50-60 % RH takes about 45 days (A. R., unpublished data). In the early phases of this dissertation work I optimized a membrane feeding technique (Montes et al. 2002) that permits a large-scale production of bed bugs (Fig. 1.2). The use of this feeding technique has major advantages in terms of convenience and productivity over direct feeding on animals, and does not require human subjects.

**Locomotor activity**

Insects move from one place to another in order to enhance their likelihood of encountering resources such as food, mates, nesting sites, and refugia (Bell 1990, Barton Browne 1993). Some insects use predictable daily changes (e.g., solar cycle, daily cycles of temperature and humidity) to coordinate their activities at a time of day when conditions are most favorable, e.g., less risk of predation, reduced competition, and protection from adverse abiotic environmental conditions (Sharma 2003). While some periodic behaviors are affected directly by these daily changes in environmental conditions, others are endogenously generated by biological oscillators (Saunders 2002, Barrozo et al. 2004). Like in other blood sucking insects (e.g., kissing bugs, some species of mosquitoes) bed bugs only become active in the dark when host activity is minimal, thus reducing the chance to be detected by their hosts (Mellanby 1939, 1940, Usinger 1966, Lorenzo and Lazzari 1998) (Fig. 1.3). Much of the locomotor activity of bed bugs concerns searching for a host, acquiring a blood meal, and returning to harborages, where
they remain while digestion takes place (Fig. 1.3). An earlier study by Mellanby (1939) noticed an increase in nocturnal activity in bed bugs that were infesting a mouse rearing facility. While Mellanby (1939) reported that bed bug activity was periodical, his experiments did not clearly determine if endogenous or exogenous factors were affecting this activity.

The factors that motivate bed bugs to leave harborages are not fully understood. Physiological conditions such as hunger or mating status are believed to be the driving forces initiating locomotor activity in bed bugs. Nearly 80% of bed bugs caught in a passive trap were unfed individuals (Mellanby 1939). A hungry bed bug can walk as far as 20 m to find a host (Kemper 1936). Field observations indicate that females are very often found away from aggregations (Pinto et al. 2007), which may represent a protective mechanism of females to costly excessive inseminations (Stutt and Siva-Jothy 2001, Pfiester et al. 2009). Field reports also indicate that bed bugs can move from one location to another through walls and ceiling voids (Pinto et al. 2007). Movement of insects can also be caused by the neurotoxic effect of some insecticides (Ebeling et al. 1966, Diotaiuti et al. 2000, Chareonviriyaphap et al. 2004).

Studies on periodicity and ecology of movement in bed bugs are extremely limited. There are no studies that reveal characteristics of the periodicity of locomotor activity in bed bugs and whether this periodicity is influenced by endogenous factors or an exogenous light cycle. A better understanding of the locomotor activity of bed bugs and of its dependence or not on an exogenous light cycle could reveal insights into the evolution of the association between blood-feeding insects and human hosts. Furthermore, studies of the influence of age, sex and feeding status on locomotor activity
could contribute important ecological information concerning their movement which will aid in the management of this pest.

**Aggregation behavior**

During daytime, bed bugs are generally found in crevices and cracks, aggregated in clusters, and in contact with their accumulated fecal matter, eggs, egg shells, and exuviae (Fig. 1.3). Bed bugs tend to reside in areas with low light intensity and between surfaces that provide them thigmotactic stimulus (Usinger 1966). In several orders of non-social arthropods, aggregations can be established by emission of auditory, visual, or chemical stimuli (Wertheim et al. 2005). Intraspecific chemical signals (pheromones) for aggregation are used by other urban insects including cockroaches and kissing bugs. Insects can obtain several benefits by aggregating, including 1) overcoming host resistance (e.g. bark beetles), 2) mating facilitation; 3) protection from predators; and 4) reduction of the impact of harsh environmental conditions (Wertheim et al. 2005).

Functional explanations concerning the benefits of aggregation behavior for bed bugs have not been fully provided. By aggregating, bed bugs might increase moisture in micro-habitats, thus reducing the impact of desiccation stress, particularly on susceptible stages such as early-instar nymphs (Benoit et al. 2007). Aggregations may facilitate multiple mating which could enhance genetic diversity and lead to fitness gains which may counterbalance the costs associated with repeated copulatory wounding (Stutt and Siva-Jothy 2001).

Earlier studies suggested that bed bugs are driven by scent gland odors and feces to return to aggregations, but no experimental evidence was provided (Marx 1955). Several chemical compounds have been identified from scent gland secretions but none
of these were associated with aggregation behavior in bed bugs (Schildknecht 1964, Collins 1968, Levinson et al. 1974). Levinson and Bar Ilan (1971) showed that *C.lectularius* aggregated under paper discs previously exposed to adult conspecifics. Similar results with the tropical bed bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) have been reported (Parashar et al. 2003). Although behavioral tests showed that water and methanol were efficient in extracting active substances, no further studies were carried out to isolate or identify the aggregating factors (Parashar et al. 2003). Recent studies by Siljander et al. (2007, 2008) showed that both contact and airborne aggregation pheromones mediated aggregation in bed bugs. The airborne aggregation pheromone was isolated from harborages and consisted of a blend of 10 compounds including nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (2E,4E)-octadienal, benzaldehyde, (+)- and (−)-limonene, sulcatone, and benzyl alcohol. The most abundant components of the aggregation pheromone, (E)-2-hexenal and (E)-2-octenal, have been previously reported as alarm pheromone (Levinson et al. 1974). However, these two compounds in the blend elicited aggregation at doses lower than alarm pheromone, which indicates that these pheromone components may be multifunctional and exert their behavioral effects in a dose-dependent manner. So far, no scientific information is available on whether or not this pheromone attracts or retains bed bugs in natural infestations.

Siljander et al. (2007) also obtained evidence for a pheromone detected by contact chemoreception and established that juvenile bed bugs preferred only to aggregate under juvenile-exposed papers. Neither juveniles, adult males, nor adult females showed a preference to aggregate under female-exposed papers, but females and males preferred to
aggregate under male-exposed papers. The sensory basis for the detection of contact aggregation pheromone was studied by Olson et al. (2009) with bioassays similar to those applied by Levinson et al. (1974). In Olson’s study, sensory organs of bed bugs, such as antenna, eyes, and proboscis, were ablated or covered and these bugs were then exposed to contact pheromones in behavioral bioassays to identify the possible location of pheromone chemoreceptors. Because aggregation was observed only in individuals whose antenna pedicels were not removed, they suggested that the sensory structures that mediate aggregation are located on this antennal segment and not in the distal antennal segment as Levinson et al. (1974) had previously reported.

Although the above studies show evidence for the presence of a contact pheromone that mediates aggregation in bed bugs, the components of this pheromone are unknown. Isolation and chemical identification of contact pheromones could add, not only important information about the ecology of aggregation in bed bugs, but also could be the basis for development of detection systems. Currently, the methods for detection of bed bugs are based on visual inspection, which is difficult, time-consuming and often impossible (Changlu et al. 2009); sticky traps, which are ineffective when infestations are small (Pinto et al. 2007); canine scent detection (Pfiester et al. 2008), which can be very expensive and inaccurate (accuracy of this method can be affected by trainer experience, dog breed, and environmental factors) (Pinto et al. 2007); and new traps that use heat and carbon dioxide which are unproven for early detection (Anderson et al. 2008, Wang et al. 2009). Thus, a pheromone-based monitoring system able to detect bed bug infestations at early stages or confirm their presence post-treatment (which are two criteria essential to effective bed bug management) is particularly important.
**Host seeking behavior**

At night, when host activity is minimal, bed bugs leave their harborages in search of a blood meal (Fig. 1.3). The manner in which bed bugs find a host is a controversial subject. Rivnay (1932) hypothesized that bed bugs search for hosts randomly and only in close proximity to hosts do they detect and orient toward heat produced by the host. Marx (1955) suggested that, besides heat, host odors, including CO₂, may play a role in attracting bed bugs to their hosts. Human sweat and body secretions were found to attract bed bugs (Rivnay 1932, Aboul-Nasr and Erakey 1968). Nevertheless, Kemper (1936) evaluated the responses to 37 chemicals and none of them attracted bed bugs. All of the above results suggest that bed bugs make use of multiple cues to find a host, some yet to be elucidated.

**Impact on human health**

Bed bug bites produce irritating and itchy skin reactions that can be severe in some cases, but emotional stress is often the most severe consequence of infestations. The Environmental Protection Agency (EPA) considers bed bugs to be a “pest of significant public health importance” (Environmental Protection Agency 2002). The current resurgence of bed bugs has been called a “perfect storm” (Potter 2006a). Some people feel discomfort, suffer from anxiety and have much difficulty sleeping (Hwang et al. 2005). The fear of bed bugs can lead a person to exhibit symptoms of “delusory parasitosis”, an emotional disorder in which people believe they are bitten by insects or that they are being infested by them (Potter 2006b).

Chronic blood loss and iron-deficiency anemia have been reported in people who have been continuously exposed to severe bed bug infestations (Venkatachalam and
Lesions caused by bed bug bites usually occur on exposed areas of the face, neck, and extremities and commonly appear as small clusters of erythematous papules or wheals (Ter Poorten and Prose 2005). The skin may exhibit immediate wheal and flare symptoms, followed by a papule, sometimes a vesicle or a blister, that suggest immediate- or delayed-type skin reactions. Anaphylactic reactions have also been reported (Ter Poorten and Prose 2005). These features clearly hint at a hypersensitivity reaction mediated by Immunoglobulin E (IgE) against C. lectularius salivary nitrophorin (Leverkus et al. 2006). Vigorous scratching and concomitant erosions predispose the skin to secondary bacterial infection (Millikan 1993).

The clinical presentation of bed bug bites substantially varies among individuals, depending on the degree of previous exposure (Leverkus et al. 2006, Reinhardt et al. 2009a). Some individuals will not react at all, even after multiple bites (Goddard and de Shazo 2009a). A recent study examined the temporal pattern of skin reactions to bites and showed that 54% of individuals showed no skin reactions after a first bite exposure. The time between the bite and the occurrence of skin reactions, of those who reacted, was between 7 days and 20 weeks post-exposure. However, this time was decreased with repeated exposure, some individuals reacting within seconds after being bitten (Reinhardt et al. 2009a). From a pest control perspective, absent or delayed responses represent a serious challenge because infestations are not reported in a timely manner and, thus infestations may remain undetected for several months allowing the population to increase to levels more difficult to control (Doggett and Rusell 2008). Delayed skin reactions to bed bug bites can also have implications in litigation issues. For example,
symptoms that appear during a stay in a hotel may represent a response to bed bug bites that occurred several days previously in another hotel, making a claim against the last hotel unsubstantiated (Reinhardt et al. 2009a).

**Bed bugs and disease agents**

The resurgence of a blood sucking insect, such as the bed bug, raises concerns about the role that these insects may have in transmitting disease agents. An early review made by Burton (1963) indicated that bed bugs may be a potential source of disease to humans as they are able to harbor more than 40 pathogens, including the disease agents that cause leishmaniasis, American trypanosomiasis, rickettsiosis, pasturellosis, tularemia, Q fever, plague, relapsing fever, leprosy and brucellosis. However, to date there is no substantial evidence of transmission of these organisms through bites from bed bugs to humans (Goddard and deShazo 2009b). In the last decades, most of the research in this area has been devoted to determining the possibility of bed bugs vectoring disease agents of public health importance, including the human immunodeficiency virus (HIV) and hepatitis B virus (HBV). The possibility of bed bugs acting as vectors was assessed in an *in vitro* study in which bed bugs were fed with HIV-infected blood through a membrane system (Jupp and Lyons 1987). Because bed bugs failed to transmit the virus to uninfected blood, mechanical transmission by bites was considered unlikely to occur under natural conditions (Jupp and Lyons 1987). A subsequent study conducted by Webb et al. (1989), by using an *in vitro* system, reported a low likelihood of HIV being transmitted mechanically by bed bugs. These authors did not find evidence of replication of HIV virus within the bed bug’s body. Numerous experimental studies have been conducted to determine the possibility that bed bugs
transmit HBV via feces. In general, bed bugs that were experimentally infected with HBV excreted viral particles in feces for at least two months, but there was no clear evidence of viral replication within the bed bug body or of transovarial transmission (Jupp and S. McElligott 1979, Blow et al. 2001). However, transtadial transmission among all nymphal instars did occur (Blow et al. 2001). Infective viral particles in feces for long periods of time could be a possible source of HBV infection as infected feces could contact skin lesions, especially because bed bugs often defecate on the skin of the host after feeding (Blow et al. 2001). This mode of transmission is widely recognized in two arthropod-borne disease systems: body lice (Pediculus humanus humanus), which vector epidemic louse-borne typhus (Rickettsia prowasekii); and kissing bugs (Reduviidae), which transmit Chagas disease (Trypanosoma cruzi) (Edman 2000). These pathogens gain entrance into hosts when infected insects defecate near abraded skin or through autoinoculation when the host scratches into the skin the contents of a crushed insect.

Ever since bed bug populations diminished in many parts of the world, there has been limited research on the relationship of bed bugs to disease-causing agents as well as the role of bed bugs as possible vectors of blood-borne diseases. The resurgence of bed bugs has not led to an increase in research because of the general belief that bed bugs are unlikely to transmit diseases, ethical issues related to conducting research trials with human subjects, and the lack of standardized laboratory methods to conduct such studies. Readdressing the potential of bed bugs to transmit disease agents is imperative because of 1) the resurgence of bed bugs in recent years, particularly in highly-populated urban areas, 2) the emergence of blood-borne, disease-causing microorganisms, whose
epidemiologies are not completely known (Harrus and Baneth 2005), and, 3) the ever evolving dynamics of some disease organisms and their potential to adapt to hematophagous arthropods (Lehane 2005).

**Insecticides and resistance**

Resistance is an evolutionary response of organisms to the presence of continued environmental changes, e.g., exposure to insecticides. Under strong insecticide selection, resistance is an unavoidable phenomenon because of preexisting variation of this trait (Conway and Comins 1979, Mallet 1989). Resistance develops through the selective survival of a few individuals that have inherited biochemical mechanisms that withstand the action of insecticides. If populations with these individuals are continuously exposed to insecticides, susceptible individuals die while resistant ones survive, breed and pass the resistant traits to their progeny (Staunton et al. 2008). Insect populations generally develop resistance to insecticides faster when these compounds have been used before or share a mode of action with other compounds (Georghiou 1986). A reduced bioavailability of insecticide residues over time can promote selection on resistant individuals that would have been killed by initial concentrations (Roush and Daly 1990). Resistance appeared in many arthropods in the 1940s, when synthetic pesticides came into wide use. By 1990, insecticide resistance had been found in > 400 species of arthropods (Roush and Tabashnik 1990), including blood-feeding insects (e.g., mosquitoes) where the loss of efficacy has led to pest resurgence and increases in rates of disease transmission (Krogstad 1996).

Insects have developed different means to withstand insecticides, including behavioral adaptations that result in avoidance; increased metabolic detoxification by
enzymes, such as cytochrome P450 monooxygenases (P450s) and glutathione transferases or esterases; decreased cuticular penetration \((pen)\), and decreased target site sensitivity of voltage–gated sodium channel (e.g., \(kdr\)) (Brogdon and McAllister 1998). Because DDT acts on the same target site as pyrethroids, cross resistance between these two classes of insecticides is common (Farnham 1977, Prasittisuk and Busvine 1977).

Historically, bed bugs have developed resistance to every insecticide used for their control (Busvine 1958, Mallis and Miller 1964). High levels of resistance to DDT and other insecticides in bed bug populations were noticed a few years after their continued use (Johnson and Hill 1948). This is believed to have caused resurgence of bed bugs in malaria eradication areas in Asia (Rafatjah 1971).

The near disappearance of bed bugs for several decades in many parts of the world and the increased concern about exposure of humans to insecticides has reduced chemical options for bed bug control. Today the mainstay for bed bug control is limited to the use of chemical formulations based on pyrethroids (Potter 2008b). Despite the broad use of these insecticides against bed bugs little is known about their efficacy. Resistance in bed bugs has been suspected based on the numerous anecdotal reports of infestations that were particularly difficult to control. According to a survey made among pest control operators, control of bed bugs often requires at least three insecticide treatments, especially in cluttered environments (Potter 2008b). The question that arises from such reports is whether the difficulty to control bed bug infestations is due to lack of insecticide efficacy because of resistance or to difficulty achieving full insecticide coverage when treating the target population. These considerations lend urgency to the need for up-to-date information on the efficacy of pyrethroids in controlling bed bugs.
Control of resistant bed bugs would require the use of non-chemical methods, the use of insecticides with new modes of action, the selection of an appropriate insecticide synergist, or improved formulations of the existing ones. Non-chemical methods for bed bug control include heat treatments, vacuuming, steaming and freezing (Pinto et al. 2007, Potter et al. 2007). Non-pyrethroid insecticides in common use for control of bed bugs include the pyrrole, chlorfenapyr and insect growth regulators. Chlorfenapyr is a halogenated pyrrole that disrupts mitochondrial oxidative phosphorylation (Hollingworth and Gadelhak 1998). Chlorfenapyr is a pro–insecticide that requires activation by cytochrome P450 monooxygenases to its more active metabolite (Black et al. 1994). Chlorfenapyr might be an important option if resistance to pyrethroids is mediated by P450s or target site insensitivity is involved. Chlorfenapyr has been used in bed bug management programs in combination with other control methods (Potter et al. 2008c, Moore and Miller 2009, Wang et al. 2009), but its effectiveness has not been fully investigated under laboratory conditions.

Use of insecticide synergists is a classical way to enhance the efficacy of insecticide treatments (Glynne–Jones 1998). Synergists make insecticides more toxic to resistant insects by inhibiting the detoxification system. Three common insecticide synergists are piperonyl butoxide (PBO), S-S-tributylphosphorotri-thioate (DEF), and 4,4’-dichloro-α-methylbenzhydrol (DMC), which inhibit cytochrome P-450 microsomal monooxygenases, hydrolases, and DDT-dehydrochlorinase, respectively (Casida 1970, Scott 1990, Hodgson and Levi 1998). Piperonyl butoxide has been extensively used not only to understand the involvement of P450s in insecticide resistance, but also to increase the effectiveness of insecticides, particularly of organophosphates and pyrethroids
(Glynne–Jones 1998). The level of synergism of pyrethroids with PBO is greater when P450s are responsible for conferring insecticide resistance (Kasai et al. 1998, Wu et al. 1998). Residual resistance after piperonyl butoxide treatment suggests other resistance mechanisms are important.

Thorough exposure of a population of insects to an insecticide is not always possible. The issue of adequate coverage is exacerbated in an insect, such as the bed bug, that seeks out hidden refuges during the day (Haynes et al. 2008). Given this cryptic behavior, insecticide efficacy relies in part on insects contacting the insecticide residues while searching for a blood meal or returning to harborages. Limited information exists about the interaction between bed bugs and insecticide residues. Insecticides can influence insect behavior via their detection by a functional sensory system, or by disrupting the normal function of the sensory or central nervous system, or hormonal system (Georghiou 1972, Haynes 1988). Insects reduce exposure to insecticides by avoidance behavior that is stimulated by some combination of irritancy and repellency; irritancy occurring after physical contact and repellency occurring without physical contact with the insecticide (Chareonviriyaphap et al. 1997). There is limited information about sub-lethal or secondary effects of insecticides on host-finding and harborage-seeking behavior. Knowledge of these behavioral effects may ultimately provide a better understanding of the overall impact of insecticide treatment in bed bug management programs.
Objectives

The research objectives of this dissertation are designed to provide not only practical information for the pest control industry and its stakeholders, but also to provide basic knowledge that stimulates new research avenues on this urban pest.

In Chapter 2, I study the characteristics of the periodicity of locomotor activity and examine if this activity is influenced by an endogenous clock. I also monitor activity of bed bugs under reversed dark cycle regimes to determine if light entrains the circadian rhythm of locomotor activity. In addition, I provide insights about how age, sex and feeding status influence locomotor activity.

Chapter 3 of this dissertation addresses hypotheses related to resistance of bed bugs to various pesticides. I examine the susceptibility of some populations collected in Ohio and Kentucky to commonly used insecticides for bed bug control. In addition, I use a discriminating dose to test for the presence of deltamethrin resistance in samples collected across the USA.

In Chapter 4, I use a set of bioassays to evaluate the behavioral responses of bed bugs to deltamethrin and chlorfenapyr.

In Chapter 5, I use the P450 inhibitor piperonyl butoxide to assess the role of P450s in deltamethrin resistance. I also evaluate piperonyl butoxide–synergized pyrethroid formulations in two pyrethroid resistant strains to determine the utility of this synergist.

In Chapter 6, I measure with laboratory assays the toxicity of chlorfenapyr to several bed bug strains, including two strains highly resistant to pyrethroids. I also compare the effectiveness of two chlorfenapyr–based formulations.
Two of my original objectives were exploratory in nature therefore the results are present in Appendices. In Appendix A, I monitor the fate and persistence of an orally-ingested *Borrelia hermsii* (a relapsing fever spirochete) within bed bugs. In Appendix B, I examine the aggregation responses of bed bugs to papers exposed to conspecifics and methanol extracts of these materials.
Figure 1.1. Unfed and fed nymphs and adults. Unfed first instar nymphs (A); fed and unfed fifth instar nymphs (B); fed and unfed females (C); fed and unfed males (D).
Figure 1.2. Artificial feeding system for bed bugs. The feeding apparatus consisted of a glass container with a stretched parafilm membrane placed at the bottom (A). Animal blood in the blood feeder is warmed to 40°C using a circulating water bath (B). Bed bugs are in containers covered with fine-mesh cloth (B). Insects have to pierce the cloth and the parafilm membrane to get a blood meal (C). Several life stages of bed bugs recently fed (D).
**Figure 1.3. Diagram showing the series of events occurring in a day-night cycle.**

During the day the insect remains inactive inside its refuge. At night, when activity of its host is minimal, the insect leaves the refuge and moves (randomly?) until influenced by host cues. Once a host is detected, feeding occurs. The insect returns to harborages, attracted or arrested by aggregation pheromones. This aggregation pheromone together with thigmotaxis keeps the insects inactive in crevices and cracks during the day hours.
Introduction

Insects are exposed to predictable changes of light associated with the solar cycle, and to less predictable daily cycles of temperature and humidity. Some insects use these predictable daily changes to coordinate their activities at a time of day when conditions are most favorable, e.g., less risk of predation, reduced competition, and protection from adverse abiotic environmental conditions (Sharma 2003). While some periodic behaviors are affected directly by daily changes in environmental conditions, others are endogenously generated by biological oscillators (Saunders 2002, Barrozo et al. 2004). Evidence of endogenous rhythms of activity is revealed when the animal is experimentally exposed to constant dark (DD), or constant light (LL), where temperature and any possible external factor (Zeitgeber) are kept constant (Aschoff 1960). Endogenous clocks have the potential to be synchronized to daily cycles of photic and non–photic influences (Mrosovsky 1996, Refinetti 2005).

The bed bug, Cimex lectularius L. (Heteroptera: Cimicidae), is a flightless blood–sucking parasite that usually feeds at night (Usinger 1966). Usinger (1966) suggested that the ancestors of C. lectularius fed on cave–dwelling bats. According to this hypothesis, our ancestors became an alternative host when they shared caves with bats. Interestingly, a shift from bats to humans would require an evolutionary shift in endogenous control of host-seeking behavior or perhaps more simply a facultative response to host stimuli. A more permanent association between bed bugs and humans might have occurred later
during the period when humans established villages and transported bed bugs with them (Usinger 1966). Use of heat to warm houses made these habitats suitable for bed bugs throughout the year (Johnson 1941). Bed bugs are not evenly distributed throughout human habitations, but tend to live close to their hosts (Usinger 1966, Potter et al. 2006c). During daytime, bed bugs remain hidden in cracks or crevices. This state of immobility is induced by contact stimuli with surfaces or bodies of other insects (Johnson 1941) and aggregation pheromones (Siljander et al. 2007, 2008). Bed bugs usually become active during dark, when human activity is minimal (Usinger 1966). Much locomotor activity of bed bugs concerns searching for a host, acquiring a blood meal, and returning to harborages, where they remain while digestion takes place. Bed bugs walk relatively long distances (at least 20 m) in search of a host (Usinger 1966). Long periods of activity of bed bugs during host search can negatively impact their survival (Mellanby 1938).

Studies on periodicity and the ecology of movement in bed bugs are extremely limited. The aim of this study is to reveal characteristics of the periodicity of locomotor activity, to determine if the periodicity is influenced by endogenous factors or an exogenous light cycle. In addition, we evaluate the influence of age, sex and feeding status on locomotor activity. A better understanding of the locomotor activity of bed bugs will advance knowledge of the functional properties of the circadian system in this insect, could reveal insights into the evolution of the association between a blood-feeding insect and a human host and will contribute important ecological information concerning their movement which will aid in the management of this pest.
Materials and Methods

Insects

Bed bugs were obtained from a colony maintained at 27°C, 50 ± 5% RH, and a photoperiod of 14:10 (L:D) h. Insects were fed in the laboratory through a parafilm–membrane feeder with citrated rabbit blood heated to 40°C by a circulating water bath (Montes et al. 2002). Adults used in the experiments were unmated, and this status was assured by separating engorged fifth–instar nymphs before the adult molt occurred. Experiments were conducted using six groups consisting of the following sexes, ages and feeding histories: females and males held unfed one week after molting to adult, nymphs held unfed for one week after molting to the fifth instar, females and males held unfed five weeks after molting to adults and nymphs held unfed for five weeks after molting to the fifth instar. Additionally, two more experimental groups: one–week old females and males fed a blood meal two days before the initiation of the experiments were also evaluated. Adults were fed separately to prevent mating.

Experimental conditions and recording method

Experiments were conducted in a programmable environmental chamber maintained at 25°C, and 50% ± 5% RH. During lights–on the arenas were illuminated with fluorescent tubes from above with a light intensity ca. 50 lumens per square foot (lm/sqf) at arena level. During lights–off, two infrared lights (Strategic Vista, Markham, Ontario) provided the homogeneous illumination on the arena required for recording without disturbing the insects. Activity was monitored in insects individually placed into arenas. Arenas consisted of individual cells of 6–well cell culture plates (Corning, Corning, NY) (well diam. 3.48 cm, height 2 cm) whose surfaces were covered with white
filter paper (Whatman no. 2) (Fig. 2.1). The tight fit of the filter paper prevented the bed bugs from going underneath, keeping them on the arena surface, and Fluon AD–1 (polytetrafluoroethylene; Northern Products, Woonsocket, RI) coating on the walls of each cell prevented individuals from climbing out of the arena. An array of these arenas was formed by two rows of four 6–well cell culture plates (48 arenas) laid on a tray (30 × 50 cm) (Fig. 2.1). An infrared sensitive digital camera (Sony Handycam, San Diego, CA USA) was positioned at 0.5 m above the arena level and a photograph was taken every 10 min. The digital photos (2016 × 1512 pixels) were analyzed at 10 min intervals (observation of two consecutive pictures allowed the detection of positional changes). Based on a preliminary contrast with videorecording I concluded that a 10 min sampling interval captured the pattern of daily movement. Insects were acclimatized to the chamber conditions by placing them in the arenas two days before the beginning of the recording. Six bed bugs (1:1 sex ratio) from each experimental group were randomly assigned to the arenas. The experiment was repeated twice, thus each experimental group was represented by 12 individuals. At least 864 photos registered the activity of each insect.

**Periodicity of activity**

Locomotor activity of each experimental group of bed bugs was monitored under a photoperiod of 14:10 (L:D) h for six days. Actograms were constructed from positional changes expressed as a proportion of insects moving at each 10 min interval.

**Temporal patterns of activity during scotophase**

Further characterization of nocturnal activity of all experimental bed bug groups was made by estimating the onset and offset of activity during seven scotophases. Onset
and offset of locomotor activity was defined as the average time when the first and last positional change occurred, respectively. The onset and offset of activity was determined for each individual and for each scotophase, and the mean for each group was then computed. Onset and offset of activity during the scotophase was analyzed as a repeated measure using the Mixed Procedure with autoregressive covariance structure (Littell et al. 1998, SAS Institute 2002). Tukey’s multiple comparison test was used (SAS Institute 2002).

**Activity in continuous conditions of light or dark**

The insect groups used in this experiment were adult females, adult males and fifth–instar nymphs held unfed for one week after molting to the appropriate stage. Bed bugs were acclimated to the chamber and arena conditions for two days under a photoperiod of 14:10 (L:D) h. before exposure to continuous conditions. On day three, insects were exposed to continuous dark (DD) or continuous light (LL) and their activity monitored for six days. Free–running period (τ) was calculated with pooled data from each group by the chi square periodogram procedure (Sokolove and Bushell 1978) using the software LSP version 2.6 (Refinetti 2005). There was no detectable difference in temperature at arena level due to infrared or fluorescent lights inside the chamber during these experiments.

**Entrainment**

The photoperiod of females, males, and nymphs entrained to 12:12 (L:D) h was reversed to D:L or L:D on day two of the experiments by extending 12 h of darkness or 12 h of light, respectively. Activity was monitored up to day six since phase shift. Number of days that insects required to synchronize to the new photoperiod was based on
times of peak of activity (acrophase) calculated with pooled data from each group by the method of the single cosinor (Nelson et al. 1979) using the software ACRO version 3.5 (Refinetti 2005). Re–synchronization to the new phase was defined as the cycle number where a shifting acrophase falls into the 95% confidence interval of the acrophase estimated prior to the phase shift (e.g., it took between two and four 24 h cycles to re-establish the original acrophase, see below).

**Intensity and fluctuation of activity**

Total movement for each group was calculated by adding the number of positional changes detected during the light and dark period during the six days of monitoring. Daily variation of activity was also calculated. Data were analyzed as a repeated measure using the Mixed Procedure with autoregressive covariance structure (Littell et al. 1998, SAS Institute 2002). Multiple comparisons were Tukey–adjusted (SAS Institute 2002).

**Results**

**Periodicity of activity**

Bed bugs began to move shortly after lights–off with a rapid increase in the number of insects changing position during the first two hours (Fig. 2.2). After this initial increase of activity, insects remained relatively active for several hours before declining toward the end of scotophase. In the transition from dark to light, the insects showed a sudden increase in activity, but the frequency of movements rapidly decreased, persisting at low levels throughout the photophase (Fig. 2.2). Out of all recorded positional changes occurring during both the scotophase and photophase, the percent occurring at night was
85.4, 86.6, and 65.2% for females, males, and nymphs held unfed for one week, respectively. Half of the photophase movements in all groups occurred one hour after lights-on. Adult bed bugs and nymphs held unfed for five weeks were also more active during the scotophase (83.9, 80.2, and 63.0% for females, males and nymphs). Adult bed bugs that had recently fed did 82.0 and 84.7% (females and males) of the movements during night time.

**Temporal patterns of activity during scotophase**

The average time of onset of activity was about 1.7 h into the scotophase (Fig. 2.3). No significant differences in the onset of activity were observed between genders ($F = 0.09; \text{df} = 1, 70; P = 0.76$) or among groups categorized according to feeding history ($F = 2.36; \text{df} = 2, 70; P = 0.10$). However, the average time of offset of scotophase activity was influenced by gender ($F = 3.75; \text{df} = 1, 98; P = 0.05$), females stopping activity later than males ($8.3 \pm 0.14$ vs. $7.9 \pm 0.14$ h, respectively). Offset of activity also varied according to feeding status of bed bugs (Fig. 2.3) ($F = 13.5; \text{df} = 2, 97; P < 0.0001$). Offset of activity was detected significantly later in the scotophase in adults held unfed for one week ($8.7 \pm 0.14$) as opposed to those held unfed for five weeks ($7.5 \pm 0.18$) ($t = 5.2; \text{df} = 98; P < 0.0001$), or those fed recently ($8.1 \pm 0.17$) ($t = 2.6; \text{df} = 95; P < 0.05$). Adults that had fed stopped moving significantly later than those held unfed for five weeks (Fig. 2.3) ($t = -2.5; \text{df} = 101; P < 0.05$). No interactions between gender and feeding history were detected ($F = 1.91; \text{df} = 2, 98; P = 0.15$).

**Activity in continuous conditions**

All developmental stages showed an endogenous rhythm of activity under continuous light (LL) or continuous dark (DD) (Fig. 2.4) with the free–running period ($\tau$)
longer in DD than LL. Under DD continuous dark, the free–running period in females was 26.0 h, in males it was 25.8 h, and in nymphs it was 25.0 h (Fig. 2.4, A). Under LL, the free–running period in females and nymphs was 24.0 h, while in males it was 23.6 h (Fig. 2.4, B).

**Entrainment**

Females, males and nymphs of bed bugs entrained initially to a 12:12 L:D cycle re–synchronized their locomotor activity with phase shifts of the L:D cycle (Fig. 2.5). When the scotophase was extended for 12 h before resuming a 12:12 L:D cycle there was a gradual shift of locomotor activity toward the new scotophase (Fig. 2.5, A). Females and males with D:L shifted phase required four 24 h cycles to have their new peaks of activity (acrophase) (5.6 and 6.4 h, respectively) fall into the confidence interval of the acrophases estimated prior to the phase shift (6.2 h [5.1 – 7.3] and 6.3 h [5.3 – 7.4]). Peak of activity post shift for nymphs (8.5 h) recovered to the same time of the scotophase within 3 d (7.9 h [4.8 – 11.0]). When the photophase was temporarily extended by 12 h, bed bugs shifted their activity to the dark phase of the new photoperiod (Fig. 2.5, B). Females, males and nymphs with shifted phase required two 24 h cycles to have their new peaks of activity (5.2, 5.2 and 7.9 h, respectively) in the same time of the scotophase prior to phase shift (5.5 h [3.7 – 7.2], 6.7 h [5.1 – 8.2], and 5.7 h [3.2– 8.1]).

**Intensity and fluctuation of activity**

The number of positional changes per day varied according to the feeding history of bed bugs ($F = 10.55; \text{df} = 2, 78; P < 0.0001$) (Fig. 2.6). Adults held unfed for one week (averaging females and males) moved 18% more often than those unfed for five weeks (36.0 and 25.2 movements per day, respectively) ($t = 3.5; \text{df} = 78; P < 0.05$) and
22% more than adults that had fed (36.0 and 23.0, respectively) \( t = 4.3; \ df = 77; \ P < 0.05 \) (Fig. 2.6). There was no significant difference in the number of positional changes between adults held unfed for five weeks and those that had fed \( t = 0.77; \ df = 78; \ P = 0.72 \). Overall, females changed position significantly more than males (30.4 and 25.5 movements per day, respectively) \( F = 3.9; \ df = 1, 78; \ P = 0.05 \). No interaction between gender and feeding status was detected \( F = 1.4; \ df = 2, 78; \ P = 0.24 \). The number of times bed bugs changed position in a 24 h period changed over time \( F = 5.75; \ df = 5, 290; \ P < 0.0001 \) (Fig. 2.7), but no interaction was detected between daily movements and feeding history \( F = 1.30; \ df = 10, 290; \ P = 0.23 \). Adults that had fed showed a different daily fluctuation in the pattern of movements (Fig. 2.7, A). By day three, males had increased movements with respect to day one by 61%, but female movement only increased 10% (Fig. 2.7, A). However, after day three, males moved gradually less until the end of the experiment at day six, reaching levels similar to those recorded on day one. In contrast, females increased their activity after day three until day six, having an overall increase of 46% compared to movements recorded on day one. Adults held unfed for one week showed a marked fluctuation in daily activity over the six days of recording (Fig. 2.7, B). Bed bugs from this group increased movements during the first two days (21 and 30%, females and males, respectively). After day three the number of movements gradually decreased. By the end of the recording period, females and males from this group reduced daily movements by 18 and 13%, respectively, in relation to day one, and 32 and 33% from the peak (Fig. 2.7, B). A less pronounced fluctuation of activity was displayed by bed bugs held unfed for five weeks (Fig. 2.7, C). During the first days, the activity of females and males remained relatively stable; then after day three, the daily
rate of movements began to decrease until the end of the experiment. Females and males from this group reduced overall the number of daily movements by 33% and 29%, respectively (Fig. 2.7, C).

**Discussion**

**Periodicity of activity**

Locomotor activity of all groups of bed bugs monitored was periodical and showed a clear nocturnal pattern. Although a peak in activity was observed at the beginning of photophase, minimal activity was detected throughout the rest of this phase period. A previous study by Mellanby (1939) also noticed an increase in nocturnal activity in bed bugs that were infesting a mouse rearing facility. However, while we observed bed bugs having the highest levels of activity during dark hours, Mellanby (1939) reported a peak of activity of bed bugs during the transition between night and day. These differences in periods of high activity may be attributed to the conditions under which each study was conducted. We monitored activity of bed bugs under controlled conditions of humidity and temperature, and in absence of a host with a photographic technique that detected any movement. Mellanby (1939) determined activity based on catches of bed bugs in passive traps that were set up in a room containing mouse cages. In addition, their traps were checked for bed bugs at three–hour intervals, and the presence of human activity might have disturbed the bugs’ normal activity. The peak of activity observed by Mellanby could be explained by a reaction caused by the lights–on transitional signal (Aboul–Nasr and Erakey 1968) which we also
detected in our study. Alternatively, lights-on may be an indication to wandering bed bugs that it is time to return to the less exposed harborages.

**Temporal patterns of activity during scotophase**

Bed bugs started to move shortly after the onset of darkness and no variation among stages or groups with different feeding status was found. These results suggest that bed bugs use change of ambient illumination as a time–cue to start their daily phase of activity. Light signal has been recognized as a primary *Zeitgeber* for locomotor rhythm in nocturnal insects, including cockroaches, and kissing bugs (Mellanby 1940, Settembrini 1984, Saunders 2002). The level of activity in all stages gradually increased as the dark advanced and remained relatively high for several hours. Then, intensity of movements gradually decreased during the last third of the scotophase with minimal activity before lights–on. I speculate that a return to the harborage before the risk of exposure increases could be adaptive, as was suggested by Moore-Ede (1986) for other insects. Bed bugs with a short period of starvation had offsets of activity later in the scotophase than other groups and this might be associated with an extension of their exploratory behavior in search for a host.

**Activity in continuous conditions**

Periodic locomotor activity in all stages persisted for at least six cycles under constant conditions of light (LL or DD) in the absence of other obvious time cues (such as temperature or humidity), suggesting that it has an endogenous circadian nature. Endogenous control of locomotor activity has been reported in triatomine hemipterans (Schofield 1979, Settembrini 1984, Lazzari 1992). Results of these studies reported $\tau$ in DD shorter or equal to 24 h. Unlike triatomines, we found that bed bugs are an exception
to “Aschoff’s rule” which states that in nocturnal animals their τ in DD is shorter than their τ in LL (Pittendrigh 1960). Aschoff (1979) extensively reviewed patterns of circadian rhythms for various animals groups and concluded that many arthropods do not follow the rule.

**Entrainment**

As in other insects, the bed bug is able to synchronize its circadian clock according to environmental light cycles. Entrainment of endogenously generated rhythms by external periodic factors enables organisms to maintain a stable phase relationship with cyclical environmental factors (Sharma 2003). Entrainment was demonstrated when bed bugs, having transferred to a reverse cycle of light and dark (i.e. from L:D 12:12 to D:L 12:12), adopted the new pattern within four cycles, as expected for an endogenously controlled system. If locomotor activity were under exogenous control, i.e. in direct response to the environmental cycle of light and temperature, adaptation to the new reversed DL regime would occur within one cycle. Entrainment of circadian rhythm of locomotor activity by environmental light cycles may be crucial for bed bug survival (less conspicuous activity) considering the frequent changes in light conditions to which bed bugs are exposed in human environments. Locomotor activity was also entrained by environmental cycles in *Triatoma infestans* Klug (Settembrini 1984, Lazzari 1992). Lazzari (1992) evaluated the relative importance of light and temperature as *Zeitgebers* and found that both signals might interact to assure appropriate phase of circadian clocks. In addition to light and temperature, other environmental cycles may be responsible for the entrainment of circadian rhythms (Mrosovsky 1996). Further experiments are required to fully characterize the adjustment of phases of the circadian rhythm of
locomotor activity in bed bugs to light\dark cycles as well as potential involvement of other Zeitgebers.

**Intensity and fluctuation of activity**

Insects move from one place to another in order to enhance their likelihood of encountering resources such as food, mates, nesting sites, and refugia (Bell 1990, Barton Browne 1993). However, in insects such as bed bugs that engage frequently in host searching behavior, not finding a host quickly can exhaust nutritional reserves compromising their survival (Mellanby 1938). Thus, as bed bugs can survive at least one year without feeding (Johnson 1941) it would be expected that these insects possess mechanisms that could reduce the impact of long periods without food. In this study, the intensity of movement was influenced by feeding history of the bed bugs. Bed bugs that were subjected to a short period without food (one week after emergence) were more active than those starved longer (five weeks). This relationship between length of time of food deprivation and activity level suggests that the intensity of activity is a measure of the nutritional state of bed bugs, and that metabolic reserves play a role in modulating activity level. As locomotion requires high energy consumption, it is reasonable to assume that bed bugs held without food for a short period of time were more active than long–starved ones because of the availability of metabolic reserves from blood consumed as fifth–instar nymphs. Insects with a low likelihood of finding resources might employ energy–conserving strategies such as reducing movements when resources are not quickly available (Reynierse 1972). A decrease in activity has been reported in tsetse flies under deprivation conditions which precedes starvation death (Brady 1972). Because bed bugs survive several months without feeding, reduced levels of their activity may be
adaptive to the extent that this conserves internally stored resources. Recently-fed adults showed activity but their activity was less intense than unfed groups. Limited activity in engorged bed bugs was also observed by Johnson (1941) in studies conducted in experimental huts. Activity in recently fed bed bugs might not be associated with host searching behavior as the insects had been recently fed. In fed males, this activity might be a strategy to increase mating opportunities. In the case of the fed females, activity might be a mechanism by which females escape from high frequency male mating attempts which negatively impact longevity and reproductive success because of traumatic insemination (Stutt and Siva–Jothy 2001, Reinhardt et al. 2009b).

The relation between levels of spontaneous activity and feeding is best seen when activity of groups with different feeding status is examined over time (Fig. 2.7). Recently fed bed bugs showed overall low levels of activity (Fig. 2.7, A). However, the time-course of changes in daily activity in fed bed bugs differed between genders. Males’ activity increased during the first days of monitoring followed by a rapid decrease. In comparison, fed females initially had a period of low activity followed by an increase in activity until the end of the experiment, suggesting a more complex relationship between feeding status and movement. This difference from the fed males seems to have little to do with sex per se since females and males held unfed displayed patterns which were indistinguishable from one another (Fig. 2.7, B and C). On the other hand, these differences between fed females and males cannot be attributed to a masking effect of reproduction and egg development as the females used in the study were unmated. A more compelling reason for differences in activity might be the fact that fed females weighed on average 20% more than males, and this extra weight might reduce their
ability to move, as was discussed above. Bed bugs subjected to a short period without food (one week) initially increase their activity and this may be an expression of exploratory behavior to locate a food source (Lehane 2005) (Fig. 2.7, B). However, if the absence of a host is prolonged, the insects might suffer from a gradual deterioration of metabolic reserves that might compromise their survival. Here, bed bugs would need to counterbalance this effect by reducing activity (Fig. 2.7, B and C). I suggest that during prolonged periods of starvation (no host available) bed bugs make a transition from exploratory behavior to host–stimulus dependent searching. During this latter period long–starved insects might reduce the thresholds of sensory abilities in order to be able to detect quickly host stimuli (Barton Browne 1993). This suggestion remains speculative but it produces the testable prediction that bed bugs under the presence of host cues (e.g., temperature, carbon dioxide) increase activity. In conclusion our data provide evidence that locomotor activity of bed bugs is endogenously generated by circadian clocks and can be entrained by light conditions. We also show that activity of bed bugs fluctuates as insects alternate between states of food deprivation and satiation. Additional studies are required to identify other physiological and environmental factors that can affect movement in bed bugs.

The putative switch of hosts from bats to humans (Usinger 1966) may require little or no adaptation to a change in blood characteristics, but perhaps a significant change from diurnal to nocturnal feeding. This change may be facultative, i.e, respond whenever host cues are present or require an evolved response of a circadian clock. Our results indicate that the bed bugs we studied had an endogenous circadian clock that was well adapted to a diurnally active host. It would be interesting to contrast the biological
rhythms of activity of *C. lectularius* from human environments or bird roosts with other populations of this species infesting caves, or populations of bat bugs inhabiting caves.
Figure 2.1. Arenas in scotophase (A) and photophase (B). Each photograph captured position of individuals in each arena (a total of 48). Change in positions was noted by comparing consecutive images. Each block of arenas included individuals from each insect category (see text). The experiment was conducted inside an environmental chamber at 25°C and 50 ± 5% RH. Infrared photography was used to record nocturnal movements.
Figure 2.2. Periodicity of locomotor activity of adult female, male, and fifth-instar bed bugs. Insects were kept in a photoperiod of 14:10 (L:D) h for six days. The fed group (one week post-emergence) received a blood meal two days before initiation of the experiment. The unfed groups had emerged one or five weeks before initiation of the experiment. Dark and striped horizontal bars indicate the periods of lights-off and-on, respectively. Vertical bars represent the percentage of bed bugs that changed position at each 10 min interval (n=12).
Figure 2.3. Mean time of onset (± SE) (\(\bullet\)) and offset (\(\bigcirc\)) of locomotor activity of bed bugs with different feeding history recorded for seven scotophases. The unfed groups had emerged one or five weeks before initiation of the experiment. The fed group (one week post–emergence) received a blood meal two days before initiation of the experiment. Insects were kept under a photoperiod of 14:10 (L:D) h (n=12). Bracketed means with the same letter indicates that adults (combined female + male) from any group are not significantly different (\(P > 0.05\)). Xs indicate the mean of all adults in the group.
Figure 2.4. Free–running activity recorded in unfed adult female, male, and fifth–instar bed bugs one week post–emergence for six days. Insects were initially kept in a photoperiod of 14:10 (L:D) h for two days and then transferred into A) continuous darkness (DD), or B) continuous light (LL). Dark and striped horizontal bars indicate the periods of lights–off and–on, respectively. Vertical bars represent the percentage of bed bugs that changed position at each ten min interval (n=12).
Figure 2.5. Re-entrainment of locomotor activity of bed bugs after 12 h-phase shift of the L:D cycle. The phase shift was implemented extending the initial scotophase (A) or photophase (B). Insects were initially entrained to a photoperiod of 12:12 L:D cycle. Dark and striped horizontal bars indicate the periods of lights–off and–on, respectively. Vertical bars represent the percentage of bed bugs that changed position at each ten min interval (n=12).
Figure 2.6. Daily mean number of positional changes (± SE) of adult female and male bed bugs with different feeding histories. The unfed groups had emerged one or five weeks before use. The fed group (one week post-emergence) received a blood meal two days before initiation of the experiment. Insects were kept under a photoperiod of 14:10 (L:D) h (n =12). Bracketed bars with the same letter are not significantly different ($P > 0.05$).
Figure 2.7. Fluctuation of locomotor activity of bed bugs with different feeding history over six days. The fed group (one week post–emergence) (A) received a blood meal two days before initiation of the experiment. The unfed groups (B and C) had emerged one or five weeks before use. Insects were kept under a photoperiod of 14:10 (L:D) h (n =12).
Chapter Three: Insecticide resistance in the bed bug: a factor in the pest’s sudden resurgence?

Introduction

The bed bug, *Cimex lectularius* L. (Heteroptera: Cimicidae), is a flightless blood-sucking parasite that usually feeds at night (Usinger 1966). Lesions caused by bites usually occur on exposed areas of the face, neck, and extremities producing small clusters of erythematous papules or wheals (Ter Poorten and Prose 2005, Thomas et al. 2004). Although they are not known to be a vector of human diseases, bed bugs severely reduce quality of life by causing discomfort, anxiety, sleeplessness, and ostracism (Hwang et al. 2005). Bed bug infestations often require expensive ongoing inspections and treatments, disposal and replacement of infested beds and other furnishings, and quarantine of infested areas. In public facilities they may result in adverse publicity, and litigation by persons who are bitten (Doggett 2005, Potter 2005).

Bed bugs have a long association with humans; e.g., they were found in Egyptian tombs dating back over 3000 years (Panagiotakopulu and Buckland 1999). Bed bugs were part of life before chlorinated hydrocarbons and other synthetic insecticides became widely used in the 1940s and 1950s. While bed bugs never completely disappeared, they were so uncommon throughout much of the world that even pest control professionals rarely encountered them (Potter 2005). A resurgence of bed bugs has occurred in North America, Europe, and Australia over the past ten years (Boase 2001, Doggett et al. 2004, Potter 2005). Infestations are now common in the urban environment, including single
family dwellings, apartments, rooming houses, hotels, health care facilities, and college dormitories (Hwang et al. 2005).

Several hypotheses have been proposed to explain the sudden resurgence of bed bugs including increased travel to and from areas of the world where bed bugs remained common, increased exchange of second-hand furniture, a shift from premise-wide use of broad spectrum insecticides to more selective control tactics for other urban pests, and insecticide resistance (Doggett et al. 2004, Potter 2005). Evolution of resistance is a common outcome of use of a single insecticide, or insecticides with a common mode of action, against populations for consecutive generations (Georghiou 1986). Insecticide resistance has been found in over 400 species of arthropods (Roush and Tabashnik 1990) including blood-feeding insects (e.g., mosquitoes) where the loss of efficacy has led to pest resurgence and increases in rates of disease transmission (Krogstad 1996).

Our observations that some infestations were difficult to control (Potter et al. 2006c) and that some field-collected bed bugs survived direct spray applications with label-rate, formulated deltamethrin (A.R. unpublished data) led to this investigation. The results presented here show very high levels of resistance to two widely used pyrethroids in populations of bed bugs collected from human dwellings from across the United States.

**Material and Methods**

**Insects**

Four colonies were initiated from infested dwellings from Lexington, Kentucky (LEX1) and Cincinnati (CIN1, CIN2, and CIN3). Dwellings were separated by at least
6.1 km. Two laboratory colonies were also established that had never been exposed to pyrethroids; one from Fort Dix, NJ had been collected more than 30 years earlier (Bartley and Harlan 1974), and the second from Gainesville, FL had been collected over 20 years earlier. An F1 generation was produced by crossing virgin female bed bugs from the Fort Dix laboratory colony with males from CIN-1. Crosses in the other direction were less successful, and therefore were not evaluated in this study. Other field-collected bed bugs came from Los Angeles, CA (LA-1 and LA-2 populations), Kissimmee, FL (KIS-1), Frankfort, KY (FRA-1), Lexington, KY (LEX-1), Kalamazoo, MI (KAL-1), Troy, MI (MI-1 and MI-2), Dover, NJ (DOV-1), New York, NY (NY-1 and NY-2), Smithtown, NY (SMI-1), Cincinnati, OH (CIN-1, CIN-2, CIN-3, and CIN-5), Vienna, VA (VIE-1), and Worcester, MA (WOR-1). Samples were either collected by us or shipped to us by collaborators.

Colonies of bed bugs were reared in laboratory conditions using a parafilm-membrane feeder. Heparinised chicken blood was heated to 40°C with a circulating water bath (Montes et al. 2002). Colonies were maintained at 27°C, 50 ± 5% RH, and a photoperiod of 14:10 (L:D) h.

Residual assay

Adults from the Fort Dix colony or recent adult descendents from the LEX-1, CIN-1, CIN-2, CIN-3 (1:1 sex ratio, three replicates of 20 insects) were exposed for 24 h to insecticide residues on filter papers (Fig. 3.1). Insects were evaluated 7-12 d after adult emergence, and had not been fed. Based on a preliminary range test the concentration of deltamethrin (99% purity, Chem Service, West Chester, PA) was adjusted to $4.4 \times 10^{-5}$ to $1.3 \times 10^{-2}$ for Fort Dix, $1.3 \times 10^{-2}$ to 3.96 for LEX-1, CIN-1, CIN-2, CIN-3, and $4.4 \times 10^{-4}$
to 3.96 mg active ingredient (a.i.) per cm² for F1. Tested concentrations of \(\lambda\)-cyhalothrin (99% purity, Chem Service) ranged from \(6.6 \times 10^{-6}\) to \(6.6 \times 10^{-3}\) for Fort Dix and \(6.6 \times 10^{-3}\) to \(1.32\) mg/cm² for CIN-1 (only this field-derived colony was used with this compound). An insecticide-acetone solution of 50 μl was applied to each filter paper disc (Whatman #2, cut to 2.27 cm² [1.7 cm diam]) and allowed to dry completely before being placed in the bottom of individual cells of 24-well cell culture plates, which were then covered (Fig. 3.1). Control discs received acetone only. There was one individual bed bug per cell. Continuous exposure to the upper surface of the filter paper was ensured by the tight fit of the paper, and by a Fluon AD-1 (polytetrafluoroethylene; Northern Products, Woonsocket, RI) coating on the walls of each cell that prevented individuals from climbing off the treated surface. Temperature was maintained at 25°C after initiation of the exposure. After 24 h exposure in the culture plates, mortality was assessed by gently touching each individual with a fine paint brush and categorizing them as alive (coordinated avoidance movement) or dead (no response, usually on backs with no movement of any body parts). The few moribund individuals that were unable to maintain balance and showed uncoordinated twitching were recorded as dead in these assessments, and did not recover.

The LC_{50} (concentration that kills 50% of individuals at 24 h) was determined for Fort Dix and F1 using probit analysis (Finney 1971, Minitab 2005). LC_{50} values for other colonies could not be calculated because the highest tested concentrations resulted in little mortality. It was not practical to use higher concentrations (i.e., the highest concentrations [18% a.i. in acetone = 3.96 mg/cm²] left a visible residue on the filter paper disc; it was impractical to make more concentrated solutions). For these colonies.
the LC\textsubscript{50} was greater than the 3.96 mg a.i./cm\textsuperscript{2} for deltamethrin and 1.32 mg a.i./cm\textsuperscript{2} for \(\lambda\)-cyhalothrin. Resistance ratios calculated on this basis (LC\textsubscript{50} resistant colony/LC\textsubscript{50} susceptible colony) underestimate the actual ratio, and therefore are conservative.

In another experiment 12–20 third to fifth instar nymphs from colonies or directly from apartments were evaluated with acetone alone or 0.13 mg/cm\textsuperscript{2} technical grade deltamethrin (10\texttimes high labeled rate of active ingredient in commercial product). The latter treatment concentration was selected to discriminate between resistant and susceptible populations based on our earlier assays with adults. Control and treatment mortality was compared with a chi-square analysis (Minitab 2005). A significant chi-square value indicates that the population was susceptible.

**Results and Discussion**

There was a dramatic difference in susceptibility to deltamethrin between the Fort Dix colony and the four field colonies from the Kentucky-Ohio area. The Fort Dix colony suffered 100% mortality at \(4.4 \times 10^{-3}\) mg/cm\textsuperscript{2} and higher tested concentrations (Fig. 3.2; \(n = 60\); slope = 0.97 \(\pm 0.09\); LC\textsubscript{50} = \(3.10 \times 10^{-4}\) mg/cm\textsuperscript{2} [95\% CI \(2.51 \times 10^{-4}\) to \(3.82 \times 10^{-4}\)]; \(\chi^2 = 8.26\); df = 4). There was no control mortality in any of our assays. For the field-derived colonies, the highest concentration that we evaluated (3.96 mg/cm\textsuperscript{2}) killed only a few individuals (LEX-1, 5\%; CIN-1, 1.7\%; CIN-2, 3.3\%; CIN-3, 3.3\%; \(n = 60\) for all,), and no mortality resulted from lower insecticide concentrations. The resistance ratio of these four colonies relative to the Fort Dix colony was >12,765. The practical upper limit of solubility of deltamethrin in acetone prevented us from determining the LC\textsubscript{50} for these four colonies, and therefore presentation of probit values is not appropriate. The F1
offspring of matings between CIN-1 and Fort Dix showed intermediate levels of resistance (n = 60; slope = 0.35 ± 0.034; LC$_{50}$ = 0.46 mg/cm$^2$ [95% CI = 0.28 to 0.78]; $\chi^2$=8.59; df = 4; resistance ratio = 1,481) (Fig. 3.2). This result indicates that the genetic basis of resistance was not a single dominant-recessive gene, but was influenced by one or more genes with incomplete dominance. In addition, the fact that viable offspring were produced indicates that the genetic differences that influenced resistance were superimposed on a genetic background that was similar.

The results with λ-cyhalothrin paralleled those with deltamethrin (Fig. 3.2). The Fort Dix colony was susceptible to λ-cyhalothrin (n = 60; slope = 0.45 ± 0.048; LC$_{50}$ = $2.16 \times 10^{-4}$ mg/cm$^2$ [95% CI = $1.39 \times 10^{-4}$ to $3.38 \times 10^{-4}$]; $\chi^2$=10.63; df = 2; no control mortality). Mortality was 100% at $6.6 \times 10^{-3}$ mg/cm$^2$. The CIN-1 colony showed no control mortality, and only 21.6% mortality at the highest concentration tested (1.32 mg a.i./cm$^2$). Therefore, the resistance ratio was at least 6,123. Although the resistance ratios for deltamethrin and λ-cyhalothrin are underestimates, they appear to be of the same order of magnitude as the highest levels of resistance seen with other species of insects (Guerrero et al. 1997, Liu and Yue 2000).

Resistance to pyrethroid insecticides is not a local phenomenon, nor is it universal (Table 3.1, Fig. 3.3). We assessed presence or absence of resistance in third to fifth instar nymphs from 20 populations using a discriminating dose. Two laboratory colonies that have never been exposed to pyrethroids were susceptible (Table 3.1; 100% mortality at the discriminating dose). Populations collected in California, Florida, Kentucky (two colonies), Massachusetts, Michigan (three colonies), New York (three colonies), Ohio (four colonies), and Virginia were resistant (0% mortality at the discriminating dose)
(Table 3.1, Fig. 3.3). The population collected in Dover, NJ (DOV-1), showed intermediate level of resistance. One California population collected from the same building as a resistant population was susceptible (100% mortality at the discriminating dose) indicating independent source populations, a founder effect, or rapid evolution of resistance. An alternative explanation of the latter result is that this population could have been pre-exposed at the collection site to some other environmental stress (such as a different insecticide). To rule out this possibility I reared the LA-1 population in the laboratory and assessed the impact of our discriminating dose. These offspring were also determined to be susceptible (100% mortality at 24 h, n = 20; no control mortality).

Evolution of resistance to insecticides is the expected outcome of their repeated use. An interim report by Boase et al. (2006) suggests that cypermethrin resistance is present in the United Kingdom. Because DDT-resistance was reported decades ago (Busvine 1958, Mallis and Miller 1964), and cross-resistance between DDT and pyrethroid insecticides is common (Farnham 1977, Prasittisuk and Busvine 1977), resistant alleles may already have been present in populations. Failure of pyrethroids to quickly control infestations of resistant populations increases the opportunity for their spread. Spread of resistant populations is facilitated by the transport of bed bugs from one building to another and by unintended recycling of infested mattresses and furniture. Attempts to dispose of infested items may be more frequent when insecticides alone have failed to eliminate the problem. Acquisition of used furniture is a common source of new infestations (Potter 2005).

Inability to control bed bugs with pyrethroids may necessitate development of products with new modes of action, re-labeling of existing efficacious products, and
greater reliance on alternative tactics such as heat treatment, vacuuming, mattress encasements, or barriers. In addition, future investigations into the mechanisms of pyrethroid resistance could provide useful information to enhance existing insecticides or point to alternate compounds with different modes of action. Increased public awareness is also needed to minimize the risks of acquiring or transporting bed bugs. The options for chemical control of bed bugs were diminished by regulatory restriction of chlorinated hydrocarbon, organophosphate, and carbamate insecticides in many countries of the world. Resistance to pyrethroids, the largest remaining insecticide class, further limits these options. Without safe and effective alternatives, the continuing escalation of this serious pest problem seems inevitable.
Table 3.1. Mortality of bed bug nymphs (third to fifth instars) exposed for 24 h to a discriminating dose (0.13 mg/cm²) of technical grade (99% active ingredient) deltamethrin (n = 20 unless otherwise noted).

<table>
<thead>
<tr>
<th>Population&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Origin</th>
<th>Control</th>
<th>Deltamethrin</th>
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<tbody>
<tr>
<td>LAB-1</td>
<td>Fort Dix, NJ</td>
<td>0</td>
<td>100&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAB-2</td>
<td>Gainesville, FL</td>
<td>0</td>
<td>100&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>0</td>
<td>100&lt;sup&gt;*&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>0</td>
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</tr>
<tr>
<td>SMI-1</td>
<td>Smithtown, NY</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VIN-1</td>
<td>Vienna, VA</td>
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</tr>
</tbody>
</table>

<sup>a</sup>LAB-1 maintained >30 yr without exposure to insecticides; LAB-2 colony maintained >20 yr without exposure to insecticides; nymphs collected in apartments were evaluated for LA-1, LA-2, DOV-1, KIS-1, FRA-1, KAL-1, MI-1, MI-2, NY-1, NY-2, SMI-1, and VIN-1; nymphs from colonies initiated in 2005 were used for LEX-1, CIN-1, CIN-2, CIN-3, CIN-5, and WOR-1.

<sup>b</sup>LA-2-Control, n = 12; LA-2-Deltamethrin, n = 14; KIS-1-Control, n = 19
Figure 3.1. A dry residue bioassay to measure susceptibility of bed bugs to insecticides. Filter papers were impregnated with 50 μl of an insecticide-acetone solution (A) and allowed to dry completely before being placed in the bottom of individual cells of 24-well cell culture plates (B). One individual bed bug was placed per cell (C). Coating on the walls with Fluon prevented individuals from climbing off the treated surface (C). 20 bed bugs were evaluated per each plate (D).
Figure 3.2. Log dosage versus mortality on probit scale for adult bed bugs exposed to deltamethrin (upper graph) or λ-cyhalothrin (lower graph). Populations tested were Fort Dix (●), a susceptible colony; Cincinnati, OH; CIN-1 (■), a field-collected resistant colony, and F1 offspring of crosses between the two colonies (▼). F1 offspring were only tested with deltamethrin. For CIN-1 only one data point with mortality greater than 0% could be obtained because we were near the practical upper limit for dilution of deltamethrin in acetone. There was about four orders of magnitude difference between an insecticide dose that kills the susceptible and resistant colonies for both deltamethrin and λ-cyhalothrin.
Figure 3.3. Location and susceptibility to deltamethrin of bed bugs collected across the United States. Third to fifth instar nymphs from each place were exposed to a discriminating dose (10× high labeled rate of active ingredient in commercial product) that differentiate between resistant and susceptible populations.
Chapter Four: Behavioral responses of the bed bug to insecticide residues

Introduction

The recent resurgence of bed bugs, *Cimex lectularius* L. (Heteroptera: Cimicidae), has renewed interest in effective control tactics, particularly on the efficacy of insecticides on different bed bug populations. Several studies have focused on the determination of susceptibility of bed bug populations to commonly used insecticides (Boase et al. 2006, Moore and Miller 2006, Karunaratne et al. 2007, Romero et al. 2007a, b). However, behavioral responses of bed bugs to insecticide residues could also influence their efficacy.

Bed bugs have cryptic habits and are usually found assembled along mattress and box spring seams, or cracks, crevices or edges of furniture and other locations (Potter et al. 2006c). A typical harborage contains adults, nymphs, eggs, egg shells, shed skins, and feces. Aggregation seems to be promoted by thigmotaxis (Usinger 1966) and/or the presence of chemical cues (Marx 1955, Levinson and Bar Lan 1971, Siljander et al. 2007). Even with thorough inspections, some bed bugs go undetected, and therefore are not directly treated. Thus, insecticide efficacy relies in part on insects contacting the insecticide residues while searching for a blood meal or returning to harborages. Little is known about the interaction between bed bugs and insecticide residues.

Insecticides can influence insect behavior via their detection by a functional sensory system, or by disrupting the normal function of the sensory or central nervous system, or hormonal system (Georghiou 1972, Haynes 1988). Insects avoid prolonged exposures to insecticides by moving away from the treated area either due to repellency
(after perceiving insecticides at some distance) or due to irritancy (after contacting the treated area).

Sensory-mediated “repellency” or “irritancy” of sublethal neurotoxic effects can complicate the interpretation of laboratory evaluations of insecticide residues. These behavioral responses can increase or decrease exposure to potentially lethal residues. The role of irritancy and/or repellency of pyrethroid insecticides has been studied in other medically-important insects e.g., cockroaches, kissing bugs, and mosquitoes (Ebeling et al. 1966, Diotaiuti et al. 2000, Chareonviriyaphap et al. 2004), as well as in urban and structural pest species e.g., ants, termites (Knight and Rust 1990, Su and Scheffran 1990).

The objective of this study was to evaluate the behavioral responses of bed bugs to residues of a pyrethroid (deltamethrin) and a pyrrole (chlorfenapyr), two of the most commonly used insecticides in bed bug control in the US.

**Material and Methods**

**Insects**

Colonies of bed bugs were maintained at 27°C, 50 ± 5% RH, and a photoperiod of 14:10 (L:D) h. The bed bug strains were collected from human dwellings in Los Angeles, CA (LA-1), Cincinnati, OH (CIN-1, CIN-3), and Lexington, KY (LEX-1). The strain LA-1 is susceptible to deltamethrin while the other strains are highly resistant to the same pyrethroid (Romero et al. 2007a, b). Insects were fed with a parafilm-membrane feeder containing citrated rabbit blood which was heated to 40°C with a circulating water bath (Montes et al. 2002). Bed bugs were evaluated in behavioral bioassays 7-12d after adult emergence. Bed bugs were not fed as adults. This procedure standardized both age
and hunger level. Because bugs are adapted to survive long periods (months) without taking a blood meal (Usinger 1966), our test insects were hungry but not severely stressed.

**Choice tests**

Insects were offered two tents made of filter paper (15 × 12 mm, Whatman no. 2) folded in the middle to offer a tent-like shelter of 15 mm length × 5 mm height with two open ends. Group responses were carried out in flat-bottomed Pyrex bowls (12.4 cm diam × 6.0 cm height) (Corning Incorporated, Corning, NY) whose surfaces were covered with a white filter paper (110 mm diam; Whatman no. 2), fixed to the glass with double-sided tape. After each assay, papers were removed and bowls rinsed with acetone. During the photophase, arenas were illuminated with 40 W fluorescent tubes placed 60 cm above the arena surfaces which provide a light intensity of approximately 660 lux. Under these conditions bed bugs would seek harborages during the day.

Individual responses were carried out in 500-ml glass beakers whose bottom-inside surfaces were covered with white filter paper (70 mm diam.; Fisherbrand, quality P4). Paper was fixed to the glass with double-sided tape to prevent bed bugs from crawling under the paper. After each assay, beakers were cleaned as mentioned earlier for bowls. During the photophase, each one of three blocks of 16 arenas was illuminated with a 19 W fluorescent light which was placed 60 cm above the arena surfaces (light intensity of approximately 300 lux at arena level).

In the first experiment, ten bed bugs (1:1 sex ratio) were offered tents which had been sprayed until the paper was saturated with a suspension of 0.06% Suspend SC (deltamethrin, Bayer Environmental Science, Montvale, NJ), 0.5% Phantom
(chlorfenapyr, BASF, Research Triangle Park, N.C.) or distilled water (estimated volume in each tent after spray was 60 µl). Tents were allowed to dry for 2 h before being placed in arenas. Six replicates were performed for each insecticide.

In a second experiment, assays were conducted to measure individual responses to deltamethrin or chlorfenapyr (technical grade) that corresponded to the maximum labeled rate (Suspend), or the regular recommended labeled rate (Phantom) of the commercially-available formulations. Individual insects were offered a tent impregnated with 50 µl of an insecticide-acetone solution of 0.06% deltamethrin (99% purity; Chem Service, West Chester, PA,) or 0.5% chlorfenapyr (99.3% purity; Chem Service, West Chester, PA,) and a control tent treated with 50 µl of acetone. The acetone was allowed to evaporate. Unless otherwise stated, 60 replicates (30 males and 30 females) were performed.

In a third experiment, individuals from the CIN-1 pyrethroid-resistant strain (1:1 sex ratio, 48 replicates) or groups of ten insects (1:1 sex ratio, 12 replicates) were exposed to arenas (beakers for individuals or bowls for groups) which contained an established harborage and a clean tent. The established harborage was obtained by placing 10 fed insects (1:1 sex ratio) in a tent for 48 hours to allow them to produce body secretions, fecal matter and eggs. In follow-up experiments, established harborage were sprayed with Suspend and offered to 10 insects (1:1 sex ratio, 12 replicates) along with a water-treated tent. Finally, 20 insects (1:1 sex ratio, six replicates) were offered two established harborage, one treated with Suspend and the other treated with distilled water.

All assays lasted about 16 h (from about 4:30 p.m. to 8:30 a.m of the next day) with the following light-dark regimen: lights off at 9 p.m. and lights on at 7 a.m. (the
same light cycle used during rearing). Room temperature remained at 24 ± 2º C. Insects were acclimated to the environment for 15 min by restricting them in a shell vial (21 diam. × 70 mm height) which was placed inverted in the center of the arena. Insects were released by lifting up the shell vial. At the end of the test, the location of insects resting on a tent or wandering in the arena was recorded. Number of responses was analyzed by a binomial test with exact two-tailed \( P \) values, with the null hypothesis that tents were chosen with equal probability. Thus the response of each individual was assumed to be independent.

**Video recording of bed bug activity**

Activity of individuals from the pyrethroid-resistant CIN-1 and susceptible LA-1 strains was recorded while exposed to a single tent treated with a dry deposit of 0.06% deltamethrin, 0.5% chlorfenapyr, or an acetone-treated (50 µl) control tent. The susceptible strain LA-1 was also exposed to a lower concentration of deltamethrin (0.006%) on a tent. Evaluations were conducted in glass-bowl arenas as described in the group choice response experiments. The recording was carried out with a black-and-white video camera (Pelco, model MC3651H-2) suspended 1 m above the arena surfaces and a time lapse recorder (Panasonic, model AG-RT850). Six arenas were simultaneously recorded. Individual insects in shell vials were acclimated for 15 min in the arena. During the photophase, three 19 W fluorescent lights were placed above arenas pointing toward the walls and ceiling of the room where assays were conducted. The light intensity at arena level was about 40 lux. During the scotophase, a dim red light (20 W) pointing toward the ceiling provided a homogeneous illumination required for recording. Time that insects spent outside tents was divided by total time of the test period and the square
roots of these proportions were arcsine transformed before analysis of variance and Tukey's pair-wise comparison (at 5% level of significance) with Minitab (2005). Twelve replicates were performed for each strain.

Responses of host seeking insects to insecticide barriers

This experiment was conducted in plastic containers (5 cm diam. × 5 cm height); the open end of each container was covered with a fine mesh fabric (organza). This fabric top made contact with a parafilm-membrane feeder described earlier. Insects reached the heated surface by crawling up a strip of cardboard (20 mm wide × 70 mm height). A paper strip (20 mm wide × 50 mm height, Whatman no. 2) was impregnated with an insecticide-acetone solution (200 µl of 0.06% deltamethrin or 0.5% chlorfenapyr) or acetone, and was wrapped around the cardboard strip and attached with staples. To reach the blood source bed bugs would need to cross the insecticide-treated band. Ten insects (1:1 sex ratio, four replicates) were released and allowed to respond for 15 min. Then, the number of fed and unfed insects was recorded for each treatment and analyzed with a chi-square goodness-of-fit test (Minitab 2005).

Results

Choice tests

In assays with groups of insects, three out of four strains (LA-1, CIN-1 and CIN-3) significantly preferred to settle in water-treated tents rather than in tents treated with Suspend ($P < 0.05$) (Fig. 4.1, left side). LEX-1 strain showed no preference. Insects from LA-1 were least likely to occupy Suspend-treated tents as they were often found either
residing in insecticide-free tents (53%) or wandering in the arena (45%). No individuals from any strain avoided resting in tents treated with Phantom (Fig. 4.1, right side).

Assays with individuals showed that three out of four strains (LA-1, CIN-3, and LEX-1, but not CIN-1) significantly preferred to settle in acetone-treated rather than in deltamethrin-treated tents, at a ratio of at least 2:1 (acetone:deltamethrin) ($P < 0.05$) (Fig. 4.2, left side). In the deltamethrin assays, >93% of insects settled in one or the other of the offered tents with the exception of individuals from the susceptible strain LA-1. At the end of the test period, 58% of the individuals from this susceptible strain were wandering in the arena with symptoms of pyrethroid poisoning, including ataxia or increased locomotor activity. Insects from all strains did not significantly avoid resting in tents with dry deposits of 0.5% chlorfenapyr ($P > 0.05$) (Fig. 4.2, right side).

Individuals and groups of bed bugs from CIN-1 assembled significantly more in established harborages ($P < 0.05$) than in control tents (Fig. 4.3, A and B). Similarly, groups assembled significantly more in Suspend-treated established harborages than in control tents ($P < 0.05$). (Fig. 4.3, C). No preference was observed for the established harborages treated with Suspend or the one treated with water ($P < 0.05$) (Fig. 4.3, D).

**Video recording of bed bug activity**

Overall, the amount of time that individuals from CIN-1 spent away from tents differed significantly among treatments ($F = 6.33$; df = 2, 33; $P < 0.005$). CIN-1 individuals spent significantly more time away from deltamethrin-treated tents (36.7%) than acetone-treated control tents (6.2%) ($P < 0.05$), but no differences were found in the amount of time outside of tents in arenas with deltamethrin and chlorfenapyr-treated tents (36.7 vs. 16.4%) (Fig. 4.4 A).
The mean percentage of the time spent outside tents varied with treatment for LA-1 \((F = 7.52; \text{df} = 2, 15; \ P < 0.05)\). Interaction with deltamethrin-treated tents caused individuals from this strain to spend most of the time outside the treated tents (72.7\%) in comparison to time spent outside the treatments with control (20.0\%) or chlorfenapyr tents (24.6\%) (Tukey’s test \(P < 0.05\)) (Fig. 4.4 B). Individuals from the LA-1 strain also spent more time outside in the arena when exposed to tents treated with lower doses of deltamethrin (0.006\%; one-tenth maximum label rate) \((F = 5.03, \text{df} = 1, 16, \ P < 0.05)\) (Fig. 4.4 C).

**Response of host-seeking bed bugs to insecticide barriers**

In both CIN-1 and LA-1 strains, there were no significant differences in the percentage of fed insects between control and deltamethrin (for CIN-1, 55 vs. 52.5\% respectively, \(\chi^2 = 0.05, \ P = 0.82\); for LA-1, 52.5 vs. 57.5\%, \(\chi^2 = 0.02, \ P = 0.65\)) and between control and chlorfenapyr (for CIN-1, 55 vs. 50\%, \(\chi^2 = 0.201; \ P = 0.65\); for LA-1, 52.5 vs. 35\%, \(\chi^2 = 2.489, \ P = 0.20\)). No mortality was observed among the fed individuals from the resistant and susceptible strain in the chlorfenapyr assays (7 days after exposure).

Exposure to deltamethrin barriers was also insufficient to cause significant mortality in fed individuals from both resistant and susceptible strain (0\%, and 5\% mortality 7 days after exposure, respectively).

**Discussion**

Bed bug locomotor activity occurs mostly during the night, and they tend to remain hidden in refuges during the day. Given their cryptic behavior, it is not always
possible to find and treat all of their harborages. Thus, when bed bug control methods include the application of insecticides, it is advantageous that insecticide formulations leave toxic residues for insects to encounter when seeking blood meals or returning to their refuges. Insecticide residues can cause changes in behavior of bed bugs. The study of behavioral responses is important because these responses can affect insecticide efficacy and ultimately provide a better understanding of the overall impact of insecticide treatment in bed bug management programs.

We evaluated the behavioral responses of bed bugs to deltamethrin and chlorfenapyr, two of the most commonly used insecticides for their control. Deltamethrin is a pyrethroid which acts at sodium channels and is widely-used against urban and agricultural pests (Casida and Quistad 1998). Chlorfenapyr is a halogenated pyrrole that, unlike pyrethroids that disrupt the nerve transmission, uncouples oxidative phosphorylation processes in mitochondria (Hollingworth and Gadelhak 1998).

Insects tended to avoid dry residues of Suspend and preferred to rest in insecticide-free tents. Although LA-1 susceptible individuals avoided the insecticide (Fig. 4.1 and 4.2), about 50% of individuals were found wandering in the arena either alive or with irreversible symptoms of pyrethroid poisoning. On the other hand, no evidence of acute intoxication was observed in individuals from the resistant strains (CIN-1, CIN-3, and LEX-1), even in those insects that were resting on the deltamethrin-treated tent (Fig. 4.1 and 4.2). All these individuals survived. Avoidance responses displayed by insects indicated that bed bugs have behavioral mechanisms that reduce exposure to insecticides. These behavioral responses to insecticides have been reported in cockroaches, which
along with physiological resistance may be partly responsible for insecticide treatment failures (Lockwood et al. 1984, Rust et al. 1993, Hostetler and Brenner 1994).

The responses of bed bugs to the active ingredient of Suspend, deltamethrin, were consistent with those found with the formulated material, indicating that deltamethrin is at least one constituent of the formulation responsible for the avoidance responses of bed bugs. If bed bugs avoid insecticides and retreat to insecticide-free areas it would reduce not only the efficacy of insecticide treatments but also might encourage dispersal of bed bugs from the areas where they initially reside to other areas within the same dwelling and/or adjoining areas. Avoidance behavior to pyrethroid insecticides has been documented in other crawling household pests such as ants, termites, cockroaches, and kissing bugs (Knight and Rust 1990, Su and Scheffran 1990, Hostetler and Brenner 1994, Diotaiuti et al. 2000).

Bed bugs spend most of their time aggregated in harborages and only abandon them when the time comes to search for a blood meal. Marx (1955) suggested that bed bugs are driven by scent glands, odors and feces to return to harborages. Levinson and Bar Ilan (1971) demonstrated that bed bugs aggregated in filter papers previously exposed to adult conspecifics and suggested the existence of an aggregation pheromone. Aggregation may be mediated by contact pheromones produced by males and immature stages (Siljander et al. 2007). In our study, filter papers that had been previously occupied by adults attracted both individual and groups of bed bugs (Fig. 4.3, A and B). Harborages remained attractive to bed bugs after being treated with Suspend (Fig. 4.3 C). This indicates that either attracting or arresting factors were unaltered after insecticide treatment. These factors could include pheromones or physical characteristics of frass or
eggs (thigmotactic cues). Our laboratory findings of non-avoidance behavior by bugs to treated harborages correspond with field observations following Suspend applications in which insects are found resting in treated harborages (Romero et al. 2007b). The continued occupancy of insects in such treated areas might increase exposure of bugs to the insecticide. This effect, though, might have limited benefits when populations are resistant to pyrethroids. Insecticide assays showed mortality of less than 30% in individuals from the CIN-1 pyrethroid resistant strain when they were exposed continuously for more than a week to dry deposits of Suspend (A. R., unpublished data).

Insects from strains that avoided deltamethrin deposits in the absence of any aggregating stimuli (see choice tests, Fig. 4.1 and 4.2), would crawl over deltamethrin or chlorfenapyr barriers to reach a heat source and take a blood meal. Fed insects from the resistant and susceptible strain survived the exposure to chlorfenapyr residues. Insect mortality in deltamethrin barrier assays was minimal for both strains. Moore and Miller (2006) reported no avoidance behavior of bed bugs to insecticides when a heat source was nearby. Our studies confirm that responses of bed bugs to a close-range heat source may take precedence over avoidance responses to deltamethrin.

Video-recordings over the course of a night and day allowed us to observe the activity of individual insects while they interacted with a tent impregnated with dry deposits of insecticides. A highly pyrethroid-resistant and a susceptible strain showed an increase in locomotor activity as a result of contacting the deltamethrin-treated tent. An indication of this increase in activity was demonstrated by the longer time insects spent outside of treated tents (Fig. 4.4). Hyperactivity has been reported as the first symptom of poisoning by some types of pyrethroids in blood-feeding insects, including *Aedes aegypti*.
(L.) and *Anopheles maculipennis atroparvus* van Thiel (Kennedy 1947), and *Triatoma infestans* Klug (Alzogaray et al. 1997). Hyperactivity for type I pyrethroids could be caused by repetitive discharges of nerves and associated muscular contraction (Scott and Matsumura 1983). However, because deltamethrin is a type II pyrethroid, which does not cause such repetitive discharges, the avoidance of residues may involve other mechanisms. An increased rate of movement caused by a sublethal dose of insecticides can have beneficial or adverse effects depending on the circumstances. For example in control programs of *T. infestans* in South America, a spray with certain pyrethroids is part of pre and post- insecticide inspections as these insecticides are routinely used to flush out bugs from harborages. Among different pyrethroids tested, deltamethrin proved to be the most active agent in stimulating this response for *T. infestans* and *Rhodnius prolixus* Stål (Wood et al. 1993). On the other hand, locomotor hyperactivity caused by insecticides can increase the chance of insects to move across insecticide-treated surfaces which would accelerate the acquisition of lethal doses of insecticide (Kennedy 1947). However, little benefit of this effect can be expected when individuals are resistant to the insecticide used, when individuals encounter sublethal doses or there is poor insecticide coverage which allows bugs to move into insecticide-free areas. This might explain, in part, patterns of bed bug spread observed under field conditions where adjoining locations become infested over time.

Individual insects did not avoid dry deposits of chlorfenapyr. Similar results were achieved when groups were exposed to dry deposits of Phantom (a.i. chlorfenapyr). These results indicated that neither the technical nor the formulated material prevented individual or groups of insects from establishing residence in chlorfenapyr-treated tents.
These findings are not surprising as other studies report non-repellent effects of chlorfenapyr in other insects, e.g., ants, termites (Buczkowski et al. 2005, Rust and Saran 2006). No avoidance by bed bugs of chlorfenapyr might lead them to pick up a greater dose of the insecticide, and eventually this exposure causes mortality (Chapter Six).

Behavioral responses of bed bugs to insecticides and their implications for control may vary depending on several factors including insecticide susceptibility of populations, insecticide coverage, and the presence of other stimuli in the environment. The presence of aggregating factors in harborages and attraction to a heat source might reduce the avoidance behavior of bed bugs to deltamethrin. Survival of bed bugs after their contact with deltamethrin residues, with the subsequent increase in locomotor activity, represents a potential problem for the spread of bed bugs to adjoining areas. This is a behavioral effect that should be considered with all insecticides used for bed bug management.
Figure 4.1. Preference of a group of bed bugs (5 females, 5 males) from four strains for a paper tent sprayed with an insecticide (Phantom, 0.5% a.i., or Suspend, 0.06% a.i.) or a tent sprayed with distilled water. After a 16 h test period, the number of insects resting on any of the treatment harborage or wandering on the arena was recorded. Asterisk indicates significant differences between the control and insecticide treated tents (Binomial test, $P < 0.05$). N=60, however, wandering insects were not included in the analysis.
Figure 4.2. Preference of individual bed bugs from four strains for a paper tent impregnated with insecticide-acetone solution (0.5% chlorfenapyr or 0.06% deltamethrin in 50 µl acetone) or a tent treated with acetone (50 µl, control). After a 16 h test period, the place where the insect was found was recorded (resting on insecticide or acetone treated tent or wandering in the arena). Asterisk indicates significant differences between the control and insecticide-treated harborages (Binomial test, $P < 0.05$). Wandering insects were not included in the analysis.
Figure 4.3. Bed bug preference (CIN-1 strain) to different treatments in two-choice tests. A. Individual females offered a tent with feces, eggs and body secretions (harborage products) and a tent sprayed with distilled water (control); B. Groups (5 females, 5 males) offered harborage tents and control tents; C. Groups offered Suspend (0.06%)-treated harborage and a tent sprayed with distilled water. D. Group offered a Suspend (0.06%)-treated harborage or harborage tent sprayed with distilled water. In all experiments, harborage products were obtained by placing ten recently fed bed bugs (1:1 sex ratio) on tents during the 48 hrs prior to assays. After a 15 h test period, insects resting on any of the treatment harborages or wandering in the arena was recorded. Asterisk indicates significant differences between treatment and control (Binomial test, $P < 0.05$). Wandering insects were not included in the analysis.
Figure 4.4. Mean ± SE percentage time that insects from CIN-1 (A) and LA-1 (B and C) strains spent away (wandering in the arena) from insecticide treated tents (deltamethrin or chlorfenapyr) during the 15 h-testing period in no-choice tests. Bars with the same letter are not significantly different (analysis of variance followed by Tukey’s test, $P > 0.05$).
Chapter Five: Evaluation of piperonyl butoxide as a deltamethrin synergist for pyrethroid–resistant bed bugs

Introduction

Control of bed bugs, Cimex lectularius L. (Heteroptera: Cimicidae), is primarily based on intensive application of a limited number of insecticides, mainly pyrethroids (Doggett and Rusell 2008, Kilpinen et al. 2008, Potter 2008). Recent laboratory studies have found that some bed bug populations have become resistant to pyrethroids (Boase et al. 2006, Romero et al. 2007ab; Kilpinen 2008, Yoon et al. 2008). In the USA, evaluations of field–collected populations showed that bed bugs have the ability to develop very high levels of resistance to the pyrethroids deltamethrin and \(\lambda\)-cyhalothrin, with resistance ratios (RR) of at least 6,000–fold (Romero et al. 2007a, b). The presence of pyrethroid–resistant bed bug populations represents a major limitation because there are few insecticidal options. Furthermore, failure to eliminate resistant bed bugs could be a contributing factor for the spread of this pest (Romero et al. 2007a).

Cytochrome P450 monooxygenases (P450s) in insects are important in the metabolism of endogenous substrates as well as the catabolism of xenobiotics such as plant toxins, drugs and insecticides (Feyereisen 2005). Enhanced oxidative metabolism of insecticides by P450s is one mechanism by which insects become resistant to insecticides (Casida 1970, Scott 1999). Pyrethroid resistance in some insects has been associated with changes in the levels of expression of certain P450s (Liu and Scott 1998, Kasai et al. 2000, Pridgeon et al. 2003) and/or with structural changes in the enzyme that lead to an increase in their catalytic activity (Zhu and Snodgrass 2003). Piperonyl butoxide (PBO) enhances insecticide toxicity by inhibiting the oxidative metabolism by P450s (Casida
1970, Hodgson and Levi 1998). The synergistic effect of PBO has been extensively used not only to understand the involvement of P450s in insecticide resistance but also to increase the effectiveness of insecticides, particularly of organophosphates and pyrethroids (Glynne–Jones 1998). The level of synergism of pyrethroids with PBO is greater when P450s are responsible for conferring insecticide resistance (Kasai et al. 1998, Wu et al. 1998). Synergism with PBO has been proposed to increase the efficacy of pyrethroids against bed bug infestations that are difficult to control. Nevertheless, currently there is no adequate evidence about the effectiveness of these mixtures against resistant bed bugs. In this study I show P450s mediated detoxification in deltamethrin–resistant bed bugs. I also demonstrate that dry residues of piperonyl butoxide–synergized pyrethroids have limited effectiveness with some resistant strains suggesting that other resistance mechanisms are also involved.

**Material and Methods**

**Bed bug strains**

Insects were obtained from colonies maintained at 27°C, 50 ± 5% RH, and a photoperiod of 14:10 (L:D) h. Two strains used were highly resistant to deltamethrin (Romero et al. 2007a, b), CIN–1 (collected in 2005 in Cincinnati, OH) and WOR–1 (collected in 2007 in Worcester, MA), and two were susceptible (Romero et al. 2007a, b): LA–1 (collected in 2006 in Los Angeles, CA) and Fort Dix, which has not been exposed to insecticides for > 30 yr (Bartley and Harlan 1974). Insects were fed in the laboratory through a parafilm–membrane feeder with citrated rabbit blood which was heated to 39°C with a circulating water bath (Montes et al. 2002). Evaluations began 7–12d after adult
emergence, and insects had not been fed as adults. We found more consistent results with this group of insects than those that had more recently emerged or fed.

**Application of PBO**

Insects were placed in individual cells of 24–well cell culture plates (Corning Inc., Corning, NY). The bottoms of the cells were covered with tightly fitting filter papers. Insects were temporarily immobilized by placing plates on ice for ~ 5 min. Adults (1:1 sex ratio; three replicates of 20 insects) were treated topically with a sublethal dose of PBO (Sigma–Aldrich, St. Louis, MO) 2 h before a 24-h exposure to dry deposits of deltamethrin. Topical applications (1 µl) of a piperonyl butoxide solution in acetone (50 µg / µl) were made onto the dorsal surface of the abdomen with a microapplicator (Hamilton Co., Reno, NV) equipped with a 25 µl–glass syringe (Hamilton Co., Reno, NV). Control insects received 1 µl of acetone alone. After treatment, insects were kept at 26°C for 2 h prior to deltamethrin exposure.

**Exposure to deltamethrin**

In dry residue assays, the range of concentrations of deltamethrin (99% purity; Chem Service, West Chester, PA) used against each strain was determined by preliminary tests. Tested concentrations ranged from $4.4 \times 10^{-5}$ to $1.32 \times 10^{-3}$ mg of active ingredient (A.I.) per cm$^2$ (mg/cm$^2$) for Fort Dix, from $1.32 \times 10^{-5}$ to $1.32 \times 10^{-2}$ for LA–1, and from $1.32 \times 10^{-4}$ to $1.32$ mg/cm$^2$ for CIN–1 and WOR–1. For each strain, the deltamethrin concentrations used in the PBO trials were the same as those in non–PBO trials. An insecticide–acetone solution (50 µl) was applied to each filter paper disc (Whatman no. 2; cut to 2.27 cm$^2$ [1.7 cm in diameter]). The disks were allowed to dry completely before being placed in the bottom of individual cells of 24–well cell culture
plate. Control discs received acetone only. Individual insects treated with PBO were transferred to individual wells containing deltamethrin–treated filter papers and then, culture plates were covered. Continuous exposure to the upper surface of the filter paper was ensured by the tight fit of the paper, and by a Fluon AD–1 (polytetrafluoroethylene; Northern Products, Woonsocket, RI) coating on the walls of each cell that prevented individuals from climbing off the treated surface. Topical applications of deltamethrin in acetone (1 µl containing 0.0182-182 µg of A.I) were made onto the dorsal surface of the abdomen of individuals from the strain CIN–1. Temperature was maintained at 26ºC after initiation of the exposure. After 24–h exposure, mortality was assessed by gently touching each individual with an entomological forceps and categorizing bed bugs as alive (coordinated avoidance movement) or dead (no response, usually on backs with no movement of any body parts).

**Test with formulated insecticides**

Adults (1:1 sex ratio; three replicates of 20 insects) were exposed continuously to dry residues of a commercial formulation of piperonyl butoxide synergized pyrethrins (Kicker, Bayer Environmental Science, Research Triangle Park, NC), deltamethrin (Suspend SC, Bayer Environmental Science, Research Triangle Park, NC), a mixture of deltamethrin plus piperonyl butoxide–synergized pyrethrins, or deltamethrin plus a commercial formulation of PBO (Exponent, McLaughlin Gormley King Co., Minneapolis, MN). Deltamethrin (0.06%) and the synergized pyrethrins (0.2% pyrethrins, 2 % PBO) were used at the maximum labeled rates. Deltamethrin was mixed with the synergized pyrethrins at a 1:1 ratio, and with PBO at a 1:10 ratio (maximum labeled ratio) (final concentration of PBO = 0.5%). An insecticide–distilled water solution of 50
µl was applied to each filter paper disc and allowed to dry for 3 h. Control insects were exposed to filter paper discs treated with distilled water only. Insects were exposed continuously to the treated surfaces and mortality was recorded daily for at least 7 d.

**Statistical analysis**

Dose–mortality data were analyzed by using probit analysis (Finney 1971, SAS Institute 2002). The goodness–of–fit of the probit model to the data was compared using a χ² statistic (SAS Institute 2002). The RR (LC$_{50}$ of the resistant strain divided by LC$_{50}$ of the susceptible strain) and the synergist ratio (SR) (LC$_{50}$ of insecticide alone divided by LC$_{50}$ of the insecticide plus PBO) were calculated for each strain. Time–mortality data for assays with formulated insecticides were fitted to log–probit or the complementary log–log (CLL) regression model (Throne et al. 1995, Robertson et al. 2007), to estimate the time required to kill 50% (LT$_{50}$) of the exposed insects (Throne 2009). We chose these regression models from six possible transformations based on χ² values (lower values better describe the data) and on the examination of the fitted regression lines compared with the transformed observations (Throne et al. 1995). The LT$_{50}$ values of any two treatments with formulated insecticides were compared using lethal time ratios, as described by Robertson et al. (2007). This method was chosen because it is a more powerful method for comparison of lethal times than examination for overlaps of 95% confidence intervals (Payton et al. 2003). According to Robertson et al. (2007), two LT$_{50}$s are not significantly different if the 95% confidence interval (CI) of the LT$_{50}$ ratio includes 1.
Results and Discussion

The strains CIN–1 and WOR–1 exhibited high degrees of resistance to deltamethrin compared with the Fort Dix susceptible strain (Fig. 5.1; Table 5.1). These strains were >2,500-fold resistant, and an accurate estimate of toxicity was not possible because the mortality level did not exceed 50%, even at a very high exposure rate of 1.32 mg (A.I.)/ cm² (100 times recommended rate of formulated deltamethrin). Lack of fitness of the regression lines \( (P < 0.05; \text{goodness–of–fit tests}) \) also prevented us from presenting probit values and conducting further statistical analysis with these two strains. In contrast, the strain LA–1 demonstrated a high level of susceptibility to dry residues of deltamethrin (Table 5.1; RR = 1.3; goodness–of–fit tests, \( \chi^2 = 4.2; \text{df} = 2, P = 0.12 \)).

In all strains, treatment with PBO increased mortality of all exposure rates of deltamethrin (Fig. 5.1). However, no statistical differences were observed between the regression lines (with and without PBO) from the susceptible strain Fort Dix (log–likelihood ratio test, \( \chi^2 = 3.1, \text{df} = 1, P > 0.05 \)). Regression lines from LA–1 were significantly different \( (\chi^2 = 10.5, \text{df} = 1, P < 0.05) \); treatment with PBO reduced slightly the RR (from 1.3 to 1.0-fold) (Table 5.1). Regression lines from assays with PBO had an acceptable fit for CIN–1 \( (\chi^2 = 6.7, \text{df} = 3, P = 0.08) \) and WOR–1 \( (\chi^2 = 3.6; \text{df} = 3; P = 0.3) \) (Fig. 5.1). The highest exposure rate of deltamethrin (1.32 mg [A.I.] / cm²) with PBO caused 73% and 91% mortality in CIN–1 and WOR–1, respectively (Fig. 5.1). The degree of enhancement of deltamethrin toxicity by PBO can be determined by comparing the resistance ratios calculated from mortalities of assays with and without the synergist. In CIN–1, treatment with PBO reduced the RR from > 2,500 to 174, whereas in WOR–1 the RR was reduced from > 2,500 to 39 (Table 5.1). We did further evaluations with the
strain CIN-1 to determine whether topical applications of deltamethrin to PBO-treated bed bugs would lead to higher mortality because of greater bioavailability of the pyrethroid. In assays with deltamethrin alone, the highest exposure dose (182 µg) killed 52.5% of individuals (n = 40). In assays with bed bugs pretreated with PBO, the same dose killed 72.5% (n = 40). Pretreatment with the synergist reduced the LD$_{50}$ from 36 µg (deltamethrin alone) to 16.7 µg (PBO + deltamethrin).

The residual resistance after PBO treatment indicates that P450s contribute to but are not wholly responsible for deltamethrin resistance in bed bugs. Our data also suggest that other resistance mechanisms not mediated by P450s might be involved, e.g., increased metabolic detoxification by glutathione transferases or esterases, decreased target site sensitivity of voltage–gated sodium channel (e.g., kdr), or decreased cuticular penetration (pen) (Brogdon and McAllister 1998). Recently, Yoon et al. (2008) examined the resistance profile of a strain of bed bugs collected from New York City (NY–BB) and showed that they were 264–fold resistant to deltamethrin. DNA sequencing of these individuals identified two point mutations, V419L and L925I in the voltage–gated sodium channel gene; whereas L925I mutation has been reported in pyrethroid–resistant strains of whitefly (Morin et al. 2002), V419L is a novel mutation. Further analysis of this New York bed bug sample showed no detectable differences in the metabolic activity of glutathione transferases, esterases, and 7–ethoxycoumarin O–deethylases (Yoon et al. 2008). However, these results do not exclude the possibility that deltamethrin is metabolized by increased activity of one or more specific P450s.

SRs measure the relative involvement of resistance–related mechanisms. A greater SR in field–derived strains than in a susceptible reference strain would indicate
the degree that the metabolic mechanism detoxifies an insecticide. In this study, the synergist ratios varied among strains. The highest SR was estimated for WOR–1 (176), followed by CIN–1 (40), LA–1 (5.6), and Fort Dix (2.7) (Table 5.1). The fact that SRs in WOR–1 and CIN–1 were higher than the SR estimated for the susceptible strain Fort Dix, suggests that P450s play an important role in detoxifying deltamethrin. Quantitative expression (concentration of enzymes) or qualitative differences (structure of enzymes) in P450s of the resistant strains need to be investigated.

To investigate the potential of the synergist piperonyl butoxide to improve the efficacy of insecticide treatments, we evaluated the effect of the addition of commercial formulations of PBO (Exponent) or synergized pyrethrins (Kicker) to deltamethrin (Suspend). Piperonyl butoxide has been widely used in agricultural and urban systems to enhance the performance of pyrethroids in insecticide resistance management programs (Glynne–Jones 1998). Suspend is the most widely used insecticide formulations for bed bug management in the United States (Potter 2008b) in part because its label is permissive for use in mattresses, box springs, upholstered furniture and other areas where both humans and bed bugs reside. The addition of PBO as contained in formulations like Exponent and Kicker has been recommended where resistance to pyrethroids is suspected (e.g., Bayer Environmental Science, 2007). Field studies suggest that inclusion of synergized pyrethrins can increase the efficacy of deltamethrin against bed bugs (Barile et al. 2008). The mixture Suspend plus Kicker is used based on the assumption that natural pyrethrins would cause fast knockdown, and PBO would enhance the activity of insecticidal components by inhibiting the detoxifying action of monooxygenases. Previous laboratory studies showed that spraying Kicker onto bed bugs directly caused
relatively high mortality to CIN–1 (73%) and WOR–1 (85%) strains in 24 h (A. R. unpublished data). However, dry residues of the same formulation were not as effective (< 2% mortality after 7 d of continuous exposure). In contrast, a susceptible strain (Fort Dix) was killed by these dry residues (92% mortality).

Analysis of time–mortality results with Suspend or Suspend in combination with Exponent or Kicker showed acceptable to good regression fits ($\chi^2$ not significant; $P > 0.05$) (except CIN–1 with Suspend + Kicker) (Table 5.2). The rate of mortality of bed bugs exposed to Suspend, measured by LT$_{50}$ values, was much slower in CIN–1 ($LT_{50} = 15.2$ d) and WOR–1 ($LT_{50} = 5.4$ d) than in the susceptible strain Fort Dix (100% mortality after 5 h exposure; data not shown). Slow acting effects of dry residues of Suspend were also reported in laboratory studies by Moore and Miller (2006) with a field strain of bed bugs that required 14 d and 8 h of continuous exposure to reach 50% mortality. The mixture of Suspend and Kicker required 36% less time than Suspend alone to cause 50% mortality in CIN–1 (9.7 and 15.2 d, respectively) and 50% less time in WOR–1 (2.7 and 5.4 d) (Table 5.2); however, significant differences between the two treatments were observed only in WOR–1 (Table 5.3). In the same manner, the combination of Exponent and Suspend was as effective as Suspend and Kicker in WOR–1 ($LT_{50} = 2.6$ d), but not in CIN–1 ($LT_{50} = 16.4$ d) (Table 5.2 and 5.3). Results with formulated insecticides parallel those obtained in the assays with topical application of PBO and residues of technical grade deltamethrin, described above. Lack of enhancement of mortality with dry residues of deltamethrin by PBO against CIN–1, a pyrethroid–resistant strain, indicates that addition of PBO, either alone or in combination with pyrethrins, is not a comprehensive solution to deltamethrin resistance. Dry residues of
piperonyl butoxide synergized–pyrethroid formulations might only be effective against susceptible bed bugs or against pyrethroid–resistant bed bugs in which P450s are primarily responsible for the detoxification of the insecticide. Presence of a kdr–like insensitivity or any other resistance mechanism that is not inhibited by PBO might limit the effectiveness of dry residues of synergized pyrethroids against resistant bed bugs.

Failure of piperonyl butoxide synergized pyrethroids to control pyrethroid resistant southern cattle tick, *Boophilus microplus* (Canestrini), was reported in Australia (Crampton et al. 1999). They suggest a minor role of P450s in detoxifying pyrethroid insecticides, and implied that other mechanisms might be conferring resistance.

Additional testing in our laboratory showed that direct application to individuals of CIN–I with the combination of Suspend and Kicker caused three–fold higher mortality than sprays with Suspend alone during the first 24 h (A. R. unpublished data) and similar results are reported in other studies (Bayer Environmental Science 2007). These results suggest that the overall efficacy of treatments with mixtures of synergized pyrethrins and deltamethrin can be enhanced by directly spraying bed bugs, highlighting the importance of detecting and spraying aggregation sites whenever possible. However, the cryptic habits of these insects make this very difficult. Thus, there is a need for residual insecticides that can kill bed bugs as they search for blood meals or return to hidden harborages.

Given the potential of bed bugs to evolve more than one resistance mechanism (Karunaratne et al. 2007, Yoon et al. 2008), a sound strategy for pest management must be based on a thorough understanding of the resistance mechanisms involved. The frequency and the relative importance of such resistance mechanisms will determine the
potential of synergists for effective control of resistant bed bugs. The presence of metabolic detoxifying mechanisms may be countered by selection of appropriate synergists or insecticides with different modes of action. The latter may be critical when target site insensitivity or penetration resistance is involved.
Table 5.1. Toxicity of deltamethrin without and with piperonyl butoxide (PBO) to the field–collected strains LA–1, CIN–1 and WOR–1 strains and the susceptible–laboratory Fort Dix strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$n^a$</th>
<th>LC$_{50}^c$ mg a.i. cm$^2$ (95% CI)</th>
<th>Slope ± SE</th>
<th>RR$^d$</th>
<th>(95% CI)</th>
<th>Slope ± SE</th>
<th>RR</th>
<th>SR$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fort Dix</td>
<td>60</td>
<td>$5.1 \times 10^{-4}$ (3.3x$10^{-4}$–7.6x$10^{-4}$)</td>
<td>1.41 ± 0.26</td>
<td>–</td>
<td>$1.9 \times 10^{-4}$ (8.3x$10^{-5}$–3.3x$10^{-4}$)</td>
<td>1.28 ± 0.22</td>
<td>–</td>
<td>2.7</td>
</tr>
<tr>
<td>LA–1</td>
<td>60</td>
<td>$6.7 \times 10^{-4}$ (2.0x$10^{-4}$–1.3x$10^{-3}$)</td>
<td>0.97 ± 0.20</td>
<td>1.3</td>
<td>$1.2 \times 10^{-4}$ (1.6x$10^{-5}$–3.3x$10^{-4}$)</td>
<td>0.85 ± 0.16</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>CIN–1</td>
<td>60</td>
<td>&gt;1.32</td>
<td>–</td>
<td>&gt;2,588</td>
<td>$3.3 \times 10^{-2}$ (4.8x$10^{-3}$–2.3x$10^{-1}$)</td>
<td>0.62 ± 0.10</td>
<td>174</td>
<td>40</td>
</tr>
<tr>
<td>WOR–1</td>
<td>60</td>
<td>&gt;1.32</td>
<td>–</td>
<td>&gt;2,588</td>
<td>$7.5 \times 10^{-3}$ (4.4x$10^{-3}$–1.2x$10^{-2}$)</td>
<td>1.01 ± 0.10</td>
<td>39</td>
<td>176</td>
</tr>
</tbody>
</table>

$^a$ Total number of insects used

$^b$ Insects were pretreated with PBO (1 µl of a 50µg/µl solution in acetone) 2 h before were exposed to dry residues of deltamethrin.

$^c$ LC$_{50}$ = The LC$_{50}$ (concentration that kills 50% of individuals at 24 h)

$^d$ Resistance Ratio (RR) = LC$_{50}$ trial/LC$_{50}$ of susceptible strain (Fort Dix).

$^e$ Synergist Ratio (SR) = LC$_{50}$ without PBO/LC$_{50}$ with PBO.
Table 5.2. Time–mortality regression estimates for pyrethroid–resistant bed bugs exposed to dry residues of various pyrethroid treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Regression model</th>
<th>CIN–1</th>
<th>WOR–1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Slope ± SE</td>
<td>LT$_{50}$ (days)</td>
</tr>
<tr>
<td>Suspend</td>
<td>60</td>
<td>Log–Probit</td>
<td>1.54 ± 0.24</td>
</tr>
<tr>
<td>Suspend + Kicker</td>
<td>60</td>
<td>Log–Probit</td>
<td>1.21 ± 0.19</td>
</tr>
<tr>
<td>Suspend + Exponent</td>
<td>60</td>
<td>Log–Probit</td>
<td>1.06 ± 0.18</td>
</tr>
</tbody>
</table>

* Significant ($P < 0.05$) (goodness–of–fit test).

$^a$ Values for goodness–of–fit of the regression models to the observed mortality data.
Table 5.3. Pairwise comparisons of LT$_{50}$ values among treatments in pyrethroid–resistant bed bugs.

<table>
<thead>
<tr>
<th>Treatments compared</th>
<th>CIN–1 LT$_{50}$ ratio (CI 95%)</th>
<th>WOR–1 LT$_{50}$ ratio (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspend vs. Suspend + Kicker</td>
<td>1.5 (0.7–3.2) NS</td>
<td>2.0 (1.2–3.1) *</td>
</tr>
<tr>
<td>Suspend vs. Suspend + Exponent</td>
<td>1.1 (0.5–2.5) NS</td>
<td>2.1 (1.5–3.0) *</td>
</tr>
<tr>
<td>Suspend + Kicker vs. Suspend + Exponent</td>
<td>1.7 (0.7–4.1) NS</td>
<td>1.1 (0.6–1.8) NS</td>
</tr>
</tbody>
</table>

* The LT$_{50}$ between the two treatments are significantly different ($P < 0.05$) if the CI of their ratio does not include 1 (Robertson et al. 2007).

*a Ratio = larger LT$_{50}$/smaller LT$_{50}$.
Fig. 5.1. Log dosage versus mortality on probit scale for adult bed bugs exposed to deltamethrin. Closed circles (●) are responses from bed bugs pretreated with PBO, and open circles (○) from bed bugs not pretreated with this synergist. Populations tested were Fort Dix a susceptible long–maintained laboratory colony; LA–1, another deltamethrin susceptible strain; and WOR–1 and CIN–1, highly resistant strains to deltamethrin. For the latter two strains, deltamethrin alone did not cause mortality >50%.
Chapter Six: Evaluations of chlorfenapyr for control of the bed bug, *Cimex lectularius* L.

Introduction

Recently, the bed bug, *Cimex lectularius* L. (Heteroptera: Cimicidae), has reemerged as a serious and growing problem, not only in North America, but globally (Krueger 2000, Boase 2001, Doggett et al. 2004, Potter 2006, Kilpinen 2008). The bed bug resurgence has renewed interest in effective control tactics. Chemical options for bed bug management, however, are limited because of precautions about treatment in areas where human exposure is possible and regulatory restriction of chlorinated hydrocarbons, organophosphates, carbamates, and other insecticides. Furthermore, the near disappearance of bed bugs in many parts of the world reduced the interest of industry for registering insecticide products against this pest. The most commonly used insecticides for bed bug control today in the USA are pyrethroids (Potter 2008). Recent laboratory studies reported that some bed bug populations have become highly resistant to this group of insecticides (Boase et al. 2006, Romero et al. 2007ab, Kilpinen 2008, Yoon et al. 2008, Lilly et al. 2009). Molecular and biochemical studies suggest that target site insensitivity and metabolic detoxification are involved as resistance mechanisms to pyrethroids in some bed bug populations (Yoon et al. 2008, Romero et al. 2009b). Failure to eliminate resistant bed bugs could be a contributing factor for the spread of this pest (Romero et al. 2007b). Therefore, alternative effective insecticides for bed bug control are of great importance. Chlorfenapyr is an option that is registered for bed bug control and is increasingly being used commercially (Potter et al. 2008, Moore and Miller 2009,
Wang et al. 2009). However its effect on bed bugs has not been fully investigated under laboratory conditions. A previous study showed bed bugs did not avoid resting on chlorfenapyr–treated surfaces (Romero et al. 2009a). Chlorfenapyr is a halogenated pyrrole that disrupts mitochondrial oxidative phosphorylation (Hollingworth and Gadelhak 1998). It is a pro–insecticide that must be activated by cytochrome P450 monooxygenases to its more active metabolite (Black et al. 1994). Chlorfenapyr has proved to be an effective non–repellent insecticide against other medically important and household insects, e.g., cockroaches, ants, horn flies, subterranean termites, and mosquitoes (Ameen et al. 2000; Guglielmone et al. 2000, Buczkowski et al. 2005, Rust and Saran 2006, N'Guessan 2007). The objective of this study was to evaluate the effectiveness of chlorfenapyr against both susceptible and pyrethroid resistant strains of bed bugs. We also examined the toxicity of aged residues of chlorfenapyr to bed bugs and compared the effectiveness of two chlorfenapyr–based formulations.

**Material and Methods**

**Bed bug strains**

Insects were obtained from colonies maintained at 26º C, 65 ± 5% RH, and a photoperiod of 12:12 (L:D) h. Two strains used were highly resistant to deltamethrin (Romero et al. 2007ab): CIN–1 (collected in 2005 in Cincinnati, OH) and WOR–1 (collected in 2007 in Worcester, MA); and two were susceptible (Romero et al. 2007ab): LA–1 (collected in 2006 in Los Angeles, CA) and Fort Dix which has not been exposed to insecticides for more than 30 years (Bartley and Harlan 1974). Insects were fed in the laboratory through a parafilm–membrane feeder with citrated rabbit blood which was
heated to 39º C with a circulating water bath (Montes et al. 2002). Evaluations began 7–12d after adult emergence, and insects had not been fed as adults. We previously found more consistent results with such bed bugs than those that had more recently emerged or fed.

**Evaluations with chlorfenapyr technical grade**

Adults (1:1 sex ratio; three replicates of 20 insects) were continuously exposed to a dose of 0.5% chlorfenapyr technical grade (99.3% purity; Chem Service, West Chester, PA) that corresponded to the labeled rate of the commercial formulations. An insecticide–acetone solution of 50 µl was applied to each filter paper disc (Whatman no. 2; cut to 2.27 cm² [1.7 cm in diameter]) and allowed to dry completely before being placed in the bottom of individual cells of 24–well cell culture plates. A bed bug was placed in each cell and the culture plate was then covered. Control discs received 50 µl of acetone only. Continuous exposure to the upper surface of the filter paper was ensured by the tight fit of the paper, and by a Fluon AD–1 (polytetrafluoroethylene; Northern Products, Woonsocket, RI) coating on the walls of each cell that prevented individuals from climbing off the treated surface. Temperature was maintained at 26ºC after initiation of the exposure. Mortality was assessed daily for at least one week by gently touching each individual with an entomological forceps and categorizing bed bugs as alive (coordinated avoidance movement) or dead (no response, usually on backs with no movement of any body parts).

**Evaluations with aged residues of Phantom SC**

Adults (1:1 sex ratio; three replicates of 20 insects) were continuously exposed to filter papers that were treated with a water-based formulation of chlorfenapyr (Phantom
SC Termiticide-Insecticide, BASF, Research Triangle Park, N.C.). Discs received label rate equivalents (5 g AI L\(^{-1}\) in 50 µl of distilled water) and were allowed to dry (24 ± 2°C) for three hours (referred as fresh dry residue hereafter) or for one, two or four months. Control insects were exposed to filter paper discs treated with distilled water only. Insect mortality was recorded daily for at least seven days.

**Comparison of efficacy of two chlorfenapyr formulations**

In direct contact assays, a group of ten male (or female) bed bugs from the CIN–1 and WOR–1 strains were directly sprayed with Phantom SC or an aerosol formulation (0.5% Phantom Pressurized Insecticide; BASF, Research Triangle Park, N.C.). Groups treated with Phantom SC received two pumps with a fine mist sprayer (4 oz. fine mist spray bottle; SQB.4FMS from ProChemical and Dye, Somerset, MA) which was adequate to wet each individual. Other groups of bed bugs received two brief discharges (about 0.5 s) with the aerosol formulation. Controls consisted of sprays of distilled water or aerosol without active ingredient (blank formulation). The distance between spray nozzle and treated bugs was approximately 20 cm. Treated individuals were immediately transferred into individual wells of a 24–well cell culture plate lined with filter paper. Insect mortality was recorded 4h post–spray and then daily for at least seven days.

In residual assays, discs of filter paper were treated with 50 µl of Phantom SC, or two 0.5 sec discharges from Phantom aerosol or aerosol blank. Each of these applications left the filter paper visibly wet, but there was no runoff. These discs were allowed to dry for 3 h before they were inserted into the 24–well cell culture plate. At this time untreated individual bed bugs were added onto these filter paper discs. Observations of mortality were the same as above.
Behavioral responses to surfaces treated with chlorfenapyr aerosol

Responses of bed bugs were carried out in 500–ml glass beakers whose bottom–inside surfaces were covered with white filter paper (70 mm diam.; Fisherbrand, quality P4), henceforth referred to as arenas. Paper was fixed to the glass with double–sided tape to prevent bed bugs from crawling under the paper. After each assay, papers were removed and beakers rinsed with acetone. Individual bed bugs were offered two tents made of filter paper (15 × 12 mm, Whatman no. 2) folded in the middle to offer a tent–like shelter of 15 mm length × 5 mm height with two open ends. One tent was treated with a 0.5 sec discharge of Phantom aerosol while the other remained untreated and served as a blank. The treated tent was allowed to dry for 3 h before being placed in arenas. Forty eight individuals (1:1 sex ratio) from CIN–1 or WOR–1 were evaluated. Behavioral assays lasted about 16 h (from about 4:30 p.m. to 8:30 a.m. of the next day) with the following light–dark regimen: lights off at 6 p.m. and lights on at 6 a.m. (the same light cycle used during rearing). During the photophase, each block of 16 arenas was illuminated with a 19 W fluorescent light which was placed 60 cm above the arena surface (light intensity was approximately 300 lux at arena level). Room temperature remained at 24 ± 2º C. Insects were acclimated to the arena for 15 min by restricting them in a shell vial (21 diam. × 70 mm height) which was placed inverted in the center of the arena. Insects were released by lifting up the shell vial. At the end of each assay it was noted whether the test individual was resting on a treated or untreated tent or wandering in the arena. Number of responses was analyzed by a binomial test with exact two–tailed P values, with the null hypothesis that the tent were chosen with equal probability. Bed bugs that were wandering were not included in this analysis.
Data analysis

Time–mortality data were fitted to the probit, complementary log–log (CLL), or log–probit regression model (Robertson et al. 2007, Throne et al. 1995), to estimate the time required to kill 50% (LT$_{50}$) of the exposed insects (Throne 2009). We chose these regression models from six possible transformations based on chi–square values (lower values better describe the data) and on the examination of the fitted regression lines compared with the transformed observations (Throne et al. 1995). The LT$_{50}$ values of any two treatments with formulated insecticides were compared using lethal time ratios, as described by Robertson et al. (2007). This method was chosen because it is a more powerful method for comparison of lethal times than examination for overlap of 95% confidence intervals (Payton 2003). According to Robertson et al. (2007), two LT$_{50}$s are not significantly different if the 95% confidence interval (95% CI) of the LT$_{50}$ ratio included 1.

Results

Evaluations with chlorfenapyr technical grade

Time–mortality data from bioassays with dry residues of chlorfenapyr technical grade showed good regression fit to the probit model for the strains Fort Dix and WOR–1 ($\chi^2$ not significant; $P > 0.05$), but poorer fit for LA–1 and CIN–1 ($P < 0.05$). The rate of mortality of bed bugs exposed continuously to chlorfenapyr, measured by LT$_{50}$ values, was slower in the susceptible laboratory strain Fort Dix (LT$_{50}$ = 6.6 days [5.9–7.3]; $n = 60$; slope = 0.41± 0.048; $\chi^2 = 5.0$; df = 6) than the field–derived strains WOR–1 (LT$_{50}$ = 5.3 days [4.9–5.8]; $n = 60$; slope = 0.56± 0.056; $\chi^2 = 8.2$; df = 5), LA–1 (LT$_{50}$ = 5.8 days
[2.4–10.1]; n = 60; slope = 0.30± 0.039; χ² = 28.5; df = 5), and CIN–1 (LT₅₀ = 5.4 days [3.9–7.2]; n = 60; slope = 0.30± 0.037; χ² = 13.3; df = 6). However, no significant differences of LT₅₀s were detected among these strains.

Evaluations with aged residues of Phantom

The χ² values were not significant (P > 0.05) for 13 of the 16 time–mortality regressions, indicating good fit of data to the regression models (Table 6.1). Within each strain, LT₅₀ and LT₉₀ values estimated with mortality from assays with residues aged for different periods of time were not significant from one another (Table 6.1). Overall, all bed bug strains needed to be exposed continuously more than 3 days to dry residues in order have 50% mortality, and at least seven days to reach mortality over 90% (Table 6.1).

Comparison of efficacy of two chlorfenapyr formulations

A good fit to the regression models was observed in all assays, with the exceptions of the mortality regression of direct sprays of CIN–1 with Phantom SC and dry residue exposures of WOR–1 to the same formulation (χ² significant; P < 0.05) (Table 6.2). Aerosol treatments with chlorfenapyr, both as direct sprays and as dry deposits, consistently killed CIN–1 and WOR–1 faster than the liquid formulation (Phantom SC) (at a ratio of 1:2, approximately) (Fig. 6.1, Table 6.2). Direct sprays with aerosol had significantly shorter LT₅₀s than dry residues of the same formulation in evaluations in CIN–1 (1.2 vs. 2.6 days) and in WOR–1 (0.9 vs. 2.0 days) (Fig. 6.1). Similar rates of mortalities were observed when individuals from CIN–1 were sprayed directly with Phantom SC (LT₅₀ = 5.3 days) or when they were exposed continuously to dry residues of the same formulation (LT₅₀ = 4.9 days). In assays with WOR–1 with
Phantom SC, no significant differences were observed between LT$_{50}$s of direct sprays (LT$_{50}$ = 3.1 days) and exposure to dry residues (LT$_{50}$ = 3.3 days).

**Behavioral responses to surfaces treated with chlorfenapyr aerosol**

Individuals from strains CIN–1 and WOR–1 did not avoid resting in tents with dry deposits of chlorfenapyr aerosol (Fig. 6.2). At the end of the test period, 20% of the individuals from the WOR-1 strain were moving sluggishly in the arena, a typical symptom of chorfenapyr intoxication.

**Discussion**

Management of pyrethroid–resistant bed bugs requires selection of appropriate synergist, insecticides with new modes of action, improved formulations of existing ones, or incorporation of non-chemical control tactics (Romero et al. 2007b, Romero et al. 2009b). Addition of the synergist piperonyl butoxide to pyrethroids has been attempted to control pyrethroid–resistant bed bugs, but its effectiveness varies among populations (Romero et al. 2009b). Bed bugs with $kdr$–like insensitivity or any other resistance mechanism that is not inhibited by PBO would limit the effectiveness of this synergist (Romero et al. 2009b). Insect growth regulators (IGR), such as hydroprene and methoprene, are potential alternatives to pyrethroids. Laboratory results showed that dry residues of IGRs can cause production of infertile adults, morphological malformations, incomplete ecdysis and supernumerary nymphs in individuals treated as nymphs (Todd 2006, Naylor et al. 2008). However, IGRs are slow acting insecticides, with limited effect on adults, and are meant to be used in conjunction with other effective fast acting insecticides (Naylor et al. 2008).
Chlorfenapyr is one of the few current insecticides with a different mode of action against bed bugs. Phantom formulated as a liquid or aerosol is labeled for indoor use as a low-pressure spot or crack and crevice spray that can be applied to places where pests are found or are likely to infest. Sprays of mattresses, a common location for bed bugs, are restricted to seams, folds and edges.

Our laboratory evaluations indicate that chlorfenapyr kills bed bugs as both a contact spray and dry residue. In dry residue assays, mortality began to occur after two days of continuous exposure with about 50% of bed bugs being killed after day three. The rate of mortality was similar in all strains, regardless of their pyrethroid susceptibility status. The slower effect of chlorfenapyr on bed bugs, compared with conventional neurotoxicants such as pyrethroids on susceptible insects, can be explained by its differing mode of action. The active metabolite of chlorfenapyr (AC 303,268) inhibits the ion transport system of the respiratory chain in mitochondria, preventing the production of the energy molecule adenosine triphosphate (ATP) which leads to insect death (Hollingworth and Gadelhak 1998). Typical symptoms of chlorfenapyr intoxication in bed bugs included sluggish movements, prostration and limited responses upon contact stimulus. Such symptoms may be less apparent in commercial practice and users should understand that bed bugs treated with chlorfenapyr succumb more slowly compared to some other insecticides.

Also of importance to bed bug management are our findings concerning the duration of toxicity resulting from chlorfenapyr residues. In this study, dry residues of Phantom SC on filter paper aged indoors for one or for two and four months remained as toxic as fresh deposits. The ability of chlorfenapyr to remain effective over an extended
period of time is encouraging because bed bugs that are not sprayed directly may still succumb after residing on treated surfaces. Very few insecticides available today have appreciable potency as a dry deposit against pyrethroid resistant bed bugs. Dry residual action of chlorfenapyr may also aid in bed bug prevention if likely areas of infestation are treated before bed bugs are first introduced into a building. Duration of residual toxicity can vary depending on the rate of insecticide migration into the substrate, or degradation on the surface (Chadwick 1985). Dry residues of chlorfenapyr, for example, were more toxic to stored product pests on concrete than on vinyl tile and plywood surfaces (Arthur 2008). In contrast, sprays of chlorfenapyr caused significant mortality to Pharaoh ants on both absorbent and non–absorbent substrates (Buczkowski et al. 2005). Further study is warranted on the longevity of chlorfenapyr on wood, fabric and similar substrates commonly occupied by bed bugs.

The aerosol formulation both as a direct spray and dry residue killed faster than the water based formulation (Phantom SC). The difference in mortality rate between the two formulations could be due to greater bioavailability of the active ingredient or synergism with other ingredients in the aerosol formulation. Direct sprays with the aerosol caused rapid mortality of both pyrethroid–resistant strains (at least 30% mortality after 4 h, data not shown) and about 50% in 24 h. In contrast, direct sprays of the water based formulation took at least three days (WOR–1) or five days (CIN–1) to cause 50% mortality. Direct sprays of the blank aerosol (formulation without chlorfenapyr) killed 25% of individuals within 4 h which indicates that some formulation ingredients also have some contact activity. Chlorfenapyr aerosol also had ovicidal properties. Direct sprays of the aerosol prevented eggs from hatching (100% efficacy, data not shown)
while 100% of nymphs emerged from eggs treated with Phantom SC (A.R. unpublished data). Insecticides that kill bed bugs quickly upon contact are widely used by pest control companies because they can quickly suppress populations and provide some relief to customers (Pinto et al. 2007). Field studies suggested that Sterifab (0.22% d–phenothrin, 60.39% isopropyl alcohol; Noble Pines Products Co., Yonkers, NY) was responsible for a large population reduction after direct application to bed bug aggregations (Moore and Miller 2009). In laboratory evaluations, direct sprays of bed bugs with Bedlam (0.4% d–phenothrin, 1.6% N–octyl bicycloheptene dicarboximide, McLaughlin Gormley King Company, Minneapolis, MN) or with Sterifab also were very effective (100% mortality within 24 h). Bedlam also had some ovicidal activity (Potter et al. 2007). A limitation of contact insecticides such as Bedlam or Sterifab, however, is their lack of residual activity against pyrethroid–resistant bed bugs (A. R. unpublished data). Sprays lacking residual effectiveness as dry deposits usually require repeated applications because some bed bugs remain hidden during treatment. Subsequent applications also are needed to kill nymphs emerging from eggs, or bugs reintroduced into the building (Romero et al. 2007b).

Chlorfenapyr aerosol had residual as well as contact activity, causing mortality at least 1.5 and 3 times faster, respectively, than when chlorfenapyr was formulated as a liquid (Phantom SC). Bed bugs from strains CIN–1 and WOR–1 showed no avoidance of surfaces treated with the aerosol. Similar responses were seen with these strains in previous evaluations with chlorfenapyr and Phantom SC (Romero et al. 2009a). Continued occupancy of harborages treated with chlorfenapyr enhances exposure to the insecticide and presumably lessens the potential spread of bed bugs to adjoining areas,
which may occur with some pyrethroids and insecticides which bed bugs tend to avoid (Romero et al. 2009a). Since the residual action of chlorfenapyr appears dependent upon extended contact of bed bugs with treated substrates (A.R. unpublished data), treating known and likely harborages may be important to the success of these applications.
Table 6.1. Lethal times for four strains of bed bugs exposed to aged deposits of Phantom SC (0.5% a.i.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aging time dry residues</th>
<th>Regression Model</th>
<th>Slope ± SE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LT&lt;sub&gt;50&lt;/sub&gt; (CI 95%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>LT&lt;sub&gt;90&lt;/sub&gt; (CI 95%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>χ&lt;sup&gt;2&lt;/sup&gt; (df)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Log–CLL</td>
<td>6.51 ± 0.70</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6&lt;sup&gt;a&lt;/sup&gt; (3.2–4.1)</td>
<td>5.6&lt;sup&gt;a&lt;/sup&gt; (5.1–6.2)</td>
<td>8.3</td>
</tr>
<tr>
<td>FORT DIX</td>
<td>1 month</td>
<td>Log–CLL</td>
<td>6.86 ± 0.73</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt; (3.3–4.1)</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt; (5.0–6.1)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>Log–CLL</td>
<td>7.25 ± 0.77</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt; (3.4–4.2)</td>
<td>5.6&lt;sup&gt;a&lt;/sup&gt; (5.2–6.2)</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>Log–CLL</td>
<td>7.28 ± 0.80</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt; (3.3–4.0)</td>
<td>5.4&lt;sup&gt;a&lt;/sup&gt; (4.9–5.9)</td>
<td>3.1</td>
</tr>
<tr>
<td>WOR-1</td>
<td>Fresh</td>
<td>Log–Probit</td>
<td>4.74 ± 0.49</td>
<td>4.0&lt;sup&gt;a&lt;/sup&gt; (3.5–4.5)</td>
<td>7.4&lt;sup&gt;a&lt;/sup&gt; (6.3–9.2)</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>1 month</td>
<td>Log–Probit</td>
<td>4.10 ± 0.41</td>
<td>3.8&lt;sup&gt;a&lt;/sup&gt; (3.3–4.4)</td>
<td>7.8&lt;sup&gt;a&lt;/sup&gt; (6.5–9.9)</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>Log–Probit</td>
<td>4.20 ± 0.43</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt; (2.7–5.0)</td>
<td>7.5&lt;sup&gt;a&lt;/sup&gt; (5.5–13.1)</td>
<td>16.4*</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>Log–Probit</td>
<td>3.90 ± 0.40</td>
<td>3.5&lt;sup&gt;a&lt;/sup&gt; (3.0–4.1)</td>
<td>7.4&lt;sup&gt;a&lt;/sup&gt; (6.2–9.7)</td>
<td>3.5</td>
</tr>
<tr>
<td>LA-1</td>
<td>Fresh</td>
<td>Log–logit</td>
<td>8.04 ± 0.93</td>
<td>4.0&lt;sup&gt;a&lt;/sup&gt; (3.5–4.6)</td>
<td>7.5&lt;sup&gt;a&lt;/sup&gt; (6.4–9.5)</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>1 month</td>
<td>Log–logit</td>
<td>9.38 ± 1.07</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt; (3.2–4.7)</td>
<td>6.7&lt;sup&gt;a&lt;/sup&gt; (5.5–9.5)</td>
<td>13.1*</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>Log–logit</td>
<td>7.37 ± 0.86</td>
<td>3.6&lt;sup&gt;a&lt;/sup&gt; (3.1–4.2)</td>
<td>7.2&lt;sup&gt;a&lt;/sup&gt; (6.0–9.3)</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>Log–logit</td>
<td>5.82 ± 0.70</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt; (2.6–5.1)</td>
<td>8.8&lt;sup&gt;a&lt;/sup&gt; (6.1–17.1)</td>
<td>13.2*</td>
</tr>
</tbody>
</table>
Table 6.1 (Continued)

<table>
<thead>
<tr>
<th></th>
<th>Log–logit</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>8.26 ± 0.94</td>
<td>4.8a</td>
<td>8.9a</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4.2–5.5)</td>
<td>(7.6–11.1)</td>
<td>(8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN–1</td>
<td>8.64 ± 0.97</td>
<td>4.8a</td>
<td>8.6a</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td>(4.2–5.4)</td>
<td>(7.4–10.6)</td>
<td>(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 months</td>
<td>9.24 ± 1.05</td>
<td>4.9a</td>
<td>8.5a</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4.4–5.5)</td>
<td>(7.4–10.4)</td>
<td>(8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 months</td>
<td>8.0 ± 0.92</td>
<td>4.6a</td>
<td>8.8a</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4.1–5.3)</td>
<td>(7.4–11.1)</td>
<td>(7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Residues were allowed to dry for three hours before insect exposure

<sup>b</sup>SE = standard error

<sup>c</sup>LT<sub>50,90</sub> = time necessary to kill 50% or 90% of individuals

<sup>d</sup>CI 95% = 95% confidence interval

<sup>e</sup>df = degree of freedom

*Significant (P < 0.05) (goodness–of–fit test)

The LT<sub>50</sub> or LT<sub>90</sub> values within strains are not significantly different from one another (P > 0.05) following method used by Robertson et al. (2007).
Table 6.2. Time–mortality regression estimates for bed bugs exposed to different formulations of Phantom.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CIN-1</th>
<th>WOR-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formulation</strong></td>
<td><strong>Type of Exposure</strong></td>
<td><strong>Regression model</strong></td>
</tr>
<tr>
<td>SC</td>
<td>Direct spray</td>
<td>Log–CLL</td>
</tr>
<tr>
<td></td>
<td>Dry residue</td>
<td>Log–CLL</td>
</tr>
<tr>
<td>104</td>
<td>Direct spray</td>
<td>Log–CLL</td>
</tr>
<tr>
<td></td>
<td>Dry residue</td>
<td>Log–CLL</td>
</tr>
</tbody>
</table>

ᵃ Values for goodness–of–fit of the regression models to the observed mortality data.

* Significant (P < 0.05) (goodness–of–fit test).
Figure 6.1. Toxicity of Phantom Aerosol and Phantom SC applied to adult bed bugs of two pyrethroid resistant strains, as direct contact sprays and as dry residues. The bars within strains with the same letter are not significantly different from one another (P>0.05) following method used by Robertson et al. (2007).
Figure 6.2. Preference of individual bed bugs from two strains for a paper tent treated with Phantom aerosol or a blank (control) tent. After a 16 h test period, the place where the insect was found was recorded (resting on insecticide, blank tent, or wandering in the arena). Asterisk indicates significant differences between the control and insecticide–treated harborages (Binomial test, $P < 0.05$). Wandering insects were not included in the analysis.
Chapter Seven: Conclusions and future directions

Few insects have resurged and spread throughout the United States at the rate that we have seen with bed bugs over the past 10 years. Bed bugs nearly disappeared after 1960, halting almost all research on this insect. As a result, a tremendous gap now exists in our knowledge of its basic biology as well as in applied research that could help to manage this pest. The most recent information, produced in the 80s and 90s, addressed the potential for bed bugs to transmit disease agents of high human health impact. Therefore, this dissertation attempts to fill part of that knowledge gap by the study of several aspects of the biology of bed bugs and by testing the efficacy and behavioral effects of insecticides currently used to treat infestations of this pest.

In Chapter 2, I showed that locomotor activity of bed bugs is periodical, endogenously generated by circadian clocks, and can be entrained by light environmental cycles. These findings not only contribute to information about the functional properties of the circadian system in bed bugs, but also provide insights on the evolution of an endogenous circadian clock that is well adapted to a diurnally active host. Future studies in this area might contrast the biological rhythms of activity of C. lectularius from human environments or bird roosts with other populations of this species infesting caves, or populations of bat bugs inhabiting caves.

I also show that activity of bed bugs fluctuates as insects alternate between states of food satiation and deprivation. Activity is reduced in satiated insects but would increase in prolonged periods of absence of the host. If the period of food deprivation is even longer, bed bugs would progressively engage in an energy saving strategy in which activity is reduced. These results provide insights about insect movement in natural
infestations. I suggest that bed bugs in recently vacated places would actively go in search of a host. These initial periods of activity could lead to relocation of bed bugs within the infested areas or to their dispersal into uninfested adjoining areas. If food deprivation does become prolonged, a reduction in insect activity would occur, limiting further dispersal. Re-occupancy of infested places with very hungry bed bugs would, most likely, generate an immediate host seeking response.

Research on insecticides is of paramount importance considering today’s scenario for bed bug control. First, the pest control industry has few chemical options with different modes of action to eliminate infestations; second, there is more caution about treatments in areas where human exposure is possible; and third, government regulatory institutions are more restrictive in the registration process of new insecticides, which make this process very expensive for registrants and the pest control industry. Little was known about the efficacy of insecticides currently used against today’s bed bugs. Previous to the initiation of this dissertation, data about the efficacy of pyrethroids was generated using susceptible bugs that had been maintained in a laboratory >20 yrs (Todd 2006). Bed bug colonies reared over long periods in the laboratory are inbred and loose original genetic characteristics over time so that they may not represent modern natural bed bug populations. Natural bed bug populations most likely were introduced into the United States from many different countries, having many differing chemical histories and selection pressures. Thus, it was imperative in my work to conduct insecticide evaluations on as many field-collected strains as possible. During 2005, I initiated bed bug colonies from samples collected in infested places in Kentucky and Ohio. Then, I standardized an artificial membrane system in the laboratory that allowed me to feed
dozens of bed bugs and establish several colonies. I used a dry residue bioassay to
evaluate dose-response analysis with deltamethrin and λ-cyhalothrin, two of the most
commonly used insecticides in the United States. These evaluations showed a dramatic
difference in susceptibility to deltamethrin between a reference susceptible laboratory
strain and four field-derived colonies. To my knowledge this is the first ever report of
pyrethroid resistance in *C. lectularius*. Furthermore, the resistance ratio estimated for four
colonies (RR >12,000) appears to be of the same order of magnitude as the highest levels
of resistance seen with other species of insects. Intermediate levels of resistance of the F1
offspring of matings between a highly pyrethroid resistant strain and a susceptible strain
revealed the genetic basis of the detected resistance. A discriminating dose test allowed
rapid testing for resistance in nearly 18 populations across the country, with results
indicating that deltamethrin resistance seems to be widespread in the United States. As
DDT-resistance was reported years ago in bed bugs, failure of pyrethroids in today’s
populations could be related to cross resistance of these insecticides and DDT.
Alternatively, pockets of susceptible bed bugs present in the United States or introduced
from other countries might have developed resistance upon insecticide exposure and
further spread. Future studies should evaluate more bed bug populations so that a better
picture about status of susceptibility to pyrethroids can be obtained. Nevertheless, I
predict that populations with high resistance to pyrethroids will predominate, given the
high chemical pressure to which bed bug populations are subjected today and the ease in
which bed bugs spread.

Management of insecticide resistance in bed bugs requires information about the
resistance mechanisms that are involved. Presence of more than one resistance
mechanism to insecticides is not uncommon in arthropod populations. I obtained insights about the potential resistance mechanisms involved in pyrethroid resistance in bed bugs by using the P450 inhibitor, piperonyl butoxide (PBO). I detected residual resistance after PBO treatment in two highly-pyrethroid resistant bed bug strains, suggesting that other resistance mechanisms not mediated by P450s (e.g., target site insensitivity, decreased cuticular penetration) might be involved. Further evaluations with piperonyl butoxide–synergized pyrethroid formulations showed limited usefulness of these formulations against some pyrethroid resistant bed bug populations. Future research needed in this area includes molecular and biochemical studies to determine the presence or absence of target-site insensitive mechanisms or other insecticide detoxifying metabolic systems. Quantitative expression (concentration of enzymes) or qualitative differences (structure of enzymes) in metabolic enzymes needs to be investigated. The frequency and the relative importance of the above resistance mechanisms will determine the potential of other synergists for effective control of resistant bed bugs. Studies have shown, however, that synergists give only temporary relief from resistance problems, and selection of insect populations to develop resistance to the combination of synergist and insecticide can occur (Roush and Daly 1990).

The presence of target site insensitivity or penetration resistance will prompt the use of insecticides with modes of action different from pyrethroids. Because chlorfenapyr targets mitochondrial phosphorylation processes in insects, use of this insecticide is an option. Chlorfenapyr might be especially useful against pyrethroid resistant strains because resistance is caused, in part, by P450s that activate the pro-insecticide, chlorfenapyr. Thus, if P450s happen to be primarily responsible for the detoxification of
pyrethroids, populations with increased activity of these enzymes would have enhanced ability to activate chlorfenapyr versus other strains. Chlorfenapyr was effective against pyrethroid-resistant strains. In addition, dry residues had long residual activity and were not avoided by bed bugs. These two characteristics are advantageous when it comes to controlling bed bug infestations. Insecticides that leave toxic residues might reduce the number of applications required to eliminate infestations so that they can be controlled more efficiently and with less human exposure to insecticides. As has happened with other insecticides, however, the continued use of chlorfenapyr or any other chemical option in the future could lead to development of insecticide resistance in bed bug populations. Thus, development of tactics to prevent, delay, or manage insecticide resistance in bed bug populations is required.

In Chapter 3, I showed that bed bugs avoided resting on areas treated with deltamethrin but not with chlorfenapyr. However, a more complex interaction between bed bugs and insecticides could occur in natural infestations. Thus, I used a set of behavioral assays that took into account several factors that can influence the responses of bed bugs to dry residues of insecticides. For example, results showed that bed bugs did not avoid resting on areas treated with deltamethrin if these areas contained feces and body secretions. Similarly, the presence of a nearby heat source did not prevent bed bugs from crossing insecticide barriers. These findings suggest that application of insecticides around areas where hosts rest might not be effective to prevent bed bug bites. Contact with surfaces treated with sublethal doses of deltamethrin caused irritation in bed bugs, which might explain dispersion of bed bugs from treated to untreated locations in adjacent areas. This behavioral effect should be considered when selecting insecticides
for bed bug management. Current insecticides or those being develop in the future should be tested for other behavioral or biological effects on bed bug populations. It would be important to understand how insecticides affect circadian rhythms of locomotor activity of bed bugs, as well as host seeking, mating, egg-laying, egg-hatching and molting.

I hope that results of this dissertation will encourage other researchers to study bed bugs. More basic and applied knowledge is critical to addressing the problem that bed bugs represent to society. Of particular urgency is the development of effective and affordable monitoring/detection tools. Without an effective method of monitoring, it will be difficult to detect infestations in the earliest stages when bed bugs are more easily eliminated. Detection tools are also needed to know if an infestation has been completely eliminated. Finally, the incorporation of alternative tactics in bed bug management programs might also help slow down the dispersal of this insect and prevent it from becoming a routinely encountered pest, as it was prior to the middle of the 20th century.

Introduction

An early review by Burton (1963) reported that bed bugs are able to harbor more than 40 pathogenic agents. However, despite the fact that many of these pathogens can survive months in bed bugs, there is no scientific evidence that infections can pass to humans through the bite of the insect. On the other hand, because some pathogens are excreted in bed bug feces, there is the potential for mechanical transmission. This mode of transmission is widely recognized in two arthropod-borne disease systems: body lice (*Pediculus humanus humanus*) that vector epidemic louse-borne typhus (*Rickettsia prowasekii*); and kissing bugs (Reduviidae), which transmit Chagas disease (*Trypanosoma cruzi*) (Edman 2000). These pathogens gain entrance into human hosts when infected insects defecate near abraded skin or through autoinoculation by scratching into the skin the contents of a crushed insect or feces. Blow et al. (2001) suggested that bed bugs can be a mechanical vector of Hepatitis B virus because infective viral particles were excreted in feces for months. This hypothesis is plausible in bed bugs as these insects often defecate either while feeding or after engorgement when leaving the feeding area (A. R. personal observation).

For hematophagous arthropods to have the capability to vector pathogens, they first must acquire the microorganism (Vaughan and Azad 1993). Once ingested, the viability of the microorganism and the possibility to be transmitted by host insect would primarily depend on: 1) the ability of microorganism to overcome the action of the insect
immune system and 2) its ability to colonize and establish in insect tissues (Lehane 2005). Few studies have investigated the fate of pathogens in the bed bug body or whether replication can occur. In the last decades, some research has been devoted to determine whether disease agents of public health importance, such as the human immunodeficiency virus (HIV) and hepatitis B virus (HBV), have the capability to replicate in bed bugs or be excreted in their feces (Jupp and McElligott 1979, Webb et al. 1989, Blow et al. 2001). Currently, information is lacking on these two characteristics of the relationship between microbial agents (e.g., protozoan, bacteria, nematodes) and bed bugs. This information is important because of 1) the resurgence of bed bugs in recent years, particular in urban areas of the world, and 2) an increased number of vector-borne protozoan and bacterial diseases in urban areas (Harrus and Baneth 2005), with the potential to be ingested by bed bugs.

*Borrelia hermsii* was used as our experimental organism to study bed bug-bacteria interactions. *Borrelia hermsii* is part of a group of closely related bacteria that causes Relapsing Fever (RF) in humans and many other mammals (CDC 2007). Relapsing fever spirochetes include *B. hermsii, B. turicatae, B. parkeri* and *B. duttoni* and have soft ticks and body lice as vectors for maintenance and transmission to humans (Dworkin et al. 2002). Earlier studies showed that orally-ingested spirochetes (species name was not reported) remained viable in the gut content of bed bugs and also appeared in hemolymph during the first 48 h post-feeding (Dunn 1923). However, in the same study, examinations of bed bugs on day 15 post-feeding detected spirochetes only in hemolymph. If spirochetes survive the conditions of the hemolymph, there is potential for them to localize in insect organs.
I provided bed bugs infectious blood meals with a membrane feeding system which has an advantage over animal models in that hundreds of bed bugs can be simultaneously fed with a known concentration of infectious agents.

The primary goal of this study was to provide information about the persistence of orally-ingested *Borrelia* within bed bugs. For this, I used a direct and culture-dependent approach so that viability of spirochetes could be determined.

**Material and Methods**

**Insects**

Insects were obtained from a colony maintained at 27º C, 50 ± 5% RH, and a photoperiod of 12:12 (L:D) h. The strain of bed bugs was collected in 2005 from an infested apartment in Cincinnati (OH, U.S.A.). Insects are fed weekly with a parafilm-membrane feeder containing citrated rabbit blood which is heated to 40°C with a circulating water bath (Montes et al. 2002).

**Bacterial strain and inoculum**

The *B. hermsii* isolate DAH used for this study was obtained from frozen aliquots. The isolate was originally procured from Dr. Tom Schwan (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT, U.S.A). *Borrelia hermsii* was subcultured in 10 ml of BSK-II medium (Barbour 1984) containing 12% rabbit serum (Pel-Freez Biologicals, Rogers, AR, U.S.A.) and incubated at 34ºC. Densities of *B. hermsii* in cultures were quantified as follows: 5 µl of bacterial suspension was diluted into 95 µl of 0.11 M sodium citrate, pH 7.2, and cells counted under dark-field microscopy using a Petroff-Hausser chamber. When cultures contained densities of $10^3$ –
10^{-5} \text{ bacteria/ml, } \sim 100 \, \mu\text{l of suspension was subcultured at 34° C in 10 ml of BSK-II medium to obtain more active and infectious spirochetes. Numbers of spirochetes were assessed beginning day 2 after inoculation and then daily until a concentration of 4.2 \times 10^{7} \text{ spirochetes/ml was reached (4-5 days, approximately). Higher concentrations were not used because it was observed that motility and viability of bacteria in cultures decreased over time. One ml of bacterial suspension was mixed with 3 ml of 0.3\% sodium citrate rabbit blood for a final spirochete concentration of 1.05 \times 10^{7}/\text{ml.}}

**Feeding of insects**

Groups of 15-day-old unmated bed bugs (adult females and adult males) were fed separately on bacteria infected blood for 15 min. Bed bugs were fed on non-infective blood meals for 3 weeks post-infection and kept in the environmental chamber at 26° C and 50 ± 5 \% RH. A group was fed on non-infective rabbit blood as a control group. Each fed individual was placed in individual cells of 24–well cell culture plates (Corning Incorporated, Corning, NY) lined with filter paper. Plates were maintained at 26° C and 50 ± 5\% RH. Unless otherwise stated, examination for spirochete infection took place at 1h and 4h post-feeding on the first day, and then once daily until day 7. After day seven, bugs were examined weekly for two additional weeks.

**Examination of hemolymph**

Each time, hemolymph from six adult bed bugs (1:1 sex ratio) was examined for the presence of spirochetes. Hemolymph from each individual was obtained by amputating the tibial segment of an appendage and a smear from the hemolymph (∼ 0.5 \mu\text{l was examined under dark field microscopy. Hemolymph from other appendages of the same individual was collected on sterilized filter paper (0.5 x 0.3 cm) and placed in BSK-}}
II medium containing 12% rabbit serum. In order to reduce contamination with other bacteria, we added rifampin (0.04 µg per ml of BSK-II) to the medium. Addition of this antimicrobial agent to BSK-II medium has shown to increase the isolation rate of *Borrelia* spirochetes from field samples because this antibiotic eliminates rifampin-susceptible microorganisms (Nelson et al. 1991). We used culturing methods to increase the likelihood of detecting spirochetes in bed bug bodies.

**Examination of whole bodies and gut contents**

Six bed bugs were surface sterilized in 70% of ethanol for one min, rinsed in sterile water, then submerged in 0.05% sodium hypochlorite for one min, and rinsed three times in sterile water. Each bed bug was homogenized in an eppendorf vial in 0.5 ml phosphate buffer saline (pH 7.2). The suspension (~0.2 ml) was transferred into BSK medium containing 12% rabbit serum and rifampin. In other groups of surface sterilized bugs, the intestinal tracts were dissected and at least 1 µl of intestinal content was obtained for examination under dark field microscopy.

**Results and Discussion**

In this study, a total of 234 previously unfed bed bugs were fed on blood containing *B. hermsii*. They were observed to be fully engorged within 15 min of exposure to blood. Mortality post-feeding was minimal and it was comparable to that of uninfected bed bugs (< 2%). Since female and male bed bugs took in on average of 7.0 and 4.2 µl of blood, respectively, it is estimated that each insect ingested a total of 7.4 to 4.4 × 10^4 bacteria. During the three week monitoring period, we did not detect spirochetes in fresh samples of hemolymph or their cultures (Table 1). In some samples,
however, spirillum-like microorganisms with intense motility were observed. When I cultured hemolymph from spirillum-positive individuals, there was no evidence of spirochete growth. Contrary to our results, earlier studies reported the presence of relapsing fever spirochetes (species are not reported) in the hemolymph of bed bugs that had fed on infected mice (Dunn 1923, Nohira 1928). Dunn (1923) examined bed bugs 7 and 15 days post-feeding and found spirochetes in hemolymph, but not in gut content.

In contrast to hemolymph, direct examination of gut contents in our study showed the presence of spirochetes. Detection of this bacterium under dark field microscopy was facilitated by the characteristic screw-like motion produced by the bacterium’s flagellum which propels the organism (Fig. A-1). All gut content samples examined 1 and 4 h post-feeding were positive for spirochetes. However, in examinations of feces one day after feeding, the proportion of positive individuals was reduced to half (Table A-1). No spirochetes were observed at 48 and 72 h and further evaluation of fecal samples was discontinued because numerous intestinal gut microorganisms made detection of spirochetes difficult. In general, spirochetes in the positive samples were present in scant number (one or two spirochetes); we observed them only after analysis of several subsamples, and exhaustive examination of the Petroff-Hausser counting chambers.

Detection of spirochetes in gut-content but not in hemolymph in this study, suggests that: 1) that the insect’s digestive physiology (e.g., peritrophic membrane, Vaughan and Azad 1993) might have prevented B. hermsii from migrating into the hemolymph, 2) spirochetes crossed the peritrophic membrane and readily localized in insect tissues, disappearing from hemolymph, or 3) an immunological response eliminated any spirochetes that may have reached the hemolymph. The possibility of
colonization and establishment of spirochetes in insect tissues can be ruled out as media inoculated with whole bodies were negative for spirochetes after day 1 (Table A-1). Contrary to other studies that report longer survival of relapsing fever borrelia within bed bugs (e.g., *B. duttoni* [at least 150 d], *Spirochateta (Borrelia) merionesi* [200 d]) (Burton 1963), our results indicate that the environment in gut or within bed bug body is not suitable for survival or persistence of *B. hermsii*.

In conclusion, *B. hermsii* spirochetes that were orally-ingested by bed bugs persisted in gut content or insect body for up to one day. These observations provide evidence that *B. hermsii* may be present in the body of bed bugs for up to 24 h if this bacterium has been ingested while feeding on an infected human or animal host. These results raise the question about the potential for transmission of this disease agent by bed bugs. Our results suggest that the bed bug is an improbable vector for this spirochete. While the pathogen was detected in feces for 24 h, defecation would likely only occur on the original bacteremic host because only one meal is required for satiation. Similarly, a crushed bed bug would only be infective if it fed and then moved to an uninfected host within 24 h. Because bed bugs will feed a second time only after a minimum of 4 days, and the bacteria only persists in the bed bug or its feces for less than 2 d, transmission from host to host seems unlikely.
Table A-1. Detection of *Borrelia* spirochetes in bed bugs after receiving an infected blood meal.

<table>
<thead>
<tr>
<th>Time after feeding</th>
<th>Hemolymph Direct</th>
<th>Hemolymph Culture</th>
<th>Feces</th>
<th>Whole body</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>Neg.</td>
<td>Neg.</td>
<td>100% (6/6)</td>
<td>33.3% (2/6)</td>
</tr>
<tr>
<td>4 h</td>
<td>Neg.</td>
<td>Neg.</td>
<td>100% (6/6)</td>
<td>16.6% (1/6)</td>
</tr>
<tr>
<td>1 d</td>
<td>Neg.</td>
<td>Neg.</td>
<td>50% (3/6)</td>
<td>16.6% (1/6)</td>
</tr>
</tbody>
</table>

*Samples from 3, 4, 6, 7, 14, and 21 d were negative.*
Appendix A-1. *Borrelia hermsii* spirochetes in BSK-II medium (dark field microscopy, 100X).
Appendix B: Aggregation activity induced by methanol feces-extracts of the bed bug, *Cimex lectularius* L. (Heteroptera: Cimicidae).

Introduction

Locomotor activity of bed bugs occurs mostly during the night (Chapter 2) and this activity has been associated with host-and harborage seeking behavior (Usinger 1966, Mellanby 1939). After foraging, bed bugs return to harborages along mattress and box springs, or cracks, crevices or edges of furniture and other locations. Very often, different developmental stages of bed bugs are found aggregated in clusters, and in contact with their accumulated fecal matter, egg shells, and exuviae. Earlier studies suggested that bed bugs are driven by scent gland odors and feces to return to these aggregations (Marx 1955). Schildknecht (1964) used chromatography to study the chemical composition of scent gland secretions and identified the compounds \( \Delta^2 - n\)-hexenal and \( \Delta^2 - n\)-octenal. Then, Collins (1968) reported that, in addition to these compounds, bed bugs produce ethanal and 2-butatone; however the biological importance in regard to aggregation behavior was not determined. In the early 1970s, Levinson et al. (1974) discovered electrophysiological activity of \( \Delta^2 - n\)-hexenal and \( \Delta^2 - n\)-octenal, but they were not able to demonstrate that these compounds had a role in aggregation behavior of bed bugs. Instead, these compounds promoted dispersion in assembled bed bugs, leading the investigators to suggest a role in alarm communication (Levinson et al. 1974).

Levinson and Bar Ilan (1971) showed that bed bugs aggregated under paper discs previously exposed to adult conspecifics and methanol extracts of these papers were also behaviorally active. Similar results were also reported by Parashar et al. (2003) with
water and methanol extracts of feces of the tropical bed bug *Cimex hemipterus* (Hemiptera: Cimicidae). More recently, studies showed that airborne pheromone (Siljander et al. 2008) and contact pheromone (Siljander et al. 2007, Olson et al. 2009) mediated aggregation of bed bugs. Even though there is evidence of a contact pheromone in bed bugs, the identity of its components remains unknown.

The present report shows results of initial activities of a long-term project aiming at isolating and identifying semiochemicals associated with bed bug aggregation. By using behavioral bioassays, I was able to demonstrate that bed bugs aggregate under papers exposed to bed bugs, and that methanol extracts the active compounds that stimulate aggregation. Similarly, I showed that a field-collected strain responded positively to aggregating factors of a long-maintained laboratory strain. Further characterization of behavioral responses of different life stages and identification of aggregating factors from harborages are currently underway in the context of a cooperative effort with private industry.

**Material and Methods**

**Insects**

Colonies of bed bugs were maintained at 27º C, 50 ± 5% RH, and a photoperiod of 14:10 (L:D) h. One colony was collected from human dwellings in Cincinnati, OH (CIN-1), while the other was the long-maintained laboratory strain, Fort Dix, which has not been exposed to insecticides for more than 30 years (Bartley and Harlan 1974). Insects were fed with a parafilm-membrane feeder containing citrated rabbit blood which was heated to 40º C with a circulating water bath (Montes et al. 2002). Behavioral assays
began 7–12d after emergence, and adults or nymphs (fifth-instar) had not been fed after emergence.

**Exposure of paper tents to bed bugs**

Tents made of filter paper (15 × 12 mm, Whatman no. 2) folded in the middle were exposed in individual cells of 6–well cell culture plate (Corning Incorporated, Corning, NY) (well diam. 3.48 cm, height 2 cm) to 5 recently fed fifth instar nymphs, 5 mated male, and 5 mated female bed bugs. Plates with insects were kept in the same rearing conditions for 48 h.

**Methanol extractions from exposed tents**

Tents exposed as above were submerged in 1.5 ml methanol (HPLC-grade) in a glass vial for ca. 2 h at room temperature. Vials were mixed every 30 minutes and the supernatant was withdrawn with a pipette and used immediately.

**Behavioral assays**

Assays were carried out in 500–ml glass beakers whose bottom–inside surfaces were covered with white filter paper (70 mm diam.; Fisherbrand, quality P4), henceforth referred to as arenas. Paper was fixed to the glass with double–sided tape to prevent bed bugs from crawling under the paper. After each assay, papers were removed and beakers rinsed with acetone. Individual bed bugs were offered two tents made of filter paper (15 × 12 mm, Whatman no. 2) folded in the middle to offer a shelter that was15 mm length × 5 mm height with two open ends. One tent corresponded to the test material (tents exposed to bed bugs or tents treated with 50 μl of methanol extracts of tents exposed to bed bugs) while the other was the control tent (untreated tent or a tent treated with 50 μl of methanol extract of unexposed tents). Unless otherwise stated, 24 individuals were
evaluated. Behavioral assays lasted about 16 h (from about 4:30 p.m. to 8:30 a.m. of the next day) with the following light–dark regimen: lights off at 9 p.m. and lights on at 7 a.m. (the same light cycle used during rearing). During the photophase, each block of 9 or 16 arenas was illuminated with a 19 W fluorescent light which was placed 60 cm above the arena surfaces (light intensity was approximately 300 lux at arena level). Room temperature remained at 24 ± 2º C. Insects were acclimated to the arena for 15 min by restricting them in a shell vial (21 diam. × 70 mm height) which was placed inverted in the center of the arena. Insects were released by lifting up the shell vial. At the end of the test I recorded whether the bed bug was resting on a treated or untreated tent or wandering in the arena. Number of responses was analyzed by a binomial test with exact two–tailed $P$ values, with the null hypothesis that the tent were chosen with equal probability. Bed bugs that were wandering were not included in this analysis.

**Results and Discussion**

Tents that had been exposed to mixed stages of bed bugs were significantly more attractive to adult female (92.6%), adult male (85.2%) or fifth instar nymph bed bugs (77.8%) than tent controls ($P < 0.05$) (Fig. B-1). These results support previous evidence that harborage products of *C. lectularius* promote aggregation (Levinson and Bar Ilan 1971, Parashar 2003, Siljander et al. 2007, Olson et al. 2009). Feces-aggregating factors have also been demonstrated in German cockroaches (Ishii, 1970) and blood-sucking kissing bugs (Lorenzo and Lazzari 1996). Aggregation activity induced by fecal material has been exploited to enhance trap catch of German cockroaches (Miller et al. 2000). Aggregation induced by feces may benefit individuals by protecting them from predators,
by increasing mating opportunities and by reducing the impact of desiccation stress (Wertheim et al. 2005, Benoit et al. 2007). In addition, aggregation causes individuals to get in closer contact with feces, favoring the acquisition of symbionts among different life stages of insects (Beard et al. 1998).

Levinson and Bar Ilan (1971) reported that the level of aggregation of bed bugs depended on the sex of the tested individual and also the sex and number of bugs that previously occupied the papers that had been exposed to bed bugs. In the Levinson and Bar Ilan study, one hundred percent assembling responses were observed in assays where insects were presented papers that had been exposed to 100 bed bugs (1:1 sex ratio), the maximum number of bed bugs that Levinson and Bar Ilan (1971) used to impregnate tents. Results from Levinson and Bar Ilan (1971) and those reported here agree that aggregation in bed bugs is greatly elicited when the source of the aggregating is feces and/or body secretions from different individuals. However, Levinson and Bar Ilan (1971) reported that fifth instar nymphs did not respond to papers previously exposed to mixtures of adults of both sexes. This suggests that nymphal specific factors are present in feces and they are necessary to elicit aggregation in fifth instar nymphs. Interestingly, Siljander et al. (2007) showed evidence for a nymph derived pheromone that elicited attraction in nymphs but not males or females. In addition, they showed that the assembling factors of bed bugs are perceived only by contact chemoreception (Siljander et al. 2007).

As reported by others, methanol efficiently extracted aggregating factors present in papers exposed to mixed-stages of bed bugs. Tents impregnated with methanol extracts from feces of the Fort Dix strain was significantly preferred by females (87.0%) and
males (94.7%) of the same strain ($P < 0.05$) (Fig. B-2). Levinson and Bar Ilan (1971) were able to extract an adult-specific contact pheromone from feces with methanol. Parashar (2003) found that methanol and water efficiently extracted aggregation pheromone from feces of mixed stages of *C. hemipterus*. Siljander et al. (2007) extracted a stage-specific contact pheromone and determined that females responded also to contact pheromone of males, but not the contrary.

I also evaluated whether the methanol extracts of harborage products from mixed-stages of Fort Dix (long-maintained laboratory strain) would elicit aggregation in adults from CIN-1, a strain collected four years ago. Although CIN-1 adults preferred resting on tents treated with methanol extracts of Fort Dix individuals (Fig. B-3), a significant level of preference was obtained with CIN-1 males (86.4%) ($P < 0.05$) but not with CIN-1 females (69.6%) ($P = 0.09$). Aggregation responses of field-collected strains to harborage products of long-maintained laboratory strains was also reported by Olson et al. (2009), indicating that these strains are an adequate source for aggregating pheromone. I conclude that the methanol extracts of bed bug feces are a good starting point to pursue the isolation and identification of the components of aggregation pheromones. Isolation and identification of biologically-active aggregating factors and their incorporation into monitoring systems could improve the management of bed bugs.
Figure B-1. Response of female, male, and nymph bed bugs of the Fort Dix strain in choice tests to tents previously exposed to mixed-stages of the same strain or to unexposed tents. Tents were exposed to 5 recently fed females, males and fifth instar nymphs during the 48 h before assays. After a 16 hour-test period, the location of the insect, either resting on one of the treatment harborage or wandering in the arena was recorded. Twenty-seven individuals from each life stage were tested. Asterisk indicates significant differences between exposed and unexposed tents ($P < 0.05$).
Figure B-2. Response of female and male bed bugs of the Fort Dix strain in choice tests to tents impregnated with methanol extracts of tents previously exposed to mixed-stages of the same strain. Tents were exposed to 5 recently fed females, males and nymphs during the 48 h before assays. After a 16 hour-test period, the location of the insect, either resting on one of the treatment harborages or wandering in the arena was recorded. Twenty-four individuals from each sex were tested. Asterisk indicates significant differences between exposed and unexposed tents ($P < 0.05$).
Figure B-3. Response of female and male bed bugs of the CIN-1 strain in choice tests to tents impregnated with methanol extracts of tents previously exposed to mixed-stages of Fort Dix. Tents were exposed to 5 recently fed females, males and nymphs during the 48 h before assays. After a 16-hour-test period, the location of the insect, either resting on one of the treatment harborages or wandering in the arena was recorded. Twenty-four individuals from each sex were tested. Asterisk indicates significant differences between exposed and unexposed tents ($P < 0.05$).
References


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