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Chlorfenapyr and Bifenthrin Susceptibility Monitoring of Field Collected Bed Bug Populations from the United States

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Chlorfenapyr and Bifenthrin Susceptibility Monitoring of Field Collected Bed Bug Populations from the United States

For the degree of Master of Science

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Head of the Departmental Graduate Program Date
CHLORFENAPYR AND BIFENTHRIN SUSCEPTIBILITY MONITORING OF
FIELD COLLECTED BED BUG POPULATIONS FROM THE UNITED STATES

A Thesis
Submitted to the Faculty
of
Purdue University
by
Aaron R. Ashbrook

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td><strong>CHAPTER 1. LITERATURE REVIEW</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Cimicids, bed bug biology, and life history</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Traumatic insemination and fitness costs</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Bed bug movement and aggregations</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Resurgence of the common bed bug</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Integrated control of bed bg infestations</td>
<td>5</td>
</tr>
<tr>
<td>1.4 Insecticide resistance and monitoring</td>
<td>9</td>
</tr>
<tr>
<td>1.5 Bed bug insecticide resistance</td>
<td>11</td>
</tr>
<tr>
<td>1.6 Chlorfenapyr and Bifenthrin</td>
<td>12</td>
</tr>
<tr>
<td>1.7 Significance and rationale</td>
<td>14</td>
</tr>
<tr>
<td><strong>CHAPTER 2. EVALUATION OF THE GLASS VIAL BIOASSAY TECHNIQUE FOR BED BUG SUSCEPTIBILITY MONITORING</strong></td>
<td>16</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>17</td>
</tr>
<tr>
<td>2.2 Materials and methods</td>
<td>20</td>
</tr>
<tr>
<td>2.2.1 Insects</td>
<td>20</td>
</tr>
<tr>
<td>2.2.2 Chemicals</td>
<td>20</td>
</tr>
<tr>
<td>2.2.3 Glass vial assay</td>
<td>21</td>
</tr>
<tr>
<td>2.2.4 Filter paper assay</td>
<td>22</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table                                      Page

2.1  Table represents the insecticide concentrations used for bioassays. Insecticide concentrations are measured in µg AI/cm². ................................................................. 23

2.2  Results of LC bioassays for each bioassay technique using Harlan strain adult male bed bugs. Formulated chlorfenapyr vial and filter paper results were scored at days 7 and 14, respectively. Bifenthrin bioassay results were scored at 3 days after exposure…... 27

2.3  Results of LC bioassays for each technique using Harlan strain adult male bed bugs. Technical chlorfenapyr vial and filter paper results were scored at days 7 and 14, respectively. Bifenthrin bioassay results were scored at 3 days of exposure................ 28

3.1  Table represents the known information of assayed bed bugs. Population name, year collected, and treatment history or known resistance of the bed bugs. Insects with unknown treatment histories were likely exposed to pyrethroids, neonicotinoid/pyrethroid combination insecticides, or chlorfenapyr. ................................................................. 44
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>13</td>
</tr>
<tr>
<td>2.1</td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td>33</td>
</tr>
<tr>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>
same letter are significantly different (Tukey’s test $P < 0.05$). Error bars represent ±SE values. For each strain (y-axis), 100 adult males were tested for each strain.

3.1 Results of the glass vial bioassays at the diagnostic LC$_{99}$ concentration for chlorfenapyr which corresponded to a 0.5% solution (556 µg AI per vial or 7.87 µg AI/cm$^2$). Bars represent percent mortality at 7 d (168 h) post treatment. Bars that are not connected by the same letter are significantly different (Tukey’s test ($P < 0.0001$). Data for Richmond and Washington D.C. strains are adapted from Chapter 1.

3.2 Results of the glass vial bioassays at the diagnostic LC$_{99}$ concentration for bifenthrin which corresponded to a 0.96x10$^{-4}$% (0.1 µg AI per vial or 0.0014 µg AI/cm$^2$). Bars represent percent mortality at 3 d (72 h) post treatment. Bars that are not connected by the same letter are significantly different (Tukey’s test ($P < 0.0001$). Data for Richmond and Washington D.C. strains are adapted from Chapter 1.
ABSTRACT

Ashbrook, Aaron R. M.S., Purdue University, December 2015. Chlorfenapyr and bifenthrin susceptibility monitoring of field collected bed bugs from the United States. Major Professors: Ameya D. Gondhalekar and Gary W. Bennett.

Complete and effective elimination of common bed bug (*Cimex lectularius*) infestations continues to be a challenge for the pest management industry. However, effective bed bug control can be achieved through integrated pest management (IPM) programs that use a variety of control techniques. An integral component of an IPM program is the type of insecticide applied. However, insecticide products for bed bug control are somewhat limited as resistance to some pyrethroid insecticides, such as deltamethrin has developed. Currently, chlorfenapyr and bifenthrin are two insecticides approved for bed bug control. Chlorfenapyr is a pro-insecticide from the pyrrole class. Bifenthrin is a type-I pyrethroid that can be applied indoors, but it is also available to the general public and therefore has the potential to be misused for bed bug control. Insecticide resistance is an inevitable consequence of widespread and continuous insecticide application when the proper strategies are not implemented. If bed bugs develop resistance to chlorfenapyr and bifenthrin, it would significantly inhibit the effective management of these notorious pests. Notwithstanding, the susceptibility levels of field bed bug populations to chlorfenapyr and bifenthrin have not been determined on a large scale. Due to the impending threat of insecticide resistance, the primary goal of this research was to screen bed bug populations from across the United States for chlorfenapyr and bifenthrin susceptibility. To screen the field populations, a diagnostic bioassay-based susceptibility monitoring program was developed and implemented.
Filter paper and glass vial bioassay methods were compared for different formulations (technical grade and formulated product) of chlorfenapyr and bifenthrin to determine the most suitable assay and formulation for susceptibility monitoring. For each treatment type, lethal concentration (LC$_{50}$ and LC$_{99}$) estimates were determined with the insecticide-susceptible Harlan strain. Statistical comparison of PROBIT analysis data for bioassay techniques found significant differences in toxicity ratios at the LC$_{50}$ and LC$_{99}$ levels. Based on the toxicity ratios and bioassay duration, glass vial bioassays using formulated insecticide product appeared more effective for chlorfenapyr and bifenthrin susceptibility monitoring. The glass vial diagnostic LC$_{99}$ concentrations for chlorfenapyr (556 µg/vial) and bifenthrin (0.1 µg/vial) were validated using the Harlan-Susceptible and field-collected (Washington D.C. and Richmond, VA) bed bug populations. Subsequently, eight additional field bed bug populations collected from geographically different regions around the U.S. were screened for chlorfenapyr and bifenthrin susceptibility with the diagnostic glass vial assay. Three of the field collected bed bug populations were resistant to chlorfenapyr when compared to the Harlan susceptible strain. Five of the ten populations had significantly different bifenthrin susceptibilities when compared to the Harlan strain at the LC$_{99}$ concentration. These findings demonstrate the need for continuous susceptibility monitoring of bed bug populations to ensure insecticides remain effective.
CHAPTER 1. LITERATURE REVIEW

1.1 Cimicids, bed bug biology, and life history

The common bed bug, *Cimex lectularius* Linnaeus, is a member of an exclusively parasitic family, Cimicidae, which contains less than 100 species (Stutt and Siva-Jothy 2001, Reinhart and Siva-Jothy 2007, Balvin et al. 2012). One unifying characteristic of this family is that its members are all obligate blood feeders of vertebrates, feeding at least once per nymphal stage in order to grow to the next life stage (Benoit et al. 2007, Reinhart and Siva-Jothy 2007, Haynes et al. 2010, Balvin et al. 2012). Cimicids usually have 5 nymphal stages prior to adulthood, and *C. lectularius* can develop to adulthood in just over one month (Reinhart and Siva-Jothy 2007, Kolb et al. 2008). Cimicid host range is determined by their ability to detect a host, puncture the epidermis, take a blood meal, and digest it (Reinhart and Siva-Jothy 2007, Benoit 2011). Only three members of the Cimicidae feed on humans: *C. lectularius, C. hemipterus*, and *Leptocimex bouti* (Pfeister et al. 2009, Benoit 2011, Balvin et al. 2012). The widespread distribution of *C. lectularius*, is likely due to their broad host range feeding on humans, bats, rabbits, rodents, chickens and many different birds (Reinhart and Siva-Jothy 2007, Benoit 2011, Balvin et al. 2012).

Bed bugs are usually nocturnal feeders, but may alter their behavior based on host availability (Benoit et al. 2007, Reinhart and Siva-Jothy 2007, Suchy and Lewis 2011). Like most blood feeding insects, bed bugs locate hosts through cues such as carbon dioxide, heat, sweat, and other human pheromones (Reinhart and Siva-Jothy 2007, Pfeister et al. 2010, Suchy and Lewis 2011). Bed bugs prefer to feed on extremities and hairless areas of hosts, feeding for approximately 10-20 minutes until they are engorged with blood (Axtell and Arends 1990, Reinhart and Siva-Jothy 2007).
1.1.1 Traumatic insemination and fitness costs

Traumatic insemination, a unique mating behavior, is another characteristic of cimicids (Reinhart and Siva-Jothy 2007, Pfeister et al. 2009, Haynes et al. 2010). The copulation event of traumatic insemination occurs when the male’s intermittent organ pierces an abdominal groove on the female called the mesospermalege (Stutt and Siva-Jothy 2001, Harraca et al. 2010). Once fed and mated, a female bed bug lays between 5-7 eggs a day and up to 500 eggs in her lifetime (Reinhart and Siva-Jothy 2007, Kolb et al. 2008, Calianno 2012). Conversely in absence of feeding or mating, an adult female stops producing eggs in approximately 35-50 days (Reinhart and Siva-Jothy 2007).

Although female bed bugs have adaptations to reduce the effects of traumatic insemination, there can be fitness costs associated with this form of copulation (Reinhart and Siva-Jothy 2007, Reinhardt et al 2008, Benoit et al. 2012). After feeding, male bed bugs will attempt to mount any large blood engorged insects in an attempt to mate (Reinhart and Siva-Jothy 2007, Harraca et al. 2010, Suchy and Lewis 2011). Males will mount other males and nymphs, therefore, as a defensive response; pheromones are released to repel the mounting bed bug (Harraca et al. 2010). The size of the blood meal prevents adult females from fending off adult males from their antagonistic mating behavior (Reinhart and Siva-Jothy 2007, Harraca et al. 2010).

Overall, female bed bugs may have reduced fitness because of traumatic insemination. It may lead to reduced egg production, wound healing, and death due to desiccation or over mating (House and Lewis 2007, Benoit et al. 2012). Experimentally, researchers have determined that life span female bed bugs can be reduced by up to 25% if over mated (Reinhardt et al. 2003). One defensive behavior adult females exhibit to avoid over mating is fleeing harborage areas (Reinhardt et al. 2003, Reinhart and Siva-Jothy 2007, Pfeister et al. 2009). In real-world settings, the tendency of females to flee population focal points may lead to the spread of bed bug infestations within or between homes and apartments. Other research suggests that female bed bugs are the most active individuals as they were readily found in climb-up traps (Wang et al 2010). Although passive dispersal is the most common mechanism that leads to bed bug spread, they are
also known to actively disperse (Reinhart and Siva-Jothy 2007, Wang et al 2010, Calliano 2012).

1.1.2 Bed bug movement and aggregations

Nymphs actively forage for food due to the high energetic requirements for molting; conversely, adults may be sedentary due to lower energetic demands (Kolb et al. 2008, Reinhart and Siva-Jothy 2007, Wang et al. 2010). In multi-unit housing, bed bugs move through entry doorways, wall voids, and electrical outlets to apartments above, below, or adjacent to the infestation, in order to reduce competition or hunger, which further complicates control efforts (Reinhart and Siva-Jothy 2007, Pfeister et al. 2009, Wang et al. 2010). Bed bugs are well-known for their ability to move passively as “hitch-hikers”. Hitch-hiking occurs when a host comes in contact with infested furniture, poultry facilities, or leaves personal belongings in an infested area and then transports them to a new location (Axtell and Arends 1990, Reinhart and SIVA-Jothy 2007, Steelman et al. 2008, Wang et al. 2010). Passive movement allows bed bugs to remain sedentary in protected areas to avoid dehydration (Benoit et al. 2007). Bed bugs may also disperse on birds or bats and create new colonies (Reinhart and Siva-Jothy 2007).

Aggregations of bed bugs, especially nymphs, spend most of their time remaining sedentary in order to reduce water loss (Benoit et al. 2007, Siljander et. al. 2008, Pfeister et al. 2009). Bed bug groups are maintained by aggregation pheromones and a positive response to physical contact i.e., thigmotaxis (Reinhart and Siva-Jothy 2007, Pfeister 2009 et al.). The bed bug aggregation pheromone is a complex blend of 10 components, mainly consisting of (E)-2-hexenal and (E)-2-octenal; however, the exact ratios are unknown (Reinhart and Siva-Jothy 2007, Siljander et al. 2008, Pfeister 2009 et al.). On the contrary, at a high enough concentration, (E)-2-hexenal and (E)-2-octenal have dual purposes of functioning as the bed bug alarm pheromone (Siljander et al. 2008, Weeks et al. 2010). Interestingly, after mating, female bed bugs do not respond to the aggregation pheromone, which may help explain their exodus from colonies (Siljander et al. 2008, Pfeister et al. 2009). Usually, bed bug aggregations are equally composed of nymphs,
adult females and adult males, but the population dynamics can shift towards adults if the infestation is aged or over-wintering (Reinhart and Siva-Jothy 2007, Pfeister et al. 2009).

### 1.2 Resurgence of the common bed bug

The resurgence of the bed bug as an urban pest has been ascribed to inexpensive travel, a growing market for second hand items, lack of awareness, their cryptic behavior, and insecticide resistance (Reinhart and Siva-Jothy 2007, Seong et al. 2010, Adelman et al. 2011). However, resistance to insecticides and incomplete elimination are the only explanations for the bed bug resurgence supported by research (Boase 2008). Incomplete elimination of an infestation may stem from application of chemicals that do not possess a broad spectrum of insecticidal activity and lack of technician experience or training in bed bug control (Weeks et al. 2010). Overall, effective elimination of bed bug infestations can be very challenging and expensive.

Bed bug infestations negatively impact human health by causing a variety of conditions, such as increased anxiety, worsening of mental conditions, lack of sleep, paranoia, and bite lesions (Reinhart and Siva-Jothy 2007, Romero et al. 2007, Wang et al. 2010, Zhu et al. 2010, Bai et al. 2011, Davies et al. 2012). Health risks associated with scratching bite lesions is infection by secondary pathogens (Bai et al. 2011, Davies et al. 2012). Bed bugs have been found to carry harmful microorganisms, but they do not vector any diseases (Reinhart and Siva-Jothy 2007, Weeks et al. 2010, Calianno 2012, Campbell and Miller 2015).

Bed bug infestations can create undesirable economic consequences due to costs associated with their eradication and, in severe cases, disposal of infested furniture is required for achieving adequate control (Hwang et al. 2005, Reinhart and Siva-Jothy 2007, Potter et al. 2008, Steelman et al. 2008). Due to bad publicity, infestations of bed bugs are also problematic for the tourist industry and hospitals, especially when individuals have sought monetary compensation for being subjected to infestations (Reinhart and Siva-Jothy 2007). In poultry facilities, bed bug infestations can decrease egg productivity, reduce egg quality due to fecal stains, increase bird feed requirements, hamper growth, cause severe anemia, and even death if an infestation is large enough.
Bed bugs are also a concern for homeless shelters and public housing due to their limited resources and residential challenges that hinder effective elimination (Hwang et al. 2005, Bennett et al. 2015).

1.3 Integrated control of bed bug infestations

Initial introduction of bed bugs into multi-family housing or a large building often goes undetected and spreads quickly due to lack of resident experience or variable bite reactions (Kells 2006, Wang et al. 2009, 2010). The lack of ability to detect low-level infestations or new introductions allows the bed bugs to spread and establish within a building or housing complex. When an infestation is established, bed bugs are problematic to eliminate because inspections often do not locate all insects (Wang et al. 2009, 2010, Steadfast and Miller 2014, Bennett et al. 2015). In order to gain control of an infestation, pest management professionals (PMPs) treat an infestation multiple times (Kells 2006, Potter et al. 2008). Due to bed bug elimination complications, it is essential for the PMPs to integrate non-chemical control techniques as well as chemical applications for effective control (Kells 2006, Wang et al. 2010, Steadfast and Miller 2014, Bennett et al. 2015). It is important to note that complete bed bug elimination cannot be attained by insecticide application as the only control measure (Kells 2006). The use of alternative techniques in combination with pesticide application is a part of an integrated pest management (IPM) program, which has been shown to be effective for bed bug elimination (Wang et al. 2010).

Integrated pest management is different than traditional chemical based control because it relies upon non-chemical elimination, education of residents, pest life cycle, and the pest’s interactions with its environment to make decisions for pest control (Brenner et al. 2003, Bennett et al. 2015). The adoption of IPM approach can also lead to management or mitigation of insecticides resistance development through the use of alternative control strategies and techniques (Hoy 1998). The use of IPM techniques reduces the selection pressure of an insecticide and therefore alters or reduces the frequencies of resistance genes (Roush and Daly 1990). Thus, resistance management is a
sub-component of IPM protocols and vice versa (Croft 1990). However, IPM strategies for the management of a pest will vary based on the setting. For example, in an agricultural setting, insecticides are applied when the population reaches an economic injury level (Hoy 1998). Since the population threshold for bed bugs is one gravid female, a low-level or incipient infestation requires immediate attention (Axtell and Arends 1990). Therefore in an urban setting the only IPM techniques available for the management of resistance are the use of non-chemical control techniques, insecticide synergists, insecticide mixtures, and product rotations. Many non-chemical control techniques are available for bed bug control (Kells 2006). Although non-chemical control techniques cannot be relied upon by themselves to completely control an infestation (Wang et al. 2012), when many techniques are combined with an insecticide, much greater control is achieved (Brenner et al. 2003). Reduction in harborage and exclusion are important non-chemical control techniques, but more are discussed later in this section (Brenner et al. 2003, Wang et al. 2012, 2014).

When an insecticide applied with a synergist (e.g., piperonly butoxide; PBO), the potency of an insecticide is increased because a resistance mechanism of an insect is blocked or reduced (Roush and Daly 1990). The resulting effect is increased potency of an insecticide and elimination of both susceptible and resistant individuals from the population (Roush and Daly 1990). In order for a synergist to be effective it must be affordable, stable, have low mammalian toxicity, and the pest must not develop resistance to the synergist insecticide combination (Roush and Daly 1990). Additionally a synergist depends upon the existence of one resistance mechanism, which is rarely the case for a pest population (Roush and Daly 1990).

Mixture formulations result in the exposure of individuals to more than one toxicant (Hoy 1998). If resistance frequencies in a population to each of the insecticides are low, it is highly unlikely that an individual will be resistant to both of the active ingredients (Roush and Daly 1990). The overall effect is that resistance to one of the insecticides is negated by the application of another insecticide (Roush and Daly 1990). Mixture formulations are controversial as they are used once a population is already resistant to a chemical and the long term effects are unknown (Roush and Daly 1990).
The rotation of insecticides is achieved by exposing a population to two or more insecticides with different modes of actions separated by times (Hoy 1998). The individuals of a population experience one chemical selection pressure at a given interval, but over time, experience multiple chemicals (Hoy 1998). Since the insecticides have different modes of action, it is likely that selection pressures for resistance to a specific insecticide are reduced (Roush and Daly 1990). It is possible that rotation of insecticides only slows development of resistance for enough time to allow for another product to be created (Hoy 1998). No matter the drawbacks of any IPM technique, the single tactic approach is unsustainable (Hoy 1998). It is also important to consider IPM and resistance management as things that work together (Hoy 1998). Luckily, there are many different sub-components of urban IPM techniques that can be used for bed bug control and elimination of infestations.

An important first step in an IPM program is the monitoring and early detection of infestations (Boase 2001, Kells 2006, Wang et al. 2010). The use of climb up traps allow bed bug populations to be better detected early, protect furniture from further infestation, and can help determine overall efficacy of control efforts (Boase 2001, Kells 2006, Wang et al. 2010). Climb up traps are a plastic double walled dish that can be placed under the legs of furniture and bed frames. The inner surfaces of climb up traps are slick to prevent insects from escaping and may also contain desiccant dusts. However, climb up traps may sometimes get dirty or full, allowing insects to escape the trap (Wang et al. 2010). Climb up traps are essential for effective IPM programs by providing a mechanism to monitor populations while also causing bed bug mortality (Wang et al. 2010).

The use of silica dust and diatomaceous earth (DE) has been a safe and effective component of bed bug control (Boase 2001, Kells 2006, Wang et al. 2010, Stedfast and Miller 2014). Dusts damage the insect cuticle, which may cause desiccation (Stedfast and Miller 2014). Diatomaceous earth can be safely applied by residents and staff to many items (Stedfast and Miller 2014). When paired with vacuuming, the use of dusts or DE can be used as a proactive treatment technique for the prevention of bed bugs in low income housing (Kells 2006, Stedfast and Miller 2014). Mattress encasements are also an effective way to trap bed bugs on a mattress and make them visible for vacuuming or
removal (Kells 2006, Stedfast and Miller 2014). However, if a mattress encasement gets torn or punctured, they become useless (Potter et al. 2008).

Freezing of infested items is another non-chemical approach used for bed bug control. But temperature requirements to cause instant death in bed bugs are quite low at their super cooling point of -20°C (Kells 2006, Benoit et al. 2009). However, bed bugs do die at temperatures higher than this, but they must be frozen long enough to ensure mortality (Kells 2006, Benoit et al. 2009).

In the late nineteenth century, a steam treatment machine was developed for bed bug control, since then portable steam machines have been updated and used to spot-treat infested areas (Kells and Goblirsch 2011, Potter 2011, Schrader and Schultz 2011, Puckett et al. 2013). To a certain extent steam penetrates cracks and furniture, but is more effective in killing all bed bug life stages when in aggregations (Kells 2006, Pereria et al. 2009, Puckett et al. 2013, Steadfast and Miller 2014). Application of steam treatments is quite labor intensive, and therefore, other heating methods are typically used (Kells 2006, Puckett et al. 2013). Heating of an entire home or infested rooms has become a common bed bug control method. Use of lethal heat can be effective for managing large bed bug infestations. Whole home heating is achieved by directly circulating heated air for several hours (Benoit et al. 2009, Kells and Goblirsch 2011). Infested furniture and items may also be put into large portable sealed heated chambers to eliminate bed bugs (Kells 2006, Kells and Goblirsch 2011).

There are, however, some drawbacks to heat treating; for example, bed bugs may flee and take shelter in cooler wall voids, deep in furniture, or neighboring unheated areas (Doggett et al. 2006, Pereria et al. 2009). Fleeing bed bugs can re-infest previously treated homes and stunned insects can fall into protected areas and then recover (Schrader et al. 2011). Relative to other insects, bed bugs are tolerant to heat exposure, with individuals surviving heat exposures of up to 50°C (Kells 2006, Benoit et al. 2009, Kells and Goblirsch 2011). Large scale heat treatments are time intensive, costly, can damage heat sensitive items, and have high energy requirements (Kells and Goblirsch 2011).

In addition to non-chemical methods, chemical control techniques (i.e. insecticide applications) are preferred by PMPs for bed bug control (Gangloff-Kauffman et al. 2006,
Despite the preference for insecticide application, few products are registered for directly treating mattresses and box springs (Potter et al. 2008). The inability to treat mattresses and box springs with insecticides limits effective control as bed bugs typically harbor in human resting areas (Potter et al. 2008). Many PMPs have been using pyrethroid or combination insecticides for bed bug remediation because organophosphate and carbamate insecticides are not available for indoor application (Moore and Miller 2008, Potter et al. 2008, Kells and Goblirsch 2011, Gordon et al. 2014). More troubling is the repeated use of insecticides with similar modes of action or a single class of pesticides, such as pyrethroids, has selected bed bug populations to evolve multiple resistance mechanisms (Zhu et al. 2012). A recent study has shown that bed bugs can develop resistance to combination products when exposed to laboratory selection, but such resistance is most likely linked to the pyrethroid component of the formulation (Gordon et al. 2014). There are few effective insecticides for bed bug control, and resistance issues are widespread among bed bug populations.

1.4 Insecticide resistance and monitoring

Resistance is defined as “the inherent ability in a strain of a pest to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population” (Tabashnik et al. 2014). Insecticide resistance has a genetic basis and may be widespread in a population, patchy, or rare (Tabashnik et al. 2014). Selection pressures from continuous chemical application can kill susceptible individuals from the population while the tolerant individuals of the population are able to survive, thus increasing the resistant genotype frequencies within a population. Resistance that results in loss of insecticide efficacy is called “practical resistance” due to its real world pest control implications (Tabashnik et al. 2014). It is much more advantageous to detect insecticide resistance in a population at low levels so that resistance management methods can be implemented before control failures occur (Busvine 1971, ffrench-Constant and Roush 1990, Varela et al. 1993).

Detection of insecticide resistance can be achieved by comparing a susceptible strain to a field strain that may or may not have control failures occurring (Tabashnik et
al. 2014). Comparisons are done via a bioassay, an experiment that determines the potency of an insecticide measured by reference to a standardized or susceptible insect colony (Busvine 1971). It is important that concentration mortality data be generated with the susceptible strain for PROBIT analysis, determining LC$_{50}$ and LC$_{99}$ diagnostic concentrations (Busvine 1971, ffrench-Constant and Roush 1990, Gondhalekar 2011). Validation of diagnostic concentrations on the susceptible strain will ensure that the concentration is accurate and that no time or individuals of a field population are wasted in resistance detection efforts (ffrench-Constant and Roush 1990). When a field population is screened at the LC$_{99}$ diagnostic concentration, the exposure method and bioassay technique should be relevant to field control (ffrench-Constant and Roush 1990). When possible, different bioassay types should be compared for large scale resistance screening (Busvine 1971, ffrench-Constant and Roush 1990). Pest arthropods come in all shapes and sizes, as do bioassay types and techniques.

Many bioassay techniques exist for testing the development of insecticide resistance or determining arthropod insecticide resistance mechanisms. *In vivo* assays allow for the documentation and identification of an insecticide resistant strain, which should occur before advanced bioassay techniques (ffrench-Constant and Roush 1990). Depending on the test organism, different *in vivo* assays can be used, such as a residual contact assay, feeding, leaf- dip assay, injection, and topical application of an insecticide (ffrench-Constant and Roush 1990). The use of different assays allows for the determination of toxicity parameters, such as lethal time, lethal concentration, or lethal dose (Cochran 1997). *In vivo* assays with different surface types should be compared for accuracy and field relevancy for future screening programs, given that the choice is critical for insecticide resistance monitoring (ffrench-Constant and Roush 1990, Fletcher 1993).

The use of different surfaces in residual bioassays among bed bugs studies, chemical trials, susceptibility, or resistance tests make the results difficult to compare between each other (Stubaker 2003). The World Health Organization has created standardized assays for many nuisance and health pests and has suggested that a glass vial assay with a treated filter paper strip inside be used for bed bug resistance monitoring.
Alternatively other researchers have also used a filter paper dish assay and a glass vial assay for bed bug susceptibility tests (Romero et al 2007, Steelman et al 2008).

1.5 Bed bug insecticide resistance

Bed bug populations have a history of developing resistance to insecticides. Bed bug infestations were relatively common until the end of World War II (Boase and Naylor 2014, Palenchar et al. 2015). Application of DDT and other organochlorines were effective in eliminating a vast majority of bed bug populations, and PMPs reported profit loss associated with great chemical efficacy (Boase 2008, Reinhart and Silva-Jothy 2007, Palenchar et al. 2015). By 1958, bed bugs were reported to be resistant to DDT, which targets the sodium channels of neurons (Brogdon and Mcallister 1998, Boase and Naylor 2014). Years later, organophosphate resistance was identified as bed bug infestations sporadically increased between the 1970s and 1980s (Reinhart and Silva-Jothy 2007, Boase and Naylor 2014). However, the causal factor in the global bed bug pandemic may be the development of insecticide resistance to the pyrethroids, which was identified in the last decade (Romero et al. 2007, Boase 2008, Boase and Naylor 2014, Planchar et. al. 2015).

Resistance to two pyrethroids, deltamethrin and \( \lambda \)-cyhalothrin, was identified using the filter paper assay (Romero et al. 2007). When the same resistant strain was exposed to piperonyl butoxide (PBO) in conjunction with deltamethrin, the insecticide caused greater bed bug mortality (Romero et al. 2009). Since PBO inhibits cytochrome P450’s, an enzyme that detoxifies chemicals, we can conclude that in this case, detoxification is an important component in bed bug resistance to deltamethrin (Adelman et al 2011, Zhu et al. 2011). However, Romero et al. (2007) accurately suggested that increased detoxification of insecticides was likely not the only mechanism of pyrethroid resistance at work in these populations.

A resistance mechanism known as knock-down resistance (kdr), results from decreased sensitivity of the target site for pyrethroids, the sodium ion channel (Adelman
et al. 2011). It is likely that the development of DDT resistance conferred cross resistance to pyrethroids since they share the same target site (Romero et al. 2007). One study found that deltamethrin resistance in bed bugs is caused by two point mutations (V419L and L925I) in the alpha sub-unit of the voltage-gated sodium channel (Yoon et al. 2008, Adelman et al. 2011). A population survey of 110 bed bug field strains from around the United States found that 88% of the strains surveyed had either one or both of these target site mutations (Zhu et al. 2010, Adelman et al. 2011). Population surveys in Australia and Israel also found these point mutations in bed bug populations (Palenchar et al. 2015). However, not all deltamethrin resistant bed bugs have the V419L and L925I mutations, therefore additional insecticide resistance mechanisms must exist in bed bug populations (Yoon et al. 2008, Zhu et al. 2010, Adelman et al. 2011).

Pyrethroid resistance mechanisms may also occur in the bed bug integument via gene up-regulation of cytochrome P450s, metabolic enzymes, cuticular proteins, and ABC transporters (Zhu et al. 2013). Increased cytochrome P450 production in the bed bug epidermis can significantly reduce insecticide penetration and could be considered a form of metabolic resistance (Mamidala et al. 2012). In order to eliminate bed bugs that may have multiple pyrethroid resistance mechanisms, PMPs are employing new non-pyrethroid insecticides for controlling infestations.

1.6 Chlorfenapyr and bifenthrin

Chlorfenapyr is a pyrrole group insecticide that is popular among PMPs because of its unique mode of action and high efficacy against bed bugs (Romero et al. 2010, Davies et al. 2012, Boase and Naylor 2014). Chlorfenapyr is a pro-insecticide, which means that it is converted to its toxic metabolite \textit{in vivo} and is generally safe for mammals. Chlorfenapyr is converted to its toxic metabolite CL 303268 or tralopyril by an insect cytochrome P450s. Activation occurs by oxidative removal of the N-ethoxymethyl group from the compound (Romero et al. 2010, Raghavendra et al. 2011). The addition of the alkyl group to the pyrrole nitrogen atom has greatly reduced the high
mammalian toxicity and phytotoxicity that would be caused by the activated product CL 303268 (Dekeyser 2005).

Figure 1.1 The image depicts the chemical structure of chlorfenapyr (left). Highlighted in red is the group that is removed from the chemical once converted by an insect cytochrome P450. The compound on the right is the toxic metabolite of chlorfenapyr also known as tralopyril.

The bioactive toxic metabolite disrupts mitochondrial oxidative phosphorylation, ultimately preventing the formation of ATP (Romero et al. 2010, Raghavendra et al. 2011). Oxidative phosphorylation is disrupted by the uncoupling of ATP production. Once uncoupled, ATP production proceeds unregulated and oxygen is rapidly depleted. The active metabolite of chlorfenapyr works because it is very hydrophobic and once inside the inner mitochondrial membrane, it destroys the proton gradient around by making it no longer energetically favorable. Since chlorfenapyr is activated by cytochrome P450 action, insecticide resistant bed bugs with increased cytochrome P450 activity are expected to be more sensitive to chlorfenapyr (Romero et al. 2010). However, this may not be the case since bioassays found no mortality differences in screened populations, regardless of the pyrethroid resistance levels in bed bugs (Romero et al. 2010). According to the product label, chlorfenapyr can be applied indoors to mattress seams, folds, and edges as well as a crack and crevice treatment (Romero et al. 2010). Chlorfenapyr residues can stay effective for up to 4 months; however, it can take approximately 9-10 days to kill bed bugs in filter paper bioassays (Romero et al. 2010).
Bifenthrin is a type-I pyrethroid, lacking the alpha-cyano group, and is an active ingredient in certain professional insecticide formulations (Talstar®), and it is also an neonicotinoid/pyrethroid combination product (Transport®) (Johnson et al. 2010). Bifenthrin is a contact insecticide that affects insects by keeping the para-homologous sodium channels open, thus causing neuro-excitation (Fecko 1999, Johnson et al. 2010). Bifenthrin can be applied indoors and in poultry facilities. However, bifenthrin cannot be applied to mattresses, and indoor application is limited to baseboards, box springs, bed frames, underside of shelves and drawers, as well as cracks and crevices. Since bifenthrin is available to the general public as an active ingredient in Ortho Home Defense, as well as other products, it has the potential to be over-used and/or misused, creating an increased potential for resistance development.

1.7 Significance and rationale

Like many problematic pest species, bed bugs are capable of becoming resistant to insecticides that are continuously used for their control (Lofgren et. al. 1958, Romero et. al. 2007, Adelman et. al 2011). Organochlorines (DDT) and certain pyrethroids such as deltamethrin and λ-cyhalothrin are some examples of insecticides to which some bed bug populations have already developed resistance (Lofgren et. al. 1958, Romero et. al. 2007, Seong et. al. 2010, Adelman et. al 2011). Given the inevitable risk of resistance development, it is likely that widely used insecticides such as chlorfenapyr and bifenthrin may lose their efficacy in the future. Cross-resistance from other pyrethroid insecticides is also likely to speed up the development of bifenthrin resistance (Romero et. al. 2007, Steelman et. al. 2008, Adelman et. al 2011). Development of chlorfenapyr and bifenthrin resistance in bed bugs could have significant impacts on overall bed bug management as there are only a handful of other insecticide options available for indoor use against bed bugs. Also, because both chlorfenapyr and bifenthrin are relatively safe, it would be advantageous to be able to continue to use these insecticides for the management of bed bug infestations.
One proven technique for delaying the onset of resistance is insecticide resistance management. Before resistance management strategies can be implemented it is important to determine insecticide susceptibility levels in field populations of the target insect pest (Brent 1986, ffrench-Constant and Roush 1990). However, to date, chlorfenapyr and bifenthrin susceptibility levels in bed bug field populations are unknown. Therefore, the central goal of this study is to develop and implement a diagnostic bioassay-based susceptibility monitoring program to measure the chlorfenapyr and bifenthrin susceptibility levels in bed bug field populations.

The rationale for determining chlorfenapyr and bifenthrin resistance/susceptibility in bed bug field populations is that once these data are available previously established resistance management techniques can be implemented without wasted effort; such as the use of non-chemical techniques, insecticide rotations and addition of synergists to formulations. The implementation of resistance management recommendations will delay the onset of insecticide resistance to both chlorfenapyr and bifenthrin. In addition, the bioassay methods and diagnostic concentrations for chlorfenapyr and bifenthrin generated in this study could serve as standard techniques for bed bug resistance detection. Furthermore, ready-to-use resistance detection kits could be developed for field use by PMPs. In this regard, glass vials treated with diagnostic insecticide concentrations (included in the resistance detection kits) could be used by PMPs to test field bed bug populations. Use of the glass vial assay would allow PMP’s to choose an effective insecticide when dealing with a bed bug infestation, resulting in more effective bed bug control. The central goal of this study will be achieved by the completion of the following two objectives:

Objective 1: Develop a simple and reliable diagnostic concentration-based bioassay method for bifenthrin and chlorfenapyr susceptibility monitoring of bed bugs.
Objective 2: Determine bifenthrin and chlorfenapyr susceptibility levels in bed bug field populations using the diagnostic bioassay.
CHAPTER 2. EVALUATION OF THE GLASS VIAL BIOASSAY TECHNIQUE FOR
BED BUG SUSCEPTIBILITY MONITORING

Abstract

Bed bugs can quickly become resistant to insecticides that are continuously or
improperly used for their control. Chlorfenapyr and bifenthrin are examples of some of
the commonly used insecticides for bed bug management. However, the susceptibility
levels of field bed bug populations to chlorfenapyr and bifenthrin are not yet know.
Initially we compared different bioassay methods (filter paper and glass vial bioassays)
and insecticide formulations (technical grade and formulated product) for their suitability
in chlorfenapyr and bifenthrin susceptibility monitoring. For both bioassay types,
formulations and insecticides, we determined lethal concentration (LC$_{50}$ and LC$_{99}$)
estimates using the insecticide-susceptible Harlan strain. Statistical comparison of
PROBIT analysis data between the two bioassays techniques found significant
differences in toxicity ratios at the LC$_{50}$ and LC$_{99}$ levels, which were likely due to
insecticide absorption by the filter papers. Based on the toxicity ratios and bioassay
duration, glass vial bioassays using formulated insecticide product were more effective
for chlorfenapyr and bifenthrin susceptibility monitoring. The glass vial diagnostic LC$_{99}$
concentrations for chlorfenapyr (556 µg /vial) and bifenthrin (0.1 µg /vial) were validated
using the Harlan-Susceptible and field-collected (Washington D.C., and Richmond, VA)
bed bug strains. Statistical comparisons of the validation assays confirm previous
findings that the glass vial assay is a simple and accurate method for monitoring
insecticide resistance in various insects and this technique can be effectively used for
chlorfenapyr and bifenthrin susceptibility monitoring in bed bugs.
2.1 Introduction

At the turn of the century the common bed bug, *Cimex lectularius*, resurged on a global scale as a major urban pest (Hwang et al. 2005, Romero et al. 2010, Wang et al. 2010, Davies et al. 2012). Bed bugs (*Cimex lectularius* L.) are blood-feeding ectoparasites mainly found in human dwellings, but have also been reported to infest poultry houses (Fletcher 1997, Reinhart and Siva-Jothy 2007, Steelman et al. 2007). Bed bug infestations and their bites negatively impact humans by causing anxiety, lack of sleep, paranoia, and bite lesions (Reinhart and Siva-Jothy 2007, Romero et al. 2007, Wang et al. 2010, Zhu et al. 2010, Davies et al. 2012). The current expansion of bed bugs is attributed to increased international travel, acquisition of infested items, and insecticide resistance (Reinhart and Siva-Jothy 2007, Seong et al. 2010, Adelman et al. 2011). Controlling bed bugs is difficult and costly because multiple insecticide treatments are required to achieve adequate control of infestations, and in severe cases, disposal of infested furniture is required (Hwang et al. 2005, Reinhart and Siva-Jothy 2007, Steelman et al. 2007, Potter et al. 2008). The cryptic behavior of bed bugs makes them difficult to manage. Approximately 76% of surveyed pest management professionals (PMPs) considered *C. lectularius* as the most difficult urban pest to control (Potter et al. 2008). Infestations of bed bugs can be eliminated by integrating chemical (insecticides) and non-chemical management techniques (Hwang et al. 2005, Potter et al. 2008, Wang et al. 2009), which also mitigates insecticide resistance risk (Gordon et al. 2014).

Insecticide resistance is “the inherent ability of a strain of a pest to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population” and has greatly contributed to the resurgence and persistence of *C. lectularius* (Zhu et al. 2010, Zhu et al. 2012, Adelman et al. 2011, Tabashnik et al. 2014). Researchers investigating bed bug control failures have identified multiple pyrethroid resistance mechanisms. One mechanism of pyrethroid resistance is knock down resistance (kdr) that results in decreased sensitivity of the target site (*e.g.*, sodium ion channels). A source of *kdr* is two point mutations (V419L and L925I) in the alpha sub-unit of the voltage-gated sodium ion channel (Yoon et al. 2008, Adelman et al. 2011). Exposing pyrethroid resistant bed bugs to deltamethrin and piperonyl butoxide (PBO), a cytochrome p450
inhibitor, synergized/increased bed bug mortality; showing the importance of metabolic resistance in the form of enhanced insecticide detoxification (Romero et al. 2007, Adelman et al. 2011, Zhu et al. 2011). Cytochrome P450s, cuticular proteins, and ABC transporters expressed in the bed bug integument have been shown to reduce insecticide penetration and contribute to pyrethroid resistance (Mamidala et al. 2012, Zhu et al. 2013). Insecticide resistance mechanisms among bed bug populations have spurred the development of new insecticide products and formulations for effective management.

Chlorfenapyr is a pyrrole group insecticide and is popular among PMPs because of its unique mode of action and high efficacy against bed bugs (Romero et al. 2010, Davies et al. 2012, Boase 2015). Chlorfenapyr is a pro-insecticide that is generally considered safe for mammals and is converted to its toxic metabolite CL 303268 by insect cytochrome P450s. Activation occurs by oxidative removal of the N-ethoxymethyl group from the compound (Romero et al. 2010, Raghavendra et al. 2011). Once activated, the metabolite disrupts oxidative phosphorylation by mitochondria, preventing the formation of ATP (Romero et al. 2010, Raghavendra et al. 2011). Bifenthrin is a type-I pyrethroid, lacking an alpha-cyano group, and is an active ingredient (AI) in certain professional insecticide formulations (Talstar® and Transport®) as well as ready-to-use retail products (e.g., Ortho Home Defense).

Within the pyrethroid group of insecticides, bed bugs display high levels of resistance to compounds like deltamethrin, but have varying susceptibility levels to others, such as bifenthrin, λ-cyhalothrin, and permethrin (Moore and Miller 2006, Romero et al. 2007, Steelman et al. 2008, Boase 2015). Thus, PMPs rely on pyrethroid and non-pyrethroid (chlorfenapyr) insecticides with low resistance levels and greater efficacy (Davies et al. 2012). Additionally, products labeled for bed bug control that include pyrethroids as active ingredients (e.g., deltamethrin, bifenthrin, λ-cyhalothrin, and permethrin) are available to the public, increasing the potential for improper use of pesticides for bed bug control and exacerbation of resistance issues.

Examples of high-level and widespread resistance to deltamethrin have clearly shown that bed bugs are capable of quickly becoming resistant to insecticides that are
continuously used for their control (Lofgren et al. 1958, Romero et al. 2007, Adelman et al. 2011, Palenchar et al. 2015). To date, both chlorfenapyr and bifenthrin are effective insecticides for the control of bed bugs (Moore and Miller 2006, Steelman et al. 2008, Davies et al. 2012, Boase and Naylor 2014). But because of the possibility of resistance development, insecticides such as chlorfenapyr and bifenthrin may lose their efficacy. Cross-resistance from other pyrethroid insecticides and improper or overuse of over-the-counter products is also likely to speed up the development of bifenthrin resistance (Romero et al. 2007, Steelman et al. 2008, Adelman et al. 2011).

Development of chlorfenapyr and bifenthrin resistance in bed bugs could have significant impacts on overall bed bug management because only few AIs are approved for bed bug control. One proven technique for delaying the onset of resistance is insecticide resistance management through the use of integrated pest management programs (IPM) (ffrench-Constant and Roush 1990, Gondhalekar et al. 2011). But, implementation of insecticide resistance management strategies requires insecticide susceptibility levels to be determined in field populations of the pest insect (Brent 1986, ffrench-Constant and Roush 1990). Currently susceptibility levels of bed bug field populations to chlorfenapyr and bifenthrin are unknown, and in order to determine this, a standard bioassay technique is required.

In 1960, the World Health Organization called for standardized techniques of resistance monitoring of malaria vectors to make results easily comparable between studies (Macoris et al. 2015). Since then, standardized techniques have been adopted for many, but not for all insect bioassays (Busvine 1971). Bed bugs still have variable bioassay techniques for insecticide susceptibility or resistance monitoring (Stubaker 2003). In recent bed bug publications, six different bioassay techniques have been used: injection, topical application, glass vial, filter paper tests with different absorptive properties (Whatman No.1 and Whatman No.2), and the WHO-recommended filter paper vial assay.

Therefore, our main goal was to develop a standardized diagnostic concentration-based bioassay method for a large-scale chlorfenapyr and bifenthrin susceptibility
monitoring. Using a laboratory susceptible bed bug strain we generated concentration-mortality data for the surface contact glass vial and filter paper bioassay methods with technical grade and formulated products. Next, insecticide, formulations, and bioassay data were statistically compared to choose a suitable method for susceptibility monitoring. By using PROBIT analysis estimates (lethal concentration values, slope, intercept, etc.) calculated for the different bioassays we determined that the glass vial bioassay with formulated insecticides is more accurate for chlorfenapyr and bifenthrin susceptibility monitoring of field-collected bed bugs. Further, two field strains were exposed to the susceptible LC₉₉ diagnostic concentration in glass vial assays to confirm the practicality of this technique for monitoring of chlorfenapyr and bifenthrin susceptibility in bed bugs.

2.2 Materials and methods

2.2.1 Insects

The insecticide susceptible Harlan strain maintained at Purdue University was used for comparing bioassays. All bed bugs were obtained from colonies maintained at 25°C temperature, 50% ±15 relative humidity and a 12:12 hour light/dark cycle in a walk-in environmental chamber (Percival Scientific, Perry, IA). Insects were fed using the Parafilm® membrane-method on defibrinated rabbit blood that was heated to 37°C using a reptile heating pad (Chin-Heady et al 2013). All insects were at least 10-14 days old and fed 4-5 days prior to the bioassay. Due to traumatic insemination-induced mortality and other negative fitness effects that occasionally occur in adult females, only adult male bed bugs were used in all bioassays.

2.2.2 Chemicals

Talstar® and Phantom® were purchased from Univar (Indianapolis, IN). Technical grade bifenthrin and chlorfenapyr (>95% purity) were purchased from Chem Service (West Chester, PA). Acetone (HPLC grade) was obtained from Fisher Scientific (Pittsburg, PA).
2.2.3 Glass vial assay

For formulated insecticides (Talstar and Phantom), stock insecticide solutions were freshly made with deionized water just before initiation of bioassays. Stock solutions were serially diluted to create a range of concentrations (Table 2.1) that provided a good distribution of mortality between 0 and 100%. Dilution factor for serially dilutions were 10 fold and then adjusted as needed for additional concentrations. Each glass vial (4 mL) (Scientific Specialties, Hanover, MD) was treated with 100 µL of a known insecticide concentration and allowed to dry on a hotdog roller (with heating element disabled) placed inside a fume hood. Complete drying of vials required ~72 h due to the use of water as solvent. Control vials were treated with deionized water. After the vials were dry, ten adult male bed bugs were placed into each vial. Vials were covered with Parafilm® that was perforated to allow aeration. Glass vials were placed upright to ensure bed bug contact with insecticide treated surfaces for the entire assay duration. Bioassay vials were held in an environmental chamber with temperature, humidity, and light conditions identical to those used for rearing. Mortality assessments for bifenthrin took place at 24, 48, and 72 h whereas chlorfenapyr assessments were made every 24 h for 168 h (7 d). All Bed bugs were scored as dead if they were unable to walk. Several concentrations that provided 75-100% mortality were used to increase the accuracy of the LC$_{99}$ estimation (ffrench-Constant and Roush 1990). Each concentration was replicated 5-6 times.

Glass vial bioassays with technical grade bifenthrin and chlorfenapyr were performed following a similar procedure as outlined above. For preparing insecticide stock solutions and dilutions, acetone was used as a solvent. Control vials were treated only with acetone. Due to use of acetone as a solvent for technical grade insecticides, treated vials were completely dry in 1 h and assays were started 24 hours after treating. Insecticide concentrations used in these bioassays are listed in Table 2.1 and each concentration was replicated 5-6 times. Bifenthrin mortality observations were taken every 24 h up to 72 h, and chlorfenapyr observations were taken every 24 h up to 7d. Initial assays with technical chlorfenapyr yielded mortality independent of concentrate
on and the cost per assay was high, therefore a complete concentration-mortality curve was not generated.

2.2.4 Filter paper assay

Stock insecticide solutions for formulated bifenthrin and chlorfenapyr were prepared in deionized water in the same manner as mentioned above for the glass vial assays. Whatman #1 (GE Healthcare, Pittsburg, PA) filter papers were cut to fit a 35x10mm plastic Petri dish (Fischer Scientific, Pittsburg, PA). Filter paper placed in the dish was treated with 200μL of insecticide solution. Preliminary studies found that 100 μL and 150 μL of a Nile blue dye solution was not sufficient to uniformly cover the filter paper disc, but the application of 200 μL resulted in total coverage. Treated filter papers were allowed to dry for 24 hours. Controls included filter papers treated with deionized water. After the filter papers were completely dry, ten adult male bed bugs were placed in each Petri dish and the lids were sealed with a Parafilm® strip. Petri dishes were held in environmental chambers used for insect rearing. Mortality was determined for the filter paper assay in the same way as the vial assay except in order to properly score mortality; bed bugs were removed from the treated surface and placed onto a clean area (Busvine 1971). The bifenthrin assays were scored daily for three days during insecticide exposure. Different from the vial assays, chlorfenapyr paper assays were scored daily for 14 days because of slower mortality observed in filter paper assays. Concentrations used in filter paper bioassays are mentioned in Table 2.1. As with vial bioassays, several concentrations providing 75-100% mortality were used to increase the accuracy of the LC$_{99}$ estimation. Each concentration was replicated 4-6 times.

Filter paper bioassays with technical grade bifenthrin and chlorfenapyr followed a similar protocol described above for formulated products with the exception that acetone was used as a solvent. Acetone blanks were used for controls. Insecticide concentrations used for generating mortality data are listed in Table 2.1 and each concentration was replicated 4-6 times. Initial assays with technical chlorfenapyr yielded mortality independent of concentration and the cost per assay was high, which prevented generation of a complete concentration-response curve.
Table 2.1 Table representing the insecticide concentrations used for bioassays. Insecticide concentrations are measured in µg AI/cm².

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Assay</th>
<th>Concentrations Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technical Bifenthrin</td>
<td>Glass Vial</td>
<td>0.088, 0.0088, 0.00088, 0.00013, 0.00011, 0.000073, 0.000059, 0.000029, 0.0000072, 0.0000035</td>
</tr>
<tr>
<td>Technical Bifenthrin</td>
<td>Filter Paper</td>
<td>0.41, 0.29, 0.19, 0.15, 0.099, 0.49, 0.25, 0.015, 0.012</td>
</tr>
<tr>
<td>Formulated Bifenthrin</td>
<td>Glass Vial</td>
<td>0.0034, 0.0017, 0.0011, 0.00085, 0.00057, 0.00028, 0.00014, 0.000071, 0.00035</td>
</tr>
<tr>
<td>Formulated Bifenthrin</td>
<td>Filter Paper</td>
<td>0.13, 0.035, 0.026, 0.0172, 0.0129, 0.0086, 0.0064, 0.0032</td>
</tr>
<tr>
<td>Technical Chlorfenapyr</td>
<td>Glass Vial</td>
<td>42441.82, 14147.27, 141.5</td>
</tr>
<tr>
<td>Technical Chlorfenapyr</td>
<td>Filter Paper</td>
<td>23.8, 11.89, 2.97, 1.49, 0.74, 0.37, 0.19</td>
</tr>
<tr>
<td>Formulated Chlorfenapyr</td>
<td>Glass Vial</td>
<td>47.2, 31.5, 15.7, 7.8, 3.9, 1.9, 0.98, 0.49, 0.25, 0.13</td>
</tr>
<tr>
<td>Formulated Chlorfenapyr</td>
<td>Filter Paper</td>
<td>80.9, 57.8, 42.2, 34.7, 23.1, 5.8, 1.4, 0.72, 0.36, 0.18, 0.91</td>
</tr>
</tbody>
</table>

2.2.5 Validation of diagnostic concentrations

Statistical analyses led to the glass vial assay being used for bifenthrin and chlorfenapyr susceptibility monitoring, which was further validated. The utility of diagnostic glass vial bioassays for bifenthrin and chlorfenapyr susceptibility monitoring was validated by testing two field-collected strains at the Harlan diagnostic LC₉₉ concentrations determined for the formulated insecticides. In addition to LC₉₉
concentrations, LC$_{50}$ concentrations for bifenthrin and chlorfenapyr were also validated. The LC$_{50}$ and LC$_{99}$ diagnostic concentrations for bifenthrin corresponded to a 0.23x10$^{-5}$% (0.000034 µg/cm$^2$) and 0.96x10$^{-4}$% (0.0014 µg/cm$^2$) solution, respectively. For chlorfenapyr, LC$_{50}$ concentration of 0.025% (3.87 µg/cm$^2$) and LC$_{99}$ diagnostic concentration of 0.5% (5.77 µg/cm$^2$) were validated. Bioassay validation was performed following the protocol described under “Glass Vial Bioassays”. The LC$_{50}$ and LC$_{99}$ concentrations for formulated bifenthrin and chlorfenapyr were validated on bed bugs of the Harlan strain, the field-collected Washington D.C. strain and the deltamethrin resistant Richmond strain. Approximately 10 to 12 replicates were performed for each strain at LC$_{50}$ or LC$_{99}$ concentrations. DI water was used as a control. Mortality observations were performed daily and bioassays lasted for 72 and 168 h for bifenthrin and chlorfenapyr, respectively.

2.2.6 Comparison of filter paper types

The effects differential insecticide absorption by Whatman No. 1 and Whatman No. 2 filter papers on insect mortality were tested by screening the Harlan strain at the susceptible LC$_{50}$ concentration for formulated bifenthrin (0.0005% solution or 0.01 µg/cm$^2$). Bioassay procedures for these tests were similar to that of the “Filter Paper Assays”. Ten adult males were used per replicate and assays were replicated 3–4 times. Controls included filter papers treated with deionized water.

2.2.7 Data analysis

The Harlan strain concentration-mortality data generated for the glass vial and filter paper bioassays and for the formulated and technical grade insecticides was analyzed in SAS 9.3 using the PROC PROBIT function (SAS, 2011). Probit analysis of the concentration-mortality data enabled determination of LC estimates and associated parameters (slope, intercept and covariance). The probit data was further used to statistically compare the toxicity profile between the glass vial and filter paper bioassays (Robertson et al. 2007). Mortality data in validatory glass vial bioassays at LC$_{50}$ and LC$_{99}$ concentrations were compared between Harlan and field-collected strains by using by
using PROC GLM and Tukey’s test (P<0.05). Filter paper bioassay data generated using the Whatman No. 1 and No. 2 filter papers was compared within strains also by using a Mann-Whitney test (P<0.05).

2.3 Results

2.3.1 Glass vial assay

Formulated bifenthrin and chlorfenapyr concentration mortality data were generated with the Harlan strain 72 and 168 hours for chlorfenapyr and bifenthrin, respectively (Table 2.2). The bifenthrin vial diagnostic concentration LC$_{99}$ was 0.1 µg /vial or 0.0014 µg AI /cm$^2$, which is 637x less than the labeled field application rate (0.06%). The chlorfenapyr vial diagnostic concentration (LC$_{99}$) was close to the field application rate at our predicted value of 503.8 µg/vial or 7.12 µg AI /cm$^2$, therefore our diagnostic concentration was rounded up to the field application rate of 0.5% (556 µg /vial or 7.87 µg AI /cm$^2$). Control mortality averaged to 2.5% for bifenthrin and 10.9% for chlorfenapyr which was accounted for in probit analysis.

Mortality data were generated for technical bifenthrin at 72 h. The chlorfenapyr diagnostic concentration was not determined for reasons previously mentioned. The bifenthrin vial diagnostic LC$_{99}$ concentration was 1.7 µg /vial or 0.024 µg AI /cm$^2$. Control mortality averaged to 2% for bifenthrin which was accounted for in probit analysis.

2.3.2 Filter paper assay

In filter paper assays the 72 h and 336 h (14 d) mortality data collected for bifenthrin and chlorfenapyr, respectively, provided best fit to the probit model. Assays with chlorfenapyr required an extra 7 d in order to generate a full range of mortality between 0 to 100% as well as higher concentrations (Tables 2.2 and 2.3) and application volumes (200 µl /filter paper) of insecticide. The bifenthrin LC$_{99}$ value for the filter paper assay was 3.42 µg /dish (0.36 µg AI /cm$^2$). The LC$_{99}$ estimate for chlorfenapyr is far over the field application rate at 21523 µg /dish (2237.1 µg AI /cm$^2$). Filter paper assays using chlorfenapyr yielded a heterogeneous mortality response and thus a heterogeneity factor of 2.14 was used for correction in probit analysis.
Control mortality averaged to <2% for both insecticides and was accounted for in probit analysis.

Tests with technical grade material, the data at 72 h for bifenthrin provided best fits to the probit model. The chlorfenapyr diagnostic concentration was not determined for reasons previously mentioned. The bifenthrin vial diagnostic LC₉₉ concentration was 25.5 µg/dish or 0.26 µg AI/cm². Control mortality averaged to 1.25% for bifenthrin which was accounted for in PROBIT analysis.

2.3.3 Comparison of bioassays for insecticide formulations

A Robertson and Preisler test was conducted on the probit output parameters (intercept, slope and covariance 1 to 3) for vial and filter paper assays (Robertson et al. 2007). Results (Tables 2.2 and 2.3) of the comparison found that filter paper LC₉₉ estimates are significantly higher than vial LC₉₉ values for both formulated and technical grade insecticides. At the LC₉₉ level, formulated bifenthrin filter paper assay required 25.6x more insecticide compared to the glass vial assay. A significant difference of 320.9x more insecticide was seen at the formulated bifenthrin LC₅₀ level. Similarly, formulated chlorfenapyr filter paper assays required 33.9x more insecticide at the LC₉₉ level in comparison to the glass vial assays. For technical bifenthrin, the filter paper assays required 114.4x more insecticide for the LC₉₉ diagnostic concentration, when compared to the glass vial bioassay (Table 2.3).
Table 2.2 Results of LC bioassays for each bioassay technique using Harlan strain adult male bed bugs. Formulated chlorfenapyr vial and filter paper results were scored at days 7 and 14, respectively. Bifenthrin bioassay results were scored at 3 days after exposure.

<table>
<thead>
<tr>
<th>Assay</th>
<th>N</th>
<th>Slope (±SE)</th>
<th>LC_{50}(95% FL)</th>
<th>TR_{50} (95% CI)</th>
<th>LC_{99}(95% FL)</th>
<th>TR_{99} (95% CI)</th>
<th>X^2 (df) (95% CI)</th>
<th>P-value</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorfenapyr</td>
<td>590</td>
<td>1.84(0.19)</td>
<td>0.39(0.5)</td>
<td>-</td>
<td>6.99(4.5)</td>
<td>-</td>
<td>4.7(8)</td>
<td>0.78</td>
<td>-</td>
</tr>
<tr>
<td>-Glass vial</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Chlorfenapyr</td>
<td>500</td>
<td>1.02(0.18)</td>
<td>1.29(2.94)</td>
<td>3.3(0.67)</td>
<td>236.6(56.6)</td>
<td>33.9(5.3)</td>
<td>40.2(9)</td>
<td>0.000</td>
<td>4.47</td>
</tr>
<tr>
<td>-Filter paper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bifenthrin</td>
<td>550</td>
<td>1.44(0.13)</td>
<td>0.000032(0.00026)</td>
<td>-</td>
<td>0.00137(0.000041)</td>
<td>0.0007</td>
<td>-</td>
<td>8.8(8)</td>
<td>0.35</td>
</tr>
<tr>
<td>-Glass vial</td>
<td></td>
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<tr>
<td>Bifenthrin</td>
<td>460</td>
<td>4.47(0.46)</td>
<td>0.010(0.11)</td>
<td>320.9(8.6)</td>
<td>0.035(0.02)</td>
<td>25.7(9.9)</td>
<td>7.3(6)</td>
<td>0.28</td>
<td>-</td>
</tr>
<tr>
<td>-Filter paper</td>
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</table>

^Lethal concentration (LC_{50} and LC_{99}) values with 95% fiducial limits, all values are expressed in µg chlorfenapyr per cm².

^Toxicity ratios at LC_{50} and LC_{99}. Toxicity ratios, confidence intervals and their significance were determined using the formula given by Robertson et al. (2007). Filter paper data was used as a baseline for toxicity ratio calculation.

^HF Heterogeneity factor.

Asterisk (*) indicates significant toxicity ratio (Robertson et al. 2007). Ratios were considered significant if the confidence intervals did not overlap with the value 1. Higher toxicity ratios were observed for the filter paper assays due to insecticide absorption by the substrate, which also caused increased assay time.
Table 2.3 Results of LC bioassays for each technique using Harlan strain adult male bed bugs. Technical chlorfenapyr vial and filter paper results were scored at days 7 and 14, respectively. Bifenthrin bioassay results were scored at 3 days of exposure.

<table>
<thead>
<tr>
<th>Assay</th>
<th>N</th>
<th>Slope(±SE)</th>
<th>LC₅₀(95% FL)ᴬ</th>
<th>TR₅₀(95% % CI)</th>
<th>LC₉₀(95% %FL)ᴬ</th>
<th>TR₉₀(95% % CI)</th>
<th>X²(df)(95% CI)</th>
<th>P-value</th>
<th>HF c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorfenapyr</td>
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<td>-Glass Vial</td>
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<td>-Filter paper</td>
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<tr>
<td>Bifenthrin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>-Glass vial</td>
<td>460</td>
<td>2.18(0.2 5±)</td>
<td>0.0141(0.0104-0.0185)</td>
<td>-</td>
<td>0.165(0.09-0.402)</td>
<td>-</td>
<td>12.77(7)</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>-Filter paper</td>
<td>710</td>
<td>1.99(0.1 6±)</td>
<td>1.7(1.4-2.1)</td>
<td>1.1(1.0-1.39)</td>
<td>25.5(18.5-39.3)</td>
<td>42.5(39.3-46.4)</td>
<td>123.8(59)</td>
<td>139.5*</td>
<td>9.64(7)</td>
</tr>
</tbody>
</table>

ªLethal concentration (LC₅₀ and LC₉₀) values with 95% fiducial limits, all values are expressed in µg chlorfenapyr per cm².

ᵇToxicity ratios at LC₅₀ and LC₉₀. Toxicity ratios, confidence intervals and their significance were determined using the formula given by Robertson et al. (2007). Filter paper data was used as a baseline for toxicity ratio calculation.

ᶜHF Heterogeneity factor.

Asterisk (*) indicates significant toxicity ratio (Robertson et al. 2007). Ratios were considered significant if the confidence intervals did not overlap with the value 1. Higher toxicity ratios were observed for the filter paper assays due to insecticide absorption by the substrate, which also caused increased assay time.
2.3.4 Diagnostic concentration validations

Higher insecticide concentrations (toxicity ratios) and experimental time required for the filter paper assays led us to choose the glass vial assay for bifenthrin and chlorfenapyr susceptibility monitoring. Determined lethal concentrations (LC$_{50}$ and LC$_{99}$) for formulated bifenthrin and chlorfenapyr were validated using the method for the glass vial assay. The Harlan; Richmond, VA; and Washington D.C. strains were used for the concentration validations. Validation of the chlorfenapyr LC$_{50}$ (0.025% solution corresponded to 27.4 µg/vial or 3.87 µg A.I per cm$^2$) with the Harlan strain yielded expected results, with mortality averaging to 50.1% (±4.3%). However, higher mortality (64.4% ± 2.3%) was observed in the Washington D.C. strain, but was lower in the Richmond, VA strain (44.0% ± 1.5%). Results from the strain comparison showed that the Washington D.C. strain had significantly higher mortality than the Harlan and Richmond, VA strains (Figure 2.1). The chlorfenapyr LC$_{99}$ (0.5% solution corresponded to 556 µg A.I. /vial or 7.87 µg A.I per cm$^2$) caused an average mortality of 94.62% (±4.3%) in the Harlan strain, but less in the Washington D.C. strain (87.4% ±1.2% mortality) and in the Richmond, VA strain (55.0% ± 1.74%). At the LC$_{99}$ rate, the Harlan strain was similar to the Washington D.C. strain, but significantly different from the Richmond VA strain (Figure 2.2). These results demonstrate the importance of a validated LC$_{99}$ diagnostic concentration for susceptibility assays because of the alternate results observed when field-collected bed bugs were exposed to the LC$_{50}$ concentrations. Control mortality was 0.7% for the Harlan strain, 1.6% for the Richmond, VA strain, and 13% in the Washington D.C. field strain therefore Abbott’s transformation was necessary for the latter mortality data.

Mortality at the bifenthrin LC$_{50}$ concentration of 0.0000023% (0.0024 µg /vial or 0.000034 µg A.I per cm$^2$) was slightly lower than expected (Fig. 2.3) averaging to 42% (±5.5%) for the Harlan strain, 17% (±4.2%) on the Washington D.C. strain, and 35 % (±4.2%) on the Richmond, VA strain. The Washington D.C. strain had significantly lower mortality than the Harlan and Richmond, VA strain. Mortality at the bifenthrin LC$_{99}$ concentration of 0.00096% (0.1 µg /vial or 0.0014 µg A.I. per cm$^2$) was also lower than
expected at 95.7% (±2.1%) for Harlan, 88% (±6.1%) for Washington, D.C. and 51% (±19.3%) for the Richmond strain (Figure 2.4). The Richmond, VA strain had significantly lower mortality compared to the Washington D.C. and Harlan susceptible strains at the LC$_{99}$ rate. Control mortality did not occur for the Harlan or Richmond strain but was 2% for the Washington D.C strain.
Figure 2.1 Results of the chlorfenapyr glass vial validation bioassays at the LC$_{50}$ concentration (0.025% solution corresponded to 27.4 µg/vial or 3.87 µg AI/cm$^2$). Bars represent mortality scored at 168 h post treatment. Bars that are not connected by the same letter are significantly different (Tukeys test P < 0.05). Error bars represent ±SE values. For each strain (y-axis), 100 adult males were tested for each strain.
Figure 2.2 Results of the chlorfenapyr glass vial validation bioassays at the LC$_{99}$ diagnostic concentrations (0.5% solution corresponded to 556 µg AI/vial or 7.87 µg AI per cm$^2$). Bars represent mortality scored at 168 h post treatment. Bars that are not connected by the same letter are significantly different (Tukeys test P < 0.05). Error bars represent ±SE values. For each strain (y-axis), 100 adult males were tested for each strain.
Figure 2.3 Results of the bifenthrin glass vial validation bioassays at the LC$_{50}$ concentration (0.0000023% solution corresponded to 0.0024 µg/vial or 0.000034 µg AI per cm$^2$). Bars represent mortality scored at 72 h post treatment. Bars that are not connected by the same letter are significantly different (Tukeys test P < 0.05). Error bars
represent ±SE values. For each strain (y-axis), 100 adult males were tested for each strain.

Figure 2.4 Results of bifenthrin glass vial validation bioassays at the diagnostic LC$_{99}$ concentration (0.00096% solution corresponded to 0.1 µg/vial or 0.0014 µg AI per cm$^2$). Bars represent mortality scored at 72 h post treatment. Bars that are not connected by the
same letter are significantly different (Tukeys test $P < 0.05$). Error bars represent $\pm \text{SE}$ values. For each strain (y-axis), 100 adult males were tested for each strain.

2.3.5  Comparison of filter paper types

We found that Harlan bed bug mortality was 22.5% higher on Whatman No.1 papers (32.5%) compared to Whatman No.2 (10%) papers when exposed to the LC50 of bifenthrin. Control mortality did not occur during assays. Bioassays were run for a total of 3 days as the same time period was used for bifenthrin LC determinations.

2.4  Discussion

Insecticide resistance in bed bug populations has been a major impediment for their effective control (Romero et al. 2007, Adelman et al. 2011, Zhu et al. 2011). In order to address this problem, proactive actions that delay or prevent the development of insecticide resistance are required. Development of an accurate and sensitive diagnostic-concentration based bioassay method that is able to determine subtle changes in susceptibility to an insecticide is an essential first step in any resistance management program (ffrench-Constant and Roush 1990, Cochran 1997).

2.4.1  Advantages of glass vial bioassay

Filter paper and glass vial bioassay techniques are two of the most commonly used bioassay methods for bed bug insecticide resistance monitoring or toxicity assays (Romero et al. 2007, Steelman et al. 2008, Gordon et al. 2014, Singh et al. 2015). Physiochemical characteristics of a treated surface influences insecticide efficacy, as toxicity of the chemical is inversely proportional to the degree to which it is absorbed into the substrate (Chadwick 1985, Rust 1995). Choice of a bioassay method for resistance monitoring also depends on the characteristics of the target chemical such as its mode of action, repellency profile and penetration rate into the insect (Rust 1995). To determine the best bioassay technique for chlorfenapyr and bifenthrin resistance monitoring in bed bugs, the toxicity parameters for the filter paper and glass vial
bioassays were statistically compared using probit data generated with the Harlan susceptible strain (Tables 2.2 and 2.3).

For formulated bifenthrin, technical bifenthrin, and formulated chlorfenapyr, the amount of insecticide required to kill 99% of test insects was higher in filter paper bioassays as compared to the glass vial technique (Tables 2.2 and 2.3). Previous research has suggested that the porous nature of the filter paper results in the increased amount of insecticide required for achieving desired insect mortality (Busvine 1971, Chadwick 1985, Roper and Wright 1985, Rust 1995, Samnon and Hall 1989). Some of the major conclusions drawn from research on interactions between pyrethroid insecticides and assay surface were that: (a) surface-type greatly affects residual activity of an insecticide and (b) absorption/migration of chemical into the assay substrate greatly reduces the insecticide availability (Chadwick 1985). These conclusions also hold true for bifenthrin and chlorfenapyr. Additionally, absorption of insecticide into the filter papers drastically increased the assay duration for chlorfenapyr. Filter paper bioassays required 14 days to achieve complete mortality, but in the glass vial bioassay, complete mortality of a chlorfenapyr susceptible strain was achieved in just 7 days (Table 2.1).

Due to the non-porous nature of the glass substrate in general, it is unlikely that it will cause a significant change in insecticide activity. However, the difference in absorptive potential of different filter papers does appear to affect insecticide toxicity. When Harlan strain bed bugs were exposed to the LC$_{50}$ concentration of bifenthrin on Whatman No. 1 and No. 2 filter papers, a 22% mortality increase was observed on Whatman No. 2 paper. According to the product specifications, Whatman No. 2 filter paper is more absorptive due to its smaller particle filtration size (8 µm) as compared to Whatman No. 1 (11 µm). Greater filtration may have caused higher absorption and thus decreased mortality in assays performed using Whatman No. 2.

Non-uniform distribution of insecticide residues on assay substrates is a major problem for a resistance detection bioassay (Busvine 1971). Uneven insecticide distribution on a substrate can allow insects to aggregate in areas that are untreated or have less insecticide residue. In the case of glass vials, however, the use of a rolling machine (hot-dog roller) provides uniform distribution of chemical residue throughout
the vial. Moreover, because bed bugs are unable to walk on vertical glass surfaces they remain confined to the treated portions of the vial for the entire assay duration. Bed bugs may have been able to climb up on each other to avoid insecticide contact at times, but complete mortality in susceptible strains was still achieved. In filter paper assays, however, the thigmotactic behavior of bed bugs (Reinhart and Silva-Jothy 2007) presents a unique challenge. Bed bugs were observed aggregating on edges of the filter paper in contact with the walls of the Petri dish, thus avoiding insecticide residues to a certain extent. Preliminary experiments indicated that if insecticide solution is applied to the center of the filter paper, the quantity of insecticide residue is highest at the point of application (i.e., center) and lowest toward the outer edges of the substrate (data not shown).

Since the ultimate goal of resistance monitoring is to detect susceptibility shifts in field populations (Busvine 1971), the use of filter paper bioassays that: (i) allow insecticide absorption into the substrate and (ii) lead to non-uniform distribution of insecticide residues reduces the precision of the assay. Furthermore, variability in resistance detection can result from use of filter papers that differ in porosity and other physical characteristics. In contrast, glass vial bioassays are not prone to any disadvantages posed by the filter papers, except for the fact that drying vials requires 2-3 days due to use of water as an insecticide solvent and non-porous substrate. Overall, vial bioassays are well-suited for both chlorfenapyr and bifenthrin resistance monitoring.

2.4.2 Use of technical or formulated product

Technical grade formulations of insecticides with >99% purity are an ideal choice for use in resistance monitoring because they do not contain any surfactants or adjuvants that enhance the toxic effect of a chemical by enhancing its absorption into the insect body. Determination of LC estimates for technical grade chlorfenapyr in filter paper or glass vial bioassays was not possible (Table 2.3). Toxicity of a chemical in surface contact bioassays is mainly dependent upon its ability to penetrate into the insect body. It is likely that the absence of adjuvants/surfactants in technical chlorfenapyr may have limited its ability to transfer and penetrate into the insect. This hypothesis appears
plausible because the LC estimates for formulated chlorfenapyr (Phantom®) were readily determinable (Table 2.2). Steelman et al. (2008), however, determined LC$_{50}$ and LC$_{90}$ estimates for field-collected bed bugs using technical grade chlorfenapyr. Nevertheless, the use of technical grade chlorfenapyr for resistance monitoring is impractical because LC$_{50}$/LC$_{99}$ values are high (617 to 996.5 mg per vial) (Steelman et al. 2008), making its use uneconomical for large-scale susceptibility screening programs. In contrast to chlorfenapyr, both technical and formulated grade bifenthrin (Talstar®) can be effectively used in resistance monitoring studies (Tables 2.2 and 2.3). However, to follow a balanced and comparative approach in this study, formulated grade bifenthrin and chlorfenapyr were used for the susceptibility monitoring program discussed below.

2.4.3 Feasibility of the glass vial bioassay for susceptibility monitoring

Diagnostic concentration-based glass vial bioassays have not been recently used for chlorfenapyr and bifenthrin resistance monitoring in bed bugs. The glass vial bioassay was used was successfully used by Steelman et al. (2008) to determine LC$_{50}$ resistance ratios in bed bugs collected from poultry houses. Other studies tested field-collected bed bug populations for resistance to certain pyrethroids and neonicotinoids using field application rates of technical or formulated grade insecticides in the filter paper bioassay (Romero et al. 2007, 2010, Zhu et al. 2010, 2013, Gordon et al. 2014).

Interestingly the diagnostic LC$_{99}$ concentration of formulated chlorfenapyr determined for the Harlan susceptible strain was slightly below the field application rate of chlorfenapyr (i.e., 0.5%). A diagnostic concentration similar to the label application rate makes the glass vial assay more relevant to control efforts in the field. Further validation of the chlorfenapyr LC$_{50}$ and LC$_{99}$ concentrations against two field-collected strains revealed the utility of this method for susceptibility monitoring. The Washington D.C. strain was significantly different from the Harlan strain at the chlorfenapyr LC$_{50}$ (64.4± 2.3%), but significantly similar at the LC$_{99}$ (87.4±1.2%) diagnostic concentration (Figs 2.1 and 2.2). To reinforce the importance of the diagnostic concentration, assays with the deltamethrin resistant Richmond strain showed significantly similar mortality at Harlan LC$_{50}$ but different at the LC$_{99}$ concentration for both bifenthrin and chlorfenapyr.
and was therefore successful in determining resistant populations (Figures 2.1, 2.2, 2.3, 2.4). It has also been hypothesized that bed bug populations with increased cytochrome P450 expression may have negative cross resistance to chlorfenapyr, as has been found in cockroaches and house flies (Scott et al. 2003, Gondhalekar et al. 2011). However, negative cross resistance to chlorfenapyr has not been reported in bed bugs and it is likely that testing bed bug strains for both bifenthrin and chlorfenapyr susceptibility might provide more insights into this phenomenon. Research by Singh et al. 2015 found that cytochrome P450 upregulation associated with blood meal digestion caused increased chlorfenapyr mortality within the first few days of exposure. The use of the glass vial bioassay would easily allow bed bugs to be fed every 3 days when being exposed to chlorfenapyr, which may make the glass vial assay even more relevant to field conditions.

The bifenthrin LC99 diagnostic concentration was ca. 630x less than the label application rate. The diagnostic validation assays (Fig. 2.4) against the Richmond, VA; Washington D.C.; and Harlan strains, the LC99 concentration for bifenthrin is accurate for determining susceptibility of field bed bug populations. Since the bifenthrin LC99 diagnostic concentration is so low, it is possible that it does not identify practical field resistance since insects in the field would be exposed to a much higher rate. However bifenthrin is also applied as a mixture formulation (Bifenthrin + Acetamiprid); therefore stand-alone monitoring for bifenthrin susceptibility is needed since it is difficult to statistically separate the individual effects of a formulated combination insecticide (Robertson et al. 2007). Additionally, laboratory selection experiments with neonicotinoid/pyrethroid mixture formulations suggest that bed bugs become resistant to the pyrethroid component of the mixture, reinforcing the importance of bifenthrin susceptibility monitoring (Gordon et. al 2014). Additionally, as bifenthrin is available to the public as an over-the-counter product, it can be misused or overused by the public and thus bifenthrin resistance screening efforts are essential to monitor resistance development. Previous bed bug susceptibility profiles had reported bifenthrin susceptibility in other field collected bed bug populations (Steelman et al. 2008, Moore and Miller 2008).
CHAPTER 3. SCREENING BED BUG FIELD POPULATIONS FOR CHLORFENAPYR AND BIFENTHRIN SUSCEPTIBILITY

Abstract

Bed bug infestations are increasingly difficult to manage due to the development of insecticide resistance. Pest management professionals are using different chemicals such as chlorfenapyr and bifenthrin to control bed bug infestations, but they could potentially lose efficacy due to resistance development. Chlorfenapyr and bifenthrin susceptibility levels of field collected bed bugs have not been determined on a wide scale. Using the glass vial assay, ten field-collected bed bug populations were screened for chlorfenapyr and bifenthrin susceptibility using validated lethal concentrations that cause 99% mortality. Three of the ten populations screened at the chlorfenapyr LC$_{99}$ concentration showed significantly lower mortality levels. Five of the ten populations had significantly lower mortality to the bifenthrin LC$_{99}$, but it is likely that practical field resistance is not developing. Regression analysis of LC$_{99}$ mortality data for the ten field strains showed a statistically significant relationship between chlorfenapyr and bifenthrin susceptibility levels. The results of the susceptibility-monitoring program reinforce the need for implementing resistance management strategies such as use of an integrated pest management (IPM) approach.

3.1 Introduction

Common bed bug (Cimex lectularius) infestations have become very challenging to eliminate due to insecticide tolerance or resistance development among populations (Romero et al. 2007, Adelman et al. 2011, Zhu et al. 2011). Bed bugs have developed
resistance to many of the insecticides once used for their control, but recent control failures were found to be associated with resistance to insecticides in the pyrethroid class (deltamethrin and $\lambda$-cyhalothrin) (Romero et al. 2007, Potter 2008, Moore and Miller 2006, Steelman et al. 2008). Further investigations into bed bug insecticide resistance found multiple resistance mechanisms among populations, such as: target site resistance (knock down resistance ($kdr$)), increased cytochrome P450 production, reduced cuticular penetration, and other metabolic resistance mechanisms (Romero et al. 2007, Zhu et al. 2011, Adelman et al. 2011, Zhu et al. 2011). In order to effectively combat the bed bugs, pest management professionals (PMPs) have been integrating both insecticide formulations and non-chemical techniques (Kells 2006, Wang et al. 2010, Steadfast and Miller 2014). Despite the availability of effective non-chemical control techniques, insecticide application is often the PMP’s first choice of treatment and is an essential technique for the elimination of bed bug infestations (Gangloff-Kauffman et al. 2006, Wang et al. 2010). Since widespread resistance to certain pyrethroids exists among bed bug populations, PMPs are now employing neonicotinoid/pyrethroid combination formulation insecticides as well as other products with different modes of action (Davies et al. 2012, Boase and Naylor 2014).

Chlorfenapyr is a chlorinated pyrrole that has been classified as slightly hazardous by the World Health Organization (WHO) (Arthur 2007, Raghavendra et al. 2011). This pro-insecticide was designed as a termiticide and for use in crop protection, but chlorfenapyr is also effective for bed bug control (Moore and Miller 2006, Raghavendra et al. 2011). Chlorfenapyr has low mammalian toxicity (Dekeyser 2005, Romero et. al. 2010, Raghavendra et al. 2011). It is pro-insecticide that is activated by an insect cytochrome P450 to its toxic metabolite (CL 303268) which acts as a mitochondrial uncoupler that prevents the formation of ATP and eventually results in insect death (Romero et. al. 2010, Raghavendra et al. 2011). The mode of action of chlorfenapyr is relatively slow and may require up to 14 days to achieve complete mortality in laboratory assays (Romero et. al. 2010, Ashbrook et al. unpublished data).
Despite its slow mode of action, chlorfenapyr is increasingly popular among PMPs because, it can be applied to mattress seams, folds, and edges (Moore and Miller 2006, Romero et al. 2010, Davies et al. 2012, Boase 2015). Unlike other insecticide formulations, chlorfenapyr can be applied more directly to bed bug harborages in human rest areas, which is a significant advantage. Some pyrethroid insecticides are repellent, but chlorfenapyr has been shown to be non-repellent to bed bugs, which results in greater insecticide uptake (Moore and Miller 2006, Romero et al. 2009). Additionally, chlorfenapyr residues can remain effective for up to 4 months (Romero et al. 2010). However, once removed from chlorfenapyr treated areas, bed bugs can feed and recover from intoxication (Moore and Miller 2006). Recovery from chlorfenapyr intoxication is problematic as it may lead to tolerance or resistance development, which has already occurred in German cockroach (tolerant) and in a house fly strain (cross-resistant) (Scott et al. 2003, Gondhalekar et al. 2011).

In spite of bed bug resistance to some pyrethroid insecticides, bifenthrin, a type-1 pyrethroid, has previously been found to be effective for bed bug control (Moore and Miller 2008, Steelman et al. 2008). Bifenthrin is an active ingredient (AI) in certain professional insecticide formulations (Talstar®) and is also used in a neonicotinoid/pyrethroid combination product (Transport®) (Johnson et al. 2010). Bifenthrin works as a contact insecticide, keeping the para-homologous sodium channels open, causing neuroexcitation (Fecko 1999, Johnson et al. 2010). Unlike chlorfenapyr, bifenthrin cannot be applied to mattress folds, and indoor application is limited to cracks, crevices, baseboards, box springs, bed frames, and the underside of shelves or drawers. In general, pyrethroid insecticides have been found to be repellent to bed bugs regardless of their resistance status (Romero et al. 2009). However, bed bugs will walk across insecticide barriers in order to obtain blood meals (Moore and Miller 2006, Romero et al. 2009). Bifenthrin is an active ingredient in many products available to the general public and therefore has the potential to be used improperly, thus increasing the potential for resistance development.
Bed bugs are capable of quickly becoming resistant to any insecticides continuously used for their control (Lofgren et al. 1958, Romero et al. 2007, Adelman et al. 2011). Organochlorines (DDT), organophosphates, and certain pyrethroids (deltamethrin and λ-cyhalothrin) are insecticides to which bed bug populations have already developed resistance (Lofgren et al. 1958, Romero et al. 2007, Seong et al. 2010, Adelman et al 2011, Boase and Naylor 2014). Given the inevitable risk of resistance development, it is likely that widely used insecticides, such as chlorfenapyr and bifenthrin, may lose their efficacy in the future. Cross-resistance from other pyrethroid insecticides is also likely to speed up the development of bifenthrin resistance (Romero et al. 2007, Steelman et al. 2008, Adelman et al 2011). Development of chlorfenapyr and bifenthrin resistance in bed bugs could have a significant impact on overall bed bug management as there are few insecticide options available for indoor use against bed bugs. It is advantageous to be able to continue to use chlorfenapyr and bifenthrin for the management of bed bug infestations since they are both relatively safe insecticides and provide high control efficacy.

In order to delay bed bug resistance development to chlorfenapyr and bifenthrin, insecticide resistance management techniques such as product rotations and use of non-chemical control techniques to reduce pesticide selection pressure should be implemented (ffrench-Constant and Roush 1991, Gondhalekar et al. 2010). However, no recent information about the susceptibility of field bed bug populations to chlorfenapyr or bifenthrin is available. Additionally, no standardized bioassay technique for chlorfenapyr and bifenthrin susceptibility monitoring of bed bug field populations was available.

The objective of this study was to determine the chlorfenapyr and bifenthrin susceptibility status of field bed bug populations using the diagnostic glass vial bioassay described in Chapter 2. Ten field-collected bed bug populations were screened at the LC99 concentrations for chlorfenapyr (0.5%) and bifenthrin (0.96x10^-4%). The information gained from this susceptibility monitoring program can be used by PMPs and other individuals to make informed decisions on insecticides optimal bed bug control and also for delaying the development of resistance to chlorfenapyr and bifenthrin.
3.2 Materials and Methods

3.2.1 Insects

Table 3.1. Table represents the known information of assayed bed bugs. Population name, year collected, and treatment history or known resistance of the bed bugs. Insects with unknown treatment histories were likely exposed to pyrethroids, neonicotinoid/pyrethroid combination insecticides, or chlorfenapyr.

<table>
<thead>
<tr>
<th>Bed Bug Colonies</th>
<th>Collection Year</th>
<th>Treatment History/resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harlan</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Indianapolis, IN-1</td>
<td>2012</td>
<td>Unknown</td>
</tr>
<tr>
<td>Indianapolis, IN-2</td>
<td>2013</td>
<td>Unknown</td>
</tr>
<tr>
<td>Indianapolis, IN-3</td>
<td>2014</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cincinnati, OH</td>
<td>2013</td>
<td>Neonicotinoid/pyrethroid resistant</td>
</tr>
<tr>
<td>Washington D.C.</td>
<td>2013</td>
<td>Neonicotinoid/pyrethroid resistant</td>
</tr>
<tr>
<td>Lafayette, IN</td>
<td>2014</td>
<td>From neonicotinoid/pyrethroid treated account</td>
</tr>
<tr>
<td>Hackensack, NJ</td>
<td>2014</td>
<td>Unknown</td>
</tr>
<tr>
<td>Knoxville, TN</td>
<td>2013</td>
<td>Unknown</td>
</tr>
<tr>
<td>Poultry House, TN</td>
<td>2013</td>
<td>Unknown</td>
</tr>
<tr>
<td>Richmond, VA</td>
<td>2008</td>
<td>Deltamethrin resistant</td>
</tr>
</tbody>
</table>

The insecticide susceptible Harlan strain and all other populations were maintained at Purdue University (Table 3.1). All bed bugs were maintained at 25°C, 50% ±15 relative humidity and a 12:12 hour light/dark cycle in a walk-in environmental chamber (Percival Scientific, Perry, IA). Insects were fed using the Parafilm® membrane-method on defibrinated rabbit blood that was heated to 37°C using a reptile heating pad (Chin-Heady et al 2013). All adult male bed bugs were recently emerged and fed 5 days prior to bioassay. Due to traumatic insemination-induced mortality that occasionally occurs in adult females, only adult male bed bugs were used in all bioassays.
3.2.2 Chemicals

Talstar® and Phantom® were purchased from Univar (Indianapolis, IN).

3.2.3 Field population screening

Ten field collected bed bug populations were screened at the diagnostic LC$_{99}$ concentrations for bifenthrin and chlorfenapyr. Formulated insecticide (Talstar® and Phantom®) stock solutions were freshly made with deionized water just before initiation of bioassays. Each glass vial (4 mL) (Scientific Specialties, Hanover, MD) was treated with 100 µL of the LC$_{99}$ diagnostic concentration for chlorfenapyr 0.5% (556.1 µg/vial or 5.77 µg/cm$^2$) and bifenthrin 0.96x10$^{-4}$% (0.1 µg per vial or 0.0014 µg/cm$^2$) and allowed to dry inside a fume hood on a hotdog roller (with heating element disabled). Complete drying of vials required ~72 h due to the use of water as solvent. Control vials were treated with deionized water. After the vials were dry, ten adult male bed bugs were placed into each vial. Vials were covered with Parafilm® that was punctured to allow aeration. Glass vials were placed upright to ensure bed bug contact with insecticide treated surfaces for the entire assay duration. Bioassay vials were held in the same environmental chamber used for rearing. Approximately 10 to 14 replicates were performed for each strain at the LC$_{99}$ concentrations. Controls included insects exposed to water-treated glass vials as well as one replicate of the Harlan strain at the LC$_{99}$ rate as a positive control. Mortality assessments for bifenthrin took place at 24, 48, and 72 h whereas chlorfenapyr assessments were made every 24 h for 168 h (7 d).

3.2.4 Data analysis

Mortality data from chlorfenapyr and bifenthrin diagnostic glass vial bioassays were arc sine transformed and analyzed by ANOVA using a general linear model (PROC GLM) (SAS 2011). Means were separated using the all pairs Tukey’s test ($P<0.05$) (Figure 3.1 and 3.2).
Figure 3.1 Results of the glass vial bioassays at the diagnostic LC$_{99}$ concentration for chlorfenapyr which corresponded to a 0.5% solution (556 µg AI per vial or 7.87 µg AI/cm$^2$). Bars represent percent mortality at 7 d (168 h) post treatment. Bars that are not connected by the same letter are significantly different (Tukey’s test ($P < 0.0001$)). Data for Richmond and Washington D.C. strains are adapted from Chapter 1.
Figure 3.2 Results of the glass vial bioassays at the diagnostic LC_{99} concentration for bifenthrin which corresponded to a 0.96x10^{-4}% (0.1 µg AI per vial or 0.0014 µg AI/cm^2). Bars represent percent mortality at 3 d (72 h) post treatment. Bars that are not connected by the same letter are significantly different (Tukey’s test \( P < 0.0001 \)). Data for Richmond and Washington D.C. strains are adapted from Chapter 1.
Results and discussion

Screening of field collected bed bugs for chlorfenapyr and bifenthrin susceptibility with the glass vial assay technique showed significant mortality variation between populations (Figure 3.1 and 3.2). Control mortality was minimal (<3%) for all strains screened, except for chlorfenapyr assays with the Indy-1 strain (17.7%), Hackensack, NJ strain (6%) and the Washington D.C. (13%) strain. Collection times were known for all populations screened, but the definite insecticide exposure history, or resistance status to other insecticides was known only for the Indianapolis 1, Indianapolis 2, Richmond, VA; Lafayette, IN; Cincinnati, OH; and Washington D.C strains (Table 3.1). However, given the common use of pyrethroid insecticides for bed bug control it is reasonable to assume that all strains with unknown insecticide exposure history were exposed to at least one type of pyrethroid insecticide.

All but three of the field bed bug populations exposed to the chlorfenapyr LC₉₉ diagnostic concentration had susceptibility levels similar to the Harlan strain. Significantly decreased mortality was found when the Poultry House, TN; Knoxville, TN; and Richmond, VA field strains were exposed to dry chlorfenapyr residues (ANOVA results for chlorfenapyr: df = 29, 90, F =4.03, P = <0.0001). Results indicate that chlorfenapyr tolerance has developed in the Cincinnati, OH; 2014 Indianapolis, IN; and Washington D.C. strains as they were similar to the Knoxville, TN strain which was significantly different from the Harlan strain (Figure 3.1). Like other studies, none of the bed bugs with known pyrethroid resistance (Richmond, VA; Cincinatti, OH; Washington D.C.) showed negative cross-resistance to chlorfenapyr (Romero et al. 2010). Increasing the time of a bioassay is not desirable, but if experimental time were longer, it is possible that complete mortality in the chlorfenapyr resistant bed bug populations would have occurred (Champ and Campbell-Brown 1970, Romero et al. 2010, Singh et al. 2015).

Half of the field collected populations exposed to the LC₉₉ diagnostic concentration of bifenthrin had significantly lower mortality compared to the susceptible Harlan strain (Figure 3.2). The Cincinnati, OH; Indianapolis-3, IN; Knoxville, TN; Lafayette, IN; and Richmond, VA, strains were found to have significantly lower mortality than the Harlan susceptible strain (ANOVA results for bifenthrin: df = 19, 90, F
=4.47, \( P = <0.0001 \)). The Lafayette, IN strain was previously exposed to a neonicotinoid/pyrethroid combination insecticide, but it did not include bifenthrin. It is unknown whether any of the other bifenthrin resistant strains had been treated with bifenthrin before; further assays should be conducted with the resistant populations. The bifenthrin field application rate (0.06%) is drastically higher than the statistically determined diagnostic \( LC_{99} \) concentration (0.96x10^{-4}%) for bifenthrin. Therefore, if any of the field populations were exposed to the label rate, they would likely die. However, screening of bed bug field populations for bifenthrin susceptibility is valuable for determining whether deltamethrin resistance confers cross resistance to other pyrethroids. In addition, stand-alone bifenthrin diagnostic bioassays are needed because of the challenges associated with determining resistance or susceptibility levels to neonicotinoid-pyrethroid combination products (e.g. bifenthrin + Acetamiprid) (Roberston et al. 2007).

Although chlorfenapyr is not repellent, bed bugs within an insecticide treated infestation will cross residual barriers and feed (Moore and Miller 2006, Romero et al. 2010, Singh et al. 2015). Bed bugs that have fed have a greater tolerance to many insecticides, including chlorfenapyr, which is likely due to upregulation of cytochrome P450s associated with blood meal detoxification (Devries et al. 2015, Singh et al. 2015). Research by Singh et al. 2015 indicates that bed bugs should be fed every three days during insecticide bioassays for more field relevant results. To accommodate this glass vial assay with a Parafilm® cap allows for \textit{in vivo} feeding of bed bugs. It is important to note that female bed bugs that have fed and are exposed to chlorfenapyr are able to lay viable eggs before mortality occurs (Singh et al. 2015). Bed bugs in future chlorfenapyr susceptibility assays could be fed every three days to determine how it influences the mortality of the tolerant populations mentioned above. The field collected bed bugs that had significantly reduced mortality to chlorfenapyr should further investigated to understand why they have decrease susceptibility to this insecticide.

Some laboratory selection bioassays suggest that bed bugs can develop resistance to the pyrethroid component of neonicotinoid combination insecticides (Gordon et al. 2014). However, practical resistance that results in control failures most likely would not
occur for the bed bug populations screened for bifenthrin susceptibility. In order to confirm such a hypothesis, in the future, the Cincinnati, OH; Lafayette, IN; Knoxville, TN; and Richmond, VA bed bug populations should be screened at the bifenthrin label application rate. Additionally, lethal concentration values could be determined for these populations to determine resistance ratios. Interestingly, the response of a bed bug strain when exposed to the bifenthrin LC$_{99}$ was a good indicator of their tolerance or susceptibility to the chlorfenapyr LC$_{99}$ diagnostic concentration and vice versa. Interestingly, the population’s response when exposed to the bifenthrin LC$_{90}$ was a good indicator of their tolerance or susceptibility to the chlorfenapyr LC$_{99}$ diagnostic concentration and vice versa (data not shown). Therefore, when proper insecticide rotations are occurring, care should be taken to avoid using bifenthrin in areas where chlorfenapyr was applied previously.

The current study is the first research project designed to screen field bed bug populations for chlorfenapyr and bifenthrin susceptibility that were collected from around the United States. The results of the study demonstrate the need for constant susceptibility monitoring of bed bug populations to ensure that insecticides remain effective. Additionally, chlorfenapyr should not be relied upon as a stand-alone chemical treatment; instead both non-chemical treatments should be used in conjunction with insecticide rotation to delay resistance development (Gondhalekar et al. 2011, Gordon et al. 2014). When chlorfenapyr or bifenthrin is applied in conjunction with IPM techniques, infestations can be effectively eliminated (Kells 2006, Wang et al. 2009, Steadfast and Miller 2014). Application of lethal heat for bed bug control is an effective alternate technique PMPs are deploying, which will also help delay the development of chlorfenapyr and bifenthrin resistance in bed bug field populations (Benoit et al. 2009, Kells and Goblirsch 2011). Bed bug infestations are still a common occurrence and are likely to remain. Development and implementation of a susceptibility monitoring program for chlorfenapyr and bifenthrin are only the first steps of a resistance management program. Adoption of an IPM approach and use of insecticide rotations/mixtures is further needed to maintain the efficacy of chlorfenapyr and bifenthrin for bed bug control.
CHAPTER 4. SUMMARY

The biology of bed bugs makes them a very challenging urban pest to control. Bed bugs are relatively long-lived, have a short development period and high fecundity, exhibit cryptic behavior, and are adapted to deal with toxic blood meals (Reinhart and Silva-Jothy 2007, Singh et al. 2015). The feeding effects that bed bugs have on individuals create a zero-tolerance threshold to their presence in most urban settings and therefore effective insecticides are required to meet this demand. However, like most pest insects, bed bugs quickly develop resistance to any insecticide that is overly or improperly used, including chlorfenapyr and bifenthrin. In order to ensure that chlorfenapyr and bifenthrin remain effective a comparative diagnostic bioassay program was implemented with this research and the major conclusions are discussed below.

Statistical comparisons of the glass vial bioassay to the filter paper bioassay found that the filter paper assay required a significantly greater amount of insecticide for LC$_{50}$ and LC$_{99}$ diagnostic concentrations that cause the desired mortality in the Harlan susceptible strain, except at the chlorfenapyr LC$_{50}$. Absorption of the insecticide by the filter paper most likely caused the increased amount of active ingredient required to achieve desired bed bug mortality. The filter paper assay also required a larger volume of insecticide to be applied in order to ensure complete coverage so that the bed bugs would not avoid residues of the active ingredient. Additionally, when bed bugs were exposed to chlorfenapyr residues in the filter paper assay, it took 14 days to achieve complete mortality. If the glass vial bioassay is used, 100% mortality of a susceptible strain can be achieved in 7 days or sooner. The glass vial assay with a Parafilm® cap would also allow individuals to be fed during the experiment, which increases the realistic value of this
exposure method. Therefore the glass vial bioassay was chosen over the filter paper assay for validation and further screening of bed bug populations.

Formulated chlorfenapyr was a better candidate for testing use because it would have required hundreds of milligrams (600 to 900 mg) of technical grade AI per assay, making it economically unfeasible to deploy in a large scale susceptibility monitoring program. The LC$_{99}$ diagnostic concentration determined for formulated chlorfenapyr was 0.42%, which is close to the label application rate and was therefore, rounded up to 0.5%. This made the chlorfenapyr glass vial bioassay using formulated product more relevant to field control and easy to deploy by PMPs. In contrast to chlorfenapyr, it is possible to use both formulated and technical grade bifenthrin in resistance or susceptibility monitoring studies. However, in order to have a balanced approach, formulated bifenthrin was used instead of technical product. Validation of the LC$_{50}$ and LC$_{99}$ diagnostic concentrations determined for the glass vial bioassay against the Harlan-susceptible strain, and the field collected Richmond, VA strain, and the Washington D.C. strains confirmed the utility of this technique to detect susceptibility shifts in bed bug strains. Mortality values and statistical comparisons can be seen in Chapter 1.

The next phase was to deploy the glass vial LC$_{99}$ diagnostic concentrations for chlorfenapyr and bifenthrin and screen eight additional bed bug populations (ten total). Three of the ten field bed bugs screened, had significantly lower mortality at the chlorfenapyr LC$_{99}$ diagnostic concentration when compared to the Harlan strain. Half of the strains had significantly lower mortality than the Harlan strain at the bifenthrin LC$_{99}$ diagnostic concentration. However, the LC$_{99}$ diagnostic concentration for bifenthrin is relatively low as compared to the label application rate and therefore it is likely that if any of the bifenthrin tolerant populations were exposed to the label application rate, complete mortality would occur. Development of chlorfenapyr resistance in the Knoxville, TN, Poultry House, and Richmond strains in reference to our Harlan susceptible strain is much more problematic. Interestingly, resistance to chlorfenapyr was a good indicator of bifenthrin resistance. Results confirm that chlorfenapyr should not be solely relied upon for control of bed bug infestations, but is instead paired with
insecticides that have a faster mode of action and combined with non-chemical control techniques.

The use of integrated pest management (IPM) programs is an effective way to eliminate bed bug infestations while also mitigating resistance development by reducing insecticide selection pressure (Kells 2006, Wang et al. 2010, Steadfast and Miller 2014). Care should be taken when choosing a product to rotate to because strains displaying chlorfenapyr resistance may also have reduced susceptibility to bifenthrin. Diagnostic glass vial assays for chlorfenapyr and bifenthrin should be regularly performed to closely monitor susceptibility levels in bed bug populations. If PMPs use the diagnostic glass vial assays developed for chlorfenapyr or bifenthrin, they could choose an insecticide for bed bug control that is more effective, while also achieving adequate control and increasing customer satisfaction. Only through consistent susceptibility monitoring programs and implementation of IPM protocols can the efficacy of insecticides such as chlorfenapyr and bifenthrin be preserved for effective bed bug remediation.
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