2016

THE CHEMICAL ECOLOGY OF BED BUGS (*CIMEX LECTULARIUS*, L.) AND THE IMPACT OF A NEUROTOXIC INSECTICIDE ON PHYSIOLOGY AND BEHAVIOR

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Digital Object Identifier: https://doi.org/10.13023/ETD.2016.499

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THE CHEMICAL ECOLOGY OF BED BUGS (*CIMEX LECTULARIUS*, L.) AND THE IMPACT OF A NEUROTOXIC INSECTICIDE ON PHYSIOLOGY AND BEHAVIOR

DISSERTATION

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

By

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ABSTRACT OF DISSERTATION

THE CHEMICAL ECOLOGY OF BED BUGS (*CIMEX LECTULARIUS*, L.) AND THE IMPACT OF A NEUROTOXIC INSECTICIDE ON PHYSIOLOGY AND BEHAVIOR

Following a decades-long hiatus in many nations, populations of bed bugs (*Cimex lectularius*) have rebounded and are thriving on a global scale posing substantial challenges for pest management professionals. Insecticide resistance to both pyrethroid and neonicotinoid compounds has been identified in many populations, leading to renewed interest in alternative control methods for management of this pest. The objectives of my dissertation research were to study various, typically cryptic, bed bug behaviors in order to improve both conventional and alternative management strategies. Novel behavioral interactions among mother and offspring were characterized. Additionally, changes in behavior and physiology after treatment with a commercial insecticide were explored. In the laboratory, the presence of female bed bugs improved foraging efficacy of first instar nymphs. There is evidence that this interaction is mediated by low-volatility pheromones deposited in the feces by adult females following feeding. This discovery may lead to an improvement in trapping methods for juvenile bed bugs. A novel behavior, egg-marking, by females was characterized. After laying an egg, females rapidly move the abdomen side to side above their egg for 8-41 seconds. Although the function of this behavior is yet to be characterized, some evidence suggests a potential form of maternal care. Finally, laboratory assays indicate numerous detrimental effects of sublethal exposure to the commercial insecticide, Temprid® SC, which could impact management practices in the field. Sublethal exposure to Temprid® SC led to decreases in egg viability, feeding efficacy, locomotion, and mating success. Other behavioral changes were more variable, such as the first and median day of egg laying. Aggregation behavior and eclosion of fifth instars, however, were not impacted by treatment. In this dissertation, I show that i) females benefit offspring by enhancing feeding efficacy of first instars, and ii) sublethal impacts of Temprid® SC affect bed bug behavior and physiology in ways that could impact current management strategies. These results demonstrate that alternative management strategies disrupting mother-offspring interactions are possible for bed bug control. Bed bug behavior, especially in response to insecticide treatment, should also be thoroughly evaluated to enhance conventional control practices for *C. lectularius* in the field.

KEYWORDS: *Cimex lectularius*, bed bug, behavior, semiochemical, aggregation, sublethal effects

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2016

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I would like to dedicate this dissertation to my father, Dallas Wayne Crawley who passed away June 17, 2015, before I could celebrate this achievement with him. Thank you for taking so many of my wild softball pitches to the ankles and for believing in me. Rest peacefully.

Acknowledgements

I would like to begin by thanking my advisor, Dr. Kenneth F. Haynes, for allowing me to join his lab and for making me a better entomologist, and scientist. I would not have a fraction of the success I have achieved without his input and guidance. I could not have asked for a more supportive, understanding, or fair mentor. Without him, I likely would not have identified chemical ecology as an interest of mine, and thus many of my accomplishments both now and in the future can be credited to his influence and knowledge. Looking ahead, I anticipate and look forward to continued collaboration as well as friendship for years to come.

I must also thank Dr. Michael F. Potter for piquing my interest in bed bugs and urban entomology. If Dr. Potter had not allowed me to accompany him to a fumigation in Louisville, Kentucky, I am not sure I would have had the inspiration to pursue a graduate career studying bed bugs. Similarly, I'd like to thank Dr. James Wagner for steering me toward a career in animal behavior. His courses at Transylvania University made learning enjoyable, and for years he has believed in my potential. I also appreciate his willingness to serve on my committee, advise me on statistics, and to travel here (often by bicycle) from another institution. I would also like to thank my other committee members, Drs. Jennifer White and David Westneat. Each of them made obscure statistical techniques seem more understandable and contributed many worthwhile ideas to projects that might otherwise have been ignored. All of their input helped make my work more interesting and robust. I would also like to thank all of the faculty members in this department because they have each given me advice (either personal or professional) at one time or another.

I would like to express sincere gratitude to my lab members, both past and present. Mark Goodman taught me how to work with bed bugs, and was such a calming presence in our lab. Both Mark Goodman and Shelby Stamper provided technical help on many of my introductory experiments that otherwise would not have been possible. Scott Bessin has provided many laughs, in that we never understand jokes the other makes. Additionally, Scott was always willing to help with overflow when I had taken on too much to do alone. Dr. Katelyn A. Kowles was a constant source of fun and laughter, and made some of my later, tedious experiments much more enjoyable. Last, but absolutely not least, Dr. Jennifer R. Gordon. Jennifer rapidly became my best friend and I could not have finished this degree without her. Also, thank you to each graduate student who has been my friend during my tenure in the Department of Entomology. Our many hikes, races, and brewery outings provided much needed relief from the pressures of graduate school. I would especially like to thank Dr. Elizabeth Andrews for being one of my first friends here, and for encouraging me to pursue a Ph.D.

I would like to take the time to thank the various connections I have made in the pest management industry that have given me networking advice, allowed me to visit infestations, or helped me attend conferences. Specifically, I would like to thank Mark Vanderwerp and Rose Pest Solutions for inviting me to present at one of their training conferences. This was a very worthwhile experience for me.

Additionally, I very much appreciate each of my funding sources. Thank you to the Department of Biology for allowing me to teach Microbiology as a Teaching Assistant for so many years. Without this experience, I never would have met Dr. John

P. Seabolt, yet another amazing mentor to me. I am also thankful to the Department of Entomology for funding, Rollins Inc., FMC, and MGK.

Finally, I would like to express my gratitude to my friends and family outside the department. I would not be the accomplished public speaker I am today without my mother, Kimberly Crawley, who encouraged/forced me to participate in theatre. My sister, Olivia Crawley, who always asks the tough questions like, “Really, bed bugs?” Cody Ellis, for unwavering support, lots of laughter, and motivation to keep pushing. You make difficult times much more bearable. Thank you to my best friend, Brandi Monnett, who has steered me through both life and graduate school. Without all of their support, there is no way I would be here today.

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Chapter 1. Introduction

Bed bug resurgence

Following a decades-long hiatus in many nations, populations of bed bugs (*Cimex lectularius,* L.) have rebounded and are thriving on a global scale (Doggett et al. 2004, Romero et al. 2007). Although bed bugs have been defined as parasites of humans since the beginning of recorded time (Usinger 1966), their presence was initially intermittent due to the nomadic tendencies of early hominids (Potter 2011). However, the eventual establishment of immobile villages and communities allowed for a more prolonged, intimate association between bed bugs and humans. For instance, bed bugs have been exhumed from ancient archeological sites dating back in excess of 3,500 years (Panagiotakopulu and Buckland 1999). Gradual expansion of civilization, urbanization, and the development of international commerce and travel allowed bed bugs to reach new borders that were previously uninhabitable. Bed bugs entered Europe and Asia in 77 CE, China in 600 CE, and Germany and France in the 11th and 13th centuries, respectively (Usinger 1966, Potter 2011). Populations persisted mostly intermittently and seasonally around the world until the 1900's, when large booms in population numbers occurred alongside the advent of central heating. Consistent temperatures allowed bed bugs a reproductive advantage that led to epidemics in many major cities (especially in developed nations) in the 1930's and 1940's (Johnson 1942). At this time, bed bug infestations were often treated with various home remedies and control methods focused on improving sanitation. Not surprisingly, low-income areas were hit the hardest since there were fewer resources available to discourage infestation (e.g., reduced access to

fresh bedding and linens). However, without effective insecticides, privileged and poor alike struggled to control populations of bed bugs during this era.

A renowned and dramatic shift in both bed bug control and pest management emerged in the 1940's after the discovery of dichloro-diphenyl trichloroethane (DDT). Application by both professionals and civilians during this time became routine. The success of DDT can be attributed not only to its mode of action, but also to its ability to target cryptic bed bug behavior. For the first time in history, bed bugs hidden in harborages during treatment, as well as newly emerged first instars, were effectively targeted by a residually active product (Potter 2011). The use of DDT and chlorinated insecticides, as well as the development and use of pyrethroids, organophosphates, and carbamates also helped reduce populations of bed bugs to the point of near elimination, especially in developed nations. Sporadic infestations were only occurring in areas where individuals did not practice proper sanitation practices or where there was little access to chemicals (Krueger 2000). However, DDT, and carbamates are no longer acceptable for use against bed bugs in most countries due to safety concerns and regulatory restrictions. Organophosphate use is also more tightly regulated. In addition, as with any chemical that kills and applies selection pressure to the population, resistance to DDT was reported by military personnel even before it was readily available for civilian use. Along with increased travel, the reuse of furniture, and an overall loss of vigilance, insecticide resistance is proposed as one of the main factors in the resurgence of populations of bed bugs that we have observed worldwide for the last fifteen years (Doggett et al. 2004, Romero et al. 2007).

Bed bug biology

The bed bug is a flightless, nocturnal insect that will also parasitize birds, bats, and other domesticated animals (Usinger 1966). They are classified in the order Hemiptera and belong to the family Cimicidae. Adult bed bugs are approximately 3-5 mm in length, dorso-ventrally flat and oval in shape, with brownish-red coloration. Unfed nymphs are yellow-brown, transparent, and turn dark red after taking a blood meal. After hatching, bed bug nymphs develop through five separate stages with each instar requiring at least one blood meal before molting to the subsequent instar (Usinger 1966, Davies et al. 2012). As adults, both sexes of bed bugs will require blood meals; females will need the nutrition from the blood meal to produce a clutch of eggs, and males for spermatogenesis (Reinhardt and Siva-Jothy 2007). Optimally, adult bed bugs will feed at least once per week for their entire lifespan (approx. 150 days) (Polanco et al. 2011). However, frequency of feeding is variable and depends on temperature, host availability, and life stage of the individual bug. After feeding, bed bugs return to refuges (also termed "harborages" in the literature) where they will aggregate throughout the day until feeding occurs again during the scotophase (Reinhardt and Siva-Jothy 2007). Locomotion is regulated by a circadian rhythm, with most exits from the aggregation occurring at night in 24-hour intervals (Romero et al. 2010a). Aggregations of bed bugs consist of bugs in all life, feeding, and mating statuses (Pfiester et al. 2009). Within aggregations bed bugs copulate, lay eggs, defecate, and molt. Bed bugs remain in these aggregations during daylight hours, and leave predominantly during the night at intervals of several days (Kilpinen et al. 2013). However, frequent random movement during daylight hours has been documented (Cooper et al. 2015).

Much research has been directed at the activity and behavior of adult bed bugs within aggregations, especially copulatory biology and behavior. After feeding, both immediately after and up to 72 h post-feeding, adult bed bugs copulate a number of times (Stutt and Siva-Jothy 2001). Males copulate with multiple females during this time. During mating, males pierce the abdomen of the female, in opposition to insertion into the genital opening (Usinger 1966). Males pierce females using their paramere (also called the intromittent organ) and sperm is released directly into the hemolymph (Usinger 1966, Stutt and Siva-Jothy 2001, Reinhardt and Siva-Jothy 2007). This is known as 'traumatic insemination' and the imposed stress can cause reduced longevity in females, and an inability to maintain water balance (Stutt and Siva-Jothy 2001, Morrow and Anqvist 2003, Benoit et al. 2012). To counter this stress, female bed bugs evolved a paragenitalia structure called the spermalege that reduces the damage to the cuticle (Reinhardt et al. 2003, Benoit et al. 2012). Resilin, an elastic protein present in the cuticle of the female spermalege, contributes to the reduction of tissue damage and hemolymph loss caused by traumatic insemination (Michels et al. 2015). The male paramere is uniquely shaped to channel into the area of the spermalege, but miss-strikes occur and are costly to females in that they must expend energy repairing damaged cuticle, accounting for fluid loss, and there is potential for infection (Stutt and Siva-Jothy 2001, Benoit et al. 2012). Females do have a genital tract, but its only function is during oviposition. Aside from data describing the time between blood meal acquisition and oviposition (Johnson 1941), little is known about oviposition and egg laying behavior/patterns in bed bugs outside of the mating process. However, Polanco et al. (2001) did show a reduction in overall egg production due to excessive mating events

imposed on females. Sexual conflict and traumatic insemination are two related aspects of bed bug biology and behavior that have prompted extensive interest (Stutt and Siva-Jothy 2001, Morrow and Arnqvist 2003, Reinhardt et al. 2003, Reinhardt and Siva-Jothy 2007, Reinhardt et al. 2009a, Reinhardt et al. 2009b, Benoit et al. 2012).

Chemical ecology and olfaction

Although it has generated widespread interest, an understudied aspect of bed bug biology is bed bug olfaction and chemical ecology. Chemical ecology deals with the chemical mechanisms that regulate both intra- and interspecific interactions between living things (Eisner and Berenbaum 2002, Wertheim et al. 2005). A wide array of arthropods use common chemical compounds, or have modified common chemical compounds, into unique semiochemicals that can serve multiple functions in very diverse environments (Blum 1996). In fact, chemical communication is arguably one of the most widely used modalities for communication across all of the kingdoms (Wyatt 2009). Semiochemicals (derived from the Greek *semeion*, sign), are compounds responsible for transmitting information between individuals. One class of semiochemicals, pheromones, are chemicals released by one individual that trigger changes in behavior and physiology of a conspecific (i.e., intraspecific) (Karlson and Lüscher 1959). Pheromones are grouped based on the behaviors they elicit. For example, aggregation pheromones attract and/or arrest conspecifics to the location of their release point (Wertheim et al. 2005) while alarm pheromones alert conspecifics of potential threat and may initiate dispersal (Bowers et al. 1972). Allomones and kairomones regulate interspecific interactions benefitting the emitter but not the receiver (e.g. defensive

compounds), or the receiver but not the emitter (e.g., many attractants or phagostimulants), respectively (Brown, Jr. et al. 1970).

Pheromones and other semiochemicals often originate from specialized secretory glands, but they may also arise from body cavities, as well as organs central to digestion and reproduction (mouth, anus, etc.) (Wertheim et al. 2005). Adult bed bugs have a paired, specialized set of scent glands as well as a central reservoir situated in the metathorax at the base of their abdomen (Usinger 1966). The contents of the scent glands are released through a scent channel and an area where evaporation occurs, on the ventral side of the first abdominal segment (Usinger 1966, Weeks et al. 2010). The perception of pheromones by insects in general requires chemosensory organs that are most often located on the antennae, tarsi, or appendages of the mouth (Wertheim et al. 2005). For bed bugs, the antennae serve as the main olfactory organ (Steinbrecht and Muller 1976), but there is much left to elucidate regarding their role in semiochemical detection. From what is understood, perception of semiochemicals by bed bugs is similar to other hematophagous insects; volatile semiochemicals are detected after entrance into the antennae through pores in olfactory sensilla. They then move through the hemolymph (inside the sensilla) and bind to dendrites on olfactory receptor neurons (ORNs). Odorant-binding proteins (OBPs) are typically responsible for the transport of semiochemicals across the hemolymph. Genomic sequencing has identified 11 highly species-specific OBPs for bed bugs (Benoit et al. 2016), however, no functional studies have been conducted to date. Once semiochemicals have navigated through the hemolymph, they bind to receptors on the dendrites of olfactory receptor neurons (ORNs) and an action potential is interpreted by the insect's central nervous system (CNS),

initiating the behavioral response. Two olfactory regions (ORs) have been identified on bed bug antennae, but gustatory sensilla (which likely mediate some behaviors) on the antennae are still unidentified. Recent sequencing of the bed bug genome identified 24 genes encoding 36 gustatory receptors (GRs) (Benoit et al. 2016). Like OBPs, their location and function still require elucidation, but it is known that four of the bed bug GRs are related to a lineage of carbon dioxide receptors found in some species of flies, moths, beetles, and a termite (Benoit et al. 2016). In comparison to other blood-feeding insects, bed bugs have fewer olfactory-like sensilla on their antennae (50-fold fewer than *Triatoma infestans*) which authors propose could be due their close physical association with human hosts (Harraca et al. 2012, Hansen et al. 2014).

Semiochemically-mediated behaviors exhibited by bed bugs are well documented in the literature and many have been well studied. There has been much interest in alarm pheromones and dispersal, aggregation behavior, and some emphasis has been placed on sex and juvenile recognition. All of these interactions are mediated by pheromones produced by bed bugs. Additionally, kairomones likely play a role in host-location and have also been explored to understand bed bug ecology, as well as for their potential use in monitors and traps.

Alarm pheromone

Typically, alarm pheromones are known for their beneficial role in allowing individuals to escape predation (Bowers et al. 1972). This is because alarm pheromones typically cause the rapid dispersal of insects away from a potential threat. The bed bug alarm pheromone has many additional functions, however, and only one study has tested the role of the alarm pheromone against predators in the field (Reinhardt 2012). The

chemical structure of and compounds comprising the bed bug alarm pheromone were identified in the 1970s. We know that the bed bug alarm pheromone is responsible for the characteristic aroma associated with bed bug infestations. In a state of distress or alarm, bed bugs expel the contents of their scent glands, which stimulates locomotion of conspecifics (Levinson and Bar Ilan 1971). Early studies of scent gland composition revealed a concentration of (*E*)-2-hexenal and (*E*)-2-octenal in very high proportions, as well as acetaldehyde, butanone, and some minor components in smaller quantities (Levinson et al. 1974a,b). Later, headspace analyses revealed that (*E*)-2-hexenal and (*E*)-2-octenal could comprise as much as 78% of the 15 substances found in whole body eluates (Siljander et al. 2008). Additionally, a correlation can be observed between the rates of dispersal of bed bugs when exposed to their alarm pheromone; the delay of movement away from the area decreases as the concentration of alarm pheromone increases (Levinson et al. 1974a,b, Weeks et al. 2011). Levison et al. (1974a) also identified the behavioral threshold of response to (*E*)-2-hexenal and (*E*)-2-octnenal: 6×10^{15} and 9×10^{14} molecules mL^{-1}, respectively. However, this pheromone blend is multi-functional, with multiple behavioral responses to these compounds observed. For example, a recent paper described the ability of low concentrations of (*E*)-2-hexenal and (*E*)-2-octnenal to attract bed bugs (Ulrich et al. 2016).

Beyond repellency and attraction, Ryne (2009) elucidated one of the first additional behavioral functions for the bed bug alarm pheromone. As previously stated, mating in the bed bug is closely associated with the completion of blood feeding. During feeding, female bed bugs become too engorged to protect their exposed abdomen from males. Thus, bed bug mating is primarily based on vision and males are attracted to

engorged bugs (Stutt and Siva-Jothy 2001, Reinhardt and Siva-Jothy 2007). In fact, males will mount any engorged bug in close proximity, regardless of life stage or gender. However, homosexual mating attempts by males would not only be fruitless (i.e., no offspring produced), but there would be wasted energetic output (misused sperm) as well as abdominal injuries since males do not have the spermalege structure to protect against traumatic insemination. Males (both signaler and receiver) should benefit, then, by producing and responding to a signal that identifies their sex to other males. By using a blend of alarm pheromone, males are able to signal their identities to other males, avoiding erroneous mating attempts (Ryne 2009). Males with blocked scent glands are mounted more frequently than males that retain their ability to release alarm pheromone. Additionally, these treatment males held with other males showed decreased longevity in comparison to control males that were held on their own. Although the author stated that the cause of decreased longevity is unknown, it is likely that mating attempts were a contributing factor since multiple mating scars were observed on the abdomens of treatment males. Accordingly, Ryne (2009) proposed that a blend of (*E*)-2-hexenal and (*E*)-2-octenal serves as an effective sex identification signal in addition to a locomotor stimulant.

Adult male bed bugs are not the only life stage that can utilize alarm pheromone to signal identity. After feeding, late instar nymphs overlap in size with adult females and their size makes them a target for males that are mounting based on vision. To combat this, dorsal abdominal secretions of nymph-specific alarm pheromone prevent males from traumatic extragenital insemination of late instar nymphs (Harraca et al. 2010b). The alarm pheromone blend released by juveniles is unique when compared to

the blend released by adults. Not only does the ratio of (*E*)-2-hexenal:(*E*)-2-octenal differ (2:5 instead of 1:1), but nymphs have two additional juvenile-specific compounds in their alarm pheromone blend: 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal (Feldlaufer et al. 2010, Harraca et al. 2010b). The blend and the additional two compounds were first elucidated by Feldlaufer et al. (2010), but the behavioral function was not identified until later that same year (Harraca et al. 2010b). Researchers also came to the conclusion that males have specific receptors on the antennae that detect the juvenile-specific alarm pheromone, indicating the importance in avoiding mating attempts between juveniles and adult males.

Finally, the bed bug alarm pheromone also has anti-fungal properties (Ulrich et al. 2015). A study testing (*E*)-2-hexenal and (*E*)-2-octenal against an isolate of *Metarhizium anisopliae* sensu lato (*s.l.*) (Metsch.) Sokorin (Hypocreales: Clavicipitaceae) resulted in a significant inhibition of conidial viability (Ulrich et al. 2015). Direct contact, as well as fumigation for as little as 0.5 h, resulted in complete inhibition of fungal growth. Additionally, bed bugs held on filter papers with the fungus present had lower mortality rates if the alarm pheromone was present, versus insects held on infected papers and no pheromone (Ulrich et al. 2015). This indicates that the alarm pheromone may also be effective against potential pathogens that infect aggregations of bed bugs.

Aggregation pheromone

Aggregation pheromones mediate the formation of aggregations by attracting and/or arresting all conspecifics to the point of pheromone emission (Wertheim et al. 2005). The responders to emission of aggregation pheromone can be the same sex or opposite sex of the emitter. This is in contrast to sex pheromones, which only influence

the behavior of the opposite sex. Attempts to identify the "nest odor" (aggregation pheromone) of bed bugs first attracted attention many decades ago (Marx 1955). It was thought that nest odors were responsible for promoting the gregarious habits of bed bugs (Marx 1955). Bilateral antennectomy caused a loss of the aggregation response, indicating that the antennae served as the primary location for receptors of aggregation pheromone (Marx 1955). Filter papers with feces from other bed bugs, or even papers that have had contact with other bed bugs, can promote aggregation behavior. Additionally, thigmotaxis helps maintain aggregations (Levinson and Bar Ilan 1971, Siljander et al. 2007, Olson et al. 2009). Extracts of filter paper that have had previous contact with bed bugs also initiated aggregation behavior, however, removal of the ability to make direct contact with the papers causes a cessation of response (Siljander et al. 2007). Although some studies have shown no bias in bed bugs that respond to aggregation pheromone (Olson et al. 2009), others have shown that females are less likely to aggregate than males, and that the more males are present in the population, the less likely that mated females are to aggregate (Pfiester et al. 2009). Siljander et al. (2008) demonstrated that only virgin females responded to aggregation pheromone, prompting the development of the hypothesis that female bed bugs aggregate less often in order to avoid traumatic insemination by males. However, this hypothesis has yet to be appropriately tested in the laboratory or field (Crawley et al. 2015).

One of the first attempts to elucidate the airborne bed bug aggregation pheromone resulted in the finding that there were ten 'essential' components in the blend: (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, (*RS*)-limonene, benzaldehyde, nonanal, decanal, sulcatone, and benzyl alcohol (Siljander et al. 2008). Authors could not

distinguish between attraction versus arrestment in these studies due to their experimental design. Following the identification of the airborne aggregation pheromone, it was hypothesized that an additional contact, substrate-borne pheromone leading to arrestment had yet to be identified. The contact pheromone, histamine, was elucidated in 2015 (Gries et al. 2015). Additionally, these authors proposed only five essential volatile components: dimethyl disulfide, dimethyl trisulfide, (*E*)-2-hexenal, (*E*)-2-octenal, and 2-hexanone. The five volatile components are responsible for attracting bed bugs to the point of release, and histamine is responsible for bed bug arrestment. This blend was effective in attracting and arresting all life stages, sexes, and mating statuses of bed bugs. A more recent study claimed that low concentrations of (*E*)-2-hexenal and (*E*)-2-octenal alone were enough to attract (but not arrest) adult bed bugs, however juveniles were not tested for responses, and females and males differed from one another in their responses (Ulrich et al. 2016).

It should be noted that aggregation behavior is not unique to bed bugs, it is a general phenomenon found within many insect taxa and regulates a vast number of ecological interactions (Wertheim et al. 2005). Aggregation behavior and the production of aggregation pheromone is common in the order Hemiptera (Wertheim et al. 2005), and is prevalent in other hematophagous hemipterans (Lorenzo et al. 1996, Lorenzo et al. 1998). Benefits for forming aggregations include (but are not limited to) the more efficient utilization of resources, an increased ability to find mates, and enhanced protection from predators and/or adverse environmental conditions (Wertheim et al. 2005). Indeed, it is known that aggregation behavior can be beneficial for groups of bed bugs; aggregating helps bed bugs conserve water and prevents desiccation (Benoit et al.

2007). There are also benefits for juveniles: nymphs reared in groups develop faster than nymphs reared in isolation (Saenz et al. 2014) and first instar nymphs find hosts more effectively in aggregations with females present, versus aggregations where no females are present (Crawley, personal observation). There is preliminary evidence that the latter is mediated by components of bed bug aggregation pheromone (Crawley, personal observation). This is not unusual; adult insects often utilize aggregation pheromone to influence the feeding efficacy of juveniles within aggregations (Wertheim et al. 2005, Lin 2006, Wong et al. 2013). This interaction is considered a form of subsociality: "post-ovipositional parental care behavior that promotes the growth, survival, and development of juveniles" (Tallamy and Wood 1986). Historically, there has been little focus on adult-juvenile interactions within aggregations of bed bugs. In the future, it may become evident that aggregation pheromone, like the bed bug alarm pheromone, has multiple functions—including the regulation of subsocial adult-offspring interactions.

Kairomones and host-location

In opposition to mutual benefits for conspecific emitters and responders, kairomones are occasionally called the "backfire" of pheromone production. Kairomones are an interspecific class of semiochemicals where the receiver benefits from the cue, not the emitter. Kairomones are common mediators of predator-prey or parasite-host interactions, especially for blood-feeding insects such as mosquitos and sandflies (Kelly and Dye 1997, Takken and Knols 1999). The use of kairomones by bed bugs is probably the area of bed bug chemical ecology that is least understood. Although it is evident that bed bugs exhibit tendencies toward random movement and random searching (Cooper et al. 2015), there is also evidence of directed orientation towards host cues such as heat

(Haynes et al. 2008). Orientation toward areas warmed to human body temperatures have been observed, and a temperature increase of 1-2° C is enough to elicit probing behavior (Rivnay 1932, Aboul-Nasr and Erakey 1967). Aboul-Nasr and Erake (1967) showed that responses to heat were lost at distances greater than 3.5 cm. However, both of the two previously mentioned studies were conducted without proper replication or statistical analyses, so interpretation of observations should be undertaken with some reservation. The use of heat has been shown to increase trap catch (Anderson et al. 2009, Wang et al. 2009b). However, studies on the orientation behavior of bed bugs to heat, and the role of heat as an attractant, should be pursued further.

It is hypothesized that carbon dioxide also plays a role in host location and detection for bed bugs (Marx 1955, Aak et al. 2014) and carbon dioxide is a well-established kairomone for other hematophagous insects such as mosquitoes and sand flies (Smallegange et al. 2010, Hoel et al. 2011). Traps that incorporate carbon dioxide catch significantly more bed bugs of all life stages than traps without carbon dioxide (Anderson et al. 2009, Wang et al. 2009b). One laboratory study showed no positive relationship between trap catch across four release rates (200, 300, 400, 500 mL CO_2/min) (Singh et al. 2012). In essence, all traps baited with carbon dioxide caught more bed bugs than control traps, but trap catches across different release rates were not significantly different from one another (Singh et al. 2012). However, field studies have demonstrated a positive relationship between carbon dioxide release rates and bed bug trap catch (Singh et al. 2013). This difference could possibly be attributed to the presence of human hosts in field settings. Authors hypothesized that when hosts are present, release rates of the traps may need to be higher than that released by human hosts (which is 250 mL/min

for adults) to be attractive (Singh et al. 2013). It is clear that carbon dioxide is a cue that stimulates bed bug movement, but it remains to be confirmed whether carbon dioxide is an attractant.

The extent of other kairomone usage for host location by bed bugs is not well understood. The conclusion of one of the most recent studies on this topic was that host odor has a very weak impact, if any impact, on bed bug behavior (Harraca et al. 2012). Only five compounds associated with human odor were detected by olfactory receptor neurons in the smooth-peg sensilla of bed bug antennae in this study. Behaviorally, bed bugs were actually repelled by high concentrations of odors associated with humans. However, in field trials, unheated traps with carbon dioxide and a bait comprised of L-lactic acid and (*RS*)-1-octen-3-ol successfully attracted bed bugs, but the study lacked a control (i.e., trap with no kairomones) (Wang et al. 2009b). Additionally, screening of bed bug sensilla showed no olfactory response to $_{L}$-lactic acid, or (*RS*)-1-octen-3-ol (Harraca et al. 2010a). A trap with a chemical lure consisting of nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil was attractive to bed bugs in the laboratory (Singh et al. 2012). However, this study confounds the role of human odor in attraction, since spearmint and coriander Egyptian oil are not human odors. Responses to other more complex blends of human odors such as sweat have also generated conflicting results in the literature (Aboul-Nasr and Erakey 1968, Levin 1975, Weeks et al. 2011). Similarly to heat and carbon dioxide, our knowledge of other kairomonal responses by bed bugs is incomplete. Further studies on host cues and kairomone use should be developed in order to better understand bed bug ecology and host-finding behavior.

Semiochemicals and bed bug management

There is widespread interest, especially due to the recent elucidation of the less-volatile component of the aggregation pheromone, in developing traps and lures baited with semiochemicals to detect and monitor bed bugs. Over the past few years, there have been a number of monitors that have attempted to incorporate the use of aggregation pheromone, heat, carbon dioxide, and other host kairomones in order to monitor potential and existing infestations. However, these devices have not been adopted on a widespread scale. This is probably due to poor trap design, a lack of attractiveness of chemical lures, high production costs, or insufficient/unattractive release rates of carbon dioxide (Weeks et al. 2011, Singh et al. 2013, Crawley et al. 2015). Additionally, traps utilizing kairomones are only attractive to the portion of the bed bug population seeking a host. The current issues with trap design do not imply that there is no future in semiochemically-baited traps. Slight modifications of products currently on the market, or low-cost alternatives, could result in more widespread use.

There are two types of monitors that pest management professionals, tenants, homeowners, etc. commonly use to detect bed bugs: passive and active. Passive monitors do not have the addition of an alternative heat or chemical source (Vaidyanathan and Feldlaufer 2013). Passive monitors are traditionally placed behind the bed, under furniture legs, or beside the furniture in order to intercept bed bugs as they are walking to and from the host, or to and from a harborage. Active monitors incorporate heat and/or a chemical lure and should be attractive to bed bugs in order to be effective. There are currently only three monitors on the market that are classified as active: Bed Bug Alert, Bed Bug Beacon by PackTite, and the SenSci Active Volcano. These incorporate heat

and carbon dioxide, carbon dioxide, and kairomones other than CO_2, respectively, in order to attract bed bugs. The relative efficacy of these products has not been scientifically evaluated in the field. The cost of one these traps ranges anywhere from approximately $5-$55, and multiple traps are usually necessary to effectively monitor one room. Thus, these monitors are often cost prohibitive to the general public, especially when factoring in the cost of the labor that will be required for pest management professionals (PMPs) monitoring the traps periodically. As a lower cost option, Singh et al. (2015) demonstrated the effectiveness of a sugar-yeast monitor (for carbon dioxide production) in attracting bed bugs in the field. However, these homemade traps are not available for purchase, and many argue the practicality of carbon dioxide release from traps used simply for routine surveillance (Weeks et al. 2011). This, combined with the relative effectiveness of passive monitors, and the high cost of active monitors, makes the future of semiochemical-bated traps uncertain (Crawley et al. 2015). New traps incorporating bed bug aggregation pheromone have been tested and proposed (Gries et al. 2015), but have not yet advanced to the marketplace. Semiochemicals may be used to train canines to detect bed bug infestations, and a recent study demonstrated the ability of trained dogs to discriminate between active and inactive refuges (Pfiester et al. 2008). However, inconsistencies among teams from different companies is a concern (Wang and Cooper 2011) indicating the importance of identifying the semiochemical(s) that dogs alert to—which is currently unknown. Ultimately, it is clear that there is vast potential for the use of semiochemicals in bed bug management, but equally clear is that this potential has not been realized to date.

Conventional bed bug control

Bed bug elimination and detection with semiochemical-baited traps is not yet conventional in bed bug management. More often than not, management of populations of *C. lectularius* is achieved through a series of more reliable approaches, including: mechanical removal by vacuuming, the use of heat and steam, application of insecticides, and detection using passive monitors. Early detection is crucial for preventing the establishment and spread of bed bugs. The use of passive monitors can aid in monitoring and early detection, and can be more effective than visual inspections alone. In the early stages of infestation, mechanical removal through vacuuming and small scale usage of heat and steam can be useful in controlling or even eliminating a bed bug introduction/infestation. Bed bug populations can also be reduced through the use of mattress encasements, which entomb some of the bed bugs (Koganemaru and Miller 2013). However, for the reduction/elimination and residual control of large populations of bed bugs, comprehensive heat treatment and insecticides are the cornerstone for treatment (Potter et al. 2011).

Multiple insecticides from a number of classes are currently used to control bed bugs globally (Romero et al. 2009, Davies et al. 2012, Doggett et al. 2012, Potter and Haynes 2014). Pyrethroid insecticides, applied alone or in combination with a neonicotinoid, are most commonly used by pest management professionals in the United States (Potter et al. 2015). Also used are pyrroles, desiccant dusts, and insect growth regulators (Romero et al. 2010b, Doggett 2013, Goodman et al. 2013, Gordon et al. 2014). Silica gel, a desiccant, is a very effective product gaining popularity in recent years (Potter et al. 2014). However, combination products containing both a

neonicotinoid and a pyrethroid component are regarded as the most effective, practical choice currently on the market (Potter et al. 2015). Unfortunately, insecticide resistance to combination products is a growing concern (Gordon et al. 2014), as formerly effective products are often failing to control bed bugs in the laboratory, and occasionally in the field.

Insecticide resistance in the bed bug

Studying insecticide resistance in the bed bug is crucial in order to avoid loss of efficacy of commercially available products. The evolution of insecticide resistance is a common outcome when only a single insecticide, or multiple insecticides with a similar mode of action, are used against heterogeneous insect populations for multiple generations (Forgash 1984, Guerrero et al. 1997). Insecticide resistance in the bed bug is one of the most prominent hypotheses for their recent global resurgence (Doggett et al. 2004, Potter 2005, Romero et al. 2007). Bed bugs have developed, and are continuing to develop resistance to multiple products including both pyrethroid and neonicotinoid insecticides; two of the most common classes of insecticides registered for use against this pest (Romero et al. 2007, Adelman et al. 2011, Koganemar et al. 2013, Zhu et al. 2013, Gordon et al. 2014a, Gordon et al. 2014b, Benoit et al. 2016, Romero and Anderson 2016). It is known that multiple molecular mechanisms are responsible for high levels of insecticide resistance in the bed bug.

A unique and effective mode of resistance to insecticides in bed bugs involves changes in the bed bug cuticle that permit delayed absorption of insecticides into the hemolymph due, in part, to the production of metabolizing enzymes in epidermal cells (Mamidala, et al. 2012, Koganemaru et al. 2013, Zhu et al. 2013, Benoit et al. 2016).

Koganemaru et al. (2013) showed that genes associated with the epidermal layer of the cuticle of bed bugs were upregulated in resistant strains, as well as that susceptibility to pyrethroids significantly increased when injections were performed (as opposed to topical application). Furthermore, Zhu et al. (2013) demonstrated that not only were cuticular genes upregulated in resistant populations, but the epidermal cells of the cuticle were generating enzymes that actively metabolized insecticides. This localized mechanism of resistance has not been identified in any other insect to date.

Additionally, although not unique to bed bugs, many populations of *C. lectularius* possess point mutations in genes coding for voltage-gated sodium ion channels—the target site of DDT and pyrethroid insecticides. Termed "knock-down resistance" (kdr) and "super-knockdown resistance" (super-kdr), single point mutations in genes coding for sodium ion channels usually result from a switch from leucine to one of three other amino acids (histidine, serine, or phenylalanine) at the S6 hydrophobic segment at domain II of the protein (Williamson et al. 1996, Martinez-Torres et al 1999). Super-kdr refers to an addition of a secondary point mutation alongside the leucine switch (Guerrero et al. 1997). These mutations result in an inability of pyrethroids to act lethally at the target site. V419L and L925I mutations in the voltage-gated sodium ion channel α-subunit are responsible for deltamethrin resistance in the bed bug (Yoon et al. 2008). Additionally, molecular analysis of populations of bed bugs in the United States and Europe showed that >80% and >95% of populations, respectively, contained V419L and/or L925I mutations, which indicates extremely widespread distribution of target-site based pyrethroid resistance (Zhu et al. 2010, Booth et al. 2015).

Finally, multiple studies have demonstrated that many resistant populations of *C.*

lectularius have a heightened ability to detoxify insecticides (Yoon et al. 2008, Romero et al. 2009, Adelman et al. 2011, Mamidala et al. 2011, Zhu et al. 2012, 2013, Romero and Anderson 2016). This enhanced metabolism of insecticides in resistant insect populations stems from the altered (usually enhanced) expression of one of three major classes of detoxifying enzymes: P450 monooxygenases, esterases, and glutathione S-transferases (GSTs). For instance, four P450 genes (CYP397A1, CYP398A1, CYP4CM1 and CYP6DN1) are overexpressed in deltamethrin-resistant populations of bed bugs (Zhu et al. 2013). When these genes are knocked down using RNA-interference, there is a reduction in deltamethrin resistance levels (Zhu et al. 2012, 2013) indicating the importance of P450s for the ability of bed bugs to resist pyrethroids. Additionally, relative to a susceptible population, the activity of cytochrome P450s, GSTs, and esterases in neonicotinoid-resistant bed bug populations is elevated, likely conferring resistance to neonicotinoids—however other mechanisms of resistance may be involved as well (Romero and Anderson 2016). Two different esterases have been implicated in detoxification of pyrethroids (Adelman et al. 2011, Zhu et al. 2013). However, another study found no association of this class of enzymes with insecticide resistance in the bed bug (Yoon et al. 2008). Authors of a recent study proposed that these inconsistencies may be due to the lack of an effective bioassay that separates metabolic activity of esterases from potentially competing oxidases (Lilly et al. 2016). Using a PBO analogue, Lilly et al. (2016) provide evidence for a role of detoxifying esterases in deltamethrin (and thus pyrethroid) resistance in bed bugs. To date, more consensus has been reached regarding the involvement of P450s and reduced cuticular penetration in resistance to pyrethroids and neonicotinoids in the bed bug (Romero et al. 2009, Bai et al. 2011,

Koganemaru et al. 2013, Zhu et al. 2012, 2013, Booth et al. 2015). However, upon the development of a novel, effective bioassay (Lilly et al. 2016), more work should be aimed at the study of the role of esterases in the metabolic detoxification of insecticides for bed bugs. Additionally, although the lethal effects of insecticides have clearly been studied extensively, there has been far less work conducted on sublethal effects of insecticides and the potential influence of these effects in bed bug management. Knowledge of sublethal effects of insecticides in the bed bug could enhance/alter insecticide application in the field.

Sublethal effects of insecticides

Sublethal effects are physiological or behavioral effects occurring after exposure to an insecticide that do not directly induce mortality (Desneux et al. 2007). Sublethal doses/concentrations induce no apparent mortality in the experimental population. However, when evaluating insecticide efficacy, the standard measure of effectiveness is direct observation of mortality after exposure to the active ingredients (Stark et al. 1995). As a consequence, sublethal effects of insecticides, as well as the impact of insecticides on insect behaviors, are typically overlooked during the evaluation of chemical products for efficacy (Stark and Banks 2003). Since many common insecticides, like the pyrethroids and neonicotinoids, act at the neuronal level to disrupt the typically well-orchestrated behavior of insects, any disruption in function could cause a reduction in survival (aside from direct lethal effects) and decreased reproductive output (Haynes 1988). Thus, the sublethal effects of insecticides could have critical population-level outcomes that should not be ignored in pest management, where resultant changes in pest behaviors after treatment could impact control (Maltby 1999, Desneux et al. 2007). This

is especially true for urban pest management, where biological and other methods of control are typically not a practical alternative to chemical treatments.

Sublethal exposure to insecticides can induce multiple behavioral and/or physiological changes in insect life history parameters that could lead to population-level outcomes, such as: a reduction in feeding or searching time (Miao et al. 2014, Uhl et al. 2015), alteration in predator avoidance behavior (Tan et al. 2014), a reduction in life span (Ahmad et al. 2013), longer development time (Alinejad et al. 2014), and a reduction in fecundity (Stark and Banks 2003, Shi et al. 2011). Shifts in fecundity may occur through variety of mechanisms, such as alteration of spermatogenesis, sperm motility, oogenesis, ovulation, and egg fertilization after exposure (Haynes 1988). Not only can fecundity be affected physiologically, but in many instances, sublethal exposure can change behaviors that indirectly affect egg production and output. For instance, a reduction in the time spent engaging in sex pheromone signaling could reduce the overall number of mating events (Haynes and Baker 1985). Additionally, when foraging behavior is reduced/altered, the number of eggs produced usually decreases (Duncan 1963). If locomotor movements decrease, insects may encounter fewer mates and make fewer mating attempts. Behavior and physiology are tightly coupled, and sublethal effects often have negative impacts on each that could cause pest populations to decline over time, even if mortality is not immediately observable.

As previously stated, populations of bed bugs are commonly managed via the application of insecticides. In the midst of rapid evolution of resistance to commonly used products, it is more important than ever to fully assess the impact of such measures. Sublethal effects of insecticides have historically been overlooked in bed bug

management. Only one study to date has investigated the potential of sublethal effects to impact populations of bed bugs. Jones et al. (2013) demonstrated that sublethal exposure to permethrin-impregnated fabric, which is commonly sold as a liner for beds, negatively affected both feeding behavior and fecundity. Significantly fewer female bed bugs laid eggs after sublethal exposure to the fabric, and exposure times as short as one minute were enough to induce behavioral changes. These results, as well as results discussed in this dissertation, point to the potential of population-level consequences of sublethal effects in bed bugs. Numerous studies with other insect species point to potential sublethal effects on bed bug locomotion, fecundity, host-finding, and mating behaviors that could impact management strategies.

Goals and objectives

The goal of my research was to increase our knowledge of bed bug behavioral ecology, including understudied aspects of various behavioral interactions between bed bugs and their environment that could potentially impact or enhance management. I took two broad investigative approaches to achieve this goal. First, I considered it important to investigate the chemical ecology mediating interactions among juvenile and adult bed bugs, or among adults and their eggs, as these interactions are typically overlooked. Second, I wanted to better understand the interactions between bed bugs and their environment, which often includes interaction with insecticides. Therefore, I wanted to investigate the sublethal effects of a neurotoxic insecticide on bed bug behavior and physiology. I addressed my research goal through the completion of four main objectives. In Chapter 2, I describe the first objective: to investigate the chemical ecology mediating interactions among juvenile and adult bed bugs. My second objective,

outlined in Chapter 3, was to investigate and characterize oviposition ecology and behavioral patterns in female bed bugs. In Chapter 4, my objective was to describe the sublethal effects of a pyrethroid-neonicotinoid combination insecticide (Temprid® SC) exposure on bed bug behavior. Finally, in Chapter 5, I expanded upon this study by describing the sublethal effects of exposure to Temprid® SC on bed bug physiology. In my final chapter, I discuss the conclusions and implications of my research in bed bug management and as well provide an explanation of the impact of my work in the field of bed bug and insect ecology on a broad scale.

Chapter 2. Chemically-mediated interactions between female bed bugs (*Cimex lectularius*) and first instar nymphs result in increased feeding efficacy for nymphs

Introduction

Chemical signals are of profound importance in many insect systems (Wertheim et al. 2005). In fact, chemical communication is arguably one of the most widely used modalities for communication across all of the animal phyla (Wyatt 2009). An extensive array of arthropods use common chemical compounds, or have modified common chemical compounds, into unique semiochemicals that can serve multiple functions in very diverse environments (Blum 1996). Semiochemicals, or infochemicals, are compounds responsible for transmitting information between individuals. One class of semiochemicals, pheromones, are chemicals released by one individual that trigger changes in behavior and physiology of a conspecific (i.e., intraspecific) (Karlson and Lüscher 1959). Pheromones are grouped based on the type of behavior that they elicit. For example, pheromones that induce group formation are termed 'aggregation pheromones'. These pheromones elicit aggregation behavior among conspecifics.

Aggregative behavior is central to the life history and ecology of many insects (Prokopy and Roitberg 2001, Wertheim et al. 2005). The formation of aggregations, which are a positive response of individuals to stimuli culminating in an association between two or more conspecifics for a sustained time period, can have both costs and benefits for participating individuals. For instance, benefits to aggregating may include more efficient resource use, an increased ability to find mates, protection from natural enemies, and/or alteration of the microclimate allowing for protection from environmental conditions (Prokopy and Roitberg 2001, Wertheim et al. 2005). However,

there are also potential costs for insects living in crowded conditions. These may include competition, disease transmission, deteriorating resources, or conspicuousness to natural enemies (Wertheim et al. 2005). Exhibition of aggregation behavior by a group of insects is shaped when individuals balance these potential costs and benefits.

The exhibition of aggregation behavior, its costs and benefits, as well as the mechanisms of their use, are similar within taxa—including within species, families, and even orders (Wertheim et al. 2005). Within the Coleoptera, for instance, tend to exhibit aggregation behavior when their aggregation pheromone and food odor is present (Bashir et al. 2001). Often, only males produce aggregation pheromones, which facilitates mate-finding. In the Hemiptera, aggregation pheromones are often accompanied by defensive compounds, and/or alarm pheromones (Cruz-López et al. 2001). Additionally, many hemipteran aggregation pheromones have a dual function—facilitating feeding or host-finding for nymphs (Wertheim et al. 2005, Lin 2006, Wong et al. 2013). Bed bugs, (*Cimex lectularius* L.: Hemiptera: Cimicidae) produce both alarm and aggregation pheromones, but their role in bed bug ecology, specifically, the benefits of their use and production, has been relatively unexplored.

Bed bugs are obligate hematophagous nocturnal ectoparasites that parasitize many mammals, and some birds (Usinger 1966, Reinhardt and Siva-Jothy 2007). Like many other species of Hemiptera, bed bugs exhibit aggregation behavior. Aggregations of bed bugs consist of bugs in all life, feeding, and mating statuses (Pfiester et al. 2009). Within aggregations bed bugs copulate, lay eggs, defecate, and molt. Bed bugs remain in these aggregations during daylight hours, and only leave during the night to feed at intervals of several days (Kilpinen et al. 2012). The interactions between male and female bed bugs

within an aggregation have been extensively studied due to the interest in traumatic insemination and sexual conflict, but the interactions between adult bed bugs and their offspring, as well as whether these interactions are semiochemically mediated, have not been thoroughly characterized. There are studies that suggest some benefits of aggregating for juveniles, such as a reduction in water loss and increased development rate (Benoit 2007, Saenz et al. 2014). However there has been little focus on the potential role that adult bugs play in juvenile development within an aggregation. Due to the frequency with which adult insects within aggregation facilitate feeding of juveniles (Wertheim et al. 2005, Lin 2006, Wong et al. 2013), I hypothesized that adult bed bugs (specifically females) may facilitate juvenile feeding and host-finding through the production of aggregation and/or alarm pheromone during feeding.

In this study, I used a wide array of behavioral assays as well as gas chromatography-mass spectrometry (GC-MS) to determine whether female bed bugs improve the feeding efficacy of first instar juveniles. I hypothesized that in the presence of females, first instar bed bugs would feed more frequently than in their absence. If females did increase the frequency of first instars that fed, I predicted that this interaction would be mediated by semiochemicals.

Materials and Methods

Insect Rearing

Approximately 100 bed bugs were collected in an apartment in Plainview, NY in 2008 (NY-1 colony). Bed bugs from this colony were used in all of the following experiments. Bugs were reared in standard laboratory conditions (27°C, 70% RH, photoperiod of 14:10 L:D) and weekly blood meals were administered with an artificial

blood feeding system (Montes et al. 2002). In this system, defibrinated rabbit blood (Quad Five, Rygate, MT) was pipetted into glass feeders **(**Kimble Chase Custom Glass Shop, Vineland, NJ**)** and heated to 39° C with a circulating water bath. A piece of parafilm that lined the bottom of the glass feeder served as a barrier between the insects and the blood. In order to feed, bed bugs contained in 59 mL plastic jars sealed with organza (Consolidated Plastics, Stow, OH) had to pierce the organza and the parafilm membrane with their mouthparts.

Adult influence on juvenile bed bugs in an artificial feeding system

Adult bed bugs were fed 24 hours after eclosion. Six days after the blood meal was obtained, adult bed bugs were placed in 25 cm (height) x 10 cm (diameter) glass cylinders lined with organza at the open, upper end. Each cylinder had a 25 cm (height) x 2.5 cm strip (width) of contact paper adhered to one side to allow bed bugs to climb the vertical surface. The base of the tube contained three 4 cm x 4 cm pieces of blotter paper stacked horizontally that served as a harborage. In order to feed, a bed bug needed to leave the harborage, walk up the vertical surface of the contact paper, and then move to the organza covered upper surface. Bed bugs in glass cylinders were held in red light conditions, in a room at standard room temperature, at a 14:10 hour L:D cycle. Bed bugs were allowed to acclimate to this room for 24 hours prior to experiment initiation.

In order to select groups for treatments, bed bugs were randomly allotted to one of four cylinders. Treatments included ten adult males, ten adult females, or both (five males and five females) with 30 first instars, or 30 first instars alone. First instars were the offspring of the females and/or males contained in the cylinder with them. Each of the cylinders was placed on a level table and covered with organza, which was held in

place with multiple rubber bands. The cylinders were then placed underneath feeders, which comprised the parafilm-membrane feeding system previously described. A small tube delivering 5% CO_2 in air was attached to each feeder and directed through the organza coverings via a small plastic tube. The CO_2 was used to promote movement and foraging behavior. This gas cycled on for 30 minutes at a flow rate of 1 L/minute to each arena, and off for 15 minutes. Trials began approximately three hours into the scotophase, and lasted for four hours total. Afterward, the number of bed bugs that successfully fed versus the number of bugs that did not feed was recorded.

Using Statistix 10.0, a two-factor ANOVA was used to discern whether there was an effect of adult female bed bugs on first instar feeding frequencies. Proportions were arcsine square-root transformed prior to analysis. Normality was confirmed using a Shapiro-Wilk test. Mortality during the treatment was rare but if observed, those bugs were omitted from statistical analysis. Twenty replicates of all four treatments were conducted and included in the model.

Female influence on the feeding efficacy of second to fifth instar bed bugs

The effect of female bed bugs on juvenile feeding frequencies was tested in this experiment. The same methods as previously described were used, with the following modifications. Instead of contact paper, each cylinder had a 25 cm (height) x 2.5 cm strip (width) of blotter paper lined with filter paper (adhered to blotter paper with double sided sticky tape) was used to allow the bed bugs to travel from the bottom to the top of the cylinder. The blotter paper added rigidity to the filter paper. Rather than a 24 hour acclimation period, bed bugs were allowed to acclimate to this room for five minutes prior to experiment initiation. To select groups for treatments, bed bugs were randomly

allotted to cylinders. Treatments included 20 of the instar of interest (second through fifth) either held alone, or with ten females. Juveniles were seven days past eclosion and had not yet taken a blood meal. One replicate included individual groups of second through fifth instars, with or without groups of females present. Each of the organza-covered cylinders was placed on a level table. The cylinders were then placed underneath glass feeders, which comprised the artificial feeding system previously described. Trials began approximately three hours into the scotophase, and lasted for 30 minutes. The number of bed bugs that successfully fed versus the number of bugs that did not feed was recorded.

Using Statistix 10.0, a multi-factor ANOVA was used to test whether there was an effect of female bed bugs on the feeding frequencies of second to fifth instar nymphs. Proportions were arcsine transformed prior to analysis. Normality was confirmed using a Shapiro-Wilk test. Mortality during the treatment was rare but if observed, those bugs were omitted from statistical analysis. Ten replicates were conducted.

Influence of fifth instars on first instars in an artificial feeding system

To test the effects of fifth instars on first instar feeding efficacy, the previous experiments were repeated with one modification. Bed bugs were permitted to feed for one hour under laboratory conditions during the scotophase, rather than four hours. Ten fifth instars were held with twenty first instars, or twenty first instars were held alone. Using Statistix 10.0, a Wilcoxon Signed Rank test was used to look for an effect of fifth instar bed bugs on the proportion of first instars that fed in an artificial feeding system. A non-parametric test was used as a Shapiro-Wilk test indicated that these data were not normally distributed. Twenty replicates were conducted.

Time until feeding in an artificial feeding system

The mean time of arrival at the host and initiation of feeding for female bed bugs versus first instars was tested in this experiment. If females were producing a signal that allowed first instars to find the host, they should arrive at the host prior to first instars. Insects were reared as previously described. Small cylinders comprised the arena of a feeding apparatus composed of three plastic pieces with an overall dimension of 12 cm (height) x 10 cm (width). Each plastic piece slid snugly into a slightly wider piece to comprise a complete cylinder. Each test cylinder was held vertically above the lens of an infrared sensitive digital camera **(**Sony CyberShot, San Diego, CA, USA**).** A strip of contact paper measuring 12 cm (height) x 3 cm (width) was placed on the side of the cylinder which served as a substrate that allowed the bugs to move from the base, to the top for feeding, and back to the bottom for aggregation. Organza divided the upper opening of the cylinder from the glass feeder (previously described). In order to take a blood meal, a bed bug had to pierce through both the organza and parafilm. Fifteen female bed bugs and fifteen first instars were placed into each cylinder. Females had not been offered a blood meal for seven days, while first instars were never permitted to take a blood meal after hatching. Ten minutes after bed bugs were added to cylinders, a stream of 5% CO_2 in air flowing at 1 L/minute was supplied at the top of the cylinder, next to the glass feeder containing heated defibrinated rabbit blood. Bugs had access to the feeder during the scotophase for one hour. An infrared sensitive camera was used to record feeding activities of female bed bugs and first instars in each container throughout the course of the experiment. A photograph was taken every 30 seconds by programmed remote trigger (Palm m125 with OmniRemote Software) to determine the time of

feeding. The time that bugs initiated feeding was recorded for both females and first instars. These data were verified by viewing pictures taken throughout the experiment. During the course of the experiment, bed bugs in the cylinders were held in red light conditions in a room at standard room temperature (previously described).

A paired t-test was used to look for differences in the amount of time it took female bed bugs to feed from an artificial feeder versus first instars. Normality was confirmed using a Shapiro-Wilk test. Eight replicates were conducted and analyzed.

Volatile compounds released during feeding

In this experiment, I tested whether different volatile compounds or blends of volatile compounds were released during feeding by different groups of bed bugs. All collections occurred in a dark room at standard temperature (previously described) to mimic the natural foraging behaviors of bed bugs. For volatile collections during bed bug feeding, an automatic interval timer (CNT Digital Time Delay Relay/Counter) was used to activate a series of solenoid valves (Valcor Engineering Corp., Kenilworth, N.J.) at six consecutive ten-minute intervals. The valves permitted air flow (1 L/min) for six consecutive ten minute intervals through six collector tubes filled with approximately 20 mg of Super-Q. The small glass tubes were attached and opened into a cuboid, plastic container measuring 23 cm (height) x 10 cm (width) x 10 cm (depth) with a removable bottom that was held vertically above the lens of a digital camera with the infrared filter removed **(**Canon EOS 300D, Canon INC., Taiwan**).** Bed bugs were held in this container throughout the experiment. The camera was connected to a remote (JJC TM Series Timer/Remote Controller) that automatically took a picture once every minute during the collection period and captured images of bed bug feeding behaviors. Within the

container, a strip of filter paper measuring 10 cm (height) x 3 cm (width) was placed at the bottom that served as a substrate to allow the bugs to move from the base, to the top to feed, and back. Organza divided the upper opening of the box from a glass feeder filled with defibrinated rabbit blood (previously described). The time of feeding and the number of bed bugs feeding was recorded. Photos taken during the course of the experiment were used to confirm the accuracy of the behavioral observations.

The six collector tubes trapped compounds that bed bugs released during six consecutive ten-minute intervals. After the one hour period, the glass tubes were removed from the plastic container, and 100 μL of methylene chloride was pipetted through each tube for compound elution. Extracts were collected in a 1 mL conical vial. One hundred μL of methylene chloride containing 1 ng/μL of (*E*,Z) 4,7-tridecadienyl acetate (100 ng of internal standard) was also pipetted through the tube to serve as a reference that was used to calculate the concentration of compounds emitted by the bed bugs.

The samples were reduced to approximately 5 μL with a nitrogen stream. One μL of the sample was injected into an Agilent 6890 gas chromatograph coupled to Agilent 5974 mass spectrometer. The GC temperature program used was 40°C for two minutes, with an increase of 10°C per minute up to a final temperature of 220°C. A split-splitless injector was used with a DB5 GC column (0.25 mm diameter; 30 m length; 0.25 μL film thickness). A carrier gas (helium) with a flow rate of 1 mL per minute moved volatilized materials through the column. Mass spectra were obtained using electron ionization mode at 70 eV, and scanning was completed for the range *m/z* 35-400. A purge valve

opened after one minute into the sample run. When possible, replicates were conducted on the same day to reduce variation caused by time.

A multi-factor ANOVA was used to compare the quantity of alarm pheromone emitted during feeding by females, males, and fifth instars. These data were highly positively skewed, requiring log transformation prior to analysis. A post-hoc Tukey HSD test looked for pairwise differences between each treatment. Five replicates were conducted and included in the model.

Effect of substrate exposed to females on first instar feeding

I tested whether substrates walked across by females during feeding stimulated first instars to feed. Twenty adult females one week of age were added randomly to glass cylinders (described in experiments one and two). Each cylinder contained a ramp composed of 25 cm (height) x 2.5 cm (width) pieces of filter paper taped to both sides of blotter paper of the same dimension. Filter/blotter paper ramps were used in lieu of contact paper for ease of removal and reuse. Cylinders also contained a filter paper harborage measuring 4 cm x 4 cm. The filter paper was folded 1 cm from the edge to create a harborage for bed bugs to aggregate underneath. Female bed bugs had access to the membrane feeding system for thirty minutes.

Immediately after feeding, females were gently removed from the ramp or the harborage with soft forceps. Both the ramps and harborages containing female feces and other residues from feeding were placed in clean glass cylinders that housed 20 first instar bed bugs. First instars were given ten minutes to acclimate to these cylinders prior to the addition of a ramp. As a control, 20 first instars in a separate cylinder were given a ramp and harborage with no previous exposure to female bed bugs. The cylinders were

sealed with organza and immediately placed under glass feeders. First instars were permitted to feed for 40 minutes in a dark room. Although CO_2 was used to promote feeding behavior in other experiments, bed bug feeding frequencies were still quite high without this stimulus. Therefore, CO_2 was omitted from this and all subsequent experiments.

A paired t-test was used to compare the proportion of first instars that fed when exposed to ramps used by females, versus control ramps. Normality was confirmed using a Shapiro-Wilk test. Ten replicates were conducted and analyzed.

Effect of substrate exposed to males on first instar feeding

I tested whether substrates walked across by males during feeding stimulated first instar bed bugs to feed. The experiment was conducted identically to methods used for female bed bugs (above). Statistical analysis was also identical. Ten replicates were conducted.

Production of fecal spots on substrates during feeding

The number and placement of fecal spots produced during feeding by male and female bed bugs was compared in this experiment, with the hypothesis that compounds deposited in the feces was attractive. One week after molting, male and female bed bugs were separated and placed in glass cylinders (described in previous experiments) in groups of ten. Each cylinder contained a filter paper harborage measuring 4 cm x 4 cm, and a ramp composed of 25 cm (height) x 2.5 cm (width) piece of filter paper taped to both sides of a piece of blotter paper of the same dimension. Each group was permitted to feed from a glass feeder for one hour. After one hour, the ramps were removed from the cylinders and the number of fecal spots was counted. The ramp and the organza

layers were also removed and the number of fecal spots counted to determine the proportion of fecal spots left on the harborage, versus the ramp, versus the organza during feeding.

A two-factor ANOVA was used to compare the number of fecal spots excreted during feeding by male and female bed bugs. These data were square-root transformed prior to analysis. A chi-square test was used to determine whether the location of fecal spot placement differed between the two treatments. Ten replicates were conducted and analyzed.

Methanol extracts from ramps

I tested whether female bed bugs produce a putative contact pheromone that stimulates first instars to feed. Males were also tested to confirm previous behavioral observations. Thus, either 45 males or 45 females were permitted to feed for one hour in glass cylinders with filter paper ramps (previously described). After feeding, the ramps were cut into small pieces using cleaned scissors and placed into 10 mL glass vials containing 4 mL of methanol. Samples were vortexed in order to mix them evenly. Samples were placed in a freezer at -80°C for 24 hours before use.

After 24 hours, 1 mL of the extraction was removed using a sterile pipet, and the mixture was pipetted onto filter paper ramps (previously described). As a control, 1 mL of methanol was pipetted onto one ramp as well. Twenty first instars were exposed to one of the treatment ramps for one hour. Differences in feeding frequencies between treatments were recorded and compared.

A multi-factor ANOVA was used to compare the arcsin square-root transformed proportions of first instar bed bugs that fed from methanol extracts of treatment ramps. A

Tukey HSD post-hoc analysis identified differences between treatments. Eight replicates were conducted and analyzed.

Identification of putative pheromone using GC-MS

Histamine was hypothesized to potentially play a role in mediating the interaction between adult females and first instars because it had previously been identified as a contact aggregation pheromone (Gries et al. 2015). A group of 305 adult females from the Fort Dix colony were provided with access to rabbit blood using glass feeders. These females needed to climb 25 cm up a filter paper ramp (1.25 cm width x 25 cm height) to reach a glass feeder. Females were given 30 minutes to feed. Of 305 females, 233 successfully fed. The filter paper ramp, which was marked with feces by engorged bed bugs, was cut into small pieces using clean scissors and soaked for one hour in 10 mL of methanol, with frequent agitation of the vial. The solution was passed through a C18 Sepak Column (Waters Chromatography, Dublin, Ireland) allowing more polar compounds (such as histamine) to pass. Two mL was transferred to a conical bottomed vial, and the methanol was completely evaporated under a nitrogen stream. Two hundred μL of methylene chloride was added to the vial, along with 20 μL of N-methyl-bis(trifluroacetamide) (MBTFA). The new solution was heated for thirty minutes at 60°C, after which 20 μL of methanol was added to quench the reaction. MBTFA reacts with compounds with an amine or alcohol moiety, replacing active hydrogen of these groups with a trifluroacetyl moiety. This makes the derivatives of amine and alcohol containing compounds less polar, and thus more suitable for GC-MS analysis. These GC-MS analyses used the same instruments and column as the previously described

methods for volatile analyses. The starting GC temperature was 100°C and the final temperature was 300°C.

Histamine dose-response assay

After identifying histamine in female fecal spots, the "methanol extracts from ramps" experiment was repeated to test the effect of histamine on first instar feeding frequencies. For this experiment, however, various concentrations of histamine were added to filter paper ramps, as opposed to methanol extracts of fecal spots. The effects of 5, 50, and 500 µg of histamine in 100 µL of methanol, as well as a methanol only control, on the feeding frequencies of groups of ten first instars were evaluated. A multi-factor ANOVA was used to test the effects of various concentrations of histamine on the proportion of first instar bed bugs that fed in an artificial feeding system. Proportions were arcsine square-root transformed prior to analysis. A two-sided Dunnett's test was used to identify significant differences between concentrations. Ten replicates were conducted and analyzed.

Effect of foster mothers on first instar feeding in an artificial feeding system

In this experiment, I tested whether the observed behavioral effect on first instar feeding was restricted to mothers and their offspring. Individual adult male and female bed bugs were offered a blood meal, placed in 59 mL sealed cups, and were permitted to mate for 24 hours. After this time period, females were removed and placed into petri dishes individually for ten days in order to lay eggs. When all of the eggs in each petri dish hatched, five first instars were removed and placed into glass cylinders containing ramps that could be used to climb from the bottom, up to the feeder, and back (previously described). Using a random number table, females were placed either with her offspring,

or with a foster group of nymphs. Feeding frequencies between the two groups was compared. Twenty replicates were conducted.

A Wilcoxon Signed Rank test was used to test whether mothers were more effective at increasing the proportion of first instars that feed in an artificial feeding system than foster mothers.

Results and Discussion

My first experiment was designed to examine whether adult bed bugs influenced first instar feeding frequencies. A two-factor ANOVA (factors were "males present" and "females present") was used to determine whether adult bed bugs had an effect on the arcsine transformed proportion of first instars that took a blood meal. Treatments with females (F=females, MF=males and females) had a significant effect on the proportion of first instars that fed when compared to treatments with no females (M=males, NO= nymphs only) (Fig 2.1. $p<0.0001$, df=1, $F=20.26$, n=20). There was no interaction between males and females on feeding by first instars (Fig. 2.1, $p>0.05$, df=1, $F=0.65$, n=20). Replicate had no significant effect ($p>0.05$, df=19, $F=1.75$, n=20).

To test whether this effect transcended to other instars, I conducted the same experiment, but females were placed with second through fifth instars instead. When females were housed in the artificial feeding system with groups of second through fifth instars, there was a significant effect of female presence (Fig. 2.2, $p<0.05$, df=1, $F=5.22$, n=10), however the average percentage of instars that took a blood meal was higher when females were absent, except for second instars, where the means between treatments was very similar (Fig. 2.2). There was a significant effect of replicate in the model ($p<0.0001$, df=9, $F=5.29$, n=10), necessitating blocking for this parameter. There was no

interaction between females and the life stage of the nymph ($p>0.05$, df=3, F=1.36, n=10), but there was a significant effect of life stage ($p<0.001$, df=3, F=7.74, n=10). This resulted from increased feeding rates with later instars. In summary, although adult female bed bugs had a positive significant effect on the number of first instars that fed, this effect did not translate to similar enhancement with older instars.

At this juncture, it appeared that the observed effect was specific to females and first instar bed bugs. However, in order for the positive effect of females on first instars to be classified as specific to adult females, I evaluated whether fifth instars had the same effect. This is common for other insects, such as *Oncopeltus fasciatus* (Hemiptera: Lygaeidae), where younger nymphs are attracted to odors of older, conspecific nymphs that provide lytic enzymes for seed digestion (Aller and Caldwell 1979). However, there was no significant difference between the proportion of first instars that fed in the presence or absence of fifth instars (Fig 2.3, 0.33 ± 0.06, 0.24 ± 0.07; $p>0.05$, Z=1.64, n=20). These results indicated that adult female bed bugs influenced first instar foraging behavior in a different way than bed bug nymphs and allowed me to focus on the specific influence of females in the following experiments.

To elucidate potential mechanisms for the behavioral effect I observed, I first wanted to determine whether females had time to signal the presence of a host to first instar bed bugs. If juveniles arrived at the host alongside females, this would reduce the value of signaling host presence. The mean time taken for females to reach the feeder and begin feeding was 11.42 ± 2.4 minutes versus 17.15 ± 2.9 minutes for first instars, and this difference was significant (Fig 2.4, $p<0.001$, df=7, T=(-3.89), n=8). This provided encouragement to test whether adult bed bugs released volatile chemicals while

feeding that could potentially influence first instar foraging behaviors. For other insects that aggregate, this facilitation of juvenile feeding can be stimulated by the presence of aggregation pheromone and host/food odor (Wertheim et al. 2005). I collected volatiles released during feeding from females, males, and fifth instars for comparison, with a specific interest the release of (*E*)-2-hexenal and (*E*)-2-octenal (components of the bed bug alarm and aggregation pheromone). For the release of volatile compounds to explain the behavioral effects I observed, females needed to release either different compounds, a different blend of compounds, or have a different emission rate of compounds than males and fifth instars. However, while adult bed bugs released a different blend than fifth instars (Fig. 2.5, $p<0.001$, df=2, F=9.07, n=5) a Tukey HSD test revealed there was no difference in blend components, the quantity of the components, or the quantity of the blend released by males and females.

The lack of a difference between males and females in volatile profiles associated with feeding led to an experiment to test whether substrates might be marked by females before or after feeding. A recent study demonstrated that aggregation pheromone is deposited in bed bug feces in harborages (Gries et al. 2015), and it is known that aggregation pheromone (along with alarm pheromone) plus host odor can stimulate foraging in other hemipterans (Wertheim et al. 2005). This evidence prompted me to examine the impact of female marking during or after feeding. Female-marked ramps significantly improved feeding success by first instars relative to unmarked ramps (Fig. 2.6, $p<0.01$, df=9, T=3.05, n=10). In contrast, male marked ramps led to no significant improvement (Fig. 2.6, $p>0.05$, df=9, T=(-0.52), n=20). These results indicated that

pheromones deposited onto the substrate by adult females could be responsible for the effect observed in experiment one.

As stated previously, bed bugs often deposit pheromones in their feces (Gries et al. 2015). Thus, I examined the number of fecal spots produced by males and females, and the number of fecal spots ("marks" on the ramps) produced by males and females during and after feeding differed. Females produced significantly more fecal spots (33.3 ± 3.2) than males (15.9 ± 2.0) (Fig. 2.7, $p<0.001$, df=1, F=17.19, n=10). The placement of fecal spots also differed between males and females (Fig 2.7, $p<0.0000$, df=1, $\chi^2=23.93$, df=2). Fecal spotting on the harborage accounted for most of the observed difference between males and females. The difference in fecal spotting was not due to a higher proportion of females feeding than males. However, the difference in quantity may be a result of the greater volume of blood imbibed by females—a proportionate increase of 2.19 times versus 1.46 times their body weight for females and males, respectively (Usinger 1966).

Due to the difference in fecal spot quantity and placement between male and female bed bugs, I extracted putative pheromones from the ramps used during and after feeding by both females and males in methanol, and exposed first instar bed bugs to these extracts. A significantly higher proportion of first instars fed from an artificial host when they walked on ramps marked with methanol extracts from female ramps (0.30 ± 0.05) versus ramps marked with male extracts (0.19 ± 0.05) or a control (0.21 ± 0.04) (Fig. 2.8, $p<0.05$, df=2, F=7.99, n=20). Replicate was also significant, necessitating blocking for this parameter ($p<0.0001$, df=19, F=8.89, n=20).

With the hypothesis that histamine, a component of bed bug aggregation pheromone, was present in fecal spots and methanol extracts (Gries et al. 2015), I analyzed fecal spots from females using GC-MS to look for the presence of this compound. The MBTFA-derivatized methanol extract of ramps marked by adult females contained a tailing GC peak with a retention time of 10.713 (Fig. 2.9). Derivatized histamine had a tailing peak of retention time 10.621 (Fig. 2.9). The mass spectra for both of these peaks contained m/z ions at 94 (base), 81, 69, 54, 207, 138, 126, in descending order of abundance. Both spectra gave a library match to trifluroacetyl histamine, and the mass spectra were consistent from leading edge of the peaks to tail of the peak. Because of the slight difference in retention times, both synthetic and natural derivatives were co-injected, which yield a tailing peak with a retention time of 10.758 with no apparent shoulders which, if present, could have suggested that more than one compound was present.

Based on these results, I added various concentrations of histamine in methanol to ramps (using the same procedure as ramp extracts from male and female bed bugs) and examined the feeding responses of first instars. Histamine stimulated first instar feeding similarly to females (Fig 2.10, ANOVA, $p<0.05$, $df=3$, $F=4.58$ $n=10$). A two-sided Dunnett's Multiple Comparisons Test revealed that first instars exposed to ramps containing 100 µL of a 5 µg concentration of histamine fed significantly more frequently than those exposed to a methanol control (Fig 2.10 $\alpha=0.05$).

Finally, I allowed first instars to feed either with their mother or a "foster mother" to test whether the positive influence of females on first instars was specific to mothers and their offspring. I compared the feeding frequencies between first instars from the

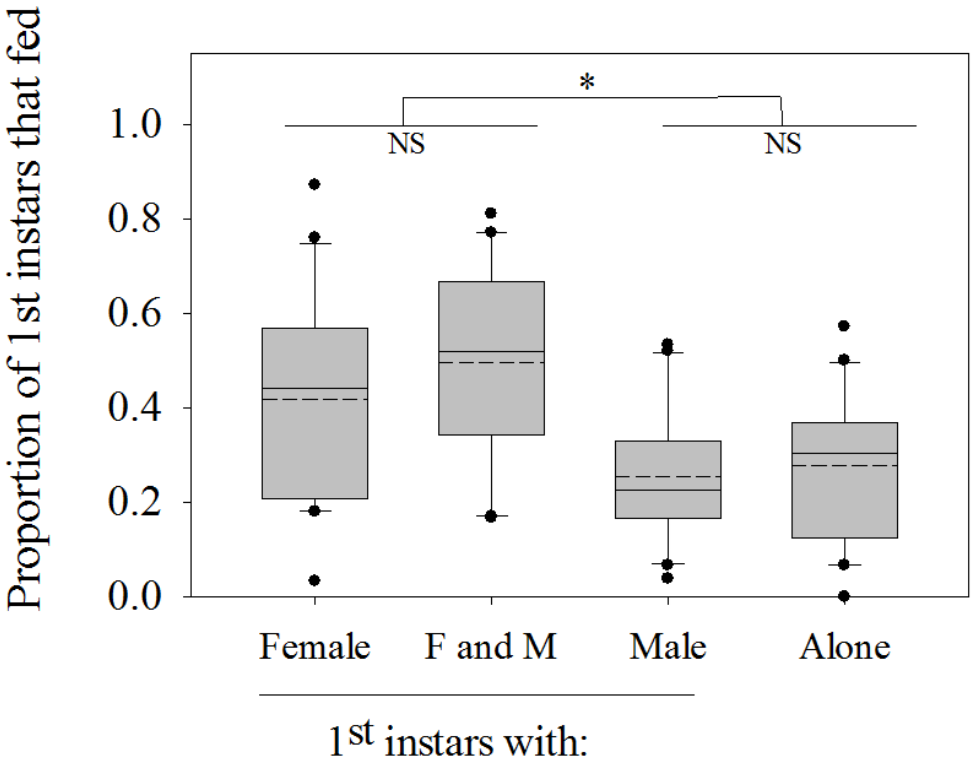
two treatment groups. An effect of 'mother' would make a stronger case for maternal care, in addition to a benefit of aggregation behavior. However, there was no significant difference between mothers and foster mothers in enhancing feeding (0.64 ± 0.08, 0.55 ± 0.06 for mothers and foster mothers, respectively) (Fig 2.11, $p>0.05$, $Z=1.08$, $n=20$).

Conclusions

The presence of female bed bugs when blood is available increases the number of first instars that take a blood meal. This effect is restricted to first instar bed bugs. In fact, female presence seems to negatively impact feeding frequencies of older instars. The finding that fifth instar bed bugs did not have an effect on first instar feeding frequencies, in addition to a lack of effect from males, provided evidence that this is a positive benefit obtained by first instars from interactions with adult females. Because females reach the host before first instars, there is time for females to signal host presence. Substrates walked across and marked by females during feeding stimulated first instar feeding behavior. This is probably due to the quantity, composition, or both of fecal spots produced after feeding by females. Methanol extracts applied to filter paper elicited higher feeding responses from first instars than methanol alone, or methanol extracts from male ramps. Using GC-MS, I detected histamine, a component of bed bug aggregation pheromone, in the methanol extracts from ramps walked across by female bed bugs. Histamine applied to ramps stimulated first instar feeding. These data provide strong evidence for an attractive, locomotor stimulatory effect of fecal spots excreted by females on first instar foraging movements. Future studies should attempt to identify the compounds and the quantity of compounds in the feces produced by both females, males, and fifth instars. If other bed bugs (aside from females) also excrete histamine, the effect

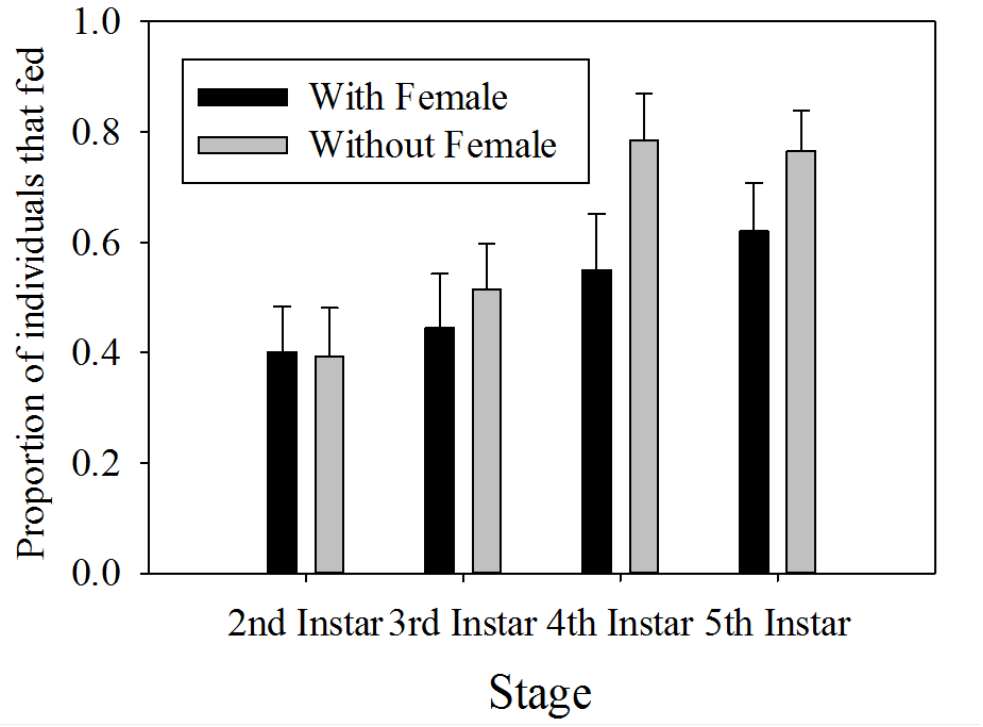
observed in this chapter could be due to quantity differences of histamine correlated to blood meal size (since females take much larger blood meals). Otherwise, there may be a unique pheromone blend produced by females that differs from males and fifth instars. The latter would make a strong case for the existence of a form of maternal care in this insect. Additionally, future studies should classify the net benefit of this interaction on first instar fitness. A positive effect would indicate that the interaction described here is a potential form of subsocial behavior (increased feeding efficacy of juveniles) in the bed bug.

Figure 2.1.



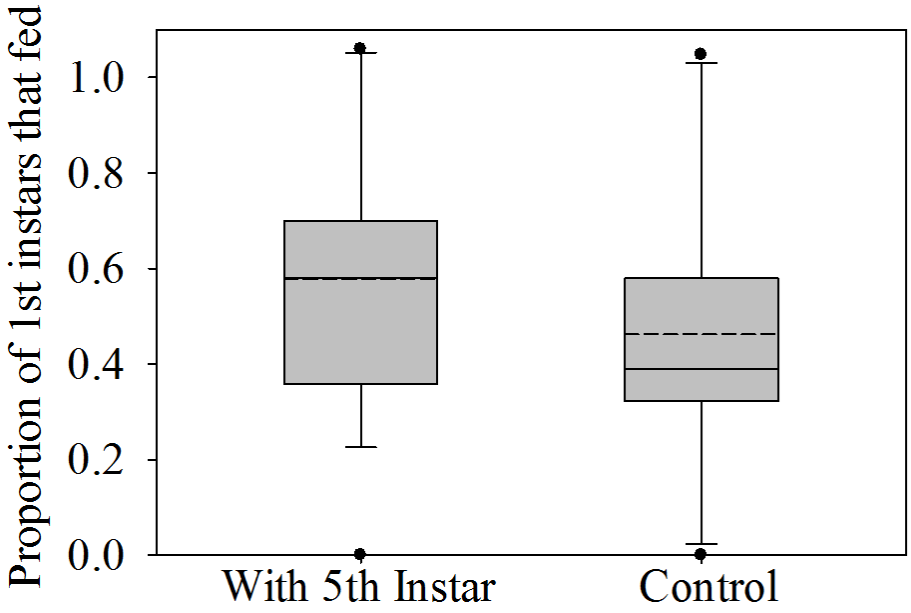
The effect of adult bed bugs on the proportion on first instars that took a blood meal in an artificial feeding system. The boxes mark the 25th and 75th percentiles, the whiskers mark the 10th and 90th percentiles, and the dots are outliers. The dashed horizontal line represents the mean, while the solid horizontal line represents the median. This description applies to all box and whisker plots that follow. The presence of females led to increased feeding while males had no effect on feeding. Twenty replicates were conducted.

Figure 2.2.



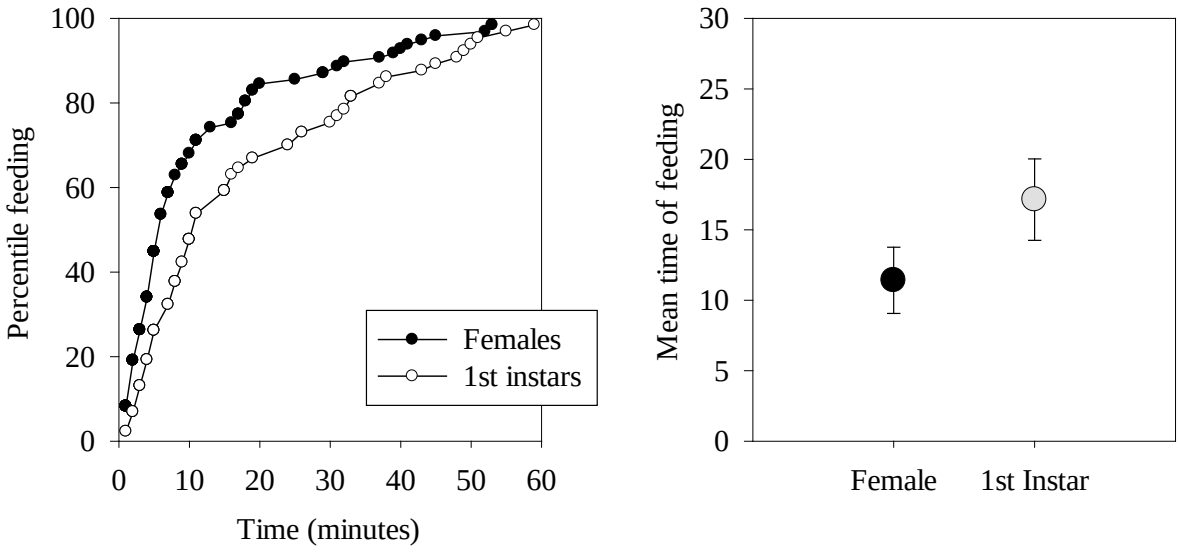
The effect of female presence on the proportion of juveniles that took a blood meal in an artificial feeding system. One replicate comprised every life stage (held individually) both with and without females present. In third through fifth instars, female presence actually corresponded with a reduction in the proportion of nymphs that fed, on average. Error bars represent standard error of the means. Ten replicates were conducted.

Figure 2.3.



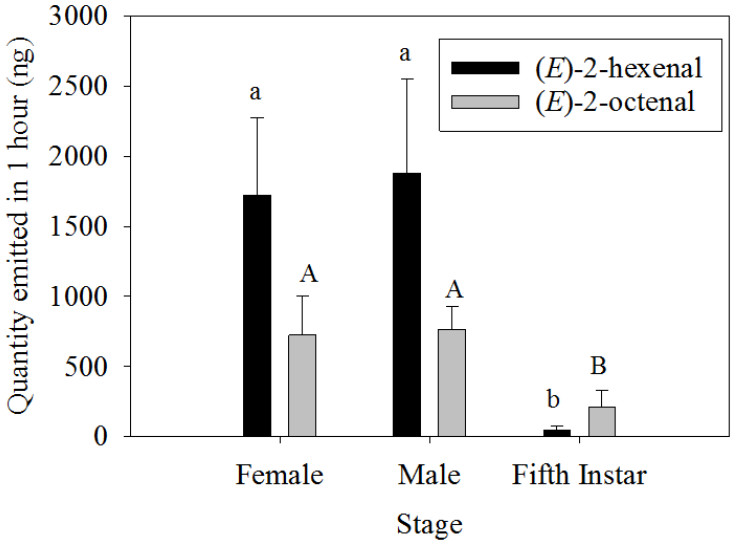
The effect of fifth instar bed bugs on the proportion of first instar nymphs that fed in an artificial feeding system. The presence of fifth instar bed bugs did not have an effect on first instar feeding rates. Twenty replicates were conducted.

Figure 2.4.



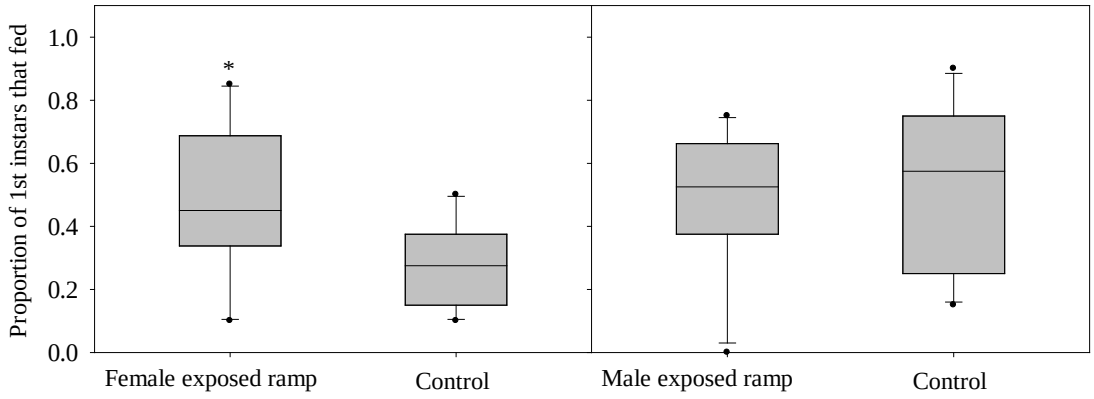
Time courses of feeding for first instar and female bed bugs. To the left, the time course of feeding for female bed bugs versus first instar bed bugs as recorded by an observer, and confirmed by time lapse photography. Females reached the artificial feeder more quickly, on average, than first instars. To the right, the mean time of feeding for each treatment is shown, with error bars representing standard errors of the mean. Data from all eight replicates are represented in the figures.

Figure 2.5



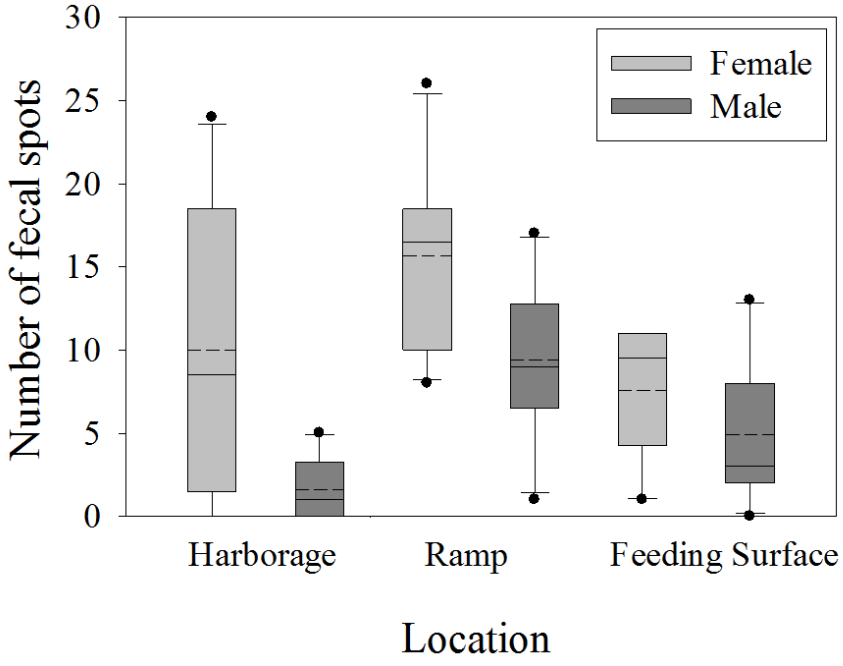
Volatile profiles of females, males, and fifth instar bed bugs during feeding. There was a significant difference in the amount of (*E*)-2-hexenal and (*E*)-2-octenal released by adult bed bugs in comparison to fifth instars. However, the blend of alarm pheromone released by adult females versus adult males was not significantly different.

Figure 2.6



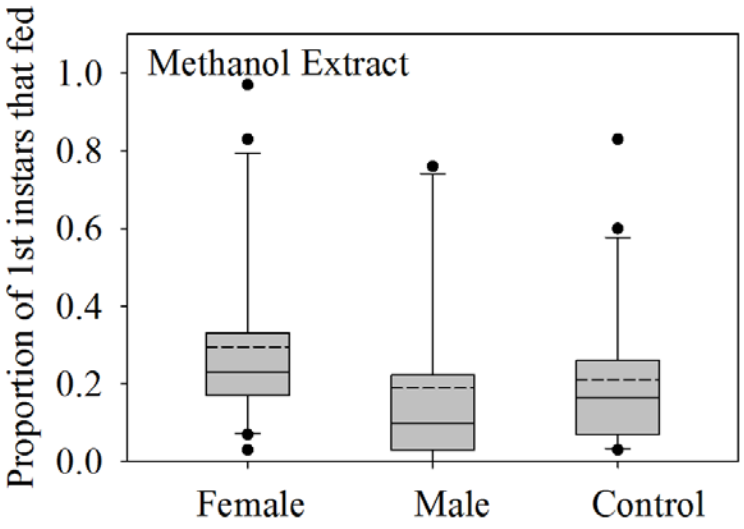
Feeding success of first instars given either male or female exposed substrates. To the left, a comparison of the proportion of first instar bed bugs that fed on ramps either exposed to females that had previously taken a blood meal, or ramps that had no exposure to females. Ramps with prior exposure to females stimulated an increase in the proportion of first instars that fed in an artificial feeding system. However, to the right, it is clear that males had no effect, with means that are nearly identical between the two groups. Ten replicates were conducted.

Figure 2.7.



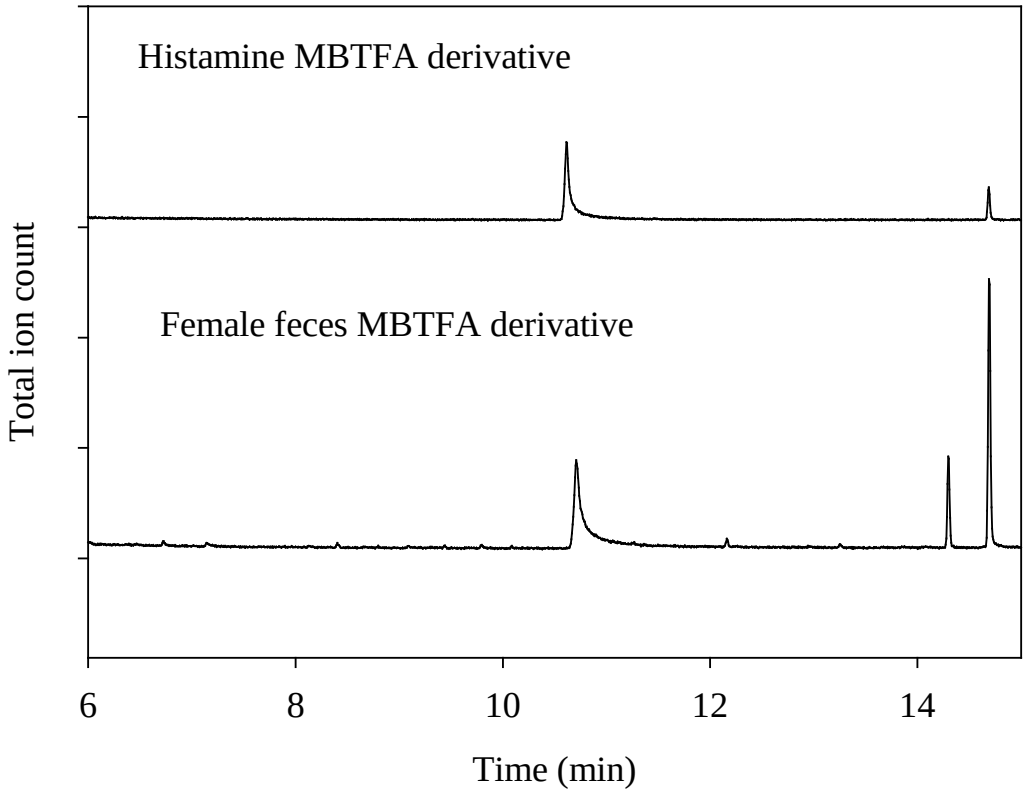
The number of fecal spots produced by males and females, as well as their location within an artificial feeding system. Females excreted significantly more fecal spots than males overall. There were differences in the placement of fecal spots between males and females as well; the largest difference is evident in the number of fecal spots placed on the harborage by each sex. Ten replicates were conducted.

Figure 2.8.



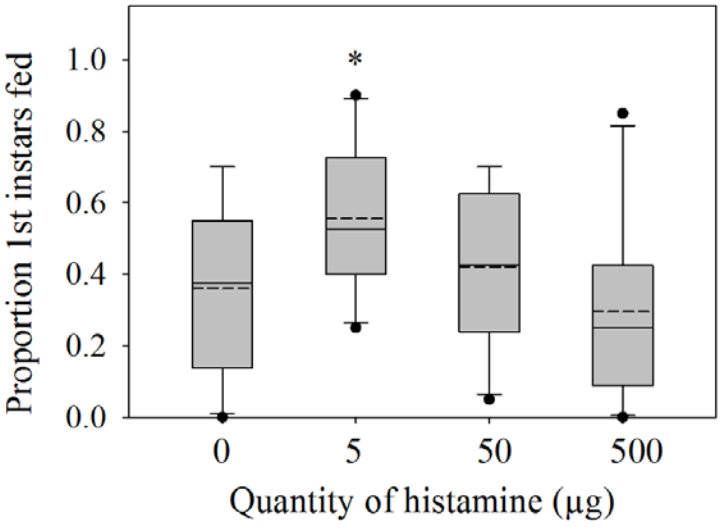
Proportion of first instars fed on ramps treated with methanol extracts. First instars were offered a ramp with methanol extracts taken from female-marked ramps, male-marked ramps, or methanol alone as a control. When first instars walked on ramps treated with methanol extracts from ramps with methanol extracts from females, their feeding efficacy increased.

Figure 2.9.



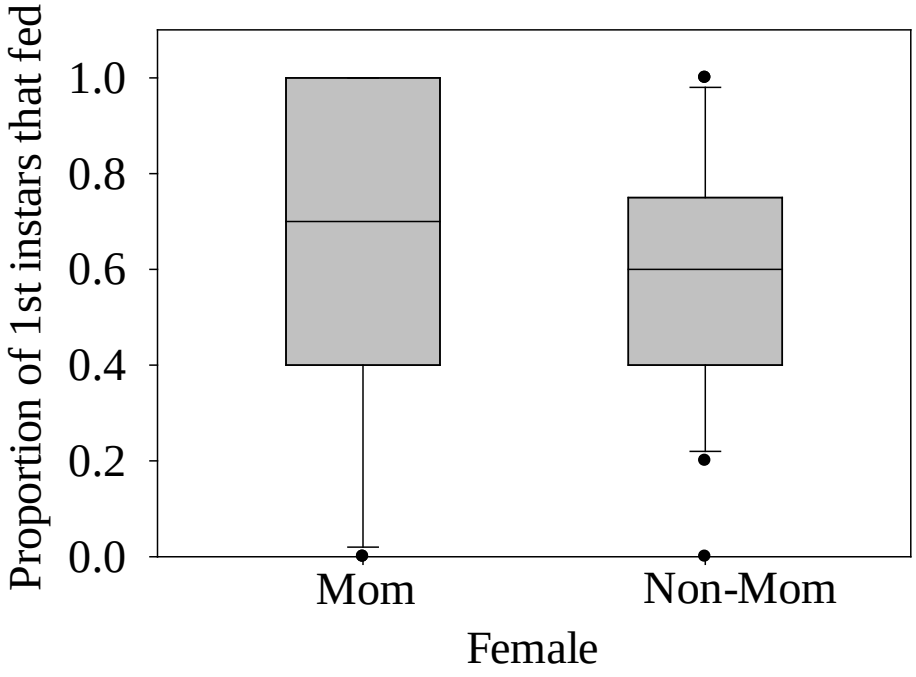
Gas chromatogram revealing corresponding peaks in derivatives of female feces, as well as a derivative of pure histamine. Peaks at 10.6 to 10.7 minutes are indicative of the presence of histamine. Analysis with mass spectrometry supported compound identity.

Figure 2.10



The effects of various concentrations of histamine on the proportion of first instars that fed in an artificial feeding system. Histamine in methanol was applied to ramps as opposed to exposure to female or male bed bugs. Histamine had an effect on the proportion of first instars that fed, and a 5 µg concentration was the effective concentration of histamine for stimulation of first instar feeding.

Figure 2.11.



The effect of mother versus a foster mother on the proportion of first instar bed bugs that took a blood meal in an artificial feeding system. The solid horizontal line represents the mean proportion fed for each treatment. Females, in general, have effects on first instar feeding rates that are not dictated by relatedness.

Chapter 3. Characterization of egg laying periodicity, clutch sizes, and a novel behavior associated with oviposition in the bed bug, *Cimex lectularius,* L.

Introduction

Oviposition depends on a complex series of physiological and behavioral events that result in an egg being placed in the environment (Ampleford and Davey 1988). The insect egg is a particularly vulnerable life stage because they are immobile and are typically laid in environments that may include egg predators, parasites and infective microbes (Kaltenpoth et al. 2005, Deas and Hunter 2013, Newcombe et al. 2013). Abiotic sources of mortality risk such as desiccation also play a role in egg hatching, thus, oviposition behavior by the mother plays an important role in offspring success (Van Dyck and Regniers 2010, Guidobaldi and Gurensten 2015). Consequently, females make extensive choices regarding appropriate oviposition sites; they choose those most likely to ensure adequate food resources, as well as protection for eggs and the hatching offspring (Gullan and Cranston 2005, Van Dyck and Regniers 2010). Oviposition site selection, as well as other behaviors associated with oviposition (such as the addition of oviposition pheromones) should be shaped by natural selection given the importance of offspring survival to overall reproductive success (Mousseau and Fox 1998, Deas and Hunter 2013).

In response to the selective influences of the parasitism and/or predation of eggs, many female insects utilize chemical defenses to protect their clutches (Newcombe et al. 2013). These defensive compounds may be synthesized by the female *de novo*, or, they can be sequestered through the host diet of the parents, usually the mother (Blum and Hilker 2002, Newcombe et al. 2013). Females often apply these defensive secretions

externally to the egg surface or environment as a barrier (Kaltenpoth et al. 2005, Matsuura et al. 2007, Hosoe et al. 2013), but they may also be incorporated into the egg's shell or internal contents (Eisner et al. 2000, Blum and Hilker 2002, Newcombe et al. 2013). Additionally, some behaviors, such as egg grooming, decrease the risk of egg mortality (Boos et al. 2014). Chemical defenses for egg protection can often be vital for hatchability and survival. For instance, eggs of *Utetheisa ornatrix* (Lepidoptera: Arctiidae) are defended with a pyrrolizidine alkaloid ultimately sequestered from host plants by caterpillars. In laboratory studies, 100% of the eggs without the alkaloid were consumed by green lacewing larvae (*Ceraeochrysa cubana*) (Neuroptera: Chrysopidae). Conversely, no eggs that contained alkaloid were consumed (Eisner et al. 2000).

Identifying oviposition behaviors and patterns that influence egg survivorship, including the chemical defense of eggs, could contribute to management of insects that pose threats to human health or resources (agricultural pests, vector insects, etc.), as these patterns will influence pest population dynamics. In fact, the ovipositional patterns of hematophagous insects have been under intense scrutiny for decades due to the public health risk the blood-sucking insects pose (Ampleford and Davey 1988, Bentley and Day 1989, Lorenzo and Lazarri 1998, Feliciangeli 2004). Interestingly, although bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae) are blood-feeding insects with public health relevance, their oviposition behaviors and/or patterns are poorly studied. Aside from data describing the time between blood meal acquisition and oviposition (Johnson 1941), little is known about oviposition and egg laying behavior/patterns in bed bugs outside of the mating process. We do not understand the factors that stimulate oviposition in this insect,

or whether females chemically protect their eggs in any way (which is common in other taxa, and within the Hemiptera).

In this chapter, I sought to characterize some of the egg laying patterns exhibited by female bed bugs. Obtaining this information could inform pest management professionals (PMPs) of the most appropriate times for follow up treatments or inspections in the field. Time-lapsed photography was used to determine when a female is most likely to lay an egg post blood meal, and whether there is periodicity to oviposition. Additionally, I characterized the number of eggs laid by groups of females every hour, over the course of ten days (following a blood meal). I recorded the number of eggs laid for two groups of females: one had previously taken a blood meal approximately seven days prior to the experiment, and then again immediately before the experiment began, while the other group took a blood meal for the first time immediately before the experiment. I hypothesized that treatment would affect egg laying, and predicted that females feeding for the first time (and thus producing their first clutch of eggs) would show a delay in egg production compared to females that were feeding for the second time. These data were collected in order to characterize a novel "egg-marking" behavior associated with oviposition. The behavior appeared to be associated with pheromone production, and the chemical defense of eggs. After determining when an egg was likely to be laid (see above), I sought to characterize the behavior by continuously recording activities associated with oviposition, monitoring one focal insect at a time. I attempted to characterize the function of this behavior using solid-phase microextraction (SPME) of the head space surround an egg, but to date, the function of

this behavior and any putative pheromones associated with it have yet to be characterized.

Materials and Methods

Insect rearing

Three strains of bed bugs were used in various experiments. The progenitors of CIN-1 colony were collected in Cincinnati, OH in 2005, NY-1 was collected from New York City, NY in 2007 and LEX-8 was collected from Lexington, KY in 2012. Bugs were housed in an incubator (Percival Scientific, Perry, IA) (27°C, 70% RH, 14:10 L:D) and weekly blood meals were administered with a blood feeding system (Montes et al. 2002). In this system, defibrinated rabbit blood (Quad Five, Rygate, MT or Hemostat, Dixon, CA) was pipetted into glass mosquito feeders **(**Kimble Chase Custom Glass Shop, Vineland, NJ**)** and heated to 39°C with a circulating water bath. Parafilm lined the bottom of the glass feeder containing the blood. Bed bugs in 59 mL plastic jars (Consolidated Plastics, Stow, OH) covered with organza (a fine mesh synthetic fabric) had to pierce the organza and the parafilm membrane to feed. Bugs were maintained weekly in this fashion until selected for experiments.

Time lapsed photography of egg laying

A Cannon 60D with an attached 100 mm macro lens was used to take photos of female bed bugs during oviposition. A Kodak Wratten gel filter (#29 Red) was held over the flash, as to not disturb bed bugs during the scotophase. The camera was held 45 cm from subjects, which were groups of 10 female bed bugs held together in four wells (10 females per well) of a six-well plastic plate (VWR, Radnor, PA) lined with Teflon to prevent movement around and outside of the well. Inside the well, the insects were able

to aggregate on one piece of blotter paper measuring 3.5 cm (width) x 1.75 cm (length). One picture was taken every sixty minutes, at the top of the hour. Bed bugs were held in an incubator at standard conditions, on a reversed light cycle of 14:10 L:D. The lights turned off at 10:00 a.m. each morning. Female bed bugs were given a blood meal at "day zero", during the scotophase, held for 24 hours, and then placed into the incubator for photos. Photographs were taken from day one (24 hours post blood meal) at 10:00 A.M. until day eleven at 10:00 A.M. I used females from the CIN-1, NY-1, and LEX-8 strains for this experiment. Each strain was tested separately. Within each strain, I observed four separate groups of females. Two groups ("fed for first time") had never mated or taken a blood meal prior to the experiment. Two other groups ("fed for second time") had previously mated and taken a blood meal, producing their first clutch of eggs c.a. seven days prior to the initiation of the experiment. This design allowed me to discern whether oviposition was either delayed, advanced, or unchanged if females were laying their second clutch of eggs as opposed to their first.

Each of the photographs obtained during the course of the experiment were analyzed using Adobe Photoshop. The cropping feature was utilized to crop only one cell of the six-well plate. Then, the "align" tool was used to automatically align each of the photographs for each individual well. After alignment and cropping, the photos were 'stacked' so that eggs could be counted from one hour to the next. A transparency sheet was mounted to the monitor of a desktop computer, and the location of each egg was marked and labeled with the time of egg laying. The egg, its number, the time, replicate, and treatment (fed once or fed twice) were logged into a datasheet for analysis.

Videography of egg laying behavior

An infrared sensitive camcorder (DNV16HDZ, Bell and Howell, New York, NY) and an infrared illuminator were used to record videos during the scotophase to capture behaviors associated with oviposition. Videos were recorded anywhere from 30 minutes to one hour during the scotophase (the optimal time for egg laying determined in the previous experiment) in order to capture females engaging in egg laying behavior. After videos were obtained, insects were continuously sampled for behaviors; all behaviors that appeared to be related to oviposition were recorded, as well as their duration.

Volatile collections using solid-phase microextraction

Solid-phase microextraction (SPME) was used to collect compounds released during egg laying by female bed bugs. The egg-laying arena was a glass test tube that had previously been rinsed with acetone and methanol. After rinsing, the tube was placed in an oven to dry. During this time, Whatman® #2 filter paper (Whatman, Maidstone, England) previously rinsed three times with acetone and three times with methanol were allowed to throroughly dry cut into 1.5 cm (height) by 1 cm (width) squares that would be placed into the bottom of each test tube for female bed bugs to sit on. The filter paper squares were placed into the bottom of each test tube. All tweezers and forceps used to insert the filter paper squares were rinsed with acetone and methanol prior to use. One female bed bug that had been fed 24 hours prior was inserted into the bottom of each tube using soft forceps. Each tube was sealed with a Teflon cap, and placed into a dark room in red light conditions (14:10 hour L:D). After females had been held for approximately four days (based on the results from the first experiment), they were observed during the scotophase for egg laying behavior. When it appeared that a female was ready to lay an

egg, which could be predicted by increased movement as well as curving of the abdomen down toward the substrate, an SPME fiber was extended into the tube, very close to (but not touching) the female. The SPME fiber was retracted after 30 minutes. At this time, the fiber was placed into the injection port of the gas chromatograph. Analysis was performed using a 60 m DB-wax column (6.5 mm diameter, 6.25 µL film thickness). The GC temperature program used started at 60°C and rose 10°C per minute for two minutes, reaching a final temperature of 250° C. A purge valve opened at two minutes into the sample run. The GC remained at constant pressure, with an initial column flow rate using helium gas of 1 mL/minute. The fiber remained in the injection port for one minute, and then removed and cleaned in a second GC inlet for 30 minutes at 250° C. Mass spectra were obtained using electron ionization mode at 70 eV, and scanning was completed for the range *m/z* 35-400.

Data Analysis

I examined the egg laying behavior and patterns of female bed bugs to elucidate the period of time post blood meal where a female was most likely to lay an egg, whether there was periodicity to egg laying, as well as to approximate how many eggs females lay depending on feeding status. However, due to small sample sizes (n=2 per treatment), statistical analyses were not applied to these data. Instead, descriptive statistics will be used to describe patterns in egg laying for each of the strains.

Results and Discussion

Time-lapsed photography allowed me to capture data that elucidated the timing of egg laying in the bed bug for three different strains, depending on their feeding status. I was also able to calculate the total number of eggs produced per group, and an average

per female. Females from the CIN-1 strain produced approximately 10.8 eggs in ten days, per female, if they had only fed once, and 12.3 eggs per female if they had fed twice. Females that fed once produced 216 eggs over the course of ten days, while females that fed twice produced 245 eggs total. It should be noted that the number of eggs laid is an underestimate, because only the side of the oviposition substrate facing the camera was analyzed. There was a strong tendency for females from both treatments to lay eggs during the scotophase (Figure 3.1). Females that had taken only one blood meal previously laid 84.2% of their eggs during the scotophase, and females that had taken two blood meals previously laid 86.5% of their eggs during the scotophase. Regardless of treatment, 50% of the total eggs laid during the scotophase were laid within 0-3 hours of scotophase initiation. With more samples, it is very likely that this would be a statistically significant difference indicating that bed bugs show periodicity in egg laying. Additionally, it appears that there is a slight delay in oviposition when females have only taken one blood meal, as opposed to two. Females that had fed twice laid 50% of their eggs by day four of the experiment. However, it took females five days to lay 50% of their total eggs if they had only taken only one blood meal.

Females from the NY-1 strain produced approximately 4.7 eggs in ten days, per female, if they had only fed once, and 10.2 eggs per female if they had fed twice. Females that fed once produced 94 eggs over the course of ten days, while females that fed twice produced 204 eggs total. Again, this is likely an underestimate. However, the large differences in egg production between the two groups in this strain deviates from that observed with CIN-1. It would be interesting to further test whether the number of eggs produced per blood meal varies as females lay more clutches/take additional blood

meals. Similarly to CIN-1, there was a strong tendency for females from both treatments to lay eggs during the scotophase (Figure 3.2). Females that had taken only one blood meal previously laid 92.5% of their eggs during the scotophase, and females that had taken two blood meals previously laid 78.9% of their eggs during the scotophase. Regardless of treatment, 50% of the total eggs laid during the scotophase were laid within 0-4 hours of scotophase initiation. This is an additional hour into the scotophase versus what I observed for CIN-1. Nonetheless, with more samples, NY-1 would likely also show a statistically significant effect of "phase" on the number of eggs produced. There was a stark delay in oviposition between treatments for NY-1. Females that had fed twice laid 50% of their eggs by day two of the experiment. However, it took females five days to lay 50% of their total eggs if they had only taken one blood meal previously. With more samples, I believe that treatment would have a significant effect on the first day of oviposition after a blood meal for this strain.

Females from the LEX-8 strain produced approximately 2.8 eggs in ten days, per female, if they had only fed once, and 6.25 eggs per female if they had fed twice. Females that fed once produced 56 eggs over the course of ten days, while females that fed twice produced 125 eggs total. Similarly to NY-1, there is an evident gap in the number of eggs produced when females have fed only once, versus twice. Additional sampling may reveal an effect of treatment on the total number of eggs laid. Similarly to the two other strains tested, however, there was a strong tendency for females from both treatments to lay eggs during the scotophase (Figure 3.3). Females that had taken only one blood meal previously laid 94.6% of their eggs during the scotophase, and females that had taken two blood meals previously laid 76.8% of their eggs during the scotophase.

Regardless of treatment, 50% of the total eggs laid during the scotophase were laid by the fourth hour of scotophase initiation. Because females from all three strains tested lay a very high percentage of their eggs during the scotophase, there is strong evidence presented here for periodicity in egg laying for the bed bug. Unlike the other strains, no delay in oviposition was evident between the treatments. Both groups laid 50% of their eggs by day four of the experiment.

Narrowing down the date and time that egg laying typically occurred allowed me to make observations on oviposition behaviors exhibited by females. I obtained 17 videos of individual females engaging in oviposition, and analyzed each of these videos (using continuous sampling for animal behavior) for consistent patterns among females. On average, females take three to six seconds to lay their eggs. This was calculated by timing the female from the moment she placed her abdomen to the substrate and the egg was present on the paper, to the moment that the egg had completely exited the female and entered the environment. It never took longer than thirteen seconds for a female to oviposit. Although I was not able to characterize any behaviors that could aid in the prediction of an egg laying event, I did observe a novel behavior following each oviposition event that has not been described to date. Following egg laying, females spend time moving their abdomen side to side above the egg for 22 seconds on average (Figure 3.5). I observed females engaging in this behavior anywhere from 18 seconds to 41 seconds. From a lateral (versus top-down) perspective, it also appeared that the females use their hind tarsi to touch the evaporative surface outside the scent glands and then her egg multiple times. I was not able to characterize the frequency of this particular behavior due to filming limitations. A top-down view prevented me from viewing the

activity of the female's legs underneath her body. I have chosen to refer to the aforementioned behavior as a "post-ovipositional waggle" (POW), and the movement of a female's abdomen rapidly from side to side always followed egg laying during observations in the laboratory. Because the nature of this behavior, along with the touching of tarsi to the scent glands, was indicative of volatile emission, I attempted to use solid-phase microextraction (SPME) to identify compounds released by females during egg laying. Other hematophagous insects, such as *Culex* spp. deposit oviposition pheromones on their eggs (Seenivasagan and Viayaraghavan 2010). Additionally, it has been proposed that similarly to *Triatoma infestans*, bed bugs use aggregation pheromones to mediate oviposition. However, to date, I have not obtained any volatile or contact pheromones associated with ovipoisition, and have been unable to characterize the specific function of the POW.

Although it is possible that females may be marking eggs with aggregation pheromones, I also propose an alternative hypothesis. It is possible that females may use the alarm pheromone as an antimicrobial compound to protect their eggs from infection—a form of social immunity. Bed bugs are susceptible to some types of fungus, and the bed bug alarm pheromone has anti-fungal properties (Ulrich et al. 2015). Social immunity (collective actions against parasites or pathogens) is a common way group-living insects reduce the risk of infection by parasites and/or pathogens (Meunier 2015). Most commonly attributed to and described for the eusocial insects, these defenses may include behaviors such as allogrooming, or corpse removal from nests. However, many non-eusocial insects also exhibit behaviors that decrease pathogen transmission among group members. Cockroaches, *Blatella germanica* avoid consumption of infected food

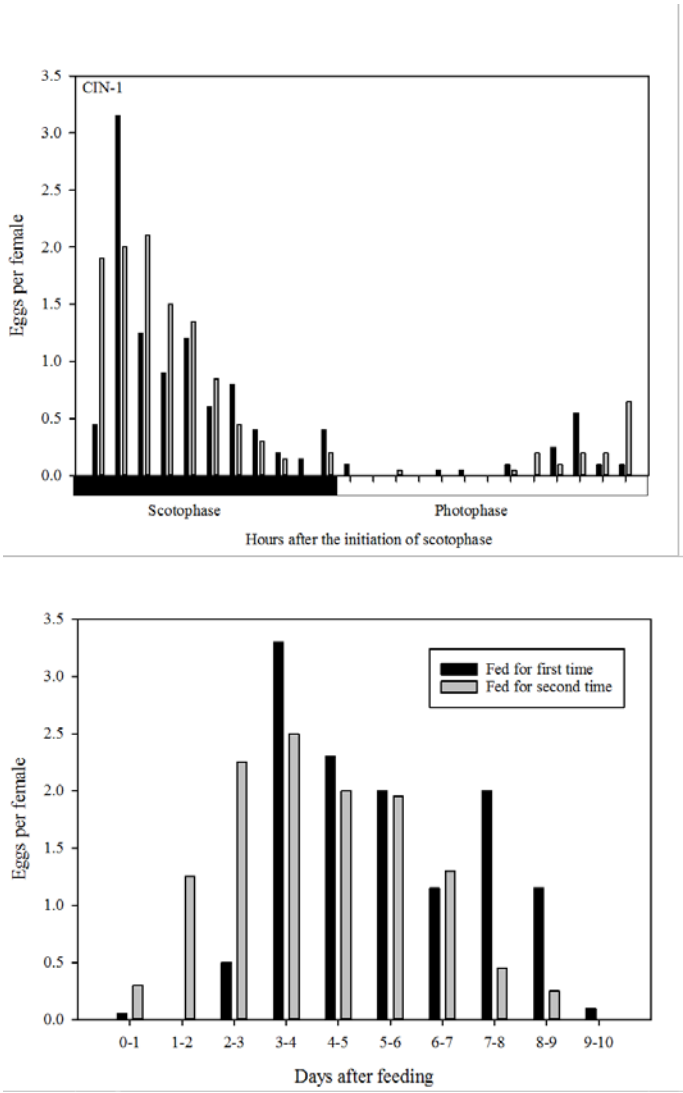
and/or conspecifics (Kaakeh et al. 1996). In earwigs, grooming by the mother prevents the colonization of pathogens (Boos et al. 2014). Earwigs also exhibit a form of social immunity by producing feces that have anti-fungal properties (Diehl et al. 2015). European beewolfs (*Philanthus triangulum)* apply bacteria with antifungal properties to brood chambers prior to oviposition (Kaltenpoth et al. 2005). Collective immune defenses are common for eusocial insects, as well as in insects that live in dense groups, like the bed bug. Future work and improvements in methodology will be necessary to elucidate the function, if present, for the POW exhibited by female bed bugs.

Conclusions

Elucidating oviposition behaviors and patterns in hematophagous insects can aid in the management of both pest insects. For instance, the development of traps/lures that contain oviposition pheromones, or the identification of potential breeding sites for insecticide application. Very little is known regarding the egg laying patterns in the bed bug, *Cimex lectularius.* This objective of this study was to characterize egg laying patterns, and the function of a novel behavior associated with egg laying in the bed bug. Female bed bugs, regardless of previous feeding experience, likely display periodicity in egg laying. Females lay the majority of their eggs during the scotophase, and the highest number of eggs are usually laid within four hours of scotophase initiation. Across the three strains tested here, females lay the most eggs anywhere from two to five days after a blood meal, with no eggs produced after ten days. Although likely an underestimate, groups of 20 female bed bugs produced anywhere from 56-245 eggs per blood meal. The large difference in the number of eggs produced depending on the treatment merits further exploration. Additionally, more samples should be tested to confirm periodicity

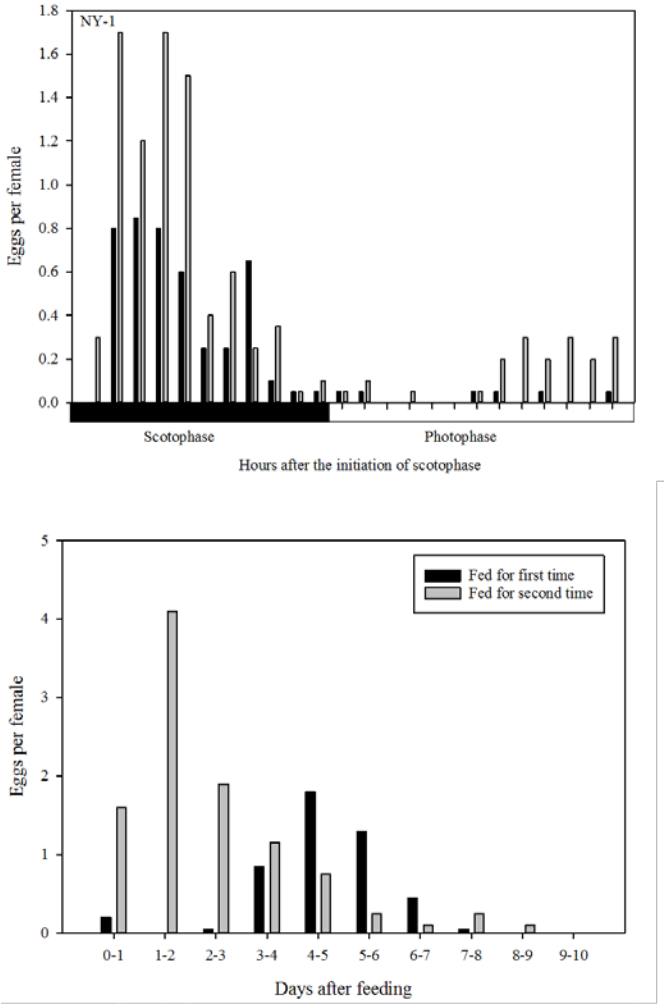
using statistical analyses. Behavioral observations revealed that once an egg is laid, a female will spend approximately 22 seconds “marking” it by waving her abdomen side to side very rapidly above and around the area where she has oviposited. A function for this behavior has yet to be identified, but I hypothesize that females are placing defensive compounds on their eggs that may protect them from microbial pathogens and, thus, could be engaging in a form of social immunity. This hypothesis should be tested in future experiments.

Figure 3.1.



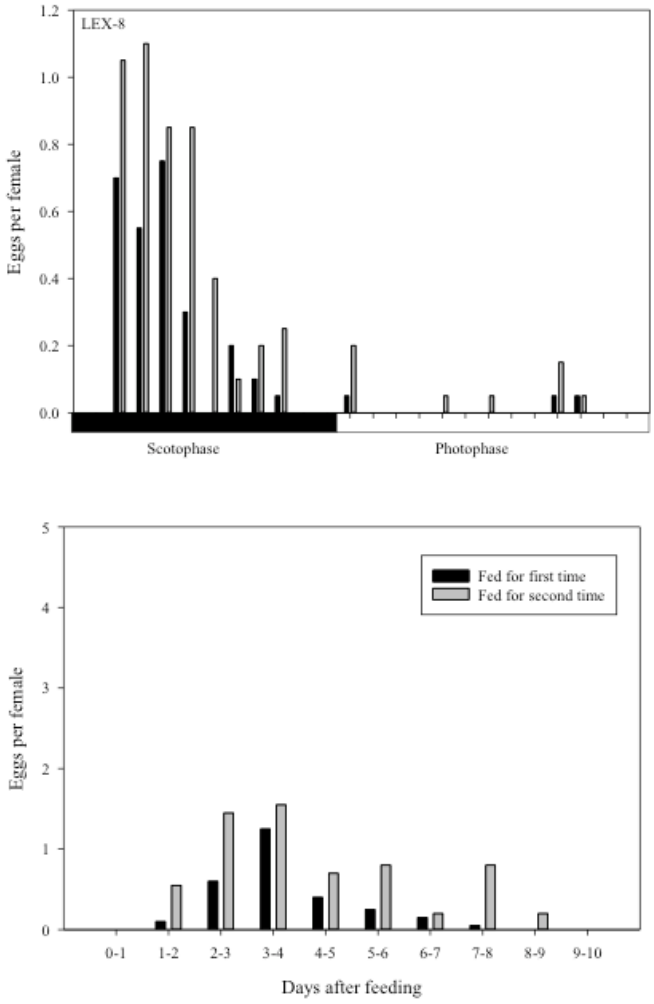
Temporal patterns of egg laying for CIN-1. There was a strong tendency for females from both treatments to lay eggs during the scotophase. Females that had taken only one blood meal previously laid 84.2% of their eggs during the scotophase, and females that had taken two blood meals previously laid 86.5% of their eggs during the scotophase. It appears that there is a slight delay in oviposition when females have only taken one blood meal, as opposed to two.

Figure 3.2.



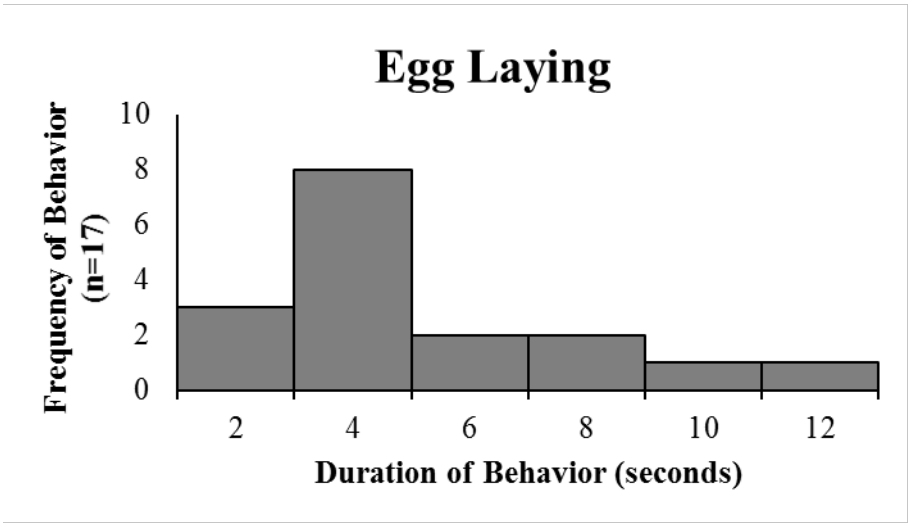
Temporal patterns of egg laying for NY-1. There was a strong tendency for females from both treatments to lay eggs during the scotophase. Females that had taken only one blood meal previously laid 92.5% of their eggs during the scotophase, and females that had taken two blood meals previously laid 78.9% of their eggs during the scotophase. There was a stark delay in oviposition between treatments for NY-1. Females that had fed twice laid 50% of their eggs by day two of the experiment. However, it took females five days to lay 50% of their total eggs if they had only taken one blood meal previously.

Figure 3.3.



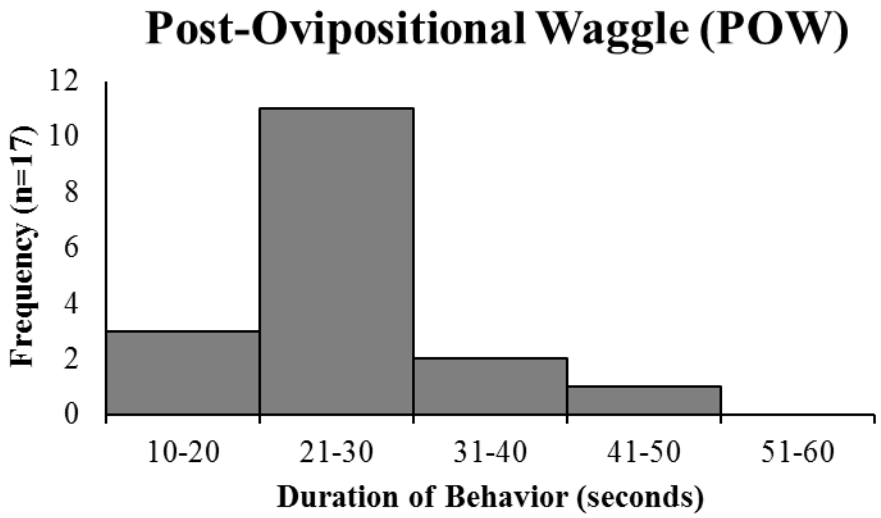
Temporal patterns of egg laying for LEX-8. There was a strong tendency for females from both treatments to lay eggs during the scotophase. Females that had taken only one blood meal previously laid 94.6% of their eggs during the scotophase, and females that had taken two blood meals previously laid 76.8% of their eggs during the scotophase. Unlike the other strains, no delay in oviposition was evident between the treatments. Both groups laid 50% of their eggs by day four of the experiment.

Figure 3.4.



The average duration of egg laying for female bed bugs. Continuous sampling for oviposition or behaviors related to oviposition revealed that egg laying typically took approximately four seconds, never lasted longer than 12 seconds, or less than one second. No predictable behaviors commonly anteceded egg laying.

Figure 3.5



Average duration of post-ovipositional egg marking (POW). Continuous sampling of focal insects revealed that females wave their abdomens side to side over newly laid eggs for approximately 22 seconds on average. The behavior never lasted longer than 41 seconds, and never less than one second. The post-ovipositional waggle (POW) always followed egg laying in all 17 observations.

Chapter 4. Behavioral effects of sublethal exposure to a combination of β-cyfluthrin and imidacloprid in the bed bug, *Cimex lectularius* L.

Introduction

Understanding the relationship between insect behavior and fitness is crucial in pest management where changes in behavior after treatment with insecticides may impact behavior, fitness, and ultimately, population control (Stearns 1976, Maltby 1999, Desnaux et al. 2007). The judicious use of insecticides remains a cornerstone of pest management. This is especially true with household and public health pests, where efficient and affordable results are needed and non-chemical tactics alone may not be an option. Many common classes of insecticides, like the pyrethroids and neonicotinoids, act at the neuronal level to disrupt the normally well-orchestrated behaviors of insects. This disruption can reduce longevity (independent of the direct lethal effects) and alter the reproductive potential for many insect species (Haynes 1988). Even so, the effects of insecticides on insect behaviors are usually overlooked during laboratory evaluations of chemical products for efficacy (Stark and Banks 2003). This oversight during laboratory product evaluation could, as a result, misrepresent product performance in the field. Much of the information collected on insecticide efficacy is based on direct observations of mortality following exposure to the active or formulated ingredients in standardized laboratory bioassays (Stark et al. 1995). This methodology does not consider the diverse ways that an insecticide could affect fitness and population growth in the field (Stark et al. 1995).

Sublethal effects are aberrant physiological or behavioral effects occurring after exposure to an insecticide (Desnaux et al. 2007). Behavioral changes from sublethal exposure to insecticide may include: stimulation of or reduction of oviposition behaviors,

alterations in the number of feeding and foraging events, inappropriate migration and dispersal, or the avoidance of insecticide residues (Haynes 1988). Evaluation of sublethal exposure and the resultant effect on fitness could provide a more comprehensive understanding of the impact of an insecticide than mortality data alone.

One pest commonly managed with insecticides is the bed bug (*Cimex lectularius*, L.). Bed bugs are flightless, hematophagous insects with public health relevance since the adults and nymphs feed on human blood (Reinhardt and Siva-Jothy 2007). Bed bugs are cryptic and nocturnal (Doggett et al. 2012). Their aversion to light and tendency to aggregate in small crevices makes detection very time consuming (Wang et al. 2009a). Although alternative non-chemical treatment methods for controlling bed bugs are available (such as vacuuming, steaming, cold treatment, and volumetric heating), surviving individuals often experience no long-term consequences (Wang et al. 2009b). Thus, utilization of residual insecticides is an important part of most pest management programs (Potter et al. 2015). Many studies have focused on the inability of insecticides to induce mortality in bed bugs due to the widespread evolution of insecticide resistance, especially resistance to pyrethroids (Romero et al. 2007, Davies et al. 2012). There is a real or perceived gap between laboratory documentation of the prevalence of insecticide resistance and continued reliance on these products by the pest management industry (Potter et al. 2015). Part of the 'disconnect' in respect to efficacy could be due to non-lethal effects on bed bug behavior. One recent study showed that sublethal exposure to permethrin-impregnated fabric affected both feeding behavior and fecundity of bed bugs (Jones et al. 2015). Significantly fewer female bed bugs laid eggs after sublethal exposure to the fabric, and exposure times as short as one minute were enough to induce behavioral changes.

These results indicate that population-level consequences are likely.

In this study, I examined bed bug behavior after sublethal exposure to Temprid®, a product that combines a neonicotinoid (imidacloprid) and a pyrethroid, (β-cyfluthrin), hereafter referred to as "the combination product". This product is the most commonly used insecticide spray in the United States for bed bug management (Potter et al. 2015). Insecticide resistance to pyrethroid, neonicotinoid, as well as combination products has recently been reported (Zhu et al. 2013, Gordon et al. 2014, Gordon et al. 2015, Romero and Anderson 2016). However, some resistance mechanisms carry a fitness cost that may enable a reversion to susceptibility in the absence of insecticide. A recent study indicated that after selection for resistance to a combination of β-cyfluthrin and imidacloprid, reversion to susceptibility occurred post-selection, most likely due to fitness costs associated with resistance (Gordon et al. 2015). Thus, in this study, I examined three populations of bed bugs that varied in their level of resistance to pyrethroids. I chose to investigate the effects of sublethal exposure on behaviors critical for bed bug survival and reproduction, including: successfully taking a blood meal, locomotion, and responses to harborages previously inhabited by conspecifics. I hypothesized that treated insects would have less success taking a blood meal; that they would lose their ability to respond to putative aggregation pheromones; that they would spend a smaller proportion of time moving; and that their nocturnal periodicity of locomotion would be disrupted. Each of these behavioral changes would have implications for the efficacy of a widely used combination product for bed bug management programs that has previously not been understood.

Materials and Methods

Insect rearing

Three strains of bed bugs were used in all experiments. The progenitors of CIN-1 colony were collected in Cincinnati, OH in 2005, and while it was initially highly resistant to pyrethroids it has become more susceptible over time (Zhu et al. 2013). NY-1 was collected from New York City, NY in 2007 and is now moderately resistant to pyrethroids. LEX-8 was collected from Lexington, KY in 2012, and is highly resistant to pyrethroid insecticides (Zhu et al. 2013). Bugs were housed in an incubator (Percival Scientific, Perry, IA) (27°C, 70% RH, 14:10 L:D) and weekly blood meals were administered with blood feeding system (Montes et al. 2002). In this system, defibrinated rabbit blood (Quad Five, Rygate, MT) was pipetted into glass mosquito feeders **(**Kimble Chase Custom Glass Shop, Vineland, NJ**)** and heated to 39° C with a circulating water bath. Parafilm lined the bottom of the glass feeder containing the blood. Bed bugs in 59 mL plastic jars (Consolidated Plastics, Stow, OH) covered with organza (a fine mesh synthetic fabric) had to pierce the organza and the parafilm membrane to feed. Bed bugs used in experiments were seven days post adult eclosion.

Residual deposit mortality bioassays

An LT_{10} (lethal time of exposure resulting in 10% mortality) was determined independently for each strain using a residual deposit bioassay (Gordon et al. 2014a). Adult bed bugs were held individually in 24-well plates (Costar, Corning, NY) with wells measuring 1.6 cm in diameter. Each well was lined with Whatman® #2 filter paper (Whatman, Maidstone, England) cut to a diameter of 1.7 cm. Each filter paper had been saturated with 50 µL (0.075% a.i., with a 2:1 ratio of imidacloprid, and β- cyfluthrin) of

the combination product diluted in water at the label rate, or water alone (as a control). Filter papers were given 24 hours to air dry prior to the bioassay. At this time, a bed bug was confined to the treated surface in one well and scored for mortality at 5, 15, and 30 minutes; 1, 4, 12, and 24 hours; 3, 7 and 14 days (if necessary). Mortality was scored at each time point by gently turning bugs onto their dorsal side with soft forceps. If the insect could not recover by turning over to the ventral side, it was considered moribund. Three replicates with ten bugs per replicate were conducted.

Feeding efficiency in an arena

I investigated the effects of sublethal exposure to the combination product on the feeding success of adult bed bugs in an artificial feeding system. Following exposure to either water or strain specific LT_{10}, insects were placed in petri dishes (100 mm x 15 mm, BD Falcon, Corning, NY) with a tent-shaped piece of blotter paper (16 cm^2 on each side) for use as a harborage. These insects were placed in an incubator (27°C, 70% RH, 14:10 L:D) for 24 hours before they were used. Following exposure to either insecticide or water, five live and apparently healthy females and males were randomly selected per replicate.

A harborage with bed bugs was placed in a glass cylinder measuring 25 cm (height) x 10 cm (diameter), sealed on top with organza. The cylinders were placed beneath glass mosquito feeders containing rabbit blood. Blood feeding followed the protocol described above. To feed, the insects needed to walk up filter paper (25 cm L x 2.5 cm W) taped to both sides of blotter paper of the same dimension (for rigidity). This ramp allowed bed bugs the opportunity to move from the bottom of the cylinder to the top to take a blood meal. After an acclimation period of 15 minutes, bed bugs were

permitted access to the feeding system for 30 minutes. I recorded the number of insects that fed, and the time of feeding of each bed bug. Ten replicates with ten insects (five males and five females) per replicate were conducted (200 bed bugs total, 100 per treatment).

Mass gain after blood meal

I investigated the amount of blood imbibed after feeding in bed bugs exposed to the combination product versus bed bugs exposed to water. Following exposure to either water or strain specific LT_{10}, insects were placed in petri dishes (100 mm x 15 mm, BD Falcon, Corning, NY) with a tent-shaped piece of blotter paper (16 cm^2 on each side) for use as a harborage. These insects were placed in an incubator (27°C, 70% RH, 14:10 L:D) for 24 hours before they were used for this experiment. After the 24-hour recovery period, eight healthy females (chosen by flipping the female to her dorsal side and assessing her ability to turn over to the ventral side) were selected and placed individually in Eppendorf tubes (Fisher Scientific, Pittsburgh, PA). Each female was weighed prior to access to a blood meal, and her starting weight was recorded using a balance at a resolution of 0.1 mg. After a starting weight was obtained, each female was placed individually in a 59 mL plastic jar (Consolidated Plastics, Stow, OH) and given access to the artificial feeding system described previously. After a blood meal was taken, females were removed and placed back into an Eppendorf tube. The final weight of each female was recorded immediately after feeding occurred. The starting weight was subtracted from this final weight in order to determine the total mass of blood imbibed. Values were compared between the treatment and control groups. Eight replicates of this assay were conducted with females only.

Locomotion assay

I tested the effects of sublethal exposure to the combination product on locomotor activity of adult bed bugs. Twenty-four hours after exposure both male and female bed bugs were randomly selected and placed in six-well plates with untreated filter paper (i.e., no further exposure to insecticide) (VWR, Radnor, PA) and housed in an incubator at the laboratory conditions previously described. Movements of these bugs were recorded over a 24-hour period using a camera (Sony Cybershot DSC H300) programmed to take one picture every ten minutes. This interval was based on an earlier study (Romero et al. 2010a). To record clear pictures during the scotophase, an LED infrared illuminator (Pinecom PN-850) was used and the camera was set to "nightshot." After 24 hours, pictures were assessed in sequential order and the bed bug in each cell scored for movement. If the insect's position differed from one frame to the next, I considered this a movement. The proportion of time intervals that a bug moved was determined independently for the day and night. Eleven replicates were conducted (eleven insects per treatment per strain).

Response of untreated bed bugs to insecticide-treated harborages

Since repellency may also account for locomotion and dispersal, I assessed the responses of untreated bed bugs to insecticide-treated harborages. In this experiment, no bed bugs were exposed to the combination product. Instead, control insects were placed in Climbup insect interceptors (Susan McKnight, Inc., Memphis TN). Interceptors were lined with 10.8 cm diam. black filter paper (Ahlstrom, West Carrollton, OH). Each Climbup contained two filter paper 'tents' (2.5 cm x 2.5 cm squares, folded down the center). One tent was saturated with 50 µL of water (control tent), and one tent was

saturated with 50 µL of the combination product (treated tent). Tent choices made by individual bed bugs were evaluated after 24 hours. Individuals that made no choice were omitted from analysis. Twenty replicates of the choice test were conducted.

Response to harborages with feces from bed bugs

I assessed the ability of bed bugs to detect putative aggregation pheromone after exposure to the combination product. Prior to the experiment, 100 adult bed bugs were placed on 2.5 cm x 2.5 cm filter paper harborages folded down the center to form 'tents' (described above). Recently fed bugs were permitted to aggregate and defecate on these tents for three days. These filter papers should contain compounds that lead to aggregation formation as shown by previous studies, since bed bug feces are known to contain their aggregation pheromone in addition to other compounds (Siljander et al. 2008, Romero et al. 2009, Gries et al. 2015). After exposure to the combination product, surviving bugs (as previously described) were placed in Climbup insect interceptors (Susan McKnight, Inc., Memphis, TN). Interceptors were lined with 10.8 cm diam. black filter paper (Ahlstrom, West Carrollton, OH). Each Climbup contained two tents: one control tent (never exposed to bed bugs) and one tent that had previous exposure to other bed bugs for three days. Within the Climbup each bed bug was given 24 hours to choose a tent, and tent choice was scored at this time. A single bug that made no choice of either tent (i.e., it was out in the arena) was excluded from the analysis. Twenty replicates of the choice test were conducted.

Data analysis

Probit analysis was used to calculate a strain-specific LT_{10} values with Minitab® 15 for Windows v2007. All other statistical analyses were conducted

using Statistix 10. Wilcoxon Rank Sum Tests were used in to examine the effects of sublethal insecticide exposure on the proportion of bed bugs that fed, the time it took for bed bugs to initiate feeding, and the mass gained after feeding. A two-way ANOVA was used to examine the impact of photoperiod and insecticide exposure on the proportion of time spent moving over the course of a 24-hour period. These raw data were arcsine-square root transformed prior to analysis. Binomial tests were used to assess whether bed bugs selected control tents versus tents that had previous exposure to bed bugs for both insecticide exposed and control bed bugs from each population. Fisher's Exact Test was used to compare the proportion choosing the tent with exposure to conspecifics in treated versus untreated bugs for each population. All analyses were conducted only within strain because LT_{10} exposure times were unique to each strain.

Results and Discussion

As expected, the LT_{10} values for this combination product differed depending on known level of pyrethroid resistance in each of the strains. The LT_{10} values (in hours) were 0.95 (0.46–1.57), 1.14 (0.43–2.11) and 5.0 (1.9–8.93) (95% fiducial confidence intervals) for CIN-1, NY-1, and LEX-8, respectively. All subsequent assays were performed after bed bugs were exposed at these LT_{10}'s. Probit analyses for each strain and all associated data tables are presented (Table 4.1).

Regardless of strain, sublethal exposure at the LT_{10} significantly decreased the proportion of individuals that successfully fed (Fig. 4.1, Wilcoxon Rank Sum Test, CIN-1: n=10, Z=2.4, $p<0.05$; NY-1: n=10, *Z*=2.51, $p<0.05$; LEX-8: n=10, Z=3.29, p=0.001). Treated CIN-1, NY-1, and LEX-8 took 58%, 68%, and 81% longer to initiate feeding than their control groups, respectively (Fig. 4.2, Wilcoxon Rank Sum Test, CIN-1: n=10, Z=2.0,

$p<0.05$; NY-1: n=10, Z=2.08, $p<0.05$; LEX-8: n=10, Z=2.04, $p<0.05$). NY-1 and LEX-8 imbibed significantly smaller blood meals with insecticide exposure (Fig. 4.3, Wilcoxon Rank Sum Test, CIN-1: n=8, Z=1.31, $p>0.05$; NY-1: n=8, Z=2.05, $p<0.05$; LEX-8: n=8, Z=2.27, $p<0.05$). Finding a host is critical to the success of a bed bug because blood allows an individual to develop from juvenile to adult, and it allows adults to produce sperm and eggs (Reinhardt and Siva-Jothy 2007). If this process were made more difficult by exposure to insecticides, then a reduction in population growth rate would be expected when bed bugs are not killed, if recovery is slow or absent. These effects may also increase the amount of time bed bugs spend exposed outside of harborages. Typically, quick forays from their hidden harborages are expected (Reinhardt 2012, Crawley et al. 2015). These findings indicate that sublethal exposure to insecticide might temporarily reduce the number of bites a host would sustain while increasing the vulnerability of bed bugs to other mortality factors (such as mechanical control) and result in gradual population decline. Conversely, rapid recovery would cause these effects to be short-lived. Thus, future studies should address recovery time after sublethal exposure. However, there is some initial evidence that recovery to the combination product used here is very gradual. I have observed negative sublethal effects up to and even after six weeks (see Chapter 5).

The percentage of time spent moving after a sublethal exposure to the combination of β-cyfluthrin and imidacloprid was significantly reduced in all three strains (Fig. 4.4, ANOVA, CIN-1: n=11, $F_{1,40}$: 7.5, $p<0.01$; NY-1: n=11, $F_{1,40}$: 21, $p<0.0001$; LEX-8: n=11, $F_{1,40}$: 22, $p<0.0001$). As expected, bed bugs moved more frequently during the night than the day (Fig. 4.4, ANOVA, CIN-1: n=11, $F_{1,40}$: 8, $p<0.01$; NY-1: n=11, $F_{1,40}$: 10, $p<0.01$; LEX-8: n=11, $F_{1,40}$: 15, $p<0.001$). There was no interaction between treatment and time of

day, indicating that the insecticide did not affect the periodicity of the response (Fig. 4.4; ANOVA, CIN-1: n=11, $F_{1,40}$: 3, p=0.10; NY-1: n=11, $F_{1,40}$: 2, p=0.15; LEX-8: n=11, $F_{1,40}$: 1, p=0.29), but did consistently reduce the movement rate. The propensity to move during part of the night before stimulation by host cues is likely to be an important part of host finding (Romero et al. 2010a). A reduction in this movement rate is likely to further reduce host-finding success.

Some insecticides cause insects to increase the time they spend moving, or to disperse in ways they normally would not (Romero et al. 2010a, Cohnstaedt and Allan 2011). When this effect is quick and acute the insecticide (e.g., pyrethrum) can be used as a "flushing agent," which can be useful in detecting the presence of cryptic pests such as cockroaches (Braness 2011). Alternatively, stimulating movement of pests can have a negative side effect of dispersing individuals to unoccupied spaces. However, we found that sublethal exposure to a combination product significantly reduced rather than enhanced movement in survivors. These results suggest that treatments with this combination product should not cause increased dispersal of bed bugs into neighboring residential units, a pressing concern for pest management professionals (Crawley et al. 2015). As an additional test for potential locomotion post-treatment, I conducted a two-choice tent assay and allowed untreated bed bugs to choose between insecticide-treated tents or control tents. I found no evidence of behavioral avoidance or repellency, which could serve as an alternative cause of dispersal in the field (Figure 4.5; Binomial Tests, CIN-1: n=20, p= 0.58; NY-1: n=20, p= 0.86; LEX-8: n=20, p= 0.25). Thus, it appears that treatments are not likely to result in increased dispersal or locomotor movements after exposure to residual deposits.

Contrary to my hypothesis, insects in the treatment or control groups for all strains selectively rested on tents that had previous exposure to bed bugs rather than control tents (e.g., both treated and control bugs chose marked tents, Figure 4.6; Fisher's Exact Test, CIN-1: p=0.23; NY-1: p=1.0; LEX-8 p=0.1) indicating that the insecticide treatment had not affected their ability to respond to aggregation pheromone. Treatment insects chose tents exposed to conspecifics at frequencies of 0.85 ± 0.08, 1.0 ± 0.00, and 0.84 ± 0.08 (proportion ± s.e.m.) for CIN-1, NY-1, and LEX-8, respectively. Control insects chose tents previously exposed to conspecifics 100% of the time. Only one insect made no choice (treated, LEX-8) and was omitted from analysis. Independent of other behavioral effects of the insecticide, bed bugs would be expected to continue to aggregate in harborages marked by other bed bugs. Thus, one would expect that treated bugs would not be more likely to leave harborages during the day. Combined with the decrease in movement caused by the insecticide, this would result in bed bugs spending more time in harborages and thus there would be a premium on treating in those spaces.

Resistance to insecticides is often documented by contrasting insecticide exposures (duration/dosages or dose) necessary to kill individuals from a field population with individuals from a population known to be susceptible. In bed bugs, resistance to pyrethroids is common and widespread, and reduced susceptibility was also reported recently to neonicotinoids (Zhu et al. 2013, Gordon et al. 2014, Gordon et al. 2015, Romero and Anderson 2016). The rapid evolution of resistance to a combination product was likewise found in one laboratory study where selection was imposed on three strains of bed bugs (Gordon et al. 2014). Recently, high levels of resistance to neonicotinoid/pyrethroid combination products were found in field populations (Zhu et

al. 2013, Gordon et al. 2014). The populations that we studied here varied in their susceptibility to combination products as measured by LT_{10}'s, suggesting lethal and sublethal effects co-vary to some extent. It is conceivable that resistance to an insecticide could result from a diminution of the deleterious sublethal effects with no change in lethal effects. However, that is not the case in the populations tested here.

Population level effects of insecticides may be underestimated by laboratory assessment of lethal effects alone. This can lead to erroneous conclusions about the aggregate effect of treatment. In theory when the gap (dose or exposure time) between lethal and sublethal effects is small then little is missed with a mortality-based assay. However, when the gap is larger, lethal assays do not reflect potential behavioral effects on individuals. In this study, the propensity to aggregate in response to aggregation pheromone is likely to have a small gap between sublethal and lethal effects (i.e., my assay did not detect a behavioral effect at the LT_{10}). However, feeding frequency, time to initiate feeding, and movement frequency follow a different pattern, with some variation among populations in the size of the gap between lethal and sublethal effects. It is clear from my study that sublethal effects on a population may be very important if duration of exposure to the insecticide is shorter than required for lethal effects. This may be the case when coverage is incomplete and aggregations are missed (leading to reduced exposure), or when resistance levels are higher leading to reduced impact of exposure. Here we studied the impact of only one insecticide product. It is very likely that insecticides with different modes of action will be characterized by distinctive suites of behavioral effects since they target different elements of the nervous system. A systematic study of the behavioral symptoms associated with insecticides with different

modes of action could be used to build predictions about how these classes will affect behavior. Future work should also investigate the effect of individual active ingredients to better understand the specific causes of the behavioral effects observed here.

Conclusions

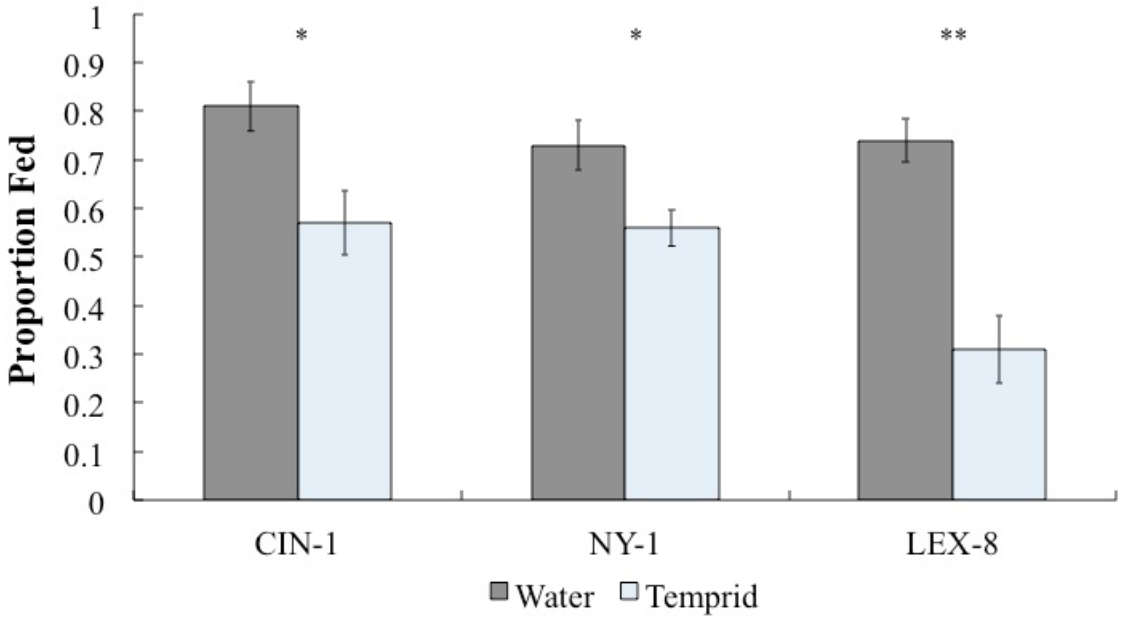
In this study, I found that some bed bug behaviors, such as feeding, and locomotion, were adversely affected by sublethal exposure to Temprid® SC, while other indicators of feeding were variable (e.g., mass gained after initiation of feeding). Aggregation behavior and periodicity of movement, however, were not affected. The net effect of this exposure has the potential to be detrimental to populations of bed bugs and, thus, helpful in managing this pest. Alternatively, there is also the possibility that sublethal effects on movement could cause small "reservoirs" of treated insects that are temporarily unexposed to ongoing contact with insecticide as they recover. This possibility could impact pest management decisions regarding timing of treatments, and the total number of necessary visual inspections. Importantly, the ultimate impact of an insecticide on a population of insects should not be assessed exclusively based on direct lethal effects. Some discrepancies between laboratory evaluations of insecticide lethality and field results could be the consequence of poorly understood behavioral effects.

Table 4.1. Probit analysis of residual exposure of three populations of bed bugs to a combination product containing β-cyfluthrin and imidacloprid. Lethal times (h) and 95% fiducial confidence intervals are shown.

Strain[a]	LT_{10} (95% CI)	LT_{50} (95% CI)	LT_{90} (95% CI)	Slope (± SEM)
CIN-1	0.95 (0.46–1.57)	8.43 (5.64–13.07)	75.14 (41.31–181.53)	0.59 ± 0.07
NY-1	1.14 (0.43–2.11)	29.42 (16.48–70.91)	760.62 (231.44–6505.62)	0.39 ± 0.06
LEX-8	5.00 (1.90–8.93)	64.15 (34.26–200.29)	822.17 (245.53–11302.70)	0.50 ± 0.10

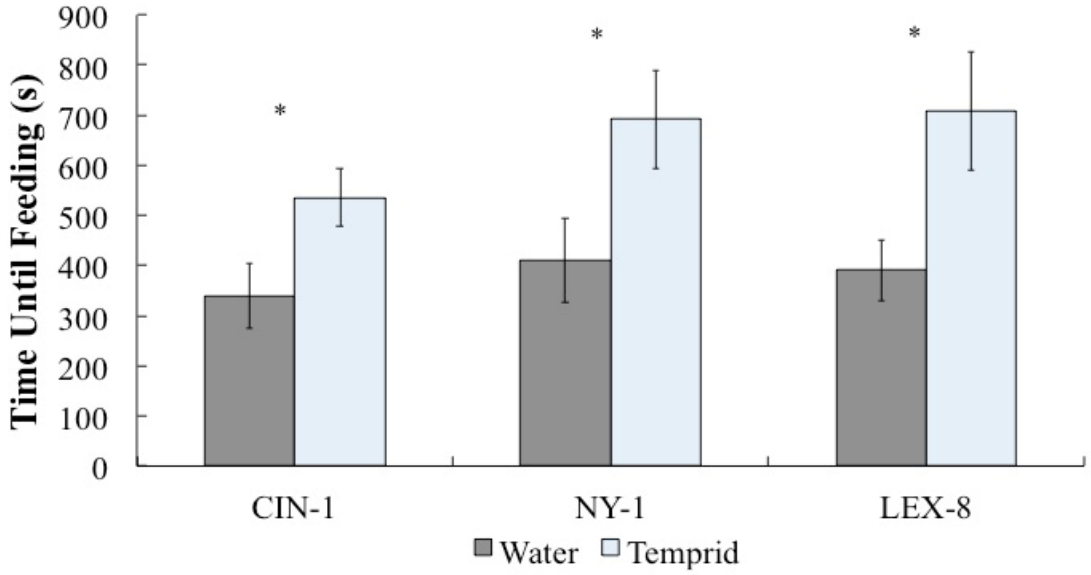
[a] 30 bed bugs were tested per strain using previous methods (Gordon et al. 2014a).

Figure 4.1.



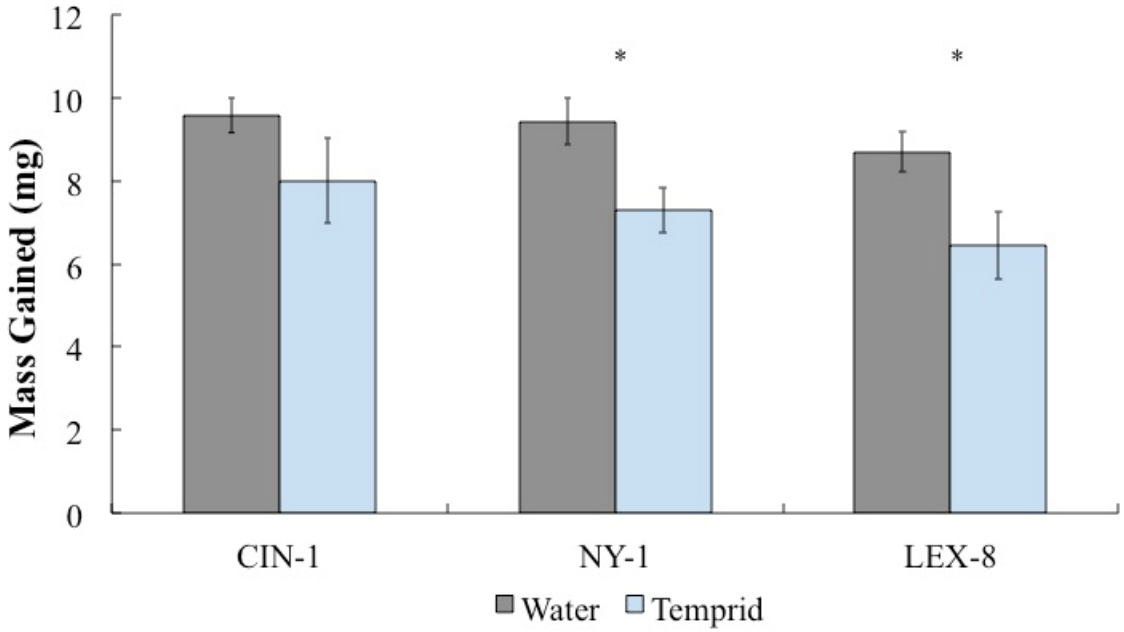
Proportion of bed bugs that took a blood meal with and without prior exposure to the combination product. Exposure to the insecticide decreased feeding by 30%, 23%, and 58% for CIN-1, NY-1, and LEX-8 respectively. Significant differences between treated and untreated groups are denoted by asterisks (*$p<0.05$, **$p<0.01$).

Figure 4.2.



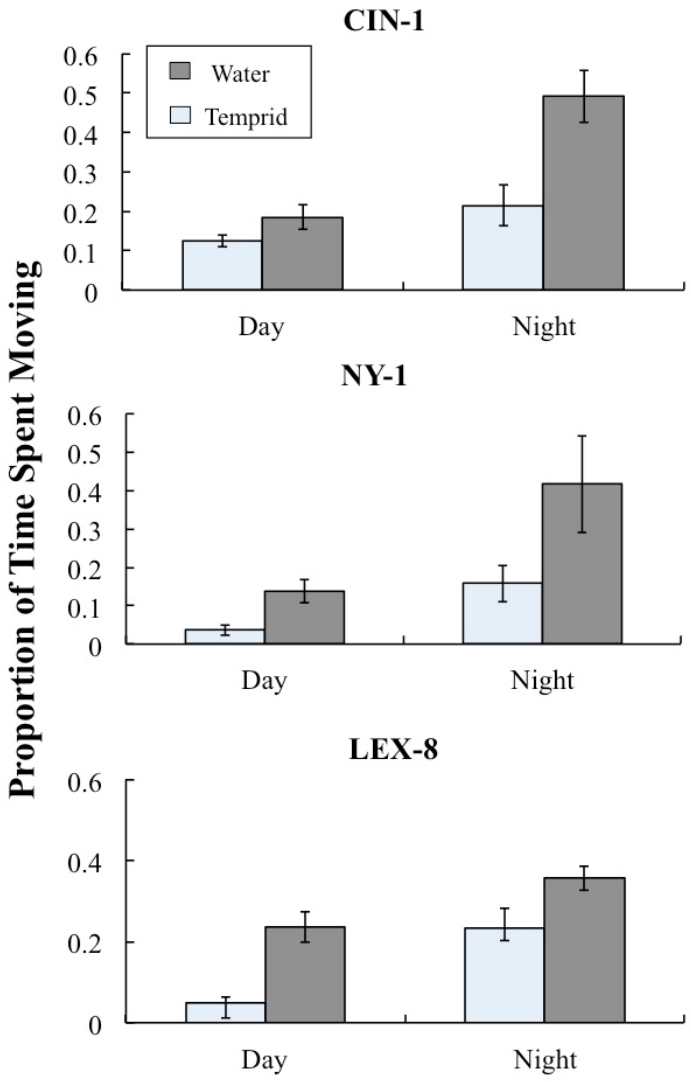
Mean time in seconds until bed bugs initiated feeding with and without prior exposure to the combination product. Bed bugs that did not feed during the assay are not included in the analysis. Exposure to the insecticide significantly increased the time taken to initiate feeding by 58%, 68%, and 81% for CIN-1, NY-1, and LEX-8, respectively. Significant differences between treated and untreated groups are denoted by asterisks (*$p<0.05$).

Figure 4.3.



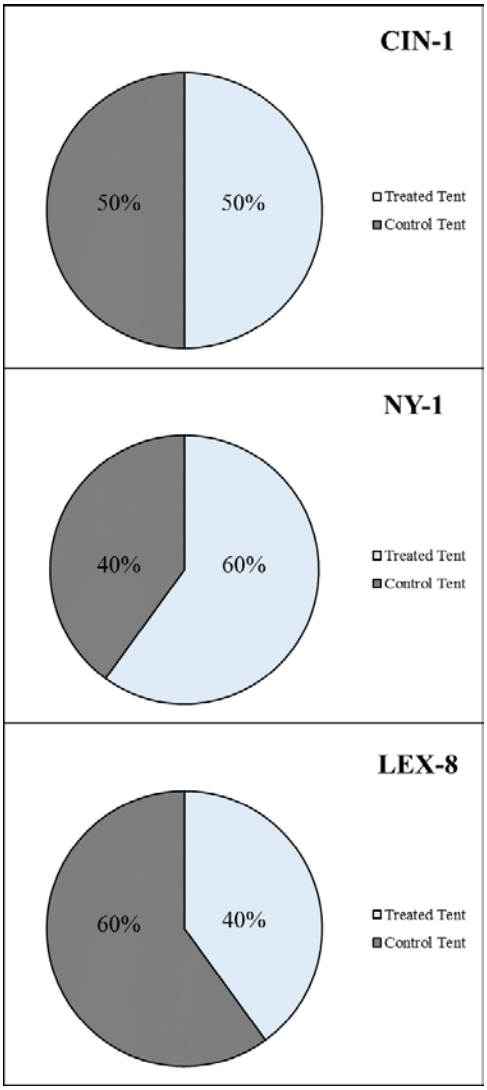
Weight gain in milligrams after initiation of feeding with and without exposure to the combination product. Exposure to the insecticide significantly decreased the amount of blood imbibed during a blood meal in NY-1 (23%) and LEX-8 (26%), but not CIN-1 (17%). Significant differences between treated and untreated groups are denoted by asterisks (*$p<0.05$).

Figure 4.4.



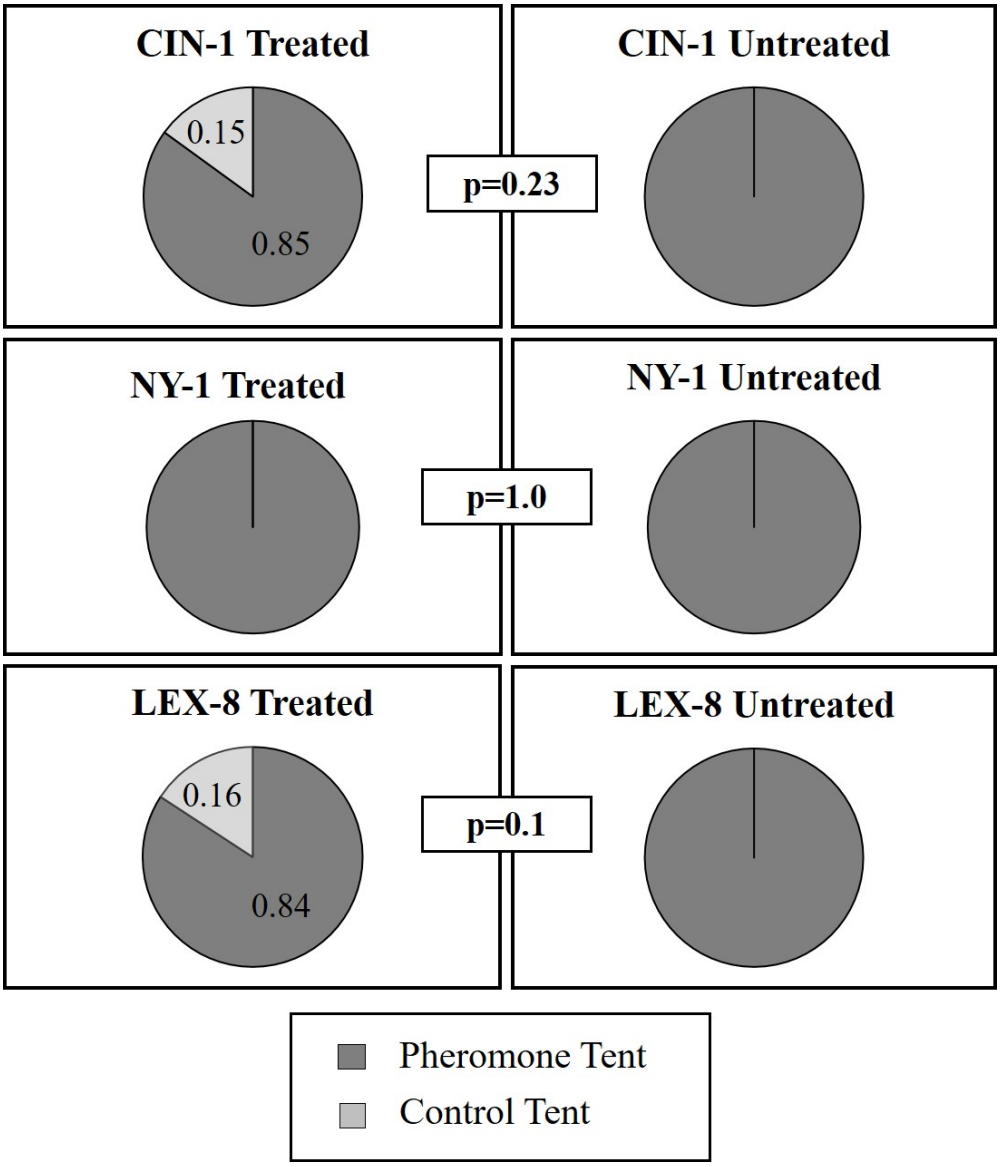
The proportion of time spent moving during both the day and night with and without exposure to the combination product. As expected, bed bugs from all three populations were more active at night than during the day ($p<0.01$). Prior exposure to the insecticide significantly reduced movement in all three populations ($p<0.01$). There was no interaction between treatment and the light cycle (scotophase vs. photophase), indicating that the periodicity of movement was not affected by exposure to the insecticide.

Figure 4.5.



A two-choice tent assay was used to evaluate repellency and/or avoidance. Untreated bed bugs had to choose between two filter paper tents, one saturated with water, and one saturated with the combination product. There was no evidence of repellency; bed bugs chose to rest on either tent with equal frequencies.

Figure 4.6.



Tent choices made by individual bed bugs either with our without exposure to the combination product. Unexpectedly, bed bugs from all three populations did not lose the ability to detect conspecific feces. Regardless of treatment or strain, bed bugs preferred to aggregate inside tents marked with feces from other bed bugs. There were no significant differences in choice for any strain.

Chapter 5. Impact of sublethal exposure to a pyrethroid-neonicotinoid insecticide on mating, fecundity and development in the bed bug, *Cimex lectularius,* L.

Introduction

Deleterious sublethal exposure to an insecticide may affect many life history parameters for insects, including: reductions in feeding or searching time, diminished life span, alterations in development time, and diminished mating and/or fecundity (Stark and Banks 2003, Desneux et al 2007, Shi et al. 2011 Ahmad et al. 2013, Alinejad et al. 2014, Miao et al. 2014). Declines in fecundity may occur through a variety of mechanisms, including alterations of spermatogenesis, sperm motility, oogenesis, ovulation, or egg fertilization after exposure (Haynes 1988). Changes in life span, development, mating, fecundity, and feeding after sublethal exposure may have negative population-level consequences (Maltby 1999). Even so, evaluation of insecticide efficacy is biased toward the use of statistics associated with mortality values alone. These mortality values are traditionally obtained from standardized laboratory assays (Stark and Banks 2003). Applying the information gleaned from these types of studies at the population-level can be problematic when there are large differences in the insecticide concentrations or exposure times leading to lethal versus sublethal effects. Thus, there is often a substantial gap in our knowledge of the sublethal effects of insecticide exposure for many arthropod pest populations. Agricultural systems are an exception, where there has been interest in the sublethal effects of insecticides on beneficial species such as the honey bee, *Apis mellifera*, and other non-target arthropods (Cresswell 2006, Desneux et al. 2007, Thompson 2010). However, there are far fewer studies on sublethal effects for urban/household pests, where consequences for non-targets are typically not directly

relevant. Bed bugs are an urban pest that are becoming increasingly difficult to manage due to widespread insecticide resistance (Romero et al. 2007, Zhu et al. 2013, Gordon et al. 2014a, Romero et al. 2016). Since insecticides remain the most common tool for treating infestations (Potter et al. 2015), understanding both lethal and sublethal effects of pesticides on populations is important.

Bed bugs (*Cimex lectularius*) are flightless, hematophagous insects with public health relevance because all life stages feed on human blood (Reinhardt and Siva-Jothy 2007). Their bites often cause itchy, painful wheals and occasionally more severe reactions (Goddard et al 2009). Additionally, infestations of bed bugs can have negative psychological outcomes from anxiety to depression, and even suicidal thoughts (Goddard and deShazo 2012). Because bed bugs are cryptic, and the majority of their activity occurs at night when the host is sleeping (Usinger 1966), they are hard to detect until an infestation is well established (Wang et al. 2009b). Missed bugs or survivors of insecticidal or non-chemical approaches could lead to further population growth and treatment failure. Thus, many pest management professionals prefer insecticides with residual activity to deter reinfestation.

Many recent studies have focused on insecticide resistance to pyrethroids and combination products (Davies et al. 2012, Gordon et al. 2015). However, very few studies have identified sublethal effects that could lead to indirect deleterious effects on bed bug populations. Two studies have explored the sublethal effects of ActiveGuard™ (permethrin-impregnated fabric) on bed bugs; exposure to the fabric alters behavior and negatively impacts their feeding and fecundity (Jones et al. 2013, Jones et al 2015). Significantly fewer females laid eggs after sublethal exposure, and exposure times as

short as one minute were enough to induce a decline in fecundity and feeding attempts (Jones et al. 2015). These results indicate a potential for detrimental population-level consequences. In the previous chapter, I demonstrated a negative impact of Temprid® SC, the most widely used insecticide for bed bug management in the USA, on bed bug feeding and locomotion, but not on aggregation in daytime harborages (Crawley et al. 2016). Here, I expand on those behavioral studies by addressing whether Temprid® SC affects mating efficacy, as well as physiological parameters such as fecundity and development. Temprid® SC contains two active ingredients, imidacloprid (a neonicotinoid), and β-cyfluthrin (a pyrethroid). I did not attempt to separate the individual impacts of these components; instead I examined the overall impact of this combination product as it is labeled for use. I hypothesized that sublethal exposure would affect various aspects of bed bug behavior and physiology, and predicted that treated insects would show decreased mating success, a reduction in fecundity, and prolonged development.

Materials and Methods

Insects

Three strains of bed bugs were used in each experiment. The progenitors of the CIN-1 colony were collected from Cincinnati, OH in 2005. The original colony was highly resistant to pyrethroids, but after generations in culture is now more susceptible (Gordon et al. 2014a). NY-1 was collected from New York City, NY in 2007 and is moderately resistant to pyrethroids (Gordon et al. 2014a). LEX-8 was collected from Lexington, KY in 2012, and is resistant to pyrethroid insecticides (Zhu et al. 2013). Bugs were reared in an incubator (Percival Scientific, Perry, IA) (27°C, 70% RH, 14:10 L:D)

and weekly blood meals were administered with an artificial blood feeding system (Montes et al. 2002). In this system, defibrinated rabbit blood (HemoStat, Dixon, CA and Quad Five, Rygate, MT) was delivered into glass mosquito feeders **(**Kimble Chase Custom Glass Shop, Vineland, NJ**)** and heated to 39° C with a circulating water bath. Parafilm lined the bottom of the mosquito feeder, and served as a barrier between bugs and the blood. Bed bugs contained in 59 mL plastic jars (Consolidated Plastics, Stow, OH) sealed with organza had to pierce the organza and the parafilm membrane to feed. Each adult was seven days past eclosion and had not yet taken a blood meal as adults when exposed to Temprid® SC (unless stated otherwise).

Residual Deposit Mortality Bioassays

The LT_{10} (lethal time of exposure resulting in 10% mortality) was determined independently for each strain using a residual deposit bioassay (Gordon et al. 2014a, Crawley et al. 2016). Methods are identical to those previously described (Chapter 4, Crawley et al. 2016).

Mating

In the first copulation choice test, females from each strain were exposed to their relative LT_{10} of Temprid® SC (as previously described). After 24 hours, insects that had fully recovered were offered a blood meal. I assessed recovery according to methods previously described (Crawley et al. 2016). Control insects were exposed to water and offered a blood meal 24 hours later. I selected insects that appeared to have fully engorged for mating experiments. Females were presented with one male in a petri dish, and were given ten minutes for copulation. For this experiment, I defined successful copulation (or a copulatory event) as prolonged mounting of a female by a male, along

with curving of his abdomen toward the female. The second, reciprocal choice test was performed identically, and examined the same effect for treated versus untreated males (with an untreated female for copulation). Each experiment was replicated 15 times. There were no instances in which copulation did not occur (i.e. no non-responders).

Fecundity with sublethal exposure before mating

This experiment was designed to examine fecundity of mixed mating pairs of bed bugs after sublethal exposure to Temprid® SC, with exposure occurring prior to mating. Males and females were separated from one another shortly after adult eclosion. At seven days post-emergence they were exposed to either water (control) or the combination insecticide (treatment). Bed bugs from each strain were randomly assigned to one of four mating pairs: a control female with a control male, a control female with a treated male, a treated female with a control male, and a treated female with a treated male (complete factorial). Each pair was placed in a 59 mL plastic jar covered with organza, fed with rabbit blood once per week, and the numbers of viable offspring (hatched eggs) were counted after six weeks. This experiment was replicated eight times.

Because there was a short period when unfed teneral adult males and females were housed together before the treatment occurred, I repeated this experiment using insects that were never exposed to the opposite sex as adults to ensure that no mating events had occurred before pairing (although mating in unfed teneral adults are not likely) (Mellanby 1939). Only the control female/control male and treated female/treated male pairs were reevaluated using this approach. To obtain bed bugs with ensured virginity, I placed fifth instars that had recently fed into individual wells of a 96-well plate (Costar, Corning, NY) until eclosion. Seven days after these adults had emerged

they were exposed to treatment or control condition for the LT_{10}. At 24 hours after the initiation of exposure, healthy survivors (assessment described previously) were assigned to a treatment. Each pair was placed in a 59 mL plastic jar covered with organza, fed with rabbit blood once per week, and the numbers of nymphs (and thus the number of eggs that hatched) were counted after six weeks. This experiment was replicated eight times.

Fecundity with sublethal exposure after mating

Prior to the start of this experiment, two-day old virgin adult bed bugs were permitted to take a blood meal. After feeding, engorged bugs were removed, and males and females were placed in 59 mL plastic jars covered with organza for three days to allow mating to occur. On day three, when they were five days post emergence, I exposed only adult females to their strain-specific sublethal dose of Temprid® SC, or to water. After 24 hours, surviving female bed bugs were placed individually into wells of a 24-well plate (Costar, Corning, NY) lined with Whatman® filter paper (Sigma-Aldrich, St. Louis, MO). Once per day (at the same time each day) numbers of eggs laid for each female were recorded. In addition, I recorded the number of eclosed first instars each day. Every 24 hours, the female was moved to an unoccupied well of a 24-well plate for accurate counts of egg germination time. Females were not fed again throughout the course of this experiment. Each female was followed until she stopped laying eggs, and until all eggs had sufficient time to hatch. Thus this assay gives information on the number of eggs laid, the time course of egg-laying, the timing of egg hatch and how these variables are affected by sublethal exposure to Temprid® SC. Twelve replicates were performed for each strain.

Development

This experiment was conducted to test whether sublethal exposure to Temprid® SC affected development time from fifth instar to adult. Directly following exposure (at two days post molt), juvenile bed bugs were kept in an incubator for recovery (27°C, 70% RH, 14:10 L:D). One day after exposure, three days old control and treatment fifth instar bed bugs were permitted to take a blood meal from the artificial feeding system. Juveniles that took a blood meal were placed in individual wells of a 96-well plate (Costar, Corning, NY) and monitored until molting occurred. Because we only used juveniles that had taken a full blood meal, any impact of the insecticide on feeding was excluded. The day of molting was recorded. Fifteen replicates of this experiment were conducted per strain.

Data Analysis

Probit analysis was used for the calculation of strain-specific LT_{10} values using Minitab® 15. All other statistical analyses were conducted using Statistix 10.0. Binomial Tests were used to evaluate differences in mating success in the behavioral mating assay. A one-way analysis of variance (ANOVA) was used to compare the number of hatched eggs from the fecundity assay where multiple mating pairs were tested. All count data were square-root transformed prior to analysis. Paired t-tests were used to analyze data from the fecundity assay when only two mating pairs were compared. A two sample t-test was used to look for differences in development time between insects exposed to the combination product versus those exposed to water. Because strain-specific exposure times to insecticide were used, all analyses were

conducted only within strain. When parametric analyses were used, normality was confirmed using a Shapiro-Wilk test.

Results and Discussion

As expected, the LT_{10} values for the combination product differed depending on the initial level of pyrethroid resistance for each strain. The LT_{10} values were 0.95 ± 0.65, 1.0 ± 0.48, and 5.0 ± 1.98 h (mean ± s.e.m.) for CIN-1, NY-1, and LEX-8 adults, respectively. All subsequent experiments using adult bed bugs were performed using these LT_{10}'s. These values have been previously reported (Crawley et al. 2016). Because strains may revert back to susceptibility as they are maintained in the laboratory, I also re-evaluated the LT_{10} values during the course of experiments. The LT_{10} values obtained from a secondary evaluation were 0.45 ± 0.14, 0.90 ± 0.33, and 4.35 ± 1.30 h (mean ± s.e.m.) for CIN-1, NY-1, and LEX-8 adults, respectively. The very close association between the data in the two separate mortality bioassays supported my decision to use the first (and previously tested) set of exposure times in all current experiments.

LT_{10} values were determined independently for fifth instar bed bugs of each strain. The LT_{10} values were 0.98 ± 0.22, 0.97 ± 0.30, and 3.33 ± 1.02 h (mean ± s.e.m.) for CIN-1, NY-1, and LEX-8, respectively. These data, for both adults and juveniles, are summarized in Table 5.1.

I was interested in whether sublethal exposure impacts mating behavior in such a way that the fecundity of adults may be reduced. I performed two experiments to test the relative mating success of treated versus untreated bed bugs. The first copulation choice test was conducted to determine if females exposed to Temprid® SC at the LT_{10} had an altered or reduced mating frequency compared to untreated females (with an untreated

male). The second, reciprocal choice test examined the same effect for treated versus untreated males (with an untreated female). For this experiment, I defined successful copulation (or a copulatory event) as prolonged mounting of a female by the male, with curving of the male's abdomen. These behaviors are known to be associated with mating in this insect (Reinhardt and Siva-Jothy 2007). Using this definition, untreated males copulated with both treated and untreated females at equal frequencies in all three strains (Fig. 5.1. Binomial Tests: CIN-1: p=0.11, NY-1: p=0.38, LEX-8: p=0.70). However, when males from the moderately resistant and resistant strains were exposed to Temprid® SC, they performed significantly fewer copulatory events on average (Binomial Tests: CIN-1: p=0.15, NY-1: p=0.05, LEX-8: p=0.01). Sublethal exposure could impact the number of offspring a male produces if behavioral alterations lead to a reduction in mating attempts compared to his untreated counterparts. This is common; sublethal effects often reduce mating success in insect populations (Linn and Roelofs 1984, Clark and Haynes 1992). However, because male bed bugs make multiple copulatory attempts over their lifetime (Reinhardt and Siva-Jothy 2007), the impact of short-term reduction in these attempts may not translate into reductions in paternity.

Insecticides can adversely affect male insects that rely on pheromones for mate-location, impacting their ability to detect sex pheromones and/or reducing behaviors crucial for mate finding—such as flight (Linn and Roelofs 1984, Clark and Haynes 1992). The role of sex pheromones in bed bug reproduction is also ambiguous; no sex pheromone has been identified to date. It is possible, however, that disruption in the ability of male bed bugs to detect alarm pheromone could lead to a reduction in fitness through superfluous homosexual mating attempts, since alarm pheromone has been

shown to prevent these interactions (Ryne 2009). The potential sublethal effects on pheromone production in bed bugs remains to be tested and should be pursued.

Two different experimental designs were used to test whether the number of hatched offspring produced by male and female mating pairs decreased after sublethal exposure. The first experiment relied on bed bugs that were selected shortly after adult emergence, while the second experiment used adults that emerged in isolation from other bed bugs, thus I could ensure that the latter insects were unmated before the experiment started. I did not expect results to change between the two designs, as other reports state that mating between teneral males and females is unlikely (Mellanby 1939).

In the first experiment, after six weeks, in two of the strains there was a significant reduction in the number of hatched eggs after insecticide exposure (Fig. 5.2, CIN-1: $F_{3,28}=13.15$, $p<0.0001$; NY-1: $F_{3,28}=1.66$, $p=0.20$; LEX-8: $F_{3,28}=4.73$, $p<0.01$). In CIN-1, an independent impact of the insecticide on both males and females was apparent, because there was a significant decrease in egg production when both males and females were treated in comparison to when only one sex was treated (Fig. 5.2a. Tukey HSD test). Similarly, in LEX-8, treatment of both males and females resulted in a decrease in the number of hatched eggs versus the control group (Fig. 5.2c). The overall trend was a sharp decline in egg production when both male and female bed bugs were exposed to insecticide, and an intermediate reduction in hatching when just one sex experienced sublethal exposure. Although not statistically significant, I observed this trend for the moderately resistant strain (NY-1) as well.

To confirm that previous results were not due to rare mating events that could have occurred between teneral males and females, I repeated aspects of the experiment

with bugs known to be virgin. Prior mating events before the experiment start could confound results of the study. However, in this experiment there was also a significant reduction in the number of eggs hatched after 6 weeks between mating pairs in all three strains (Fig. 5.3, Paired t-test, CIN-1: $t=3.59$, $df=7$, $p<0.01$; NY-1: $t=2.78$, $df=7$, $p<0.05$; LEX-8: $t=3.89$, $df=7$, $p<0.01$). The number of eggs hatched decreased substantially when both the male and female in the mating pair had sublethal exposure to Temprid® SC prior to mating (i.e., the effect of sublethal exposure can be additive), and because the overall trend in both experiments was a sharp decline in egg hatchability, I believe that sperm storage or previous mating events are not likely to change the results shown here. Mating pairs with sublethal exposure tend to consistently show stark drops in egg production, regardless of strain.

The mechanism for the reduction of viable eggs after sublethal exposure is unknown, but could include physiological or behavioral factors (Haynes 1988). One explanation is that females produce fewer viable eggs after treatment because they might imbibe less blood, which is used to produce eggs. Males may also reduce the amount of sperm they produce after sublethal exposure for the same reason. Alternatively (or in addition), because I did not observe the number of successful mating attempts in this assay, there is the possibility that male bed bugs were copulating less frequently after exposure. It should be noted, however, that in another study researchers observed successful copulation, yet still recorded no egg production after sublethal exposure to permethrin; pointing toward the potential for sublethal effects on bed bug reproductive physiology (Jones et al. 2015). More studies will be necessary to elucidate the

mechanisms that are responsible for the decreased egg viability in bed bugs with sublethal exposure to insecticides.

When I examined fecundity when the exposure to the insecticide occurred after mating, there were significant declines in the total number of eggs laid on average per female in all but one strain (Fig. 5.4, Paired t-test, CIN-1: $t=2.01$, $df=11$, $p<0.05$; NY-1: $t=2.33$, $df=11$, $p<0.05$, LEX-8: $t=0.71$, $df=11$, $p=0.25$). Although females from the LEX-8 strain did not show a decline in egg production after treatment, their median day of egg laying was delayed compared to the control group, in contrast to the other two strains (Paired t-test, CIN-1: $t=0.00$, $df=11$, $p=1.0$; NY-1: $t= -0.23$, $df=9$, $p=0.82$; LEX-8: $t= -2.35$, $df=11$, $p<0.05$). The median day of egg laying for control strains was 5.67 ± 0.99, 5.75 ± 0.28, and 5.17 ± 0.24 days (mean ± s.e.m) for CIN-1, NY-1, and LEX-8, respectively. Treated strains were not significantly different for CIN-1 and NY-1, with median days of egg laying at 5.67 ± 1.84 and 5.8 ± 1.73 days (mean ± s.e.m), respectively. However, LEX-8 was significantly delayed compared to the control group, with a median day of egg laying at 5.92 ± 1.73 days (mean ± s.e.m). In addition, the first day of egg laying was significantly delayed for LEX-8, in opposition the other two strains (Fig. 5.4, Paired T-Test, CIN-1: $t= -1.41$, $df=9$, $p=0.19$; NY-1: $t= -1.62$, $df=9$, $p=0.14$, LEX-8: $t= -3.00$, $df=11$, $p<0.05$). The first day of egg laying for control LEX-8 females was 4.16 ± 0.11 d versus 4.92 ± 0.25 days for the treatment females. The proportion of eggs that hatched was significantly lower in treatment groups for all three strains evaluated (Paired t-test, CIN-1: $t=2.62$, $df=9$, $p<0.05$, NY-1: $t=2.20$, $df=8$, $p<0.05$, LEX-8: $t=2.67$, $df=11$, $p<0.05$). The proportion of eggs hatched dropped from 0.98 ± 0.01 to 0.76 ± 0.08 for CIN-1, 1.0 ± 0.00 to 0.86 ± 0.05 for NY-1, and 0.99 ± 0.01 to 0.91 ± 0.03

for LEX-8 (means ± s.e.m). This observation is consistent with the results obtained in the first two fecundity trials. Females with sublethal exposure may be capable of laying as many eggs on average, but those eggs may not be equally viable in comparison to control insects (as in the LEX-8 strain). Taken together, these data suggest that sublethal exposure will cause significant drops in egg hatchability whether insects are exposed before or after mating. Temporal patterns in egg laying may also shift, depending on the population of bed bugs.

The delay in oviposition demonstrated by the most resistant strain (LEX-8) should be investigated further for other strains. Oviposition delays could impact the timing of follow-up treatments in the field where a secondary treatment can be applied to combat the hatching of newly laid eggs. Because LEX-8 was the most resistant strain, and thus received the longest duration of exposure to Temprid® SC, it would also be worthwhile to explore whether other strains of bed bugs also delay oviposition when lengths of exposure to insecticide are extended. It is not uncommon for sublethal exposure to alter, and often delay, the pre and post-oviposition period in other insects (Pan et al. 2014).

Contrary to my hypothesis, there was no delay in development within the three strains we tested (Fig. 5.5, Two sample t-test, CIN-1: $t=0.20$, $df= 22$, $p=0.84$; NY-1: $t=1.19$, $df=26$, $p=0.24$; LEX-8: $t=1.96$, $df=26$, $p=0.06$). In fact, on average, development rate was slightly increased for NY-1 and LEX-8 insects exposed to Temprid® SC, however this effect was not statistically significant (Fig. 5.5). There was some treatment mortality, but the difference was not significant when compared to control (Fisher's Exact Test, $p>0.05$). In some insects, sublethal exposure during a later instar can prolong development time until adulthood (Biddinger and Hull 1999), however, development rate

was not significantly affected for bed bugs. It is possible that by selecting insects that successfully fed, I had already chosen a subset of insects that recovered more readily from exposure than other fifth instars. Additionally, observations more frequent than every 24 hours may have revealed more subtle differences in development time than I detected using this methodology. However, these differences would not likely be relevant for management purposes. Future studies should address the recovery of juvenile bed bugs with sublethal exposure to insecticides, and whether they are as likely to take blood meals as control insects. This would impact the number of emerging adults in an infestation.

Conclusions

Sublethal effects cause behavioral and physiological changes in an insect after exposure to an insecticide that do not lead to immediate mortality (Desneux et al. 2007). These changes may affect fecundity, development, foraging, and other life history parameters and thus, have population-level effects. I designed this study to more thoroughly evaluate one popular combination product used against bed bugs, Temprid® SC, to determine whether sublethal effects had the potential to influence bed bug populations or their management. I hypothesized that sublethal exposure could have population-level consequences by decreasing mating success, decreasing egg output/fecundity, and lengthening maturation time of individual bed bugs, and two of these hypotheses were supported by this study. Effects on development of juveniles should be characterized further, but I saw no changes in development rate or success for fifth instars in this study. The overall detrimental effect of sublethal exposure to Temprid® SC on bed bug fecundity contributes positively to bed bug management.

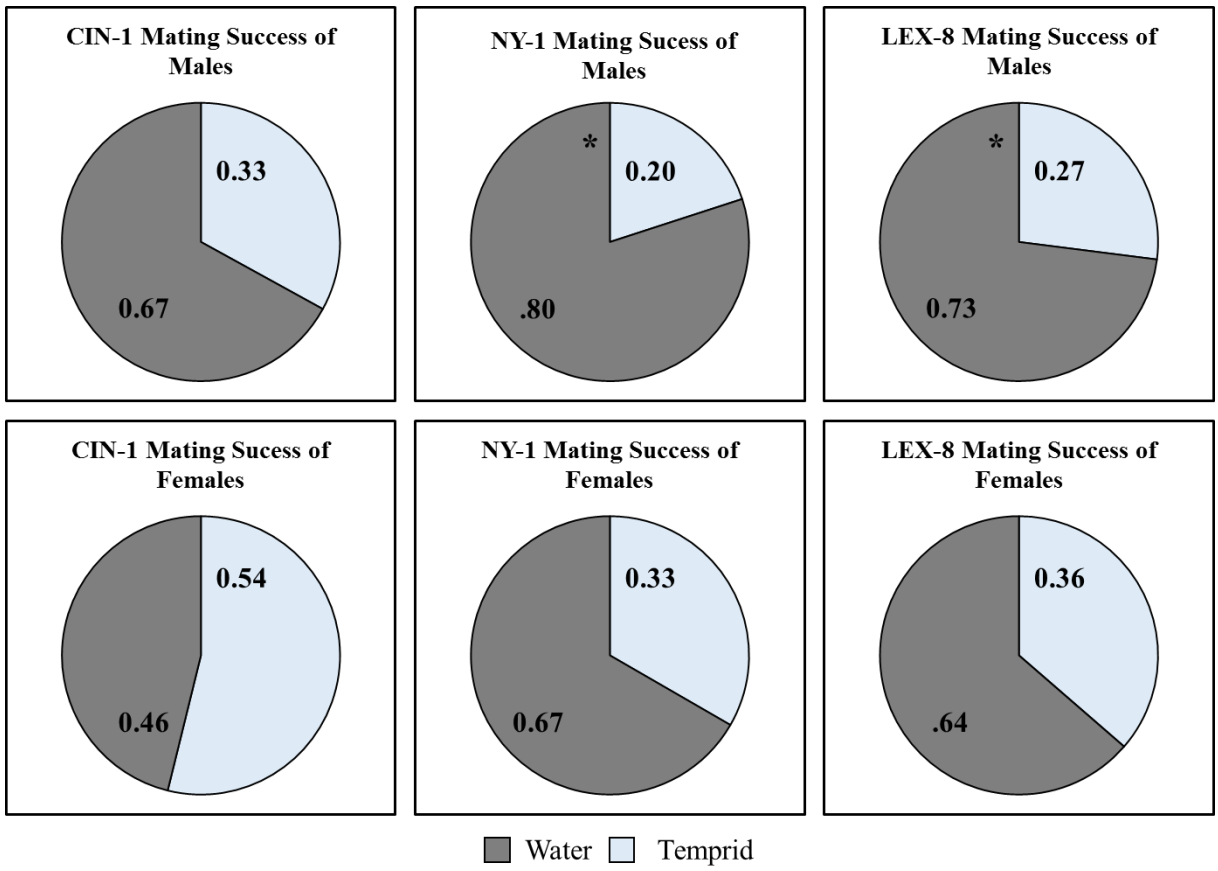
However, the potential for recovery from sublethal effects needs to be explored in depth before decisions are made based on these results. Insecticide efficacy testing that includes the evaluation of sublethal effects can complement traditional mortality based assays. In addition, these assays may help to explain the occasional incongruous results between laboratory results and field efficacy.

Table 5.1. Probit analysis of residual exposure of three populations of bed bugs to a combination product containing β-cyfluthrin and imidacloprid. Lethal times (h) and 95% fiducial confidence intervals are shown.

Strain[a]	Status[b,c]	LT_{10} (95% C.I.)	LT_{50} (95% C.I.)	Slope (± SEM)
CIN-1	**Adult 2014**	0.95 (0.46-1.57)	8.43 (5.64–13.07)	0.59 ± 0.07
CIN-1	**Adult 2015**	0.45 (0.21-0.77)	5.37 (3.68-8.03)	0.52 ± 0.06
CIN-1	**Nymph 2015**	0.98 (0.58-1.44)	4.41 (3.29-5.88)	0.85 ± 0.09
NY-1	**Adult 2014**	1.14 (0.43–2.11)	29.42 (16.48–70.91)	0.39 ± 0.06
NY-1	**Adult 2015**	0.90 (0.36-1.64)	19.13 (11.57-38.29)	0.42 ± 0.06
NY-1	**Nymph 2015**	0.97 (0.46-1.63)	10.10 (7.47-17.21)	0.53 ± 0.07
LEX-8	**Adult 2014**	5.00 (1.90–8.93)	64.15 (34.26–200.29)	0.50 ± 0.10
LEX-8	**Adult 2015**	4.35 (1.95-7.09)	45.39 (27.31-106.79)	0.55 ± 0.10
LEX-8	**Nymph 2015**	3.33 (1.48-5.50)	37.52 (23.05-80.87)	0.53 ± 0.09

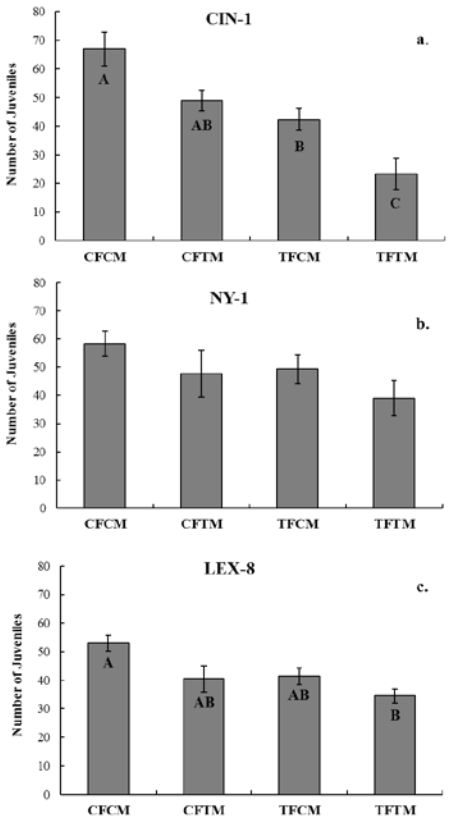
[a] 30 bed bugs were tested per strain using previous methods (Gordon et al. 2014a).
[b] Two assays were performed to ensure consistency in resistance status throughout time course of experiments. 2014 values are also described in Chapter 4 (Crawley et al. 2016)
[c] 5th instars represent 'nymphs' and were evaluated identically to adult bed bugs

Figure 5.1.



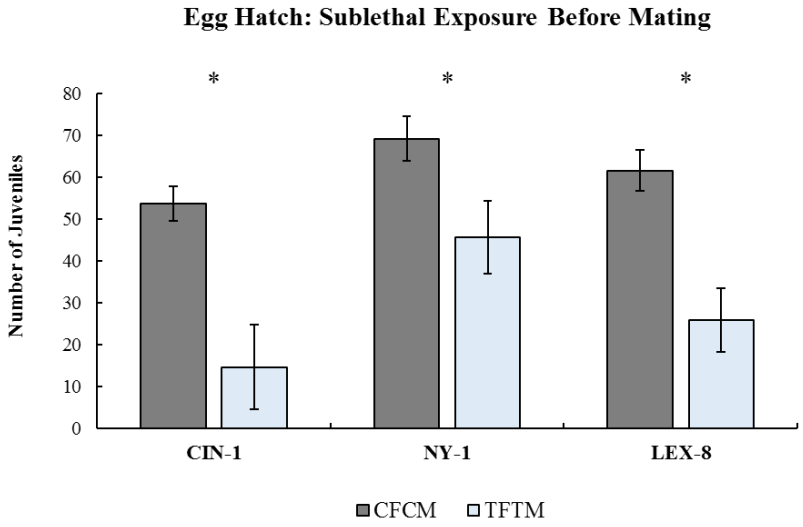
Mating success of treated and untreated males or females when an untreated member of the other sex is present. Males mated equally with treated and untreated females. However, when males were exposed to Temprid® SC, there were significant reductions in successful mating events in the NY-1 and LEX-8 strains (Binomial Tests, n=15, significant effects of treatment denoted with asterisks, *$p<0.05$). There were no non-responders.

Figure 5.2.



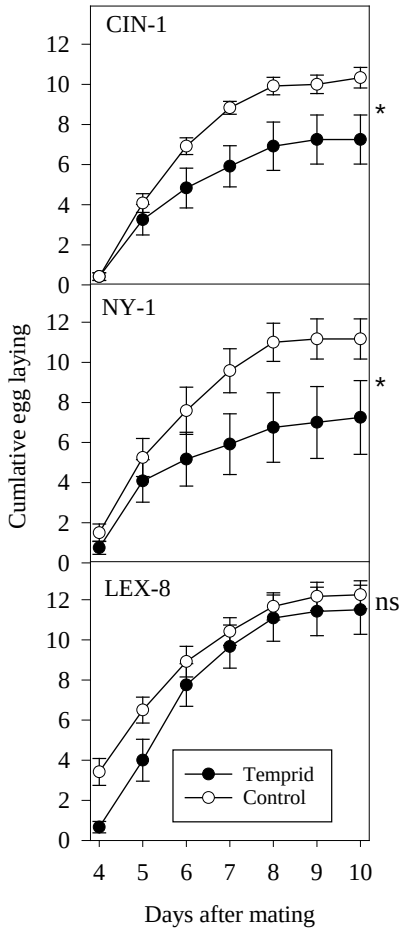
Characterization of successful egg hatch when sublethal exposure occurred before mate pairing. "CF" refers to control female, "CM" refers to control male, "TF" refers to treated female, and "TM" refers to treated male. The number of hatched eggs (obtained by counting the number of juvenile bed bugs present) is presented. Insecticide treatment had a significant impact on the number of hatched eggs over the six week evaluation in two of three strains (ANOVA followed by Tukey HSD). When treatment groups share letters in common they are not significantly different (Tukey HSD test with α=0.05). There was a steep decline in viable egg production when both male and female bed bugs in the mating pair had sublethal exposure to insecticide, and an intermediate reduction in viable eggs when either the male or the female (but not both) experienced sublethal exposure (CIN-1 and LEX-8).

Figure 5.3.



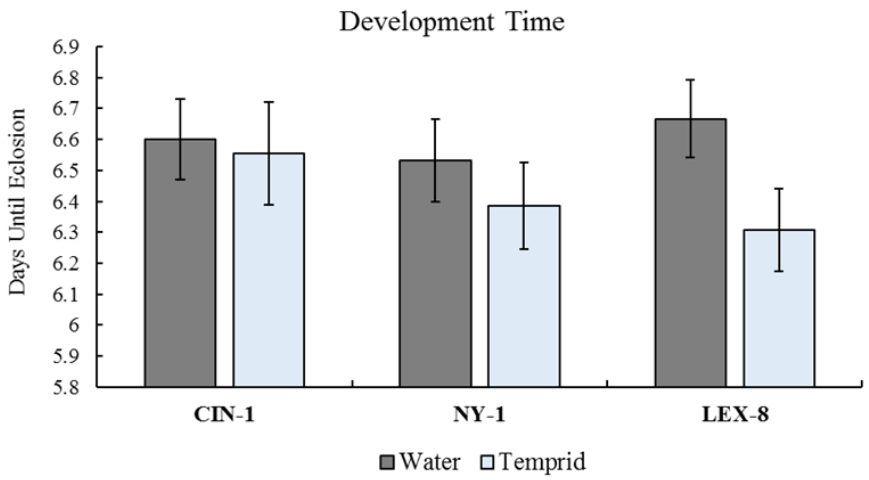
Characterization of the number of eggs hatched when sublethal exposure occurred before mate pairing. This experiment partially replicates the first fecundity test, but starts with males and females that had no previous contact with the other sex. There was significant reduction in the number of hatched eggs for every strain. Hatching dropped by 72%, 34%, and 58% for CIN-1, NY-1, and LEX-8, respectively, when both the male and female in the mating pair had sublethal exposure to Temprid® SC prior to mating. Asterisks denote statistical significance (*$p<0.05$).

Figure 5.4.



Cumulative egg laying for three strains when sublethal exposure occurred after mating. The total number of eggs laid significantly dropped for the susceptible and moderately resistant strains (CIN-1 and NY-1), but not for the most resistant strain (LEX-8) (Paired t-tests, *$p<0.05$). The median and first day of egg laying were significantly delayed in the LEX-8 strain, but not the CIN-1 or NY-1 strain. The median day of egg laying for control LEX-8 females was 5.17 ± 0.24 days, however, the treated females averaged 5.8 ± 1.73 d (mean ± s.e.m). The first day of egg laying was also delayed for LEX-8, with control females averaging 4.16 ± 0.11 d versus 4.92 ± 0.25 d (mean ± s.e.m.) for the treatment females.

Figure 5.5.



The time it took (in days) for fifth instars to molt to the adult life stage after sublethal exposure to Temprid® SC. Fifth instars exposed to water served as a control. There were no significant differences within strain, development time was not advanced or prolonged after sublethal exposure. There was mortality in the treatment groups (1 insect for CIN-1, and 2 for NY-1 and LEX-8), however the differences in mortality between the control and treatment groups were not significant. (n=15).

Chapter 6: Conclusions and future directions

Evolution of insecticide resistance (especially to pyrethroids) in conjunction with many other factors led to the resurgence of populations of bed bugs over the past two decades (Doggett et al. 2004, Romero et al. 2007). As a result, there has been much interest placed in the development of more efficient, effective management strategies, including those that depend on understanding bed bug behavior. The manipulation of bed bug behavior, especially via the use of pheromone traps and lures has been of particular interest (Weeks et al. 2011, Gries et al. 2015). However, it is unlikely that semiochemically-baited traps will work as a standalone for control and/or monitoring, as it is difficult for traps to compete with the cues of a live host and other live bed bugs (Crawley et al. 2015). Thus, a more thorough understanding of bed bug behavior and biology, more efficacious use of insecticides, along with the development of novel, cost-effective monitors and traps can be used in tandem for long-term control of bed bug populations.

One purpose of the research summarized in this dissertation was to fill a distinct knowledge gap in one area of bed bug biology and behavior: parent-offspring interactions. Because adult and juvenile bed bugs aggregate together, there is potential for parents to influence offspring development. Manipulation or interruption of these processes could serve as one alternative management strategy. Additionally, I wanted to improve our knowledge of the effects of a popular combination insecticide, Temprid® SC, on bed bug behavior and biology by studying the sublethal effects of this product at the label rate on three populations of bed bugs. The development of resistance to the

combination products is a growing concern, therefore, judicious use of our effective products is of paramount importance.

In my first study, I sought to characterize the effects of adult bed bugs on first instar feeding frequencies. The acquisition of consistent blood meals is crucial for bed bug development and propagation. In fact, obtaining consistent blood meals is considered central to population establishment and expansion (Reinhardt and Siva-Jothy 2007). In order to molt from one instar to the next, develop a clutch of eggs, or produce sperm, bed bugs must obtain blood. Since finding a host and feeding successfully are vital to bed bug development and reproduction, I wanted to explore whether adult bed bugs were enhancing offspring success by influencing feeding efficacy. Facilitation of offspring feeding is very common among insects, especially i) within the order Hemiptera, and ii) for insects that live in aggregations (Wertheim et al. 2005, Lin 2006, Wong et al. 2013). I found that adult bed bugs, specifically adult females, increase the number of first instars that find an artificial host and feed. Conversely, males and fifth instars did not have an effect on first instar feeding frequency. Although females had an impact on first instars, the effect was later lost. In fact, proportions of juveniles that fed actually decreased with female presence as instar increased.

The results from these experiments suggest that the trade-offs associated with a female remaining within an aggregation versus choosing to disperse are more complex than previously proposed. It has been hypothesized that female bed bugs are the most likely to leave an aggregation due to the costs associated with traumatic insemination (Pfiester et al. 2009). To support this, researchers have cited that mated females are less likely to respond to aggregation pheromone than virgin females (Siljander et al. 2007).

While this may be the case, my results suggest that remaining in the aggregation has benefits for newly hatched juveniles that were previously unaccounted for, and could influence a female's decision to leave. Groups of females emigrating from the aggregation too soon may risk the foraging success of their offspring. Although I have shown that first instar bed bugs feed more frequently in the presence of females, future experiments could be designed to observe the natural movements of female bed bugs among aggregations. It would be interesting to document whether females choose to i) disperse at all, ii) disperse after egg laying but before the offspring have fed successfully, or, iii) disperse after first instars have taken their first blood meal. Comparing mating scars (Ryne 2009, Benoit et al. 2012), longevity, and reproductive rates among females that made each choice could serve as a proxy for defining costs and benefits. It is also possible to artificially recreate this experiment in a laboratory setting by forcing females to make one of the three choices in an arena. Since I saw no specific relationship between mother and her own offspring (i.e., any female could enhance first instar feeding) it would also be interesting to test whether all females make similar choices regarding dispersal. Specifically, if there were a cost for staying due to excessive mating, some females could choose to leave and raise new offspring elsewhere while relying on other females to ensure that her first instars find the host.

Although future studies should address the interactions between adult bed bugs and later instars, I remained focused on untangling the interaction between females and first instar bed bugs. I found that there is an opportunity for females to signal host presence to first instar bed bugs since they reach an artificial host significantly faster. Because first instars were not typically stimulated to leave the harborage until females

began feeding (Crawley, personal observation), the impact of females is consistent with signaling. Substrates walked across by females during feeding, even in the absence of females, stimulated first instar feeding behaviors—supporting the previous observation. I hypothesized that fecal spots produced during and after feeding, both in quantity as well as their location, were responsible for stimulating first instar movement toward the feeder. Females excrete more than males during feeding; this is not surprising given the larger amount of blood imbibed by females versus males (Stutt and Siva-Jothy 2001). Extracts of these fecal spots applied to a filter paper bridge extending to the blood source elicited higher feeding responses from first instars than methanol alone, or methanol extracts of male ramps. Histamine, a component of bed bug aggregation pheromone, was present in the methanol extracts from ramps walked across by female bed bugs. Other insects also show increased foraging rates in the presence of aggregation pheromone and host cues as well (Wertheim et al. 2005). Histamine applied at biologically relevant levels to ramps stimulated first instar feeding equally as well as female fecal spots, or the presence of female bed bugs in the system.

Future studies should attempt to identify whether additional compounds are involved, as well as the quantity of compounds in the feces produced by both females and males. Peaks in the chromatograms obtained from female fecal spots indicate the presence of additional compounds. Elucidation of the compounds present in fecal spots could serve as the components of lures used to attract first instars, which are easily missed in visual inspections. Additionally, future work should explore i) the composition of male fecal spots, as well as ii) whether standardizing the amount of feces produced by males and females (since females produce more fecal spots on average) has the same

effect on first instar feeding success as female fecal spots alone. This would lend support to the hypothesis of a positive "group effect" rather than maternal care. For example, a group effect is responsible for the decreased development time of first instars within aggregations (Saenz et al. 2014).

The potential to manipulate the relationship between females and first instars via the creation of a "trail"-like pheromone would likely be limited by the low volatility of histamine, which is one of the active components of fecal spots. However, there is potential to increase the active space of a potential lure by placing multiple trails containing histamine in a circular fashion around a central trap (i.e., an "all roads lead to Rome" effect). This could be tested in the laboratory by manipulating the distance of first instars from a harborage to the trap, as well as the number of trails placed around the circumference of the central trap, and comparing trap catches between treatments. Alternatively, there is also the potential to disrupt, rather than encourage, bed bug "trail following" behavior. This method has been tested in Argentine ants (Suckling et al. 2011). By manipulating the concentration of trail pheromone, these authors were able to disrupt and discourage trail following behaviors. If there is an optimal amount of histamine required for activating first instar movement, placing more of this in the environment (sensory overload) may disrupt the ability of juveniles to find a host. This would require future studies to elucidate the optimal concentration of the pheromones in fecal spots identified here, as well as whether movement toward a host could be reduced by manipulating this concentration. If an effect were seen, this would limit the success of immigrating females to new populations. Additionally, there may also be an indirect benefit, in that high concentrations of histamine in the open environment could lead to

bed bugs aggregating in exposed places, rather than cracks and crevices. This would improve treatment efficacy and allow for the potential of mechanical control.

In my second study, I wanted to characterize the behavioral patterns associated with oviposition. This is an area of bed bug behavior that has been overlooked, but could be important for pest management professionals (PMPs) trying to gain control of bed bugs in the field. Although the physiology of oogenesis and egg laying in the bed bug has been described (Usinger 1966, Stutt and Siva-Jothy 2001) little attention has been paid to oviposition patterns in this insect. Using time-lapsed photography, I found that regardless of strain, female bed bugs exhibit tendencies toward periodicity in oviposition, similarly to *Rhodnius prolixus,* another hematophagous insect (Ampleford and Davey 1988). The bulk of eggs laid by females are produced one to four hours into the scotophase. Each female bed bug can lay anywhere between 2.8-12.25 eggs per blood meal depending on previous feeding experience. I also discovered a novel behavior associated with oviposition in female bed bugs. Immediately following egg laying, females move the abdomen side to side over top of their egg. It does not appear that this behavior is meant to seal the egg to the substrate, because it is already attached when the behavior begins. Rather, it seems that females are marking eggs with chemical compounds. I speculate that these compounds are antimicrobial in nature, similarly to other insects (Kaltenpoth et al. 2005, Boos et al. 2014, Diehl et al. 2015). It is possible that females are utilizing the alarm pheromone in the new context. The alarm pheromone produced by bed bugs has anti-fungal properties (Ulrich et al. 2015) lending further support to this hypothesis. Further studies should attempt to isolate and identify putative compounds associated with egg laying, as my attempts using solid-phase microextraction

(SPME) were unsuccessful. It is possible that my hypothesis will not be supported by future data. An alternative hypothesis is that females could be adding compounds to eggs that are attractive to self, conspecifics, or both, and serve as a way to mark harborages. If this were the case, this compound could serve as one component of an oviposition site attractant.

In my fourth chapter, I examined the sublethal effects of Temprid® SC on bed bug behavior. Sublethal effects, as well as the effects of insecticides on insect behavior are commonly overlooked during product efficacy evaluation. This oversight could lead to product differences between laboratory and field efficacy. In this study, I found that some bed bug behaviors, such as feeding, and locomotion, were adversely affected by sublethal exposure to Temprid® SC, while other indicators of feeding were variable among colonies (e.g., mass gained after initiation of feeding). Aggregation behavior and periodicity of movement were not at affected by a sublethal exposure to Temprid® SC. The net effect of this exposure has the potential to be detrimental to populations of bed bugs and, thus, helpful for overall management practices. However, there is also the possibility that sublethal effects on movement could cause small "reservoirs" of treated insects that are temporarily unexposed to ongoing contact with insecticide as they recover. This possibility could impact pest management decisions regarding timing of treatments, and the total number of necessary visual inspections. Future studies should address the potential for, and time of recovery from exposure to combination products. Additionally, the individual role of the active ingredients (imidacloprid and β-cyfluthrin) should be tested to gauge the effect of each product on the behavioral differences I observed.

Although I did not see a difference in the response to aggregation pheromone in this

study, the aggregation pheromone tents I tested were created using insects with no exposure to insecticide. An interesting future experiment could be designed to test whether aggregation and alarm pheromone production differs when bed bugs experience sublethal exposure to Temprid® SC. Because alarm pheromone is also used to signal sex and/or life stage (Ryne et al. 2009, Harraca et al. 2009b), changes in blend and/or quantity of pheromone could cause erroneous mating attempts between males and other males, as well as males and fifth instars. In addition, aberrant pheromone production may have consequences for results described in both Chapters 1 and 2. Alterations in pheromone output or time spent signaling (common in other insects) (Haynes and Baker 1985) could impact a female's ability to signal host presence to her offspring, as well as to successfully mark her eggs.

In my final research chapter, I examined the effects of sublethal exposure to Temprid® SC on bed bug mating, fecundity, and development. I hypothesized that sublethal exposure could have population-level consequences by decreasing mating success, decreasing egg output/fecundity, and lengthening maturation time of individual bed bugs. Two of these hypotheses were supported by this study. Although I saw no differences in development time between treatments, future studies could make more frequent observations to look for more subtle delays in development. This may have no bearing on the applied aspects of bed bug management, but could be an interesting physiological sublethal effect. Additionally, future studies should test the proportion of juveniles that take a blood meal after exposure, as this would impact the number of adults with the potential to eclose within a population. I was unable to study this effect, since I selected only fifth instars that fed successfully. The overall detrimental effect of

sublethal exposure to Temprid® SC on bed bug reproduction and fecundity might be beneficial for bed bug management. However, recovery from these sublethal effects should be tested before such a generalization can be broadly accepted. Additionally, intricacies such as the delay in oviposition for one strain should be further explored.

Multifaceted approaches to insecticide efficacy testing (such as incorporation of sublethal exposure assays in conjunction with mortality-based approaches) should be viewed as one way to enhance our ability to apply insecticides more efficiently and effectively. This outlook, as well as the studies described in this dissertation, could set in motion a multidimensional approach that could be applicable to other insecticides, and efficacy testing for other insect species. It is possible that as we understand more about sublethal effects associated with different active ingredients or product formulations, as well as for different pest species, behavioral syndromes will emerge that will be characteristic of the mode of action of the insecticide used. Behavioral syndromes (in the context used here) include the suite of behaviors affected by sublethal exposure to an insecticide. For instance, the behavioral syndrome identified through my research included: a reduction in foraging efficacy, locomotion, and reproductive success for bed bugs, resulting from sublethal exposure to Temprid® SC. However, there was no impact on aggregation behavior, periodicity in movement, or development time from the fifth instar to adult.

The results of my dissertation highlight the importance in understanding the basic biology and behavior of bed bugs, their ecology, as well as untangling complex aspects of the impact of common conventional treatments. The discovery of complex, potentially subsocial behaviors exhibited by female bed bugs demonstrates the importance of

studying behaviors of pest insects, as my research will further our knowledge on the behavioral ecology and sociobiology of group-living insects. From an applied standpoint, behavioral and other alternative management strategies for pest control are sometimes overlooked in favor of insecticidal approaches. This is unfortunate, as understanding and manipulating insect behaviors can increase the efficacy of chemical applications and reduce our reliance on broad-spectrum insecticides (Foster and Harris 1997). In light of rapid evolution of insecticide resistance in the bed bug, now more than ever it is important to understand bed bug biology and behavior, as well as the effects of our most commonly applied chemical products, to design more effective management programs that enhance our ability to control this notoriously resilient pest.

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Wertheim, B., van Baalen, E-J. A., Dicke, M., and Vet, L.E.M. 2005. Pheromone-mediated aggregation in nonsocial arthropods: An evolutionary ecological perspective. *Annu. Rev. Entomol.* **50**: 321-46.

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Zhu, F., Wigginton, J. Romero, A., Moore, A., *et al*. 2010. Widespread distribution of knockdown resistance mutations in the bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), populations in the United States. Archives *Ins. Biochem. Physiol.* **73**: 245-257.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., *et al*. 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. *Sci. Report.* **3**:1456. (DOI:10.1038/srep01456).

VITA

EDUCATIONAL BACKGROUND

BA: 2011. Biology (cum laude). Department of Biology, Transylvania University**,** Lexington, KY. Advisor: Dr. James Wagner. GPA: 3.6.

PROFESSIONAL APPOINTMENTS

Graduate Research and Teaching Assistant (2011-present). Departments of Entomology and Biology, University of Kentucky, Lexington, KY.

NSF Student Researcher (2010). Integrated Biomedical Sciences, University of Kentucky, Lexington, KY. Advisor: Dr. Rebecca Dutch.

Student Researcher (2010). Institute for Applied Ecology, University of Canberra, Canberra, AU. Advisor: Dr. Brian Cooke.

TEACHING EXPERIENCE

Teaching Assistant (May-December 2015). Department of Biology, University of Kentucky.

Guest Lecturer (October 2015). Department of Entomology, University of Kentucky.

Teaching Assistant (May 2011-May 2015). Department of Biology, University of Kentucky.

Guest Lecturer (April 2015). Department of Entomology, University of Kentucky.

PEER REVIEWED RESEARCH PUBLICATIONS

Crawley, S.E., Kowles, K.A., Gordon, J.R., Potter, M.F., and K.F. Haynes. Behavioral effects of sublethal exposure to a combination of β-cyfluthrin and imidacloprid in the bed bug, *Cimex lectularius* L. *Pest Management Science*. DOI: 10.1002/ps.4342.

Submitted

Crawley, S.E., Gordon, J.R., Kowles, K.A., Potter, M.F., and Haynes, K.F. Impact of sublethal exposure to a combination insecticide containing a pyrethroid and a neonicotinoid on mating, fecundity and development in the bed bug, *Cimex lectularius,* L. *Scientific Reports.*

In preparation

Crawley, S.E. and K.F. Haynes. The use of semiochemicals by adult female bed bugs (*Cimex lectularius*) during feeding enhances first instar feeding success.

Crawley, S.E. and K.F. Haynes. Characterization of egg laying periodicity and a novel behavior associated with oviposition in the bed bug, *Cimex lectularius.*

TRADE JOURNAL ARTICLES

Crawley, S.E., Potter, M.F., and K.F. Haynes. (2015). 'Think' like a bed bug. *Pest Control Technology*. Dec. 2015. 7 pgs. http://www.pctonline.com/article/think-like-a-bed-bug.

PEER REVIEWED NON-RESEARCH PUBLICATIONS

Curry, M., **Crawley, S.E.**, Kalsi, M., Saeed, A., and B. Hunt (2014). The single most promising solution to feeding the world's growing population is Entomophagy: rediscovering an ancient tradition to feed the modern world. In Paterson, J.L. & Minteer, C. (eds.) *Amer. Entomol.* **60**:4.

SCHOLASTIC AND PROFESSIONAL AWARDS AND HONORS

Shripat Kamble Urban Entomology Graduate Student Award for Innovative Research (2016). MUVE (Medical, Urban, and Veterinary Entomology) section award presented at the International Congress of Entomology Meeting. MUVE Governing Council, Orlando, FL. **$500.00**

Publication Scholarship (2016). Recipient of a departmental scholarship awarded for the acceptance of a manuscript to a peer-reviewed journal. University of Kentucky, Department of Entomology, Lexington, KY. **$250.00.**

First Place PhD Oral Paper (2016). First place award in the student paper competition held at the National Conference on Urban Entomology annual meeting in. Albuquerque, NM. **$1000.00.**

Hollandsworth Prize (2016). First place in the PhD Student Paper Competition at the American Mosquito Control Association Annual Meeting. Savannah, GA. **$1500.00.**

Symposium Organizer and Moderator (2016). Entomology without borders: The need for collaboration between medical professionals and entomologists for the betterment of global public health. Competitive application process. International Congress of Entomology, Orlando, FL.

First Place Three Minute Thesis (3MT) (2015). First place overall winner at the University of Kentucky three minute thesis competition. Advanced to regional competition in February 2016. University of Kentucky, The Graduate School, Lexington, KY. **$1000.00.**

First Place PhD Oral Paper (2015). PhD Student Paper Competition at the Ohio Valley Entomological Association Annual Meeting, Lexington, KY.

Travel Scholarship (2015). Funding for travel to the annual Entomological Society of America meeting. University of Kentucky, The Graduate School, Lexington, KY. **$400.00.**

Publication Scholarship (2015). Recipient of a departmental scholarship awarded for submitting a manuscript to a peer-reviewed journal. University of Kentucky, Department of Entomology, Lexington, KY. **$250.00.**

Travel Scholarship (2014). Funding for travel to the annual Entomological Society of America meeting. University of Kentucky, The Graduate School, Lexington, KY. **$500.00.**

MUVE Scholarship (2014). Recipient of a scholarship for demonstrated excellence in the field of medical, urban, and veterinary entomology (MUVE). MUVE Governing Council of the ESA, Portland, OR. **$500.00.**

Harrison Garman Club President (2014). Elected President of the Entomology Graduate Student Organization for the 2014-2015 academic year. Department of Entomology, Lexington, KY.

President's Prize (2013). First place in the MUVE (Medical, Urban, and Veterinary Entomology) section of the student competition at the 61st Annual National Meeting of the Entomological Society of America, Austin, TX.

Third Place PhD Oral Paper (2013). PhD Student Paper Competition at the Ohio Valley Entomological Association Annual Meeting, Indianapolis, IN.

Runner-up for the President's Prize (2012). Second place in the MUVE (Medical, Urban, and Veterinary Entomology) section of the student competition at the 60th Annual National Meeting of the Entomological Society of America, Knoxville, TN.

First Place Debate Team (2012). Entomological Society of America National Student Debate Competition. Topic: "What is the best individual solution to feeding the world's growing population?"

Third Place M.S. Oral Paper (2012). M.S. Student Paper Competition at the Ohio Valley Entomological Association Annual Meeting, Cincinnati, OH.

Senior Challenge Award Recipient (2007). Presented to one undergraduate student for demonstration of superior academic achievement and extensive involvement in extra-curricular activities. **$19,438**.

President Scholar (2007). Tuition scholarship presented to first-year undergraduates at Transylvania U. for academic excellence allotted over four years. **$56,000.**

GRANTS, FELLOWSHIPS, RESEARCH SUPPORT

Kentucky Opportunity Fellowship (2013-2014). Merit-based fellowship from the University of Kentucky, **$15,000.**

Kenan/Jones Grant Recipient (2010). Grant awarded for a research project conducted abroad alongside an international mentor. Application process was rigorous. Only 3 undergraduate students from Transylvania University received this award in 2010. **$3,000**.

National Science Foundation Research Experience for Undergraduates (2010). Biochemistry. Grant covered stipend and project materials for three months for study of research developed by the student. Integrated Biomedical Sciences, University of Kentucky, Lexington, KY. Advisor: Dr. Rebecca Dutch.

INVITED PRESENTATIONS

Crawley, S.E. and K.F. Haynes (November 10, 2016). 'Think' like a bed bug. Pest Control Short Course, University of Kentucky, Lexington, KY.

Crawley, S.E. (June 15, 2016). Sublethal effects of an insecticide on bed bug behavior. "Getting the Best of Pests" webinar series, University of Georgia, Athens, GA.

Crawley, S.E. (March 4, 2016). Conversations we can't hear: the chemical lives of bed bugs. Rose Pest Solutions Annual Conference, Troy, MI.

Crawley, S.E. and K.F. Haynes. (November 14, 2014). Conversations we can't hear: the chemical ecology of bed bugs. Entomological Society of America Annual Meeting, Portland, OR.

Crawley, S.E. and K.F. Haynes (2014). Female bed bugs (*Cimex lectularius*, L.) influence feeding behavior of first instars. John's Hopkins Vector Encounter Meeting, Bloomberg School of Public Health, Baltimore, MD.

ORAL AND POSTER RESEARCH PRESENATIONS

Crawley, S.E., and K.F. Haynes (2016). Semiochemically mediated interactions between mother and offspring in the bed bug, *Cimex lectularius*. International Society of Chemical Ecology Annual Meeting, Iguassu Falls, Brazil.

Crawley, S.E., Kowles, K.A. Gordon, J.R., Potter, M.F., and K.F. Haynes (2016). Sublethal effects of a combination product on bed bug (Cimex lectularius L.) behavior and implications for management. National Conference on Urban Entomology Annual Meeting, Albuquerque, NM.

Crawley, S.E., Kowles, K.A., Gordon, J.R., Potter, M.F. and K.F. Haynes (2016). Sublethal effects of Temprid® on bed bug (*Cimex lectularius*) behaviors and implications for control. Oral Presentation. American Mosquito Control Association Annual Meeting, Savannah, GA.

Crawley, S.E., Kowles, K.A., Gordon, J.R., Potter, M.F., and K.F. Haynes (2015). Sublethal exposure to insecticide affects multiple bed bug behaviors. Oral Presentation. Entomological Society of America Annual Meeting, Minneapolis, MN.

Crawley, S.E., Potter, M.F., and K.F. Haynes (2015). Sublethal effects of Temprid® on bed bug (*Cimex lectularius*) behaviors and implications for control. Ohio Valley Entomological Association, Lexington, KY.

Symposium Organizer (2014). Beyond Pesticides: The Conundrum of Bed Bugs. Objectives were to highlight novel bed bug research, discuss bed bug related challenges beyond insecticide resistance, and debate future needs pertaining to scientists and stakeholders. Entomological Society of America Annual Meeting, Portland, OR.

Crawley, S.E. and K.F. Haynes (2014). Semiochemically mediated adult-juvenile interactions enhance first instar feeding success in the bed bug, Cimex lectularius L. Oral Presentation. International Society of Chemical Ecology Annual Meeting, Urbana-Champaign, IL.

Crawley, S.E. (2014). Conversations we can't hear: Chemical ecology of bed bugs. Invited Speaker. Oral Presentation. Entomological Society of America Annual Meeting, Portland, OR.

Crawley, S.E., Dye, K., Gordon, J., Kowles, K., Saeed, A., and C. Stamper. From the Lab and Beyond: Entomology in Action. Poster Presentation in "Grand Challenges: Effective Science Communication" Symposium at the Entomological Society of America Annual Meeting, Portland, OR.

Crawley, S.E., Potter, M.F., and Haynes, K.F. (2013). Bed bug behavior and implications for control. Oral Presentation. University of Kentucky Pest Control Short Course, Lexington, KY.

Crawley, S.E., Potter, M.F., Haynes, K.F. (2013). Family dinner or dine alone? Do bed bug nymphs make it to the "table" on their own? Oral Presentation. Entomological Society of America Annual Meeting, Austin, TX.

Crawley, S.E., Potter, M.F., Haynes, K.F. (2013). Effects of female bed bug behaviors on host-finding ability and survival of juveniles. Oral Presentation. Ohio Valley Entomological Association Annual Meeting, Indianapolis IN.

PROFESSIONAL COMPETITION

University of Kentucky 3-Minute Thesis Competition (2015). 'Think' like a bed bug: Elucidating behavior to enhance control. **First place overall winner, advanced to regionals.**

Entomological Society of America National Student Debate (2012). Entomophagy: rediscovering an ancient tradition to feed the modern world.

SERVICE TO PROFESSION

2016 XXV International Congress of Entomology Student Debate Judge (2016). Served as a judge for the 2016 student debate competition. Topic: What would be the single best policy for improving health of *Apis mellifera* if adopted worldwide? International Congress of Entomology, Orlando, FL.

2016 XXV International Congress of Entomology Student Competition Judge (2016). Served as a judge for the undergraduate oral student paper competition. International Congress of Entomology, Orlando, FL.

2016 XXV International Congress of Entomology Volunteer (2016). Abstract editor for symposia. Reviewed 300 abstracts for clarity and grammatical errors prior to publication. International Congress of Entomology, Orlando, FL.

Entomological Society of America Annual Meeting Volunteer (2015). Helped students upload their presentations at the ESA annual meeting by volunteering in the presentation preview room. Minneapolis, MN.

University of Kentucky Agriculture Roundup (2015). Invited poster presentation geared toward educating alumni, other researchers, and the community about bed bug communication. University of Kentucky, Lexington, KY.

University of Kentucky Pest Control Short Course (2015, 2014, 2013, 2012). Set up a display to educate citizens about bed bugs, past management, and history. Lexington, KY.

Graduate Student Representative (2015). Was chosen to help the faculty members choose two new faculty candidates for the Entomology department at the University of Kentucky. Arranged faculty meetings with graduate students and wrote recommendation letters. Lexington, KY.

University of Kentucky Annual Night Bug Walk (2015, 2014,2013). Taught children and adults to identify insects, set up displays to educate the community about important insect pests. Lexington, KY.

Science Fair Judge (2014). Judged 4th grade student science fair projects at the annual Kentucky American Water Science Fair at Bryan Station High School. Lexington, KY.

Invited Speaker at Community Outreach (2013). Provided information to citizens of Shelbyville County about bed bugs. Shelbyville, KY.