

ABSTRACT

MATOS, YVONNE. Behavioral and Control Studies in Two Urban Pests, Bed Bugs (*Cimex lectularius*) and Asian Cockroach (*Blattella asahinai*). (Under the direction of Dr. Coby Schal).

The common bed bug (*Cimex lectularius*) is hematophagous insect that feeds on humans, and causes adverse effects such as embarrassing bites, secondary infections, and social isolation. Bed bugs were largely eradicated from the developed world in the 1950's with the advent of insecticides such as DDT, but are undergoing a global resurgence. Basic biology and behavior of this pest is poorly understood, and there is a need for alternative control methods due to increasing insecticide resistance.

The Asian cockroach (*Blattella asahinai*) is an invasive species introduced to the US in 1986 and has since been spreading across the Southeast. The Asian cockroach is closely related to the German cockroach (*B. germanica*), a common indoor pest, and the two species can hybridize in the lab. Asian cockroaches populations can reach very high densities, fly into homes when attracted to lights, and cause damage in fruit such as strawberries. Monitoring and control of this species have been poorly studied.

Since basic bed bug biology is poorly understood, we first review the literature on reproduction in hematophagous Hemipterans, with a focus on mate location, acceptance, and sperm handling in triatomines and bed bugs. We found that triatomines find mates through volatile and contact sex pheromones, whereas bed bugs find mates in by contact and visual cues within close aggregations. Triatomine mate choice is driven by females, who are able to resist unwanted copulation, and by males in bed bugs, since females are unable to resist copulation attempts. Triatomines require only one mating to achieve lifetime fertility,

whereas bed bugs must re-mate every thirty days, but factors affecting egg production in bed bugs are poorly understood.

In a second study, we investigated the effects of starvation, mating, sperm storage, and female and male age on egg production and egg hatch in bed bugs. We found that imposing starvation periods on females results in cyclic egg production, but does not affect percentage hatch. We found that sperm degrades quickly when stored in females, and that percentage hatch follows a similar pattern. Sperm also degrades when stored in males, but less quickly than in females, and that frequent matings can replenish fresh sperm. These findings indicate that egg production and egg hatch are governed by interactions among male age, female age, frequency of feeding and mating, and sperm condition.

In a third study, we investigated the potential for secondary kill, where an active ingredient is passed among members of a population, in bed bugs using a liquid bait approach. We found that insecticide-laden bed bug feces do not kill unfed 1st instar bed bugs or 1st instar German cockroaches. Insecticide-laden German cockroach feces killed 1st instar German cockroaches, but did not kill 1st instar bed bugs. We therefore conclude that when a liquid bait is developed for bed bugs, secondary kill with neuroactive insecticides will likely not be a significant factor in population suppression.

In a fourth study, we characterized the Asian cockroach male response to blattellaquinone, the sex pheromone of the German cockroach, in an effort to develop monitoring tools for the Asian cockroach. Electroantennogram (EAG) analysis revealed that males of both species respond electrophysiologically to blattellaquinone. In the field, male Asian cockroaches were more attracted to blattellaquinone than any other life stage, but in low numbers. These results suggest that blattellaquinone might be a component of the sex

pheromone of *B. asahinai* females, and that a blattellaquinone trap may not be most effective at monitoring for this species.

In a fifth study, we evaluated the efficacy of Zyrox Fly Granular Bait and Maxforce Complete Granular Insect Bait against Asian and German cockroaches in the laboratory. We found that both baits are palatable and non-repellent to both species. Zyrox bait also reduced Asian cockroaches in the field, and effects could last for weeks. Zyrox baits could be another tool in an IPM program for Asian cockroaches, if its label could be extended.

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Behavioral and control studies in two urban pests, bed bugs (*Cimex lectularius*) and Asian cockroach (*Blattella asahinai*).

by
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DEDICATION

To my family and friends for their support.

BIOGRAPHY

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CHAPTER 1.**Reproductive strategies across hematophagous Hemipterans**

ABSTRACT

This review covers mating systems and reproductive strategies across hematophagous Hemipterans, with a focus on bed bugs and triatomines of medical importance, and the ways these insects 1) locate mates, 2) their courtship, mate choice, and acceptance, and 3) sperm handling. We found that triatomines find mates through volatile and contact sex pheromones, whereas bed bugs find mates in by contact and visual cues within close aggregations.

Triatomine mate choice is driven by females, who are able to resist unwanted copulation, and by males in bed bugs, since females are unable to resist copulation attempts. Triatomines require only one mating to achieve lifetime fertility, whereas bed bugs must re-mate every thirty days, but factors affecting egg production in bed bugs are poorly understood.

Keywords: bed bugs, *Cimex sp.*, olfaction, sex pheromone, sperm, triatomines, kissing bugs

INTRODUCTION

Hematophagy, or blood feeding, is a life history strategy employed by a number of insect groups and has evolved independently approximately 30 times (Grimaldi and Engel 2005). Hematophagy has evolved in two major groups within the order Hemiptera and two minor groups. The subfamily Triatominae within family Reduviidae is one such group with 140 species (Schofield and Galvao 2009). Triatomines are sylvatic bugs, some of which have evolved a peridomestic relationship with humans and enter the home to feed periodically, and are of great medical importance because they vector *Trypanosoma cruzi*, the etiological agent of Chagas disease (Lent and Wygodzinsky 1979). Triatominae evolved 27-32 million years ago (Hwang and Weirauch 2012), and are primarily distributed in the Neotropics and Southern Cone (Lent and Wygodzinsky 1979). Blood feeding has either evolved once or twice from predaceous ancestors, and the first blood feeders likely cohabited mammal nests (Hwang and Weirauch 2012). All triatomines are obligatorily hematophagous in all life stages, and seek refuge close to their mammal and bird hosts in their nests or shelters, and also in tree hollows, fallen logs, and in palm fronds (Lent and Wygodzinsky 1979). In species associated with humans, they can be found in dark sheltered areas such as wall cracks, furniture, and beds (Lent and Wygodzinsky 1979). Triatomines are nocturnal insects that feed at night and the early hours of dusk and dawn correspond to their peak activity (Lazzari et al. 2013). These insects are poor fliers, and humans aid in their dispersal during travel (Lent and Wygodzinsky 1979).

Hematophagy has also evolved in the family Cimicidae, which contains about 75 species (Usinger 1966). The family Cimicidae evolved 100 million years ago (Jung and Lee 2012), and bats are considered to be the original hosts (Balvin 2008). Cimicids are also obligatorily hematophagous, and a blood meal is required to reach all stages of development and for egg production and oviposition to occur (Usinger 1966). Many species retain ancestral host associations and parasitize bats (Balvin et al. 2012b) and birds (Loye 1985), but three cimicid species have evolved associations with humans when they shared shelters with bats (Usinger 1966). These species are the common bed bug (*Cimex lectularius*), the tropical bed bug (*Cimex hemipterus*), and *Leptocimex boueti* (Usinger 1966). The cosmopolitan *C. lectularius* and pantropical *C. hemipterus* are most common and are both nuisance pests in homes. Although *C. lectularius* is primarily associated with humans, they are still commonly found in bat roosts (Balvin et al. 2012a). Cimicids live in dark cracks and crevices located near their hosts, such as mattresses, furniture, roosts, or nests, and most activity occurs at night, including feeding (Usinger 1966). Cimicids are wingless, and although bed bugs can disperse actively by walking to new habitats (Naylor 2012), they are also passively dispersed by humans in their belongings (Reinhardt and Siva-Jothy 2007). Other species rely solely on passive dispersal when the distance to new host habitats is too great to walk (Balvin et al. 2012b).

Another small family of 30 species, Polytetenidae (Grimaldi and Engel 2005), is closely related to Cimicidae and consists of flightless hematophagous bat bugs that feed upon some microchiropteran bats (Marshall 1982). Members of this family are permanent

ectoparasites on bats and are transferred from host to host via contact only (Marshall 1982). Polycetenids are blind and viviparous (Grimaldi and Engel 2005), and have not been extensively studied. Much about their reproductive ecology is unknown.

A small clade of 50 species, Cleradini, within the Rhyparochrominae subfamily of Lygaeidae has evolved a hematophagous lifestyle as well (Grimaldi and Engel 2005). Many of these species are confined to the Old World tropics where they inhabit the nests of birds and rodents and feed on their blood (Harrington 1988). The pantropical *Clerada apicicornis* is commonly associated with human structures and has been reported to feed on human blood (Harrington 1988).

In this review I will explore the reproductive strategies within cimicids and triatomines, specifically 1) mate location, 2) courtship, mate choice, and acceptance, and 3) sperm handling.

MATE LOCATION

Chemical cues

Chemical cues play a critical part in communication in insects, and reproduction is no exception (Jurenka 2004). The females of many insect species produce sex pheromones that attract males from a distance, and serve as cues for mate location and species recognition (Jurenka 2004). Other insects do not produce volatile sex pheromones that attract mates from a distance.

There has been great debate on whether there exists a sex pheromone in triatomine bugs, and evidence has been presented for and against a sex pheromone (Cruz-López et al. 2001). More recent evidence has been presented in favor of volatile chemically mediated sexual communication in these insects (Lazzari et al. 2013). Zacharias et al. (2010) showed that male *Rhodnius prolixus* initiate flight more frequently when exposed to an airstream of odors from intact virgin females than an airstream of blank or male odors. Females initiated flight similarly to airstreams with male or female odor when compared to a blank airstream. Similarly, in *T. brasiliensis*, males oriented towards adult female odor currents in a t-tube olfactometer, and less so when female metasternal glands were occluded (Vitta et al. 2009). Male *T. infestans* also aggregate around copulating pairs, but females do not (Manrique and Lazzari 1995a), and males respond electrophysiologically when exposed an airstream of mating pairs (de Brito Sanchez et al. 1995), suggesting a volatile chemical substance is at play in this interaction.

There is recent evidence that the metasternal gland is involved in sexual communication in triatomines (Lazzari et al. 2013). When female *R. prolixus* metasternal glands are occluded, males initiate flight far less frequently compared to intact females (Zacharias et al. 2010). Males of *T. brasiliensis* do not orient towards females with occluded metasternal glands, and several components in female metasternal glands elicited a GC-EAD response from male antennae (Vitta et al. 2009). In *Triatoma infestans*, (Manrique et al. 2006) collected 3-pentanone from the headspace of more than 33% of copulating pairs, and 3-pentanone was found in the metasternal glands but not the Brindley's gland. Similarly to *T.*

infestans (Manrique et al. 2006), Pontes et al. (2008a) found that compound profiles of male and female *R. prolixus* metasternal gland secretions did not differ, but females emitted these compounds more frequently than males and the emission occurred most frequently during the early scotophase when sexual activity is highest. Mating frequency decreased when the metasternal glands were occluded in both sexes. However, the authors only tested this in virgin females and not with mated females, who mate multiply in *R. prolixus* (Pontes and Lorenzo 2012). Mating frequency also decreases in *T. infestans* when female metasternal glands are occluded, as well as the tendency of non-copulating males to aggregate around the copulating pair (Crespo and Manrique 2007).

While there is mounting evidence of sex pheromones in triatomine bugs, there is little to no evidence to date of a female volatile sex pheromone in bed bugs. When volatiles released during *C. lectularius* mating were measured in real time, the main components found were (E)-2-hexenal and (E)-2-octenal, which corresponded to both homosexual and heterosexual mating attempts (Kilpinen et al. 2012). These are the major components of the bed bug aggregation pheromone (Siljander et al. 2008) and alarm pheromone (Levinson et al. 1974). Ryne (2009) showed that males release alarm pheromone when mounted by other males to prevent homosexual mating, and occlusion of the male metasternal glands resulted in longer duration of mounting by another male. Puffing male extracts at the time of mounting in a heterosexual mating reduced the total time of mountings (Ryne 2009), however, whole male extracts were taken so it is difficult to rule out the role of cuticular hydrocarbons or other male compounds that were present in addition to alarm pheromone.

Harraca et al. (2010) showed that 5th instar nymphs also release alarm pheromone to prevent unwanted matings by males, but there was no difference in frequency of mounting between females with and without occluded metathoracic glands, suggesting that females do not emit alarm pheromone during mating. However, Kilpinen et al. (2012) suggest that females do release alarm pheromone to deter unwanted copulation since they detected spikes in (E)-2-hexenal and (E)-2-octenal that coincided with termination of heterosexual mating. However, the spikes sometimes corresponded to termination of heterosexual mating and other times did not. The same was true for homosexual mating attempts. In their experiments, Kilpinen et al. (2012) had mixed males and females in containers, which resulted in numerous heterosexual and homosexual mating attempts. Performing assays of single pairs within the chamber would lead to cleaner results on which compounds are released during heterosexual and homosexual matings.

Visual cues

Visual cues are pertinent in mate location of some insects, namely diurnal insects such as butterflies (Bergman and Wiklund 2009), and are likely to be of less importance in nocturnal insects such as bed bugs and triatomines.

Visual cues, however, do play a small role in mate location in bed bugs. Male bed bugs will approach and mount any bug approximately the size of a female, including males and 5th instar nymphs (Siva-Jothy 2006), and even attempt copulation with dead females and inanimate bed bug sized objects (Rivnay 1933). It appears that males use bug size as a rough

cue in sex recognition, but are not able to discern sex until mounting occurs. Indeed male bed bugs will mount recently fed males and females at an equal rate (Siva-Jothy 2006).

Bed bug males prefer to attempt copulation with recently fed females when give a choice between unfed and engorged females (Reinhardt et al. 2009a), and the dramatically increased size of fed females compared to unfed likely provide a visual cue to males. Male preference was not tested when presented with an engorged male and an unfed female. Giving male bed bugs this choice would help discern the role of bug size in mounting preference.

The role of visual cues in mate location of Triatomines has not been extremely well studied. It has been shown that *T. infestans* will respond and track moving objects by turning away while maintaining the object in sight through their lateral vision (Lazzari and Varju 1990), and are averse to light as in most nocturnal insects (Ward and Finlayson 1982, Lazzari et al. 1998, Reisman et al. 1998, Reisenman and Lazzari 2006). It is possible that male *T. infestans* track females visually before mating attempts, since males orient their antennae towards females as they move in an arena (Manrique and Lazzari 1994). It is also possible that chemical cues are solely involved, since *T. infestans* mate in harborages during the scotophase under low visibility conditions. Indeed, occlusion of male eyes has little effect on mating success in (Rojas et al. 1990).

Tactile cues

Another possible chemical signal that could affect mating are contact pheromones or cuticular compounds. For example, cuticular hydrocarbons serve as contact sex pheromones in several species of tsetse fly (Carlson et al. 1984, Carlson et al. 1998, Carlson et al. 2005). There is recent evidence that epicuticular lipids play a role in sex recognition in *T. infestans* (Cocchiararo-Bastias et al. 2011), and it is possible that males have trouble determining the sex of an individual until contact. Behavioral evidence suggests that males of *T. phyllosoma* and *T. pallidipennis* cannot detect differences in sex until the attempt phallic insertion (Rojas and Cruzlopez 1992), and that *T. infestans* males attempt to mate with other males (Manrique and Lazzari 1995a). Since triatomines aggregate in tight spaces in refugia, it may be difficult for males to determine which insects are releasing volatile sexual signals until they achieve physical contact.

It has recently been found that cuticular compounds play a role in sex recognition in *T. infestans*. Although there is no sexual dimorphism in the cuticular hydrocarbon profile, large amounts of the fatty alcohols eicosanol and docosanol are female-specific in *T. infestans* epicuticular lipids, and pipette tips loaded with docosanol and eicosanol + docosanol elicited a mounting response from males (Cocchiararo-Bastias et al. 2011). More work is needed to elucidate whether cuticular compounds play a role in sex recognition in other species of Triatominae, and to determine how triatomines recognize species and sex when locating a mate in a mixed species aggregation. Generally, insects have species specific hydrocarbon profiles (Howard and Blomquist 2005). Triatomines are no exception – there is

variation in cuticular hydrocarbon patterns among species, even those of the same species complex (Juarez and Fernandez 2007), which could serve as a cue for species recognition. There is sexual dimorphism in amounts of cuticular hydrocarbon in some species of triatomines (Juarez and Fernandez 2007), and this could serve as a cue for sex recognition.

The cuticular hydrocarbons of bed bugs have been recently analyzed and no qualitative differences were found between extracts of adult males and females (Feldlaufer and Blomquist 2011). The authors did not test whether male or female extracts elicit a behavioral response from each sex, however. Domingue et al. (2010) found that adult males responded to the exuviae of 5th instar nymphs by arresting on paper containing extracts of the exuviae. However, adult male and female extracts were not taken and assayed to determine the responses of males and females to these extracts. This experiment could reveal if cuticular hydrocarbons of female bed bugs may play a role in sexual recognition when males mount them.

Auditory cues

Many insects use sound in sexual communication (de Mello Vigoder et al. 2013). Sound can be used as either a long range cue to attract mates, as in many crickets, or as a short range cue to elicit mating activities, as in *Drosophila* wing beating (de Mello Vigoder et al. 2013). Among hematophagous insects, sound plays a vital role in the courtship rituals of several groups including sandflies, which exhibit species-specific courtship songs (de Mello Vigoder et al. 2013). Male and female mosquitoes will harmonize wingbeats in

response to each other during courtship as mode of sexual recognition (Gibson and Russell 2006, Gibson et al. 2010). The use of auditory cues in mate location in hematophagous Hemipterans, however, has not yet been largely explored.

Triatomines do not have auditory organs that are able to perceive airborne sound, and behavioral evidence exists that suggest they are unable to hear such sounds (Lazzari et al. 2013). These insects will, however, respond to vibrations produced by conspecifics (Lazzari et al. 2013). Evidence suggests, however, that these vibrational signals are employed by females in the context of mate rejection (Manrique and Lazzari 1994, Roces and Manrique 1996, Pires et al. 2004), not in mate location. It is highly unlikely that Triatomines use auditory cues in mate location since airborne sounds do not seem to play a role in any part of their ecology.

There is no evidence either of auditory organs in Cimicids that perceive airborne sound (Usinger 1966). Additionally, Yturralde and Hofstetter (2012) found that *C. lectularius* females given two-choice tests had no preference for arenas with or without sound from ultrasonic devices. However, more bed bugs were located neutrally in the middle corridor when sound was playing than not, which the authors suggest could stimulate arrestment in bed bugs (Yturralde and Hofstetter 2012). The authors also found, however, that many bed bugs did not leave the middle corridor and venture into the arenas (Yturralde and Hofstetter 2012). Despite these findings, it could also be possible that bed bugs respond to airborne sound but not in the ultrasonic range, and that they respond to vibration.

Host availability

Both bed bugs and Triatomines are obligate blood feeders, and one of the greatest challenges of the ecology of both these groups is locating a food source, especially when hosts may also serve as predators (Lazzari et al. 2013), or may kill the insect as it is feeding. It is possible that host location mechanisms can also serve secondarily as cues for locating a mate as well. Triatomines and bed bugs require a blood meal in order to produce fertile eggs (Usinger 1966, Lent and Wygodzinsky 1979), so locating and feeding on a host is essential for females before they can oviposit, and could affect their willingness to accept a mate. Mating occurs shortly after a bloodmeal in bed bugs (Reinhardt and Siva-Jothy 2007), and males prefer to mate with fed females over unfed females (Reinhardt et al. 2009a), so bed bugs have a greater chance of locating a mate near a host than farther away. Since bed bugs are wingless, that greatly limits their ability to disperse and search widely for hosts, and tend to aggregate near the host as long as suitable harborages are available nearby (Naylor 2012).

Triatomines, in contrast, do not have this constraint and can search more widely for a host since they are able to fly. Accordingly, they have several physical adaptations that allow them to search for hosts at a greater distance. For example, *T. infestans* is extremely sensitive to differences in heat and can detect a mammal the size of a dog from a range of several meters (Lazzari et al. 2013). Additionally, numerous behaviors and one time events in the lives of Triatomines are modulated by circadian clocks, and their sensitivity to CO₂, an important host cue for all hematophagous insects, is no exception (Lazzari et al. 2013). Triatomines are most sensitive to CO₂ during the early hours of the scotophase (Barrozo et al.

2004) when they most often feed, and females are also most likely to secrete volatiles from their metasternal glands during these hours as well, at least in *T. infestans*, and fed females secrete more volatiles than unfed (Pontes et al. 2008b). These findings suggest that females may be likely to mate shortly after a blood meal during the early hours of the scotophase. However, female triatomines mate optimally 12-16 days post feeding (Manrique and Lazzari 1994, Pires et al. 2004). Much more work is needed to elucidate the relationship between host cues, feeding, and willingness of female triatomines to mate.

Ecological factors of mate location

Bed bug infestations can start with one or just a few individuals (Booth et al. 2012), and thus can face challenges in locating a mate within a home or apartment building when population levels are low. Bed bugs are often found in aggregations (Reinhardt and Siva-Jothy 2007), and female *C. lectularius* are more likely to disperse from these aggregations than other life stages of bed bug (Pfiester et al. 2009, Wang et al. 2010). In the tropical bed bug, *C. hemipterus*, blood fed adult females moved more quickly and greater distance than starved females in a Tygon tubing arena, and fed and starved adult males also moved more frequently compared to other stages (How and Lee 2010). These findings suggest that adult females are an important dispersal stage, and also that males must also disperse sometimes in order to find mates. This is confirmed by the fact that 78% of bed bug aggregations in the field are composed of nymphs (Wang et al. 2010), and that mated adult females do not respond to the airborne aggregation pheromone whereas nymphs, adult males, and virgin

females do respond (Siljander et al. 2008). Thus it is possible that aggregation pheromone could act as a long-range cue for males to locate mates (Siljander et al. 2008, Pfiester et al. 2009), particularly virgin females who have not yet been mated. What causes males to leave one aggregation in search of another is yet unknown and could lead to insights into the mate location process of these insects.

Triatomine species often overlap in distribution, and aggregation signals produced by feces are not species specific (Cruzlopez et al. 1993, Lazzari et al. 2013). It has been found that nymphs of *T. infestans* and *P. megistus* will evenly cohabit shelters in the laboratory (Mota and Lorenzo 2012), and these two species sometimes occur together in homes (Dias 1955). *T. infestans* and *P. megistus* nymphs will even aggregate in response to epicuticular lipid footprints of the opposite species (Pires et al. 2002b). However, many of these studies have been carried out with *T. infestans* and *P. megistus* nymphs, and not adults (Pires et al. 2002b, Mota and Lorenzo 2012), and it is possible that the response of adults would be different. It has even been found that crosses of several different Triatomine species will produce viable offspring through several generations, though these species all belonged to a species complex, *T. brasiliensis* (Correia et al. 2013). Other studies have revealed that infertile hybrids can be produced by closely related species (Belisario et al. 2007, Martinez-Ibarra et al. 2009, Martinez-Ibarra et al. 2011). Therefore, it is critical for Triatomines to have strong species recognition capabilities in addition to sex recognition within aggregations.

Mate location future work

Future work could include determining the role of male metasternal glands in copulation of triatomines – although occlusion of the male metasternal glands has been shown to not affect aggregation of males around a copulating pair (Pontes and Lorenzo 2012), these gland secretions could play a possible role in recognition by females or a role in mate acceptance by females. Mating success in *R. prolixus* was greatly reduced in pairs with the male metasternal glands occluded (Pontes et al. 2008b), but more work needs to be done to determine the reason for this reduction, including whether females reject males based on absence of metasternal gland compounds or if occlusion of the glands with wax interfered with the mounting behavior of males. Additionally, it has been found that emission of various *R. prolixus* metasternal gland compounds in virgin adults is modulated by temporal factors such as photoperiod and feeding status, with the most detections emitted by fed females during the scotophase (Pontes et al. 2008b). More work can be done to determine the effect of these temporal factors on pheromone release, and also the effect of mating status of females.

More work is also needed to determine the role of cuticular lipids in species and sex recognition in triatomines. Although quantitative studies have been done on characterization of these compounds which have included sexual dimorphism and species differences (Juarez et al. 2001, Juarez et al. 2002, Juarez and Fernandez 2007, Cocchiararo-Bastias et al. 2011), few studies have been done to elucidate the possible role of these compounds in the behavioral and sexual communication of these insects. Behavioral studies on the mating

sequence of triatomines have revealed that in some species males frequently antennate females before mounting (Rojas et al. 1990, Vitta and Lorenzo 2009), and significantly less mounting occurred when males had their antennae occluded (Rojas et al. 1990). More work is necessary focusing on this specific step of the mating sequence to determine the role of epicuticular compounds in mate recognition.

Additionally, it is unknown how interactions with *Trypanosma cruzi*, the etiological agent of Chagas disease, within triatomine vectors affect many aspects of the insects' behavior (Lazzari et al. 2013), including reproduction. When infected with *T. cruzi*, *Mepraia spinolai* can detect a host quicker, bites more often, and defecates faster than uninfected insects (Botto-Mahan et al. 2006). Since *T. infestans* mate more often when they ingest more blood (Nattero et al. 2011), then female triatomines infected with *T. cruzi* could have a higher reproductive output, but this remains to be investigated. It is also yet to be investigated whether females infected with *T. cruzi* are more receptive to mating attempts by males.

COURTSHIP, MATE CHOICE, AND ACCEPTANCE

The mating behavior of bed bugs has been described many times in the literature (Usinger 1966, Reinhardt and Siva-Jothy 2007). Briefly, male bed bugs will approach and mount any bed bug approximately the size of a female bed bug, especially ones that have been recently blood fed. The male bed bug then uses his hypodermic intermittent organ to pierce the right side of the female bed bug's abdomen in a specialized groove called the

ectospermalege in a process termed traumatic insemination, explained further below. The male bed bug then ejaculates into the female's spermalege, a specialized area in the abdomen to receive sperm, dismounts, and then leaves the female. He then potentially mates with other females immediately afterwards.

The mating behavior of triatomines has also been described extensively in the literature (Lima et al. 1986, Rojas et al. 1990, Rojas and Cruzlopez 1992, Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). Variation exists among species, but generally the mating sequence consists of a few steps. First the male assumes a vigilant position and moves his antennae in the direction of the female. He approaches the female and either mounts or jumps onto her. The male then positions himself dorsolaterally to one side of the female, grasps her with his legs, and exposes his genitalia. The male uses his parameres to grasp the female genitalia, inserts the aedeagus, and remains in this position during copulation. After copulation, the male either stands over the female or walks away, and the pair separate.

Female mate choice, resistance, and rejection

Mating is often costly for females, and males often have a higher optimal mating frequency than females (Arnqvist and Nilsson 2000). Females thus reject unwanted copulation attempts by males and use a number of strategies to do so, including struggles with males and habitat switching (Perry et al. 2009).

In triatomines, four types of female rejection behavior can occur: flattening, abdominal movements, evasion, and in some species, stridulation (Rojas et al. 1990, Rojas and Cruzlopez 1992, Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). In flattening, females flatten their abdomen against a substrate, preventing the male from reaching her genitalia (Manrique and Lazzari 1994). This form of rejection is the most common among species and can range from ~40-50% of instances of rejection (Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). Females reject mating attempts by abdominal movements by moving their abdomens up and down, causing the male to leave (Manrique and Lazzari 1994). Abdominal movements are typically the second most common form of rejection and account for ~20-35% of rejections in species studied (Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). Females can also evade male mating attempts by running away when he tries to mount or jump onto her (Manrique and Lazzari 1994). Evasion typically accounts for 15-25% of rejections (Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). In species where stridulation occurs, females will rub their rostrum against the prosternal groove (Manrique and Lazzari 1994). Stridulation is the least common method of rejection, occurring in typically ~15% of cases (Manrique and Lazzari 1994, Pires et al. 2004).

These male-detering stridulations differed from disturbance stridulations produced in *T. infestans* (Roces and Manrique 1996). Manrique and Schilman (2000) found a similar stridulation rejection phenomenon in *R. prolixus* females, and no copulation occurred in 61 attempts by males when females stridulated, suggesting this is a very successful form of

rejection. Stridulation in a mating context has not been found to occur in *T. brasiliensis* (Vitta and Lorenzo 2009).

It remains to be determined what causes female triatomines to choose a particular method to reject unwanted copulation by males. There could be a sequence of events according to how many attempts are by males, but in triatomines there seems to be no ordered sequence of rejection events (Manrique and Lazzari 1994, Pires et al. 2004, Vitta and Lorenzo 2009). Flattening could be the easiest to perform or the most effective method of rejection since it is most common, but evidence presented above suggests that stridulation is the most effective, at least in *T. infestans* and *R. prolixus*. If stridulation is most effective, perhaps it is utilized least often because it could be used by predators to locate the insects, as can occur in crickets (Sakaluk and Belwood 1984). A likely factor in the rejection behavior of female triatomines is that mating is costly and reduces the lifespan of females (Sulbaran and Chaves 2006). A second possibility is that females are choosing mates based on their genetic quality (Perry et al. 2009). Whether or not this occurs in triatomines, and how females assess male quality, remains to be investigated.

Female bed bugs will resist copulation attempts by males as well. When female *C. lectularius* are unfed, they will press the right side of their bodies, which contains the ectospermalege, to a substrate (Siva-Jothy 2006). Males preferentially pierce the females in their ectospermalege during traumatic insemination (Morrow and Arnqvist 2003), so these findings suggest that this is a method of resistance by unfed females. Furthermore, by pressing their ectospermalege against a substrate, 8% unfed females were able to resist copulation

attempts by males for a full 15 minutes (Reinhardt et al. 2009a). None of the fed females were able to resist copulation attempts, which the authors suggest is due to their increased size and decreased mobility (Reinhardt et al. 2009a). However, 86% of fed females released alarm pheromone during mating compared to 38% of unfed females (Reinhardt et al. 2009a). These findings contradict others that posit that alarm pheromones are rarely released in heterosexual matings (Ryne 2009, Harraca et al. 2010). It is likely that female resistance to male mating attempts is due to mitigating the costs of traumatic insemination, and not to assessment of male quality, especially because females are unable to resist male attempts at all when blood fed.

In contrast to bed bugs, female triatomines are not as receptive to mating immediately after a blood meal. In *Panstrongylus megistus*, females mated more often as time passed since the first adult blood meal, and females rejected male mating attempts less frequently, while the number of male attempts stayed constant over time (Pires et al. 2004). Similarly, in *T. infestans*, it takes 16 days after the blood meal for 100% mating success to occur (Manrique and Lazzari 1994), and 7 days after the first blood meal in *T. brasiliensis* (Vitta and Lorenzo 2009). Female *T. infestans* still displayed resistance behaviors, but were less successful, but also displayed these behaviors less frequently (Manrique and Lazzari 1994). *T. brasiliensis* females, however, displayed no rejection behavior after 11 days post feeding (Vitta and Lorenzo 2009). However, unfed females did not mate as readily as fed females, suggesting that a blood meal is necessary for acceptance in females (Vitta and Lorenzo 2009). Additionally, in *T. infestans*, the quantity of blood ingested and the number of matings

that occur are positively correlated (Nattero et al. 2011). Therefore, feeding is not only necessary for egg production in triatomines, but also for acceptance of mating attempts, as in bed bugs (see above). Interestingly, fed female *T. brasiliensis* also mated less readily with unfed males than fed males (Vitta and Lorenzo 2009), possibly because unfed males do not produce a spermatophore, at least in *R. prolixus* (Pereira-Lourenco et al. 2013). However, the effect of nutritional status on other reproductive parameters in male triatomines is not well understood.

Taste cues and nuptial gifts

Nuptial gifts play a role in the courtship behaviors of many insects, the quality of which can be a factor in the acceptance behavior of females (Vahed 1998). These gifts are any nutritious substances transferred to the female during the mating sequence, and can include prey items captured by the male, male glandular secretions, or substances in the spermatophore or ejaculate (Vahed 1998). No evidence has been presented in the literature on the mating sequences of triatomines and bed bugs (see descriptions above) to suggest that either of these groups present nuptial gifts such as prey or glandular secretions to females before or after copulation. Although more likely to occur in triatomines due to their relatively recent predaceous ancestry (Hwang and Weirauch 2012), there is no evidence to this effect. Since both bed bugs and triatomines are obligatorily hematophagous, it is unlikely they would accept and consume any substance but blood. Furthermore, since both bed bugs and triatomines seek refuge and mate close to the host (Usinger 1966, Lent and Wygodzinsky

1979), and mating occurs more frequently after a blood meal (Davey 2007, Reinhardt et al. 2009a), females likely already have all the necessary nutrients for egg production.

However, it is possible that nutrients could be transferred to females in the spermatophore (triatomines) or ejaculate (bed bugs). Bed bug ejaculate components have been shown to elevate reproductive rates in females and delay reproductive senescence (Reinhardt et al. 2009b) and have bacteriolytic and antioxidant activity (see “Effects on fecundity” for more details). The presence of nutrients that directly benefit female reproductive output has not been detected, however, although they may exist.

Many species of insects digest or eat the spermatophore after transfer (Pereira-Lourenco et al. 2013), and many species of Reduviid eject the spermatophore capsule after sperm transfer (Ambrose and Vennison 1990). Currently not much is known about spermatophore production, transfer, and capsule ejection in triatomines (Pereira-Lourenco et al. 2013), including the effects of many substances produced in the male accessory glands on female reproductive output.

Cryptic choice

Cryptic female choice occurs when females choose which competing male's sperm will take precedence either during or after mating (Albo et al. 2013). There are several mechanisms demonstrated of female cryptic choice, including sperm manipulation by the female, control of oviposition rate, female control of copulation duration and sperm transfer

(Eberhard 1996), and oviposition timing when last male sperm precedence occurs (Barbosa 2009).

There is not explicit evidence for any of the above mentioned cryptic choice mechanisms in bed bugs. It has been suggested, however, that the hemocytes in the female mesospermae function in phagocytosis of sperm as a method of female cryptic choice (Eberhard 1996). However, examination of hemocytes via SEM revealed that these hemocytes did not contain sperm (Siva-Jothy 2006). Instead, these hemocytes have an immune function (Morrow and Arnqvist 2003, Reinhardt et al. 2003) to protect the female against bacteria and fungi present on the male's intromittent organ that are invariably transmitted to her during traumatic insemination (Reinhardt et al. 2005). Transcriptomic analysis of the spermae confirms the presence of immunologically related genes (Moriyama et al. 2012). Furthermore, the first two male bed bugs to mate with a female experience equal paternity of offspring (Siva-Jothy and Stutt 2003), so cryptic female choice is likely not at play in these insects.

Few studies have been done in triatomines on the possibility of female cryptic choice, although mating with multiple males in some species could potentially be a factor leading to its evolution. Eye color in *T. infestans* is controlled by a single locus, and when recessive red eyed females are maintained simultaneously with red eyed and dominant black eyed males, a similar proportion of each phenotype can be observed in the offspring (Pires et al. 2002a). This would suggest that females are not making a cryptic choice on which male's sperm will inseminate her eggs. It also appears that the spermatheca are relatively the same length in

virgin females and recently mated females, which suggests that some of its contents must be displaced when new spermatozoa enter (Davey 1958). Whether or not females decide which spermatozoa are displaced remains to be investigated. Last male sperm precedence in *T. infestans* has also been demonstrated (Pires et al. 2002a), but since mating stimulates egg production and oviposition (Davey 1965), it is unlikely that females delay oviposition in order to use sperm from future matings.

Male-male competition

Male-male competition occurs in many insects, both over access to females and to the resources females require (Miller and Svensson 2014). This competition can be fierce and lead to sexual selection and the evolution of elaborate male weapons (Miller and Svensson 2014).

Elements of male-male competition could exist within triatomines. In some species, males will aggregate around copulating pairs (Baldwin et al. 1971, Manrique and Lazzari 1995b, Pontes and Lorenzo 2012), and will mate successively with females (Vitta and Lorenzo 2009). However, this is not true for all species, such as *P. megistus* (Pires et al. 2004). In species where males aggregate around a mating pair, the copulating male will often stand over the female (Manrique and Lazzari 1994, Vitta and Lorenzo 2009). This topic is further discussed below in “post-copulatory mate guarding.” In species where aggregated males mate successively with a female, it is unknown how males determine who gets to mate first with the female. There is no evidence in the literature of a physical struggle among these

males, simply that they aggregate. Behavioral studies on the competitive behavior of males in these aggregations, if any, are lacking.

There is no evidence to suggest there is male-male competition in bed bugs. Although two males may attempt to mate with a female at once (Kilpinen et al. 2012), no evidence of physical struggle between males has been presented. Since there is a last male sperm precedence in bed bugs of 68% (Stutt and Siva-Jothy 2001), and fed female bed bugs are unable to resist copulation attempts by males (Reinhardt et al. 2009a), and bed bugs live in close proximity in aggregations with both sexes, there is little need for males to engage in competitive behavior over females.

Male mate choice

Male mate choice has evolved in a number of insect groups, and can manifest itself through precopulatory choice, largely based on indicators of female fertility, such as size, or through male cryptic choice where males adjust the allocation of resources to females during mating based on female quality (Bonduriansky 2001). Male mate choice has not been explicitly reported in the literature for either triatominae or bed bugs (Bonduriansky 2001), and the precopulatory behavior for both groups (see above) does not suggest that males exhibit preference for females based on body size, when feeding status is equal for bed bugs. Evidence is presented below that male bed bugs have differential allocation of ejaculates to females, but this decision is not based on female quality, as explained in “Effects of female mating status.”

After feeding, male bed bugs will mate with any engorged females they happen upon (Stutt and Siva-Jothy 2001), and females show little resistance to mating when engorged, so males thus control the mating rate in bed bugs (Reinhardt et al. 2009a). The rate at which males mate, however, is constrained by availability of seminal fluids, since males use 19% of their seminal fluids during mating (Reinhardt et al. 2011). Thus males stop mating when their supplies of seminal fluid are depleted, and are further constrained by replenishment rate since more than ten days are required to fully replenish seminal fluids (Reinhardt et al. 2011). Although male bed bugs control the rate at which mating occurs, they do not appear to be choosy with their mates in terms of female quality or fitness. Instead, they simply prefer to mate with females that are recently fed, either because females are less able to resist at this time or due to immediate reproductive benefits as compared to unfed females (Reinhardt et al. 2009a), which is the prudent choice to make when constrained by the availability of seminal fluids. Indeed, males mate less often with females after more than one hour post feeding (Reinhardt et al. 2011). These findings suggest that female nutritional status is the most important factor that determines a male bed bug's decision to mate with a particular female, not relative female body size, age, or other indicators of female reproductive quality. Indeed, for species where female reproductive output peaks after a small number of matings, such as bed bugs (Stutt and Siva-Jothy 2001), the selective pressures for male choosiness are not as strong (Bonduriansky 2001).

In triatomines, males initiate the mating sequence, and repeatedly attempt copulations with unreceptive females as discussed above. This would suggest that females are the choosy

sex and control the frequency of mating, and that males do not choose to copulate with females based on indicators of female fertility, discussed above. Analysis of sperm precedence (Pires et al. 2002a) do not indicate that males do not alter resource allocation to females during mating based on female quality. Nonetheless, other physiological factors could affect mating in males – for example in *T. brasiliensis*, feeding status does not affect males' willingness to mate (unless starved for a long time), but age did affect willingness (Vitta and Lorenzo 2009).

Effects of female mating status

Females of many insect species are polyandrous, including bed bugs and triatomines which can mate with multiple males in succession. Polyandry has positive effects on female fitness, including elevated fertility and increased reproductive fitness (Arnqvist and Nilsson 2000, Jennions and Petrie 2000). While genetic diversity of offspring can be of benefit to females, it is in the interest of males to ensure paternity of the most offspring as possible, and males have evolved ways to detect the mating status of females and alter their behavior accordingly (Simmons 2001).

Male bed bugs have evolved one such strategy; they have sensillae on their intromittent organs which allow them to sense the mating status of their current mate (Siva-Jothy and Stutt 2003). When females have been recently mated, males reduce both copulation duration and ejaculate size (Siva-Jothy and Stutt 2003). However, each mating is not successively shorter – the first copulation is longest and then subsequent copulations are

about equal in length but shorter than the first (Siva-Jothy and Stutt 2003). This would suggest that there is not an adaptive advantage to successively shorten copulation duration. Although there is last male sperm precedence, it would be detrimental to reduce ejaculate size beyond a certain level when sperm may be outnumbered by the sperm of rival males.

Some species of triatomines mate successively with multiple males, and males adjust their copulation behavior in these instances. In *T. brasiliensis*, 70% of females copulated with three males over a 90 minute period, and males will shorten the duration of copulation when the female has been mated multiply (Vitta and Lorenzo 2009). It has been suggested that *T. infestans* and *R. prolixus* mate multiply as well (Manrique and Lazzari 1995b, Pontes and Lorenzo 2012), but comparative studies on mating duration in a multiple mating system are lacking in these species. There is contradictory evidence of multiple mating in *P. megistus*. Pires et al. (2004) found that although males will attempt second copulations with females, females will only mate once and reject subsequent attempts. In contrast, Lima et al. (1987) found that *P. megistus* females will mate up to seven times in their lifetime. More studies must be conducted to determine the frequency of mating in this species. Studies are also lacking in triatomines to determine how males sense the mating status of females and adjust the length of copulation accordingly. It is possible that they sensilla on their intromittent organs as well that can detect the mating status of their mates.

Post-copulatory mate guarding

Males face the dual challenge of mating with as many females as possible while preventing rival males from copulating with their mate to protect their paternal interests (Alcock 1994). Males of many insect groups have evolved post-copulatory associations with their mates as a form of mate guarding to mitigate this problem (Alcock 1994). Males employ a number of strategies to guard their mates, primarily extending copulation after insemination, mating plugs after insemination, maintaining contact with their mate after copulation, and monitoring their mate post-copulation without touching (Alcock 1994).

It has been found that males of a number of triatomine species maintain a post-copulatory association with females by prolonging copulation or maintaining their position over the female after spermatophore transfer (Rojas et al. 1990, Manrique and Lazzari 1994, Vitta and Lorenzo 2009, Pontes and Lorenzo 2012). These associations can be relatively short (~1.4 minutes) as in *T. mazzottii* (Rojas et al. 1990), or can vary in length as in *T. brasiliensis* who maintains a longer post-copulatory association in the presence of other males (Vitta and Lorenzo 2009). Since post-copulatory associations are shorter when only one male was present (maximum of ~7 minutes), these findings suggest that these post-copulatory associations are a form of mate guarding in polyandric systems (Vitta and Lorenzo 2009).

There is an association between male aggregations around the copulating pair, post-copulatory mate guarding, and polyandry. Males of species that aggregate in this situation tend to exhibit post-copulatory mate guarding, even when other males are not present (Rojas

et al. 1990, Vitta and Lorenzo 2009). Males of species that do not aggregate around a copulating pair do not exhibit post-copulatory mate guarding, and females reject second copulation attempts shortly after their first mating (Pires et al. 2004). Thus, these males have no need to guard their mate to ensure paternity of the offspring. It is also possible that sperm competition could be occurring in polyandric triatomines (Pires et al. 2004), necessitating the mate guarding behavior. Formal studies should be done to determine the strength of relationships among these three factors.

Male bed bugs, in contrast, do not exhibit post-copulatory mate guarding behavior. Upon termination of mating, male bed bugs will leave the female immediately and do not maintain an association with her (Stutt and Siva-Jothy 2001). It may be unnecessary for bed bugs to guard mates because they adjust ejaculate size according to mating status of their mate, as explained above.

Courtship, mate choice, and acceptance future work

More work needs to be done to quantify and identify the chemical substances emitted during heterosexual matings in the bed bug, if any. If females are indeed emitting alarm pheromone, and this occurs more often in fed females than unfed females, the question arises as to why heterosexual matings are successful and males do not dismount females as they would in homosexual matings. One possibility could be that there are differences in timing of alarm pheromone release. Females could be emitting alarm pheromone later during the mating process after the male has introduced the paramere, and thus a male does not get the

alarm signal upon mounting as they do when they mount males and nymphs (Ryne 2009, Harraca et al. 2010). Another possibility is that unfed females emit alarm pheromone less because they are able to achieve a greater degree of resistance, at least temporarily, to mating attempts by pressing their ectospermalege to a substrate.

A second area that needs much future work is the question of multiple mating in triatomines. It is unknown why males shorten the length of mating when females have been mated multiply and how they sense this multiply mated status. It is also unknown whether they adjust the amount of sperm or accessory gland secretions according to female mating status. Additionally, most studies that have addressed the effects of multiple matings on female reproduction have looked at multiple matings with a single male over time, or males have been replaced if the first male has died (Daflon-Teixeira et al. 2009). Studies must be done to examine the effects of mating with multiple males in succession and evaluate the paternity of their offspring. Studies such as these would help elucidate how males are adjusting their behavior according to the mating status of their mate. As mentioned above, formal studies should be done to learn more about the relationships among the behaviors of male aggregations around the copulating pair, post-copulatory mate guarding, and polyandry, and how males adjust these behaviors under different circumstances. It is also possible a link could be formed between these behaviors, adjustment of spermatophore contents, and paternity under multiple mating conditions.

SPERM HANDLING

Sperm delivery and insemination

Traumatic insemination occurs when males use a needle-like penis to penetrate the female's body wall and often ejaculates directly into the female's blood (Tatarnic et al. 2014). This type of mating system can carry extreme costs for females, such as increased introduction of pathogens (Reinhardt et al. 2003) and water loss (Benoit et al. 2012). In some instances females have evolved organs to mitigate this cost (Morrow and Arnqvist 2003, Tatarnic et al. 2014). Among terrestrial arthropods, this mating system evolved independently three times in the Cimicomorpha suborder of Hemiptera (Tatarnic et al. 2014).

In bed bugs, females have evolved a structure called the spermalege where males usually pierce the female (Carayon 1966, Morrow and Arnqvist 2003). There is great variation within the cimicids in the female paragenital system (spermalege) (Usinger 1966). On the primitive side, *Primicimex* does not have a spermalege and is pierced at random in the abdomen (Carayon 1966). The most advanced paragenital system, as seen in *Crassicimex*, has a duct connecting the mesospermalege to the common oviduct, and sperm does not have to swim through the hemocoel (Carayon 1966). *Cimex* has an intermediate paragenital system that contains a spermalege, but does not have a connecting duct (Carayon 1966). Some cimicids, such as *Leptocimex duplicatus*, all females have two spermaleges on either side of the abdomen, which both receive sperm (Carayon 1966). In *C. lectularius* and *C. hemipterus*, there is usually a single spermalege on the right side of the abdomen, but

sometimes a duplicate occurs as well on the left side (Kamimura et al. 2014). A duplicate spermalege in these species is not likely to have an adaptive advantage as it does in *Leptocimex duplicatus* – insemination never occurs in the left spermalege, even when the right spermalege is occluded (Kamimura et al. 2014). Male *C. hemipterus* likely pierce on the right side of the female's abdomen because the paramere is almost always directed towards the right side of the body (Kamimura et al. 2014).

It has been shown that the spermalege functions as a defense against pathogens introduced during mating (Reinhardt et al. 2003), and a low rate of piercing outside the spermalege can lead to a reduced lifespan and a reduction in lifetime egg production (Davis 1956, Stutt and Siva-Jothy 2001, Morrow and Arnqvist 2003). Support for this also comes from an interesting adaptation in the African bat bug, *Afrochimex constrictus*. In contrast to *C. lectularius* males, *A. constrictus* males often pierce other males when they mount them (Reinhardt et al. 2007), creating a favorable condition for evolution of an adaptation to reduce the cost of traumatic insemination. In this species, males have developed paragenital grooves (Usinger 1966) similar to the female ectospermalege, and also contain a hemocyte filled mesospermalege as females do (Reinhardt et al. 2007). These paragenital grooves are morphologically different than most female paragenital grooves, and males experience a lower rate of traumatic piercing than females do (Reinhardt et al. 2007). Some females seem to mimic the male paragenital groove morphology, and they experience less traumatic insemination than normal females but more than males (Reinhardt et al. 2007).

After females have been inseminated in the mesospermae, sperm remains there for approximately 2-4 hours before it migrates through the hemolymph to the seminal receptacles, the sperm storage organ in bed bugs (Carayon 1966). The sperm then travels up the oviducts to the ovaries where fertilization takes place (Carayon 1966).

As in many Heteroptera, the mechanism of sperm transfer in triatomines is through a spermatophore, a mass or capsule of sperm and accessory gland secretions (Lundgren 2011). During the triatomine mating sequence, males transfer a spermatophore to the female bursa copulatrix (Davey 1958). A series of contractions in the bursa copulatrix and common oviduct cause the common oviduct to dip into the spermatozoa mass, gathering up spermatozoa when the lip at the base of the common oviduct closes (Davey 1958). These spermatozoa move further up the common oviduct and are forced into the spermatheca 5-10 minutes after mating has been completed (Davey 1958). After a successful mating, the female discards the casing of the spermatophore (Davey 1965).

Sperm storage

Female insects often have the ability to store sperm for future use. Sperm is typically stored in an organ called the spermatheca, and it can range from simple tubes to an expandable circular sac (Simmons 2001). Insects may have one spermatheca or up to ten (Simmons 2001). Sperm may also be stored in seminal receptacles or bursa copulatrix in some insects (Simmons 2001). Sperm are maintained by specialized cells that provide

nutrition and an optimum environment, and can remain fertile for variable amounts of time depending on the species, even several decades (Simmons 2001).

Bed bugs store sperm in the seminal conceptacles after mating (Carayon 1966). The seminal conceptacles are of mesodermal origin and are not homologous to the true spermatheca found in other insects (Carayon 1966). When filled with sperm, the seminal conceptacles can distend, and some sperm are resorbed while in the seminal conceptacles (Carayon 1966). Female bed bugs maintain optimum fertility for about four weeks (Davis 1956). After this point the female must remate or she will lay infertile eggs (Davis 1956, YKM, personal observation). It is possible that cimicids that parasitize bats and birds may be able to store sperm longer than bed bugs due to their ecology. Since the hosts of these species often leave their nests or roosts for months or sometimes years (Loye 1985, Bartonicka and Gaisler 2007), female bugs may be stranded for this period of time and need to reestablish a population when the hosts return. The length of sperm viability in bat bugs and swallow bugs remains to be investigated.

Triatomines store spermatozoa in a paired spermatheca. One mating may be sufficient to maintain a female's fertility for life (Pires et al. 2002a), but there is contradictory evidence (Lima et al. 1987). The spermatheca contains teeth that prevent spermatozoa from migrating back into the common oviduct (Davey 1958). It appears that spermatozoa must be stored first in the spermatheca before they can fertilize eggs, and they appear to have a fixed volume (Davey 1965). In species that have a fixed spermatheca volume, it is likely that sperm from multiple ejaculates will mix because ejaculate size is smaller than the volume of the

spermatheca for most of these insects (Simmons 2001). Under these conditions, sperm competition may evolve (Simmons 2001).

Sperm competition

Sperm competition occurs in many insects due to their tendency to mate multiply and can be a driving force of evolution for reproduction in males (Simmons 2001). Sperm competition occurs when sperm of multiple males compete to fertilize a limited number of eggs (Simmons 2001). Sperm competition in males has shown to increase testes size, cause males to alter allocation of sperm and ejaculate components, influence sperm quality, modify sperm speed, and facilitate sperm cooperation within ejaculates (Simmons and Fitzpatrick 2012).

Sperm competition exists in triatomines, but has not been studied extensively. Eye color in *T. infestans* is controlled by a single locus, and when recessive red eyed females were paired with dominant black eyed males for 15 days, then paired with red eyed males, a larger than expected proportion of black eyed offspring was observed (Pires et al. 2002a). This suggests that either the black eyed males' sperm can outcompete the red eyed, or that black eyed sperm maintains its viability for longer in the female's spermatheca (Pires et al. 2002a). The fact that in some species of triatomines females will mate successively with different males (Manrique and Lazzari 1995b, Vitta and Lorenzo 2009) is an ideal situation for sperm competition to evolve, since each male would like to ensure paternity of as many offspring as possible. Since male *T. brasiliensis* adjust the duration of copulation when they

are the second or third male to mate in succession (Vitta and Lorenzo 2009), this species would be an ideal model to study whether the amount of sperm and accessory gland secretions transferred is adjusted and to evaluate which male produces the most offspring. Evidence for sperm displacement from the spermatheca has also been presented in *T. infestans* (Pires et al. 2002a), which is a strategy employed in sperm competition (Simmons 2001).

Sperm competition exists in bed bugs as well. Since female bed bugs retain sperm in the mesospermalege for 2-4 hours (Carayon 1966), and males frequently remate with females shortly after a blood meal (Stutt and Siva-Jothy 2001), it is likely that the sperm of several males will be present in the mesospermalege of females at once (Siva-Jothy and Stutt 2003). As mentioned above, males will adjust the duration of mating and ejaculate size according to the mating status of females, and this is likely due to the fact that the last male to mate with a female experiences a 68% sperm precedence (Stutt and Siva-Jothy 2001). Ultimately however, the first two males to mate experience equal paternity of offspring, so the first male must mate longer and have a larger ejaculate volume in order to balance out the sperm precedence the second male experiences (Siva-Jothy and Stutt 2003). Adjustments in the ratio of seminal fluids to sperm and ejaculate size are strategies frequently employed by males during sperm competition (Simmons and Fitzpatrick 2012). More work must be done to assess paternity in bed bugs when females have mated with more than two males to assess sperm competition and sperm precedence under stronger pressure for competition to occur. Intense sperm competition may have been present in the ancestors of bed bugs as well, since

it has been proposed as a driving force for the evolution of traumatic insemination in this group as a way of bypassing the female genital tract and thus pre and post-copulatory female choice mechanisms (Tatarnic et al. 2014).

Effects on fecundity

Polyandry and mating rate have effects on female fecundity (Arnqvist and Nilsson 2000). The mating rate of female bed bugs does not affect the overall number of eggs oviposited, as long as females are mated the minimum amount (once every 4 weeks) to maintain fertility (Davis 1956, Stutt and Siva-Jothy 2001). However, females that mate more frequently do experience a shorter lifespan, and therefore lower fecundity (Stutt and Siva-Jothy 2001). In contrast, it has been found that ejaculate components can have a positive effect on female bed bugs, including inducing a delay in reproductive senescence and increasing reproductive rates (Reinhardt et al. 2009b) and stimulates females to oviposit earlier (Otti et al. 2013). The delay in senescence may be because ejaculate components have the capacity to digest bacteria (Otti et al. 2009) and have proteins with antioxidant effects (Reinhardt et al. 2009c). Antibacterial ejaculate components do not only benefit females, however, since environmental microbes have been shown to cause sperm mortality and the antibacterial components reduce this mortality (Otti et al. 2013). These benefits to females of ejaculate components have been demonstrated in other insects as well (Avila et al. 2011).

Virgin female triatomines do produce some infertile eggs which they oviposit at a slow rate (Lent and Wygodzinsky 1979). Mating of course produces fertile eggs in

triatomines, but incurs other effects on females as well. Under laboratory conditions, multiply mated female *T. brasiliensis* produced more eggs and higher fertility than singly mated females over their lifetimes

(Daflon-Teixeira et al. 2009). However, in this study some multiply mated females were maintained with a single male throughout their lifetimes, and other were maintained with two or more males (one male at a time) if the first male died (Daflon-Teixeira et al. 2009). It would be worth investigating whether fertility and fecundity are affected when *T. brasiliensis* females are mated with multiple males in succession and maintained with multiple males. Mating also stimulates oviposition in triatomines (Davey 1965, Daflon-Teixeira et al. 2009), even in females that are unfed (Daflon-Teixeira et al. 2009). In fact, mated females require less blood than virgin females to produce eggs (Davey 2007). More studies need to be done to fully understand the relationships of feeding, mating, and egg production in triatomines.

Sperm handling future work

Since it has been found in other insects that substances transferred in spermatophores and ejaculates can have profound effects on female longevity, fitness, and fertility (Arnqvist and Nilsson 2000, Avila et al. 2011), these effects should be further studied in both triatomines and bed bugs to understand their reproductive physiology. In triatomines particularly, the secretions of the male accessory glands should be analyzed to gain an understanding of the composition of secretions and their potential effects on females, and whether female feeding alters their effects. Additionally, the intensity of sperm competition

can be evaluated in triatomine species that generally support a polyandric lifestyle, such as *T. infestans* and *R. prolixus*, vs. others that are less receptive to multiple matings, such as *P. megistus*. It is likely that sperm competition and associated male behavioral adaptations will be more intense in species that demonstrate a greater degree of polyandry.

Much is yet to be determined about sperm competition and its effects on females in bed bugs as well. Only a handful of studies, outlined above, have been conducted on these subjects. The possible role of sperm competition in the evolution of traumatic insemination in cimicids (Tatarnic et al. 2014) warrants further investigation too. If traumatic insemination did indeed evolve as a mechanism to escape sperm competition, it is clear that male bed bugs have not escaped it.

CONCLUDING REMARKS

Many insights have been gained into the reproductive strategies of hematophagous Hemipterans in recent years. The debate over the existence of a sex pheromone in triatomines is being settled. The role of the spermatheca in bed bugs has been determined as well as its role in mitigating the costs of traumatic insemination. Still much remains to be determined, however, about reproduction in these insects. A more integrated approach is necessary between the interactions of ecology, external cues, circadian control, hormonal control in their reproductive biology. Once we gain a greater understanding of all the factors that

influence reproduction in hematophagous Hemipterans, they can potentially be exploited in novel and effective control methods for these medically important insects.

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CHAPTER 2.**Effects of Cyclic Feeding and Starvation, Mating, and Sperm Condition on Egg
Production and Fertility in the Common Bed Bug (Hemiptera: Cimicidae)**

ABSTRACT

Bed bug populations have resurged globally in the past two decades, but our knowledge of their basic biology and behavior is largely limited to research done more than four decades ago. This project investigated the effects of starvation, mating, sperm storage, and female and male age on egg production and egg hatch in bed bugs. We subjected females to successive cycles consisting of a fixed 10-d starvation period followed by one, two, or three blood meals at 5-d intervals. We found that these feeding regimes imposed cycles of egg production that varied with the number of blood meals. A single blood meal followed by a 10-d starvation period resulted in higher frequency (short period) cycles of low amplitude (mean peak egg production), whereas three consecutive blood meals caused lower frequency (higher period) cycles with >4-fold greater amplitude; offering once-mated females a blood meal every 5 d resulted in constant egg production for ~75 d followed by a monotonic decline to near zero. Percentage egg hatch was independent of these treatments, but declined monotonically after ~30 d to near zero. To determine whether female age, male age, or sperm age affected these patterns, we mated newly eclosed females to 60-d-old virgin males, 60-day-old mated males, or newly eclosed males. Females produced the most eggs when mated to young males, followed by old mated males, and the fewest when mated to old virgin males; percentage hatch followed a similar pattern, suggesting that sperm stored within the male for a long time was deficient. To examine the effects of sperm age stored within the female we mated newly eclosed females, starved them for 30- or 60-d, then fed them every 5-d. The 60-d starved

group produced fewer eggs than the 30-d starved group, and both groups produced fewer eggs than young females that were mated to either old or young males. Correspondingly longer periods of sperm storage within the female resulted in lower the corresponding percentage hatch. These findings indicate that egg production and egg hatch are governed by complex interactions among male age, female age, frequency of feeding and mating, and sperm condition.

KEY WORDS Bed bug, *Cimex lectularius*, oviposition, mating, sperm quality

INTRODUCTION

Although practically eradicated from developed countries in the 1960s, bed bugs (*Cimex lectularius* L.) have resurged globally in the past 20 years (Doggett et al. 2004, Hwang et al. 2005, Reinhardt and Siva-Jothy 2007, Romero et al. 2007, Bencheton et al. 2011, Wang and Wen 2011). These blood-sucking ectoparasites can have several adverse effects on humans, including embarrassing bites, secondary infections, sleeplessness, anxiety, and social isolation (Hwang et al. 2005, Goddard and de Shazo 2012). Despite the corresponding resurgence in research on bed bugs in the past couple of decades, many elements of the basic biology and behavior of this pest are still poorly understood.

In longer lived insects, it has been shown that food quality and quantity affect egg production (Joern and Behmer 1998). All bed bugs are obligatorily hematophagous, and all life stages require a blood meal to develop and molt to the next life stage (Usinger 1966). Feeding regimes and host blood type have been investigated in several studies (Usinger 1966, Barbarin et al. 2013), and have shown that survival of bed bugs is significantly influenced by the quality of the blood diet and the female's mating status (Barbarin et al. 2014). The impacts of defined cycles of feeding and starvation on fecundity and egg hatch has not been investigated in bed bugs. We hypothesized that newly emerged adult females fed regularly would experience essentially a continuous food supply and would display constant and maximal egg production. However, if the food supply is interrupted by a starvation period, we hypothesized that egg production would be affected by the number of blood meals

between starvation periods; females fed only once would have limited resources for egg production and might allocate more nutrients to somatic functions and less to reproduction. More blood meals after a starvation period would be expected to support more egg production. The dynamics of the patterns of egg production and the effects of these treatments on egg hatch are of interest.

Bed bugs mate via traumatic insemination, where the needle-like highly sclerotized male aedeagus pierces the cuticle of the female's abdomen and delivers sperm into the female's hemocoel (Tatarnic et al. 2014). Understandably, this mating strategy can carry significant cost to the female, including the introduction of pathogens (Reinhardt et al. 2003) and water loss (Benoit et al. 2012). Bed bug females are mated more frequently than necessary to maintain fertility (Siva-Jothy 2006), and higher mating frequencies lead to a higher death rate in females (Stutt and Siva-Jothy 2001). We therefore sought to describe patterns of egg production and egg hatch in bed bugs under controlled conditions where mating frequency and the feeding cycles are clearly defined.

Sperm age and sperm storage also affect egg production and hatch in insects. Females often have specialized cells in their spermathecae that maintain sperm in an optimal environment and provide nutrition, and in some insect species sperm remains fertile for several decades (Simmons 2001). Bed bug females can store sperm for up to four weeks in the seminal receptacles, after which they begin to lay infertile eggs (Davis 1956). When males are older, sperm counts and the ability to deliver sperm to females can be reduced (Jones et al. 2006). Additionally, studies in other species have demonstrated that sperm

senescence can occur within a male before ejaculation, especially when stored for longer periods of time (Pizzari et al. 2008). These relationships have not yet been investigated in male bed bugs.

Here we investigate (1) the effects of starvation, number of feeding events, and mating, and (2) the effects of sperm age and sperm storage in males and females on egg production and egg hatch in bed bugs. Understanding these dynamics in bed bugs is important for understanding the evolution of blood-meal processing strategies and traumatic insemination. This study will also be useful for comparative reproductive biology in other hematophagous insects.

METHODS

Insects. An insecticide susceptible strain of *C. lectularius* was used (Harold Harlan strain, collected in Ft. Dix, NJ in 1973 and maintained for 35 years on human blood, then on defibrinated rabbit blood in our lab since 2008). Bed bugs were maintained in an incubator at 27°C and a photoperiod of 12:12 h (L:D). Relative humidity within colony jars was approximately 50%. Virgin females and males were obtained by separating 5th instars from the colony and keeping them in isolation as they molted into adults.

In Vitro Feeding System. Bed bugs were maintained on defibrinated rabbit blood (Quad Five, Ryegate, MT) in an artificial feeding system. Custom built water-jacketed glass feeders connected to a thermal circulator water bath (B. Braun Biotech, Inc., Allentown, PA)

heated to 37°C were used to feed bed bugs. Feeders can hold up to 4 mL of blood, which was held in place by a membrane stretched across the bottom of the feeder (NESCOFILM, Karlan, Cottonwood, AZ).

Feeding, Mating, and Starvation Assays. Virgin females 7-10 d after eclosion and males drawn at random from a colony were grouped into treatments in colony jars and allowed to feed on blood for one hour. Colony jars were constructed by removing the bottom of 5-cm diameter clear polystyrene wide-mouth threaded round jars (Consolidated Plastics Company, Inc., Stow, OH), and in its place heat-sealing plankton netting fabric (0.3-mm mesh opening, 0.2-mm fabric thickness; BioQuip Products, Inc., Compton, CA), through which bed bugs could feed. A piece of accordion-folded manila folder cardboard (6.6 x 4.4 cm) was placed inside each jar so bed bugs could crawl up to the mesh and feed. After feeding, females were placed individually into 20 ml borosilicate glass scintillation vials (Thermo Fisher Scientific, Waltham, MA) with a hole (~1 cm diameter) drilled in the cap to allow for ventilation. Plankton netting fabric was placed underneath the cap to prevent insects from escaping. An insert made from black cardstock (Staples, Farmingham, MA) cut to 40 x 14 mm was placed in each vial as an oviposition substrate. In order to ensure oviposition on only one side of the insert, a piece of transparency film (3M, Minneapolis, MN) was affixed to one side of the insert using double-sided adhesive tape (Scotch Brand, 3M). One recently fed male was paired with each female, and left in the vial for 5 d.

For feeding, individual females from each respective treatment were grouped in a colony jar and fed for one hour. After feeding, females were re-sorted individually into clean

20 ml vials with a fresh insert for oviposition. Importantly, this design precluded the identification of each individual female.

Treatment a (pink in Fig. 1A): females were fed and mated, then fed every 5 d. Every 20 d, females were re-mated as described above. There were 50 females in this treatment.

Treatment b (red): females were fed and mated, and then fed every 5 d, but only re-mated once around d 65. There were 35 females in this treatment.

Treatments c (blue), d (green), and e (black): females were fed and mated, fed 5 d later, and then subjected to a 10-d starvation period. Treatment c females were fed at three consecutive times, 1 hr every 5 d, followed by a 10-d starvation period. Treatment d females were fed only twice then experienced the 10-d starvation period, and treatment e females were fed only once between 10-d starvation periods. There were 35 females each in treatments c-e.

The experiment ran for 130 d and bed bugs were maintained in an incubator at 27°C and a 12:12 L:D cycle. Female mortality was monitored once daily and females that could no longer grasp the insert were considered dead. Eggs were maintained in the incubator for 15 d after females were removed from vials to allow all fertile eggs to hatch. Eggs and nymphs were then frozen for 24 hrs and subsequently counted.

Sperm Preservation (in Female) Assays. Virgin females 7-10 d after eclosion were grouped into two treatments and mated as described above, but not fed. Females in Treatment i (orange; Fig. 4A) were starved for 30 d after the start of the assay to prevent egg production, and subsequently fed every 5 d until d 130 as described above. Females in

Treatment j (teal) received the same procedure, but were instead starved for 60 d following the start of the assay. There were 50 females in Treatments i and j , and females were not re-mated during the remainder of the experiment. Mortality was monitored and frozen eggs and nymphs counted as described above.

Sperm Age (in Male) Assays. Virgin males 7-10 d after eclosion were separated into two groups (Treatments g and h; Fig. 4A) and fed every 10 d for 60 d. Males were fed and housed individually in 9 mm screw thread 2 ml borosilicate glass auto injection vials (Thermo Fisher Scientific). Males were allowed to feed until they fully engorged on a blood meal. In treatment h (maroon), males were maintained without females and on d 60 of the experiment, each of these “old” virgin males was mated to a 7-10-d-old virgin female as described in previous assays. In treatment g (light green), males were allowed to mate with non-experimental females every 20 d by co-housing one female with each male in the 3 ml vials for 24 hrs. On d 60 of the experiment, each of these “old” males was mated to a 7-10-d-old virgin female as described in previous assays. Treatment f (purple) served as a control, where each virgin female was allowed to mate with a “young” 7-10-d-old virgin male for 5 d. Females were housed and treated in the same manner as described in previous assays, and fed every 5 d until d 130 of the experiment. Mortality was monitored and frozen eggs and nymphs counted as described above. There were 35 males and females per treatment, and females were not re-mated during this experiment.

Data Analysis. Statistical analysis was completed using SAS version 9.4 (SAS 2013). For cumulative egg production, a negative binomial model was fit to each curve to determine

divergence points among treatments. To assess differences among treatments at a certain time point, likelihood ratios were calculated within a nested model comparison. Then pairwise comparisons were conducted where treatments were constrained to be equal. Differences over time were considered significant when $|t| > 2$. For treatments where female feeding was delayed 30 and 60 d, we considered d 30 and d 60 to be d 0 for this analysis. Standard error of cumulative means was standard error of all individuals in a given treatment through a given time point. Percentage egg hatch differences among treatments at a given time point were determined using an ANOVA and Tukey HSD test for comparisons among treatments.

To assess cyclicity in egg production, mean eggs oviposited per d were calculated for each female (replicate). In SAS, data were then Fourier transformed and a time series analysis was obtained by averaging over all replicates. Spectral densities were then estimated from the time series. To test whether a treatment effect existed, a pairwise comparison of the four periodograms was conducted. Normalized cumulative periodograms were generated as suggested by Diggle and Fisher (1991), and Kolmogorov-Smirnov distances were calculated between the normalized cumulative periodograms. A randomization procedure was used to shuffle labels for each treatment pair comparison for 10,000 random shufflings, and randomization p-values were generated.

RESULTS

The number of feeding events following a 10-d starvation period affected egg production in bed bugs. Cumulative egg production at the end of the experiment (d 130; Fig. 1A) was generally greater with more feeding events. Control females fed every 5 d and allowed to re-mate every 20 d (a. pink in Fig. 1A) group oviposited 358.31 ± 0.21 per female over 130 d. This group produced significantly more eggs than similarly fed females that were allowed to re-mate only once on d 65 (b. red in Fig. 1A) ($t = |12.1|$), which produced 262.51 ± 0.29 per d per female. Egg production in other treatment groups, which were also allowed to re-mate only once between d 65 and 75, varied by the number of feeding events before and after the recurrent 10-d starvation periods. Generally, more feeding events resulted in greater cumulative egg production. Thus, females fed three and two times (c. blue and d. green, respectively, in Fig. 1A) produced only 176.32 ± 0.33 and 172.01 ± 0.32 , eggs per d per female, respectively, significantly fewer than females fed every 5 d (b. red) ($t = |9.5|$), but these two treatments did not differ from each other ($t = |0.4|$). However, these treatment groups differed significantly from females that were fed only once between the 10 d starvation periods (e. black in Fig. 1A) ($t = |5.5|$), which oviposited 133.09 ± 0.23 eggs per d per female over 130 d. Differences among these treatments became evident early in this experiment. The effect of more frequent re-mating was evident on d 45, as females mated every 20 d (a. pink) produced significantly more eggs than females re-mated once on d 65 (b.

red) ($t = |4.5|$). Females fed less frequently (one [e. black], two [d. green], and three [c. blue] re-feedings diverged from the control group (b. red) even earlier on d 15 ($t = |5.9|$).

The 10-d starvation periods imposed a cyclic pattern on egg production, and the feeding regime appeared to modulate the frequency and amplitude of these cycles (Figs. 1B and C). When fed optimally every 5 d and allowed to re-mate every 20 d, females (a. pink in Fig. 1B) rapidly attained a steady state, producing ~ 3.5 eggs per d per female until \sim d 80, and egg production then monotonically declined to near zero by d 130. Fourier transform analysis did not detect any cyclicity in this treatment group. Preventing females from re-mating every 20 d caused a monotonic decline in egg production beginning about 40 d after the first mating, but this decline was rescued by allowing females to re-mate on d 65 (b. red in Fig. 1B). Thus, re-mating once imposed a clear second cycle in optimally fed females with a period of 65 d, coincident with the period of re-mating (Fig. 2).

The 65 d period in once-re-mated females was further disrupted by recurrent 10-d starvation periods in all three treatments, but the frequency of egg production cycles varied with the frequency of re-feeding females after each starvation period. Females fed only once exhibited rapid attenuated cycles of egg production (e. black in Fig. 1C) with a fundamental frequency at 16.2 d (Fig. 2). Females fed twice before the next 10-d starvation period had cycles of longer periods, with a fundamental frequency at 21.6 d, each with higher amplitudes, i.e., greater egg production per d (d. green in Fig. 1C, Fig. 2). Finally, females fed three times over 15 d between 10-d starvation periods exhibited even longer cycles with a fundamental frequency of 26 d (c. blue in Fig. 1C, Fig. 2). An overall model of periodograms

was significant ($D = 19.2$; $df = 2$; $P = 0.0002$). Kolmogorov-Smirnov distance post-hoc comparisons of periodograms revealed that the periods for females fed every 5 d (b. red) and females re-fed three times after each 10-d starvation period did not differ ($P = 0.998$), but the former did differ significantly from the periods for females re-fed only once (e. black; $P = 0.0104$) or twice (d. green; $P = 0.0254$) after each 10-d starvation period. Females re-fed only twice also exhibited a significantly shorter period than females re-fed three times (c. blue; $P = 0.0046$), but not relative to females re-fed once (e. black; $P = 0.7035$).

Although cyclical patterns of egg production are created by feeding, starvation, and mating frequency, the hatch rates of nymphs were largely unaffected by the different feeding regimes (Fig. 3). At d 5 the percentage hatch for all treatment groups was nearly 100% and the groups did not differ from each other ($F = 0.51$; $df = 4$; $P = 0.73$). As percentage hatch declined around d 30 in all groups that were not re-mated (i.e. a. pink group re-mated every 20 d excluded), there were likewise no differences among groups at d 55 ($F = 1.04$; $df = 3$; $P = 0.38$), when hatch rate was ~30%. Although significant divergence became apparent at d 95 ($F = 6.97$; $df = 2$; $P = 0.0017$) and a Tukey HSD test revealed that hatch rate was higher in females re-fed twice (d. green) than in the control females (the fed every 5 d [b.red] or fed every 5 d, allowed to re-mate every 20 d [a. pink]), sample sizes were small at that point due to female mortality.

To separate the effects of sperm age when stored in the male, we (1) mated young females but prevented egg production to age the sperm within the female, or (2) mated females to older virgin males. Both female age and sperm age affected egg production (Fig.

4). Frequently re-mated and re-fed females (a. pink in Fig. 4A) oviposited 256.5 ± 0.20 eggs per d per female over 70 d. However, when sperm storage was imposed on young mated females for 30 d (i. orange) or 60 d (j. teal) before they were fed, cumulative mean egg production decreased dramatically to 89.55 ± 0.28 and 22.76 ± 0.35 eggs per d per female by d 100 and d 130, respectively (Fig. 4). That female age per se was not as important as the aging sperm within the female was indicated by the observations that virgin females first mated at age 60 d produced fewer eggs per d per female over the next 70 d (see below). Likewise, the 30 d (i. orange) 60 d (j. teal) sperm stored in female groups begin to diverge very early from the male stored sperm groups and fed every five days, allowed to re-mate every 20 d group (a. pink), both at d 5 ($t = |12.3|$ and $t = |14.9|$, respectively).

A significant effect of frequent re-mating was indicated starting at d 65 ($t = |4.12|$; Fig. 4A) by lower egg production in females mated to young males and then fed regularly every 5 d (f. purple in Fig. 4A), compared with similarly fed females that were allowed to re-mate every 20 d (a. pink).

The effect of sperm age in males was also examined. Two male groups were compared: Males that were mated every 20 d, and then mated to young virgin females (g. light green in Fig. 4) were compared to same age (60 d) virgin males (h. maroon in Fig. 4). Females mated with the old virgin males produced fewer eggs by d 50 (27.8 ± 0.09) than females mated with multiply mated males (35.1 ± 0.09) ($t = |4.60|$). Cumulative egg production in females mated to young virgin males (f. purple) was higher than in females mated to old virgin males (h. maroon) starting on d 45 ($t = |4.43|$). These results suggest that

males were able to recycle their sperm through recurrent mating events produced higher quality sperm that resulted in greater egg production.

The patterns of daily oviposition in these treatments are shown in Fig. 4B,C. Females fed every 5d and allowed to re-mate every 20 d achieve a steady rate of ~3.5 eggs per d per female (a. pink in Fig. 4C). When unfed females were forced to store sperm for 30 d and then re-fed (i. orange), they achieved similar mean daily egg production on d 40 ($F = 2.11$; $df = 1$; $P = 0.15$) but declined soon after at d 45 ($F = 69.88$; $df = 1$; $P < 0.001$) and beyond. Females that stored sperm for 60 d (j. teal) oviposited up to 1.5 eggs per d per female, significantly fewer eggs than same age females that were fed every 5 d and allowed to re-mate every 20 d (at d 70, the peak of egg production for the former group: $F = 49.87$; $df = 1$; $P < 0.0001$), even though the latter had oviposited many more eggs before d 60. Percentage egg hatch followed a similar pattern, with similar maximum hatch for the 30 d stored sperm group and the control ($F = 0.53$; $df = 1$; $P = 0.47$) of females the same age (Fig. 5A). As for egg production, the maximum egg hatch for females that stored sperm for 60 d was <60%, compared to ~80% for same aged control females ($F = 7.22$; $df = 1$; $P = 0.0094$).

Male age had a less pronounced effect on egg production and egg hatch. The peaks of egg production and egg hatch rate were similar in all three treatment groups (young males, 60-d-old virgin males, 60-d-old males allowed to re-mate every 20 d; Fig. 4B). Differences among these treatments became evident around d 40 ($F = 7.02$; $df = 2$; $P = 0.0015$) when egg production was significantly lower in females mated to old virgin males. By d 50, females mated to the 60-d-old frequently mated males (g. light green in Fig. 4B) produced fewer eggs

per d per female than females mated to young virgin males ($F = 6.50$; $df = 1$; $P = 0.0135$).

These results suggest that young males produce the most fertile sperm, that sperm quality is reduced during long-term storage in males, and that mating allows males to replenish more fertile sperm. Egg hatch rate showed the same pattern, but with clearer separation among the treatments beyond d 20 (Fig. 5A). The older virgin male group diverged from the other groups around d 20 ($F = 11.74$; $df = 2$; $P < 0.0001$) and beyond, and the older mated male group diverges from the young virgin male group at d 35 ($F = 13.50$; $df = 2$; $P = 0.0005$).

DISCUSSION

Egg production in bed bugs is governed by feeding-starvation patterns, mating status and mating frequency of the female, and sperm quality, which in turn is affected by its time in storage within the male and within the mated female. Our experiments were designed to uncouple these effects. Females that were fed every 5 d and allowed to re-mate every 20 d produced eggs at a steady rate of ~ 3.5 eggs per d for ~ 70 d, followed by a gradual senescence to d 130. Preventing females from re-mating for 65 d caused a rapid decline in egg production after d 40; egg production was substantially rescued by allowing females to re-mate for 5 d between d 60 and d 65. The latter females had substantially lower cumulative egg production than frequently re-mated females. Egg hatch followed a similar pattern as egg production. These results are consistent with previous findings that the mating rate of female

bed bugs does not affect the overall rate of egg production as long as females are mated a minimum of every four weeks to maintain fertility (Davis 1964, Stutt and Siva-Jothy 2001).

Treatments that imposed a starvation period and limited the number of feeding events dramatically affected egg production. Starvation imposed a cyclic pattern of egg production. Frequent feeding events (three feedings at 5 d intervals) resulted in high amplitude long cycles of egg production, whereas infrequent feeding events (one or two feedings) attenuated the amplitude of more frequent cycles. As expected, cumulative egg production reflected the pattern of feeding frequency. Likewise, egg hatch followed the pattern of egg production so that as egg production declined, so did viability of the eggs.

A constraint of this research was that females could not be fed individually on such a rigorous feeding schedule. Therefore, female identity was lost when females were grouped by treatment for group feeding and then separated again. In future analyses, it would be instructive to follow patterns of egg production in individual females throughout her lifetime. Moreover, we binned egg production into 5 d intervals. In future studies it would be useful to understand individual variation in egg production and egg hatch per day.

Sperm age and storage location substantially affected egg production in bed bugs. When females are mated but then starved for 30 or 60 d, sperm is stored within the female until she is fed and can utilize the sperm. Under these conditions, stored sperm deteriorates rapidly, as evidenced by low egg production in these females. This finding is consistent with the observations that bed bug females become infertile as oxygen radicals increase in stored sperm (Reinhardt and Ribou 2013). Nevertheless, it may be unnecessary for bed bugs to have

a mechanism for long-term sperm storage as they live in aggregations and females are likely mated frequently by males (Stutt and Siva-Jothy 2001).

Sperm quality deteriorates at a slower rate when sperm is stored in males rather than in females. Young males have the highest quality sperm and lead to greatest egg production in both young and older females. Older 60 d virgin males that are prevented from mating presumably accumulate some senescent sperm, and females that mate with these males experience low egg production. This effect can be partially rescued by allowing males to mate every 20 d before mating with the experimental young female. These females exhibit egg production nearly at the same level as females that had been mated to young males. However, clear differences in egg production are evident ~40 d after mating, and significant differences become evident even sooner in egg hatch rate. These differences are likely due to older senescent sperm being cycled out in males during frequent matings. Indeed, the need to recycle sperm may explain the frequent observation of male-male matings, especially in male bed bugs older than 50 d (Ryne 2009).

This study could shed light on real life situations of bed bugs that experience longer periods without access to a host, such as in a hotel room that goes out of commission for a few weeks or an apartment or home that is vacated by the owners periodically. We know that even with intermittent access to a host, bed bugs can still achieve significant egg production, albeit less than when they have regular access to a host. Females can still produce fertile eggs, although few, after storing sperm for a period of 60 days. Additionally, males that have not had the opportunity to mate for up to 60 days can still fertilize females. This has

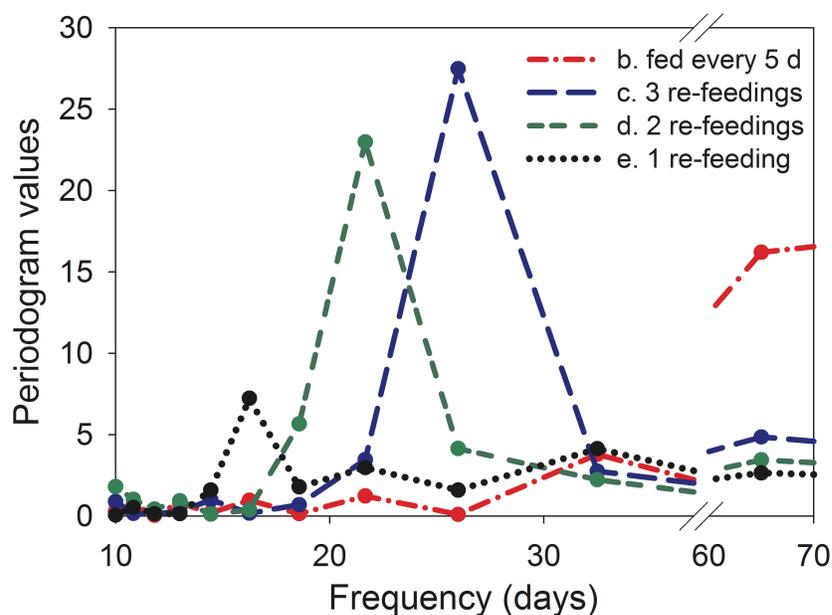
implications for control situations, such as a hotel, where staff may periodically take infested hotel rooms out of commission with the hopes that all bed bugs will die. This information could then in turn be useful for pest control operators who base their treatments on population size and change.

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continuous feeding control group (b. red) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10 d starvation periods: females received three feedings events separated by 5 d (c. blue), two feeding events separated by 5 d (d. green), and one feeding between recurrent 10 d starvation periods (e. black). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Cumulative data (A) were analyzed using a negative binomial model, and daily egg production (B, C) was analyzed with a one-way ANOVA and Tukey HSD test at given time points. Beginning sample size was 50 females for the a. pink group and 35 for all other groups.



	b. fed every 5 d	c. 3 re-feedings	d. 2 re-feedings	e. 1 re-feeding	P value
b. fed every 5 d		0.0998	0.0254	0.0104	
c. 3 re-feedings	0.6752		0.0046	0.1889	
d. 2 re-feedings	0.7375	0.5906		0.7035	
e. 1 re-feeding	0.6581	0.4244	0.2831		

Kolmogorov-Smirnov distances

Figure 2. Periodograms of eggs oviposited by *C. lectularius* females over the 130 d experiment. Each curve represents a different treatment combination, as in Fig. 1: females in the continuous feeding control group (b. red) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10 d starvation periods: females received three feedings events separated by 5 d (c. blue), two feeding events separated by 5 d (d. green), and one feeding between recurrent 10 d starvation periods (e. black). Data were

Fourier transformed and analyzed using a time series analysis. Differences among groups were assessed with Kolmogorov-Smirnov distances. Beginning sample size was 50 females for the a. pink group and 35 for all other groups.

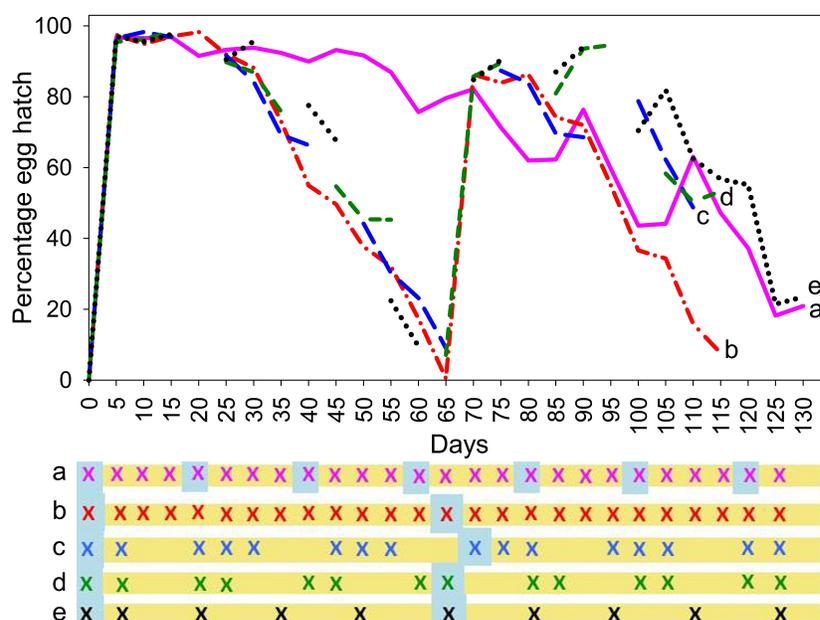


Figure 3. Egg hatch rate in *C. lectularius* females over the 130 d experiment. Treatments are as in Fig. 1: Females in the overall positive control group (a. pink) were fed every 5 d and allowed to re-mate every 20 d. Females in the continuous feeding control group (b. red) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10 d starvation periods: females received three feedings events separated by 5 d (c. blue), two feeding events separated by 5 d (d. green), and one feeding between recurrent 10 d starvation periods (e. black). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Percentage egg hatch was analyzed by a one-way ANOVA at different time points and a Tukey HSD test evaluated differences among treatments. Beginning sample size was 50 females for the a. pink group and 35 for all other groups.

males every 20 d. Young females mated to young virgin males and fed every 5 d (f. purple). Young females mated to 60-d-old males that were allowed to mate every 20 d (g. light green). Young females mated to 60-d-old virgin males (h. maroon). Females mated to young males at d 0, starved to d 30, and fed every 5 d beginning on d 30 (i. orange), and females treated as above, but fed beginning on d 60 (j. teal). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Where male treatments are not indicated, young virgin males were used. Cumulative data (A) were analyzed using a negative binomial model, and daily egg production (B, C) was analyzed with a one-way ANOVA and Tukey HSD test at given time points. Beginning sample size was 50 females for the a. pink and i. orange groups, and 35 for all other groups.

above, but fed beginning on d 60 (j. teal). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Where male treatments are not indicated, young virgin males were used. Percentage egg hatch was analyzed by a one-way ANOVA at different time points and a Tukey HSD test evaluated differences among treatments. Beginning sample size was 50 females for the a. pink and i. orange groups, and 35 for all other groups.

CHAPTER 3.**Evaluation of the Potential for Secondary Kill for Ingested Insecticides in the Common
Bed Bug (Hemiptera: Cimicidae)**

ABSTRACT

Over the past several decades, baits have become a preferred method of urban pest management in the indoor environment. Baits enable more targeted applications of insecticides with much smaller amounts of active ingredients than are used in residual sprays. Translocation of the bait by foragers, and consequent secondary kill of non-foraging members of a population has enhanced the effectiveness of these baits not only in social insects (termites, ants), but also in other species that aggregate, such as German cockroaches (*Blattella germanica* L.). We investigated the potential for secondary kill in bed bugs (*Cimex lectularius* L.), another gregarious insect pest, using a liquid bait. We hypothesized that blood engorged adults could enhance the survivorship of nymphs within aggregations by increasing the local relative humidity (RH) and providing fecal nutrients. Relatively high RH (50% and 95%) resulted in greater survivorship of 1st instars compared to 0% RH, and we thus controlled RH in the next experiment to decouple its effect on nymph survivorship from effects of fecal nutrients. The presence of either fed or unfed adults did not increase the survivorship of unfed 1st instars, suggesting that if nymphs engaged in coprophagy, its nutritional benefits were minimal. We hypothesized that adult male bed bugs fed rabbit blood fortified with fipronil or clothianidin would decrease survivorship of unfed 1st instar bed bugs housed with them. Survivorship of nymphs was unaffected in the presence of insecticide-fed adult males, suggesting that unlike in cockroaches, highly effective insecticides might not be effective as secondary kill toxicants in bed bugs. To further confirm this inference we

exposed 1st instar bed bugs and *B. germanica* to insecticide-laden adult *B. germanica* feces, because they contained substantially more active ingredient than bed bug feces. Whereas 1st instar *B. germanica* died in the presence of the insecticide-laden feces, bed bugs did not. We therefore conclude that when a liquid bait is developed for bed bugs, secondary kill with neuroactive insecticides will likely not be a significant factor in population suppression.

KEY WORDS Bed bugs, *Cimex lectularius*, secondary kill, German cockroach, *Blattella germanica*

INTRODUCTION

Baits have increasingly become a preferred and effective control method for urban pest management, largely because they have several advantages over broad-spectrum residual sprays: namely, smaller amounts of active ingredient (AI) are used in baits, they can last longer than most residual sprays, generally have lower mammalian toxicity, can be made as low-odor formulations, and can be used across a range of environmental conditions (Reierson 1995). Additionally, effective AIs that cannot be labeled in spray formulations or are photolabile have been formulated in baits (Harpaz 1987). Baits (like pharmaceuticals) can deliver much larger amounts of AI by ingestion than residual sprays do through cuticular penetration. Therefore, large amounts of AI can also be translocated by foraging individuals and cause mortality (i.e., secondary kill) in relatively more sedentary members of the population (Silverman et al. 1991, Buczkowski et al. 2001). Baits are currently used successfully against a variety of urban pests, including cockroaches, termites, wasps, ants, and rodents (Reierson 1995, Rust and Su 2012, Buckle and Eason 2015).

Baits have been highly effective against the German cockroach (*Blattella germanica* L.), a widespread urban pest, which lives in difficult-to-treat cracks and crevices (Reierson 1995). A factor contributing to the efficacy of baits is the horizontal transfer of AI to other cockroaches who have not visited the bait, via ingesting insecticide-laden feces (coprophagy) (Silverman et al. 1991, Buczkowski et al. 2001), other excretions, or feeding on dead insects (Gahlhoff Jr. et al. 1999, Buczkowski et al. 2001). Coprophagy is particularly important for

survivorship of 1st instar *B. germanica* (Kopanic et al. 2001) and it is an important mechanism for the horizontal transfer of hydramethylnon to the nymphs, leading to secondary kill (Silverman et al. 1991, Kopanic and Schal 1999). Secondary kill has been demonstrated in *B. germanica* with other AIs, including boric acid, chlorpyrifos, fipronil, and indoxacarb (Buczowski et al. 2001, Buczowski et al. 2008, Ko et al. 2016).

Bed bugs (*Cimex lectularius* L.), another urban pest, have been making a recent global resurgence (Doggett et al. 2004, Hwang et al. 2005, Romero et al. 2007, Bencheton et al. 2011, Wang and Wen 2011). These blood-sucking ectoparasites can have several adverse direct and indirect effects on humans, including bites that can lead to secondary infections, sleeplessness, anxiety, and social isolation (Hwang et al. 2005, Goddard and de Shazo 2012). Current control methods for bed bugs are limited to just a few options by efficacy, cost, and safety concerns. Residual insecticide treatments can be problematic because bed bug harborage sites are often on mattresses and other areas that are in close contact with humans (Pereira et al. 2009). Similarly to German cockroaches, bed bugs also live in cracks crevices, which may be difficult to reach with residual sprays (Usinger 1966). Moreover, there is extensive resistance to pyrethroid insecticides, which are commonly used for bed bug control, and even to the more recently introduced neonicotinoids (Romero et al. 2007, Zhu et al. 2010, Kilpinen et al. 2011). Heat treatments of a whole room, apartment, or building (Pinto et al. 2007) and fumigation of infested objects (Wang et al. 2012) often provide greater efficacy if properly implemented, but these approaches can be cost prohibitive, as heat treatment of an entire home can cost \$3,000 or more. Current control methods require

multiple visits from pest control technicians, especially to eradicate broadly distributed infestations (Pinto et al. 2007, Pereira et al. 2009).

Bed bugs are an excellent candidate for control with a liquid bait, which could provide an effective and more affordable intervention. They take very large blood meals, which would deliver a large amount of AI even when the AI is formulated in relatively low concentration. For example, bed bugs experienced high mortality when they fed on humans who had taken a prescription-based dose of ivermectin, a ubiquitous anti-parasitic drug (Sheele et al. 2013) or moxidectin, a related macrocyclic lactone (Sheele and Ridge 2016). Additionally, bed bugs can be stimulated to ingest large amounts of water augmented with phagostimulants (Romero and Schal 2014), and various insecticides, including fipronil, clothianidin, and abamectin, cause high mortality at relatively low concentrations when incorporated into an artificial feeding system (Sierras and Schal 2016). Horizontal transfer of synthetic and botanical insecticides, and biopesticides, via contact has been demonstrated in bed bugs (Barbarin et al. 2012, Akhtar and Isman 2013), but not by ingestion.

We assessed the potential for secondary kill via an ingested insecticide in bed bugs by evaluating potential benefits of 1st instars co-habiting with adults and their direct exposure to the excreta of adults fed insecticides. First, we sought to determine if adult bed bugs affect the survivorship of 1st instars by assessing (1) the potential of adults to prolong survivorship by increasing relative humidity (i.e. blood-fed adult conditioning of the microhabitat), and (2) the effects of blood-fed or unfed adults on unfed 1st instar bed bugs (i.e. do nymphs gain nutritional benefits from adult excreta?). We then (3) evaluated the direct effects of exposure

of 1st instars to the excreta of adult male bed bugs fed insecticide. If secondary kill occurs in bed bugs, it could enhance the effectiveness of a liquid bait, once one is developed.

METHODS

Insects. An insecticide susceptible strain of *C. lectularius* (Harold Harlan strain, maintained since 1973 on human blood, and on defibrinated rabbit blood in our lab since 2008) and two pyrethroid resistant strains were used: Jersey City and Winston-Salem (collected in Jersey City, NJ and Winston-Salem, NC in 2008, and maintained in culture on defibrinated rabbit blood in our lab). Bed bugs were maintained in an incubator at 27°C and a photoperiod of 12:12 h (L:D). Virgin females and males were obtained by separating 5th instars from the colony and maintaining them in isolation through the adult molt.

Blattella germanica were from an insecticide susceptible laboratory colony (Orlando Normal = American Cyanamid strain, collected >70 years ago in Orlando, FL, and maintained in our lab since 1989) and a field collected fipronil-resistant strain (PR-712, collected in 2012 from an apartment in Carolina, Puerto Rico), that was selected in the lab for higher fipronil resistance (Ko et al. 2016). Both strains of *B. germanica* were provided ad libitum with water and rat chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO).

In Vitro Feeding System. Bed bugs were fed defibrinated rabbit blood (Quad Five, Ryegate, MT) in an artificial feeding system. Custom-built water-jacketed glass feeders were

connected to a thermal circulator water bath (B. Braun Biotech, Inc., Allentown, PA) heated to 37°C. Feeders could hold up to 4 ml of blood, which was held in place by a membrane stretched across the bottom of the feeder (NESCOFILM, Karlan, Cottonwood, AZ).

Experimental Chambers and Vials. Experimental chambers for relative humidity (RH) studies were 32 oz (946 ml) glass straight-sided jars with metals caps (Uline, Pleasant Prairie, WI). Saturated salt solutions were prepared to maintain uniform RH in each chamber. Reagent grade potassium nitrate (Product no. S25494A, Thermo Fisher Scientific, Waltham, MA), which was used to maintain 95% RH at 27°C, was prepared by dissolving 38.4 g of potassium nitrate in 86 ml of deionized water on a 300 °C hot plate stirrer (Thermo Fisher Scientific). Likewise, reagent grade magnesium nitrate hexahydrate (Product no. 423885000, Acros Organics, Thermo Fisher Scientific), which was to maintain 50% at 27°C, was prepared by dissolving 165 g of magnesium nitrate in 50 ml of deionized water. After the salt solutions cooled, a 6 cm section of PVC pipe (6 cm OD, United States Plastic Corp., Lima, OH) was placed in the bottom of each chamber. The pipe held an 8.3 cm diameter circular metal screen that separated the salt solution from the vials containing bed bugs.

A glass desiccator jar (Model 3081150, Corning, Inc., Corning, NY) with 150 g of Drierite anhydrous calcium sulfate (Product no. 23001, W.A. Hammond Drierite Co. Ltd., Xenia, OH) served as the experimental chamber to maintain 0% RH. A ceramic plate on top of the Drierite separated it from the bed bug vials. Vaseline petroleum jelly (Unilever, London, UK) was used to coat the edges of the desiccator lid to create a tight seal. HOBO Temperature and Relative Humidity Data Loggers (Model UX100-003, Onset Computer

Corporation, Bourne, MA), were used to monitor the temperature and RH within all experimental chambers.

Bed bugs were contained in 7 ml borosilicate glass scintillation vials (Thermo Fisher Scientific). A ~5 mm hole in the center of each vial cap allowed for ventilation through plankton netting fabric (0.3 mm mesh opening, 0.2 mm fabric thickness; BioQuip Products, Inc., Compton, CA). Another piece of plankton netting (28 mm x 4 mm) inside the vial served as a substrate for bed bugs to grasp.

Experimental Design. Five newly hatched 1st instar bed bugs were transferred into each experimental 7 ml vial using a sliver of filter paper (Whatman number 1, GE Healthcare Bio-Sciences, Pittsburgh, PA) held in a glass 100 μ l capillary (Thermo Fisher Scientific). Each daily cohort of hatched nymphs was distributed evenly among all five treatments for a total of 50 nymphs (10 vials) in each treatment. Treatments consisted of nymphs alone at 0%, 50%, and 95% RH, nymphs with two blood-fed virgin adults, and nymphs with two unfed virgin adults at 95% RH. Mortality of nymphs was recorded twice daily. The experiment ended when all nymphs were dead.

Co-habitation with fed or unfed adults. Virgin females or males (~7 d old) were blood-fed as a group and allowed to fully engorge for 30 min. Two fully fed females or males were then placed in each 7 ml experimental vial with five unfed nymphs. Every 5 d, adults were removed from the experimental vials and fed in pairs according to their experimental vials for 30 min. Care was taken not to disturb or remove the nymphs. If an adult died or did not feed, it was replaced with another virgin adult of the same sex.

Similar experiments were conducted with ~7 d old unfed virgin females or males. Adults were not removed from vials for the duration of the experiment, but dead insects were replaced.

First instar bed bug survivorship in the presence of insecticide-fed adults. Insecticide solutions were prepared according to the procedure described in Sierras and Schal (2016). Briefly, technical grade fipronil (88.7%, Bayer CropScience, Research Triangle Park, NC) and clothianidin (99.5%, Chem Service, Inc., West Chester, PA) were dissolved and serially diluted in dimethyl sulfoxide (DMSO). The AI in DMSO solutions were then mixed with defibrinated rabbit blood, resulting in a 0.75% DMSO in 2 ml blood.

Five 1st instar Harold Harlan bed bugs that remained unfed for 5-10 d since they hatched were placed into each 2 ml autoinjection vial (Thermo Fisher Scientific). A 17 mm x 4 mm manila folder insert was placed in the vial as substrate and the Teflon septum in the vial cap was replaced with plankton netting to allow for ventilation. In a separate treatment, five German cockroach 1st instars were starved for 24 h (but provided water) and transferred into 2 ml vials. Starved adult male bed bugs of the Winston Salem and Jersey City populations were allowed to feed for 20 min on blood containing fipronil or clothianidin, respectively, and an additional control group was fed on blood only. Immediately after feeding, groups of five fully engorged adult bugs were added to each 2 ml experimental vial containing either bed bug or cockroach nymphs. Mortality of nymphs of both species was monitored daily for 7 d. Dead adults and nymphs were not removed from experimental vials.

Nymph survivorship in the presence of insecticide-laden German cockroach feces.

Approximately 60 *B. germanica* adult males from the moderately fipronil-resistant PR-712 colony were starved for 24 h and then allowed to feed for 1 h on Maxforce FC Magnum (0.05% fipronil) (Bayer Environmental Science, Research Triangle Park, NC, EPA Reg # 432-1460). The males were then transferred to a 10 x 10 cm plastic box, (Althor Products, Windsor Locks, CT) the bottom of which was covered with four accordion-folded 65 mm diameter filter paper discs (Whatman number 1, GE Healthcare Bio-Sciences), provided with water, and allowed to defecate for 24 h. All bait-fed PR-712 males died during this 24 h period. The filter paper discs and a 0.5 ml microcentrifuge tube of water (Thermo Fisher Scientific) were then placed inside a 65 mm petri dish (Thermo Fisher Scientific). Ten 1st instar insecticide-susceptible Orlando Normal *B. germanica* nymphs and 10 insecticide-susceptible Harold Harlan *C. lectularius* 1st instars were placed in each of the four petri dishes. Nymph mortality of both species was recorded every 24 h for 7 d. The same procedure was followed with another group of PR-712 males fed rat chow instead of bait as control.

To reduce coprophagy, and therefore indirectly assess the importance of contact with feces, the same experiment was conducted as described above with another group of bait-fed PR-712 males and 10 *B. germanica* 1st instars in each of four petri dishes, but no bed bugs. However, this time along with water, a 0.5 g dollop of creamy peanut butter (Jif, The J.M. Smucker Company, Orrville, OH) was placed in each petri dish as an alternative, and

presumably preferable, food source to feces. A rat chow fed, instead of bait-fed, group was again used as control. Sample size in all feces assays was 40 1st instars of each species.

Data Analysis. Data were analyzed using a Kaplan-Meier survivorship curve (Parmar and Machin 1995) generated in SPSS 22 (IBM 2013). This option for analysis was chosen over regression due to the presence of censored data. The log-rank option was selected in order to identify differences among treatment groups and pairwise comparisons were conducted across treatments.

RESULTS

Effects of RH and fecal nutrients on nymph survivorship. Relative humidity affected the survivorship of first instar nymphs. Overall, the length of nymph survivorship varied with the percentage RH with significant differences among treatments ($\chi^2 = 64.40$; $df = 2$; $P < 0.0001$; Fig. 1A). Pairwise comparisons between the 0% and 50% RH groups ($\chi^2 = 54.55$; $df = 1$; $P < 0.0001$), and 0% and 95% RH groups ($\chi^2 = 30.32$; $df = 1$; $P < 0.0001$) were highly significant, but a pairwise comparison between the 50% and 95% RH groups was not significant ($\chi^2 = 1.02$; $df = 1$; $P = 0.31$). These results suggest that under low environmental RH conditions, fresh liquid excreta from recently-fed adults could increase RH within the microhabitat and increase survival of nymphs.

However, co-habitation of blood-fed adult males or females with unfed nymphs did not increase nymph survivorship. Indeed, pairwise comparisons indicated that 1st instars

survived longer alone ($\chi^2 = 121.90$; $df = 2$; $P < 0.0001$; Fig. 1B) than with blood-fed males ($\chi^2 = 86.74$; $df = 1$; $P < 0.0001$), but not with un-fed males ($\chi^2 = 2.39$; $df = 1$; $P = 0.12$). First instars did not live longer in the presence of adult females either ($\chi^2 = 148.79$; $df = 2$; $P < 0.0001$; Fig. 1C). In fact, pairwise comparisons suggest 1st instars lived shorter in the presence of either fed ($\chi^2 = 94.52$; $df = 1$; $P < 0.0001$) and un-fed ($\chi^2 = 5.43$; $df = 1$; $P = 0.02$) females than when alone. These results suggest that unfed 1st instar bed bugs would not benefit significantly from either the water or nutrients in the excretions of recently blood-fed adults. These results also predict that secondary kill might be minimal in bed bugs.

Secondary kill effects in bed bugs. To maximize the excretion of insecticide in feces we used adult bed bugs of the Winston Salem and Jersey City populations which are moderately resistant to fipronil and clothianidin, respectively (Sierras and Schal, unpublished data). Therefore, with the concentrations of AIs we used, these bugs are expected to fully engorge and defecate without succumbing to the AIs. Indeed, only ~5% adults died in the fipronil treatment and ~65% in the clothianidin treatment during the 7 d observation period, while 100% of Harold Harlan strain bed bugs died during this period (Sierras and Schal 2016). Survivorship of insecticide-susceptible (Harold Harlan) 1st instar bed bugs, however, was unaffected by the presence of adult bed bugs that were fed fipronil or clothianidin ($\chi^2 = 2.11$; $df = 2$; $P = 0.348$; Fig. 2A).

To bioassay the relative amount of AI in bed bug excreta, we exposed insecticide-susceptible German cockroach 1st instars, which are known to be highly susceptible to these AIs, to similarly treated bed bugs and their excreta. Notably, German cockroaches are much

more susceptible to starvation, and there was a general decline in survivorship in all treatment groups over the 7-d experiment (Fig. 2B). However, the presence of insecticide-fed adult bed bugs and their excrement did not specifically affect the survivorship of 1st instar German cockroaches. For bed bugs fed three concentrations of fipronil and two concentrations of clothianidin, an overall comparison showed a marginal difference among treatments ($\chi^2 = 11.46$; $df = 5$; $P = 0.043$; Fig. 2B). However, pairwise comparisons between insecticide-fed and the respective blood-only control did not reveal any significant differences in any of the fipronil treatments ($\chi^2 = 0.27$; $df = 1$; $P = 0.60$ for 5.5 ng/ml; $\chi^2 = 0.33$; $df = 1$; $P = 0.57$ for 10 ng/ml; $\chi^2 = 1.08$; $df = 1$; $P = 0.30$ for 25 ng/ml) or clothianidin treatments ($\chi^2 = 0.08$; $df = 1$; $P = 0.79$ for 43.4 ng/ml; $\chi^2 = 3.35$; $df = 1$; $P = 0.07$ for 86.8 ng/ml).

To further increase the excretion of insecticide in feces, we used moderately fipronil-resistant German cockroaches to generate fipronil-laden feces. All treated adults died within 24 h. An overall comparison of mortality in insecticide-susceptible 1st instar German cockroaches and bed bugs revealed a highly significant difference among treatments ($\chi^2 = 136.23$; $df = 3$; $P < 0.0001$; Fig. 3). The 1st instar *B. germanica* nymphs exposed to fipronil-laden feces died significantly more quickly than those exposed to normal feces ($\chi^2 = 49.76$; $df = 1$; $P < 0.0001$). Yet, the insecticide-susceptible 1st instar bed bugs in both groups were unaffected, with only one dead.

To determine whether mortality in 1st instar *B. germanica* was due to contact with or ingestion of feces, we offered the starved experimental nymphs peanut butter. While none of

the control peanut butter-fed nymphs died, only 20% of those exposed to both peanut butter and fipronil-laden feces survived ($\chi^2 = 10.40$; $df = 1$; $P = 0.001$). There was also a highly significant difference in survivorship in 1st instar *B. germanica* between groups presented with fipronil-laden feces and with fipronil-laden feces plus peanut butter ($\chi^2 = 34.48$; $df = 1$; $P < 0.0001$). It thus appears that secondary kill due to contact with fipronil-laden feces is negligible in bed bugs and slight in cockroaches, whereas coprophagy facilitates most of the secondary kill observed in cockroaches and none in bed bugs.

DISCUSSION

The direct effect of baits on population suppression are substantial, as evidenced by the effectiveness of bait formulations in a wide array of social and solitary insects. Nevertheless, in pest management of social insects (ants, termites, wasps) and cockroaches, delivery of bait to the colony and its distribution among colony members contribute significantly to effective pest management. Three common features to all these species are (a) aggregation of foragers with non-foragers, (b) chewing mouthparts that facilitate coprophagy, trophallaxis, and/or other means of sharing nutrients and microbes, and (c) fitness benefits accrued from these behaviors. Bed bugs also live in aggregations, but their piercing-sucking mouthparts may not be conducive to coprophagy and it is not known whether non-foragers benefit from forager-mediated delivery of nutrients and microbes. First, we investigated the effects of recently blood-fed adults on survivorship of unfed nymphs, and

then the effects of adult ingestion of large amounts of insecticides on survivorship in co-habiting nymphs.

Adults might benefit co-habiting nymphs by changing in the microhabitat environment and/or by delivering to nymphs fitness-promoting materials, such as nutrients and beneficial microbes. Given the 1st instar's high surface-to-volume ratio and thin cuticle, RH is expected to be an important factor. Indeed, unfed 1st instar bed bug nymphs lived longer at medium and high RH than at low RH. Nevertheless, they did show a remarkable tolerance to desiccation. These findings are not surprising since other studies found that 1st instars were most susceptible to desiccation (Usinger 1966, Benoit et al. 2007, Polanco et al. 2011). Aggregation increases the water retention abilities in 1st instars, and it follows therefore that the presence of blood-fed defecating adults could locally increase the RH and prolong first instar survivorship (Benoit et al. 2007). Moreover, nymphs might directly imbibe and thus benefit from copious excretions of adults. However, we did not specifically investigate the potential of adult excreta to locally increase RH in experimental vials, since we were more interested in addressing coprophagy. Instead, we chose to control for RH using saturated salt solutions in subsequent experiments with blood-fed and unfed adults.

Co-habitation with either blood-fed or unfed adults, however, did not affect the survivorship of 1st instar bed bugs. On the contrary, survivorship of nymphs was lower in the presence of fed adults. It is possible that our frequent manipulation of the experimental vials had a detrimental effect on nymphs, despite our efforts not to disturb them. We tried several approaches to allow adults to blood-feed while preventing nymphs in the same vial from

feeding, including using a thicker feeding membrane, multiple membrane layers, and using multiple layers of plankton mesh; but in all cases adults did not feed as readily. Because it was imperative that we maximize the number of adults that fully engorged, we opted to remove adults from vials every 5 d to re-feed them and then re-inserted the freshly fed adults into the respective vials containing nymphs.

It is also possible that, in addition to experimental factors, biological factors might have contributed to the decline in 1st instar survivorship in the presence of blood-fed adults. For example, 1st instars could have gotten stuck in feces deposited by adults as the fecal spots dried, rendering them immobile. Indeed, this was observed in several cases, but the phenomenon did not appear to be widespread (YKM, personal observation). Another possibility is that adults could disturb 1st instars while moving around. First instars have a thin cuticle (Polanco et al. 2011) that could easily be pierced by adult tarsal claws as they move around, leaving 1st instars susceptible to desiccation. The presence of either unfed males or females (who were not removed from experimental chambers) also slightly decreased survivorship of 1st instars, and pierced cuticle is a possible explanation for this observation.

The lack of survivorship benefits to nymphs from co-habitation with freshly blood-fed adults suggests that 1st instars likely do not accrue a macronutrient benefit from adults. Behavioral observations also indicate that starved adults and nymphs do not feed on isolated droplets of blood in a petri dish, even when placed on or directly next to the drop (YKM, personal observation), but they accept and feed readily on the artificial feeding system.

Starved bed bugs on occasion, however, have been observed to drink from free standing droplets of water (Benoit et al. 2007), although this does not appear to be a common behavior. Lacking a direct benefit of coprophagy, secondary kill from ingestion of adult excretions is therefore unlikely in bed bugs. This is in contrast to the German cockroach, where starved 1st instars live longer when allowed to feed on adult feces (Kopanic et al. 2001). It is also in contrast to the situation in the closely related triatomines that engage in coprophagy, hematophagy, and cannibalism. In fact, these behaviors are mechanisms of transmission for pathogens such as *Blastocrithidia triatomae* (Schaub et al. 1989) and triatoma virus (Muscio et al. 2000). Additionally, triatoma virus can be transmitted to uninfected bugs that feed on chickens whose skin had been previously contaminated with dried and liquid feces of infected bugs (Muscio et al. 2000).

The prediction that secondary kill would be minimal in 1st instar bed bugs was confirmed by directly exposing them to adult males fed various concentrations of fipronil in blood: survivorship was unaffected compared to nymphs exposed to unfed males or 1st instars not exposed to any adult bugs. These results indicate not only that 1st instar bed bugs are not ingesting adult fecal products, but also that they appear to be unaffected by close contact with insecticide-treated adults and insecticide-laden feces. Fipronil and clothianidin are potent AIs on bed bugs both by ingestion and contact (Sierras and Schal 2016). The fipronil ingested LC₅₀ values for adults and 1st instars of the Harold Harlan strain are 13.4 and 6.84 ng/ml blood, respectively; the LD₅₀ for adult males by topical application is 2.21 ng. For clothianidin the corresponding ingestion values are 14.2 and 20.7 ng/ml of blood for

adult males and 1st instars, respectively (Sierras and Schal 2016). It is possible that the maximum amounts of these two AIs that were ingested by the adult bed bugs were insufficient to kill 1st instars via contact. Using an empirically measured ingested volume of 3.92 μ l blood per male and 0.46 μ l blood per nymph, Sierras and Schal (2016) calculated LD₅₀ values for adult males and 1st instars of 52 pg/adult male and 3 pg/1st instar nymph. Thus, at the highest concentration of fipronil that we used (25 pg AI per μ l blood), a male would ingest 98 pg of fipronil. It is unknown how much AI adult bed bugs would defecate, but even 10% would be plenty to kill nymphs, if they ingested it. By contact alone, however, this is not nearly enough fipronil to kill bed bugs. Similar calculations using clothianidin (the highest concentration of AI we used was 86.8 ng/ml blood, which was 2-fold the d 3 LC₉₀ generated from probit analysis; calculated ingested 339 pg AI/adult male; calculated 2-fold the LC₉₀ or 40 pg/1st instar nymph) we reach a similar conclusion – nymphs would likely die if they ingested the AI but there was likely not enough AI in feces to kill nymphs by contact.

We attempted to increase the amount of AI in feces by feeding adult German cockroaches fipronil bait and exposing 1st instar bed bugs to insecticide-laden feces of German cockroaches. Here too, however, 1st instar bed bugs were unaffected. As calculated by Ko et al. (2016), the LD₅₀ of fipronil was 2.12 ng AI per adult male, so a male that ingested a typical daily intake of 2.5 mg (Hamilton and Schal 1988) of the bait (0.05% AI) would ingest 1,250 ng of fipronil, ~600-fold the LD₅₀. Again, if 10% of the ingested AI were defecated, the 125 ng of fipronil would be ~40-fold the amount needed to kill 1st instar bed bugs by ingestion. Yet, while 1st instar *B. germanica* were killed by these fipronil-laden

feces, 1st instar bed bugs, which are smaller and more susceptible to fipronil, did not die. The German cockroach nymphs died because they ingested the feces, whereas the bed bug nymphs did not. This inference is supported by the observation that fewer 1st instar cockroaches died when offered peanut butter in addition to fipronil-laden feces. Finally, their survival on relatively high concentrations of fipronil in feces may suggest that bed bugs might employ some other behavioral mechanisms to avoid contact with AI-laden feces.

The concept of secondary kill has gained prominence in urban pest management. In social insects, where trophallaxis and other food-sharing tactics are central features of the social organization, horizontal transfer of AIs and secondary kill are pivotal for colony elimination of pest insects. In sub- and non-social insects, however, the magnitude and efficacy of secondary kill varies with the biology of the pest, and especially with the degree of dependence of larvae on coprophagy. For example, cat flea (*Ctenocephalides felis* Bouché) larvae feed on the feces of adults, and the relatively undigested blood excreted in adult feces contributes to larval nutrition and development (Silverman and Appel 1994, Hsu et al. 2002). In fact, larvae that do not feed on adult feces do not achieve pupation (Bruce 1948, Moser 1989). Consequently, when flea larvae consume the feces of adults that fed on host blood containing insecticide, they in turn die if enough active ingredient is present in the feces. Systemic insecticides, such as avermectins, which are ingested by vertebrate hosts and then by adult fleas of both sexes (Rust 2005), serve both as adulticides and larvicides in fleas because of the dependence of larvae on adult feces, dried blood, and infertile eggs for nutrients. German cockroach nymphs are much less dependent on coprophagy than fleas.

However, the combination of frequent (daily) large meals by adults, rapid defecation of ingested baits, mandibulate mouthparts that can ingest solid feces, and the propensity of 1st instars to engage in some coprophagy all combine to facilitate high secondary kill.

Nevertheless, the significance of coprophagy and secondary kill has never been quantified in the field (Schal 2011). Bed bugs take even larger meals than cockroaches (relative to body mass), but meals are much less frequent and the feces can be imbibed by the piercing-sucking mouthparts only transiently while it is in liquid form. Moreover, the concentration of AI in solid and gel baits is substantially greater than in systemic use of the same AI to protect vertebrate hosts. As we progress toward the design of liquid baits for bed bugs and other hematophagous arthropods, such as mosquitoes, sand flies, fleas, and some flies, these factors must be taken into account to optimize secondary kill. A major advantage to an artificial bait over the use of a live host is the concentration of AI can be elevated markedly to facilitate greater secondary kill effects.

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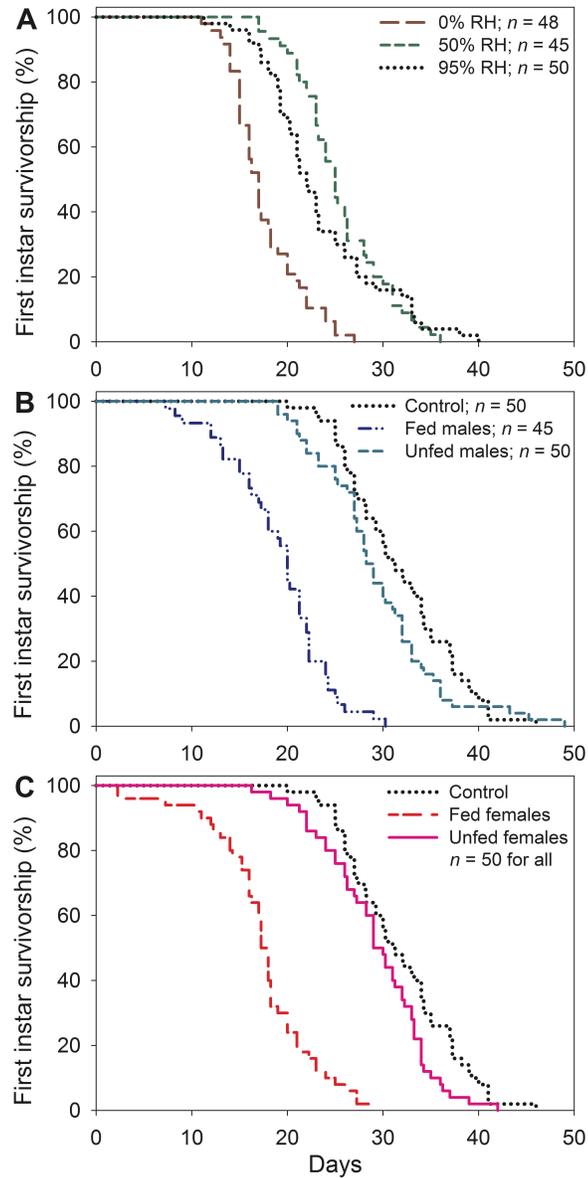


Figure 1. Kaplan-Meier survivorship curves of unfed 1st instars of *C. lectularius* alone under different relative humidity conditions (RH) conditions (A; $\chi^2 = 64.40$; $df = 2$; $P < 0.0001$), and in the presence of males (B; $\chi^2 = 121.90$; $df = 2$; $P < 0.0001$) and females (C; $\chi^2 = 148.79$; $df = 2$; $P < 0.0001$). Relative humidity was 95% in all treatments in (B) and (C).

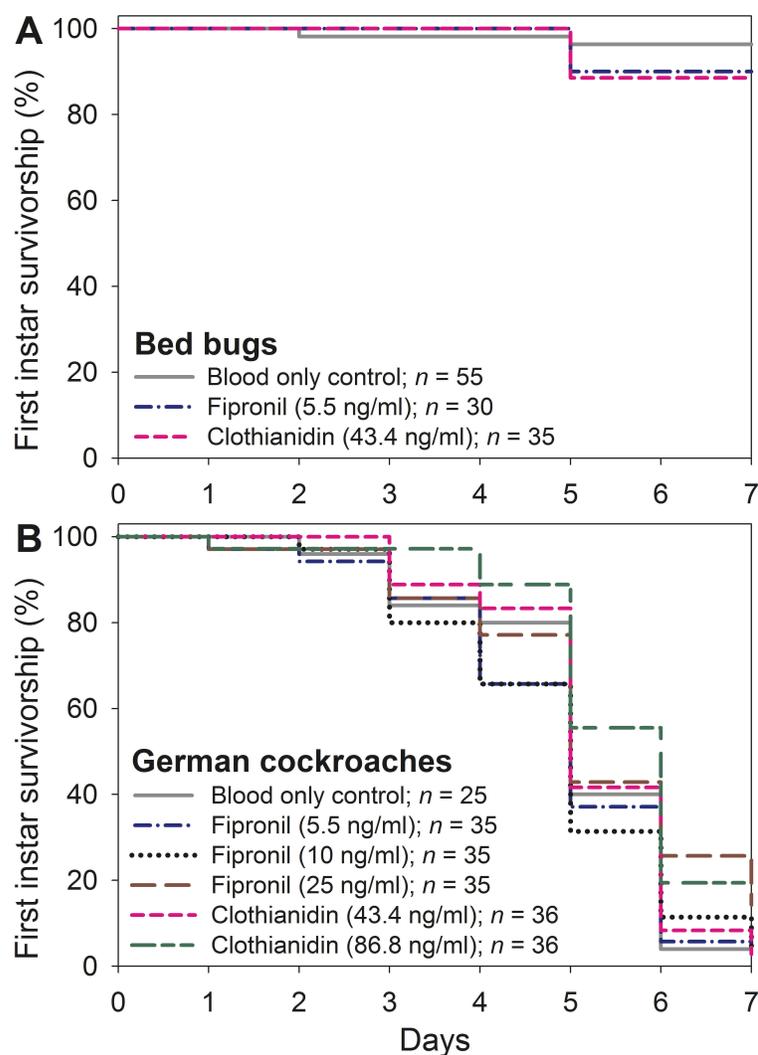


Figure 2. Kaplan-Meier survivorship curves of unfed 1st instars of *C. lectularius* (A; $\chi^2 = 2.11$; $df = 2$; $P = 0.348$) and *B. germanica* (B; $\chi^2 = 11.46$; $df = 5$; $P = 0.043$) in the presence of fipronil or clothianidin-fed adult male bed bugs. Each curve represents a different species and treatment combination. Concentrations denote the concentration of AI fed to adult male bed bugs.

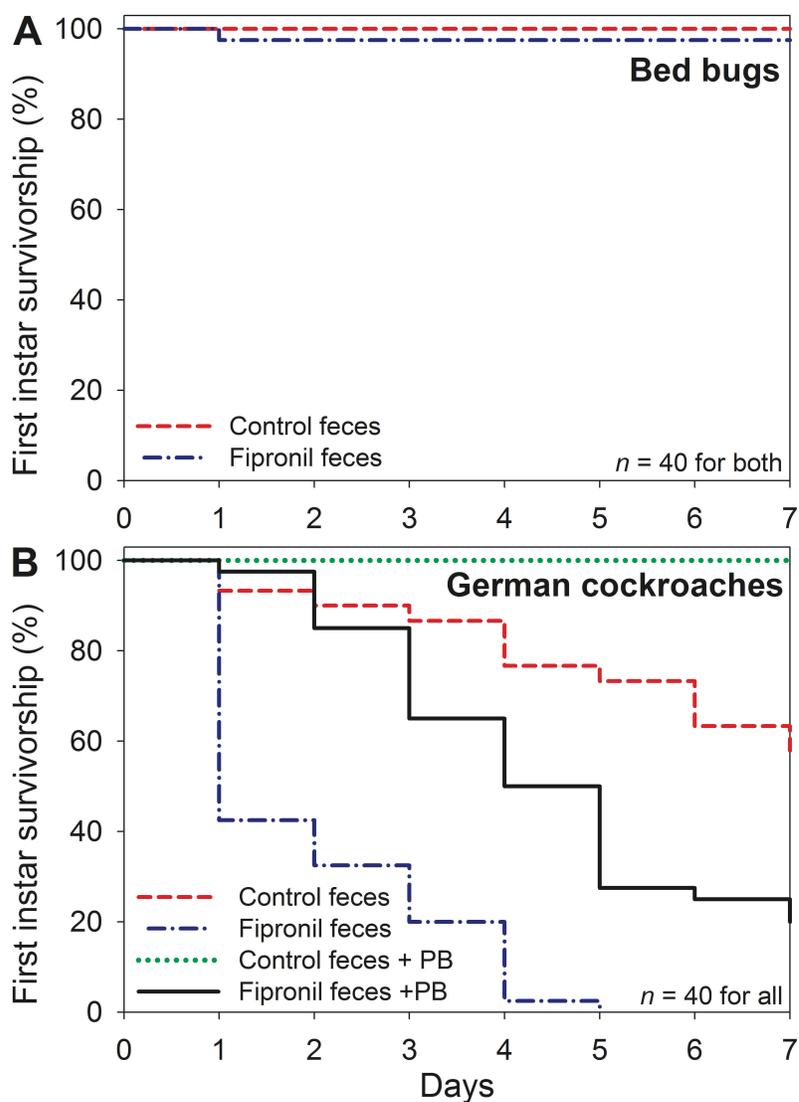


Figure 3. Kaplan-Meier survivorship curves of unfed 1st instars of *C. lectularius* and *B. germanica* exposed to insecticide-laden feces of German cockroach adult males ($\chi^2 = 136.23$; $df = 3$; $P < 0.0001$). Each curve represents a different species and treatment combination. PB is peanut butter offered as an alternative food to feces, and control feces do not contain insecticide.

CHAPTER 4.**Electroantennogram Responses and Field Trapping of Asian Cockroach (Dictyoptera: Blattellidae) with Blattellaquinone, Sex Pheromone of the German Cockroach (Dictyoptera: Blattellidae)**

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ABSTRACT

The Asian cockroach, *Blattella asahinai* Mizukubo, first introduced to Florida in 1986, has been spreading throughout the Southeastern U.S.A. Populations can reach extremely high densities and cause damage to crops as well as become a nuisance in residential settings. Since the German cockroach, *Blattella germanica* L., is its closest extant relative, we characterized the *B. asahinai* male response to blattellaquinone, the sex pheromone of the German cockroach, in an effort to develop monitoring tools for *B. asahinai*.

Electroantennogram (EAG) analysis was conducted on *B. asahinai* and *B. germanica* males and females, and revealed that the antennae of males of both species responded significantly more to blattellaquinone than females, and in both males and females absolute EAG responses of *B. asahinai* were greater than in *B. germanica* males and females, respectively. However, normalized male EAG response curves and ED₅₀ values did not differ significantly between the two species. Results of field trapping experiments demonstrated that male *B. asahinai* were more attracted to blattellaquinone than any other life stage, and 10 µg of blattellaquinone attracted the most males. These results suggest that blattellaquinone or a similar compound might be a component of the sex pheromone of *B. asahinai* females.

KEY WORDS *Blattella asahinai*, *Blattella germanica*, sex pheromone

The Asian cockroach, *Blattella asahinai* Mizukubo, first described in 1981 in Okinawa, Japan (Mizukubo 1981), was introduced into Florida in 1986 (Roth 1986) and has since been spreading throughout Florida and the U.S. Southeast along major highway routes to Alabama, Georgia, South Carolina, and Texas (Koehler 1999, Sitticharoenchai 2002, Pfannenstiel et al. 2008, Snoddy and Appel 2008). Populations of the Asian cockroach can reach high densities and adults often enter homes when attracted to incandescent lights (Brenner et al. 1988). In addition to being a nuisance in residential settings, *B. asahinai* has been found in densities of up to 54,000 per acre in strawberries and it damages fruit by creating excavations (Price and Nagle 2008). The Asian cockroach has also been observed to feed on parasitized brown citrus aphids in citrus groves (Persad and Hoy 2004), preventing the emergence of adult parasitoids. However, this invasive species has been observed to perform beneficial functions, such as feeding on the eggs of lepidopteran pests in soybean (Pfannenstiel et al. 2008).

The closest extant relative of the Asian cockroach is the German cockroach, *Blattella germanica* L. (Roth 1985). The two species are very difficult to differentiate using external morphology (Roth 1986, Lawless 1999), but can be easily distinguished by their ecology and behavior (Brenner et al. 1988). Unlike the German cockroach, which lives exclusively indoors and cannot fly, the Asian cockroach lives outdoors where it inhabits leaf litter, and can fly readily (Brenner et al. 1988).

Females of many cockroach species, including several pest species, produce volatile sex pheromones that attract males (review: Gemeno and Schal 2004). These pheromones

have been used to detect the presence of pest cockroach species, monitor population levels, and enhance attractiveness of traps containing insecticide (Bell et al. 1984, Liang et al. 1998). The major component of the female volatile sex pheromone of the German cockroach, blattellaquinone, attracts German cockroach males in olfactometer assays and lures those males to traps in the field (Nojima et al. 2005). Closely related species sometimes use similar or identical components in their sex pheromones, resulting in their co-attraction to pheromone-baited traps (Elkinton et al. 2010, Eliyahu et al. 2012). Since *B. asahinai* is most closely related to *B. germanica* and the two species hybridize in the laboratory (Roth 1986, Ross 1992), it is possible that *B. asahinai* males may respond to blattellaquinone.

The aims of this study were to (1) characterize and compare the antennal responses to blattellaquinone of *B. asahinai* and *B. germanica* males and females using electroantennogram (EAG), and (2) to determine if blattellaquinone is effective in attracting *B. asahinai* to traps in the field. If blattellaquinone is attractive to *B. asahinai* in the field, it may be a useful tool for detection and monitoring population levels.

Materials and Methods

Insects. *Blattella germanica* were taken from the lab colony (Orlando Normal = American Cyanamid strain, collected >60 years ago in Florida; in our culture since 1989) and provided continuously with rat chow (LabDiet 5001, PMI Nutrition International,

Brentwood, MO) and water. Cockroaches were raised in an incubator under a 12:12 LD cycle at 27° C and 40-70% relative humidity.

Blattella asahinai were collected from a recently established field population on the North Carolina State University main campus in Raleigh, NC, and maintained in the lab in an incubator with a 14:10 LD cycle at 27° C and 45-55% relative humidity. Cockroaches were provided continuously with rat chow and water.

Electroantennogram (EAG). Adult male and female *B. asahinai* of unknown ages and 7-9 day old adult male and female *B. germanica* were obtained from the lab colonies. Males and females of each species were isolated in the laboratory for three days. An antenna was excised above the scape and the first distal segment of the antennal tip was removed. The base of the antenna was mounted into a pulled glass micropipette containing *B. germanica* saline solution (Kurtti and Brooks 1976) and a gold wire electrode connected to ground. The antenna tip was mounted into a pulled glass micropipette containing *B. germanica* saline and a gold wire electrode connected to a high impedance universal single ended probe (Syntech, Kirchzarten, Germany). An IDAC-2 amplifier (Syntech) was coupled to a PC and EAG Pro Version 1.1 (Syntech) for data acquisition, display, and analysis.

A stock blattellaquinone (synthesized per Nojima et al. 2005) solution was dissolved in dichloromethane and serially diluted in hexane in decade steps from 10 µg/µL to 100 pg/µL. Ten µL of each solution was applied to filter paper strips (Whatman #1, GE Healthcare Bio-Sciences, Pittsburgh, PA) and allowed to evaporate. Ten µL of hexane served as negative control. Filter papers were loaded into glass Pasteur pipettes and presented to

each antenna in ascending order of dosage. A hexane control was presented first followed by blattellaquinone doses, and then hexane again. A 60 second recovery time was allowed between stimuli. Two *B. asahinai* and two *B. germanica* antennae were tested with each filter paper set, alternating each species. The species presented with odorants first alternated each time a new set of filter papers was made. Ten antennae were tested for each species and each sex.

Medical grade breathing air from a tank was routed through flow regulators, humidifiers, and a three-way electronic valve that controlled air delivery to the stimulus in the Pasteur pipette. The total flow rate was 650 mL/min, and 400 mL/min were diverted to the stimulus pipette for 0.3 sec to create a 2 mL stimulus puff. The stimulus and general airflow were recombined in an 8 mm ID stainless steel tube that delivered the stimulus to the antenna. The antenna was held 2-3 mm from the end of the tube.

Field Trapping. Blattellaquinone (100 μ L of 0.01 μ g/ μ L and 0.1 μ g/ μ L in hexane) was loaded into rubber septa (11 mm sleeve stopper, Wheaton, Millville, NJ) and allowed to evaporate to create 1 μ g and 10 μ g lures. Control rubber septa lures were loaded with 100 μ L of hexane. Lo-line traps (B&G, Jackson, GA) were used to trap *B. asahinai* in the field. Traps were placed approximately 50 cm (low traps) or 135 cm (high traps) above the ground on trees and were attached using push pins. One trap of each of three treatments (control, 1 μ g, and 10 μ g) was placed at each site, with a total of five replicates for low traps and three replicates for high traps. Traps were placed at least 7 m apart at a total of six sites, and sites were between 15 m and 80 m apart. Traps were placed at approximately 5 pm on August 23,

2011 on a clear night with little rain and were collected at approximately 8:30 am on August 24, 2011. Trapping was done in wooded areas with dense leaf litter on the North Carolina State University main campus (Raleigh, NC) along railroad tracks that run through campus between (-78.67086, 35.78490833) and (-78.66815333, 35.78394667).

Data Analysis. EAG responses (in millivolts) were obtained from the EAG Pro software as the maximum EAG amplitude. Analysis of differences in absolute EAG responses between species was carried out using SAS Version 9.4 (PROC GLM, SAS Institute 2013). Base-10 logarithms of the millivolt EAG response were taken in order to normalize the data for two-way ANOVA. The GLM procedure was used to find the least squares means with the Tukey adjustment for multiple comparisons. Analysis was run separately for males and females. Analysis of differences in absolute EAG responses within each species was carried out using JMP 11 Pro (SAS Institute 2013). Millivolt responses were log transformed and analyzed by one-way ANOVA with a Tukey HSD test to compare means. Analysis was run separately for each species and sex.

The millivolt EAG responses of males were normalized relative to the response to the hexane control for each replicate. SAS Version 9.4 was used to fit a four-parameter logistic model to the data using the NLIN procedure. A separate model was fit for each species, and ED₅₀ values (effective dose to elicit 50% of maximal response) were derived.

Field trapping data were analyzed using JMP 11 Pro. A Generalized Linear Model with Poisson distribution was fit to the data. Differences among trap position, dose, and life

stage, were determined using the Contrast platform command in a pairwise manner by setting the contrast levels to 1 and -1, respectively, for the pair being compared.

Results

Electroantennogram Responses. Antennae of both species responded to blattellaquinone in a dose-dependent manner with two clear patterns: as expected for a sex pheromone, male antennae were much more responsive than female antennae, and surprisingly the absolute EAG responses of *B. asahinai* males were higher than *B. germanica* males at all doses of blattellaquinone as well as the hexane control (Fig. 1). However, differences in absolute male EAG response between the two species were significant only at the doses of 0.001 μg ($P = 0.047$), 0.01 μg ($P = 0.026$), and 0.1 μg ($P = 0.008$) (two-way ANOVA $F = 34.29$; $df = 13,126$; $P < 0.0001$) (Fig. 1A). The dose-response curves indicated significant differences in EAG responses among doses for *B. asahinai* male antennae ($F = 19.68$; $df = 6,63$; $P < 0.0001$) and *B. germanica* male antennae ($F = 57.58$; $df = 6,63$; $P < 0.0001$; Fig. 1A). In *B. asahinai* males, the response to 10 μg and 100 μg doses was significantly higher than all other doses except 1 μg . In *B. germanica* males, the response to 10 μg and 100 μg doses was significantly higher than to all other doses. Males of both species did not respond significantly to doses $< 1 \mu\text{g}$.

After the male EAG responses were normalized relative to their respective hexane controls, the logistic fit dose-response curves of the two species were similar. The estimated

ED₅₀ of blattellaquinone for *B. asahinai* males was 0.94 µg, which did not differ significantly from the blattellaquinone ED₅₀ for *B. germanica* males (1.27 µg) (Fig. 1B; Table 1). The estimated slopes for *B. asahinai* (1.02) and *B. germanica* (1.33) were also similar, suggesting that the two normalized curves did not differ significantly.

Female antennae of both species responded much less to blattellaquinone than male antennae, but as in males, the absolute EAG responses of *B. asahinai* females were higher than *B. germanica* females to the hexane control ($P = 0.018$) (Fig. 1C) and to all doses except 10 µg (0.001 µg, $P = 0.001$; 0.01 µg, $P = 0.030$; 0.1 µg, $P = 0.0002$; 1 µg, $P = 0.002$; and 100 µg, $P = 0.007$) (two-way ANOVA $F = 16.27$; $df = 13,126$; $P < 0.0001$) (Fig. 1C). The dose-response curves had significant differences in EAG responses among doses for *B. asahinai* female antennae ($F = 10.37$; $df = 6,63$; $P < 0.0001$) and *B. germanica* female antennae ($F = 6.82$; $df = 6,63$; $P < 0.0001$; Fig. 1C). The highest doses (10 and 100 µg) elicited significantly greater responses in *B. asahinai* and *B. germanica* females than all other doses except 1 µg. Females of both species did not respond significantly to doses < 10 µg blattellaquinone.

Field Trial. Traps were attached to tree trunks at two heights, 50 cm (low traps) and 135 cm (high traps) above the ground (Fig. 2). The highest mean number of *B. asahinai* captured in traps was males at the 10 µg dose of blattellaquinone, both in the high and low trap positions (Fig. 3). The generalized linear model fit to the trapping data was highly significant ($\chi^2 = 134.67$; $df = 17$; $P < 0.0001$). Pairwise comparisons of each life stage captured at each dose indicated that significantly more males were captured at the 10 µg dose than almost any other life stage and dose ($P < 0.0001$ for most). However, male trap catch at

the 10 µg dose high positioned traps was not significantly more than nymphs captured by the 10 µg dose low traps ($P = 0.075$). Additionally, although more males at the 10 µg dose were captured in traps at the low position than the high position, the difference in mean trap catch for males was not significant ($P = 0.093$). Few nymphs and females were captured, and only in the low traps at 50 cm.

Discussion

Blattellaquinone is a major component of the volatile sex pheromone of *B. germanica* females that attracts males (Nojima et al. 2005). Since *B. germanica* is the closest extant relative of *B. asahinai* and the two species hybridize in the laboratory (Roth 1986, Ross 1992), we hypothesized that blattellaquinone might elicit EAG responses from the antennae of *B. asahinai* in a sex-specific manner. If so, we would also expect that blattellaquinone might attract males in the field and therefore could be developed as a tool to detect and monitor *B. asahinai* populations. A recent invasion and persistence over several years (YKM, personal observation) of a highly localized *B. asahinai* population near railroad tracks and a construction site at North Carolina State University allowed us to test these hypotheses.

Antennae of adult male *B. asahinai* and *B. germanica* exhibited clear dose-dependent EAG responses to blattellaquinone (Fig. 1A). The antennae of adult females of both species also responded to blattellaquinone, but with much lower EAG responses than males (Fig. 1C). These results show that the *B. germanica* sex pheromone also elicits sex-specific EAG

responses from *B. asahinai* males. Surprisingly, the absolute EAG responses of *B. asahinai* males and females were significantly higher, respectively, than the EAG responses of male and female *B. germanica*. However, when male responses of both species were normalized to the respective hexane control responses, there was no significant difference in the ED₅₀ values or slopes of normalized curves of the two species (Fig. 1B). Thus, both species have similar antennal responses to blattellaquinone, suggesting that blattellaquinone might be a component of the female volatile sex pheromone of *B. asahinai*. However, the reproductive biology and chemical ecology of *B. asahinai* have not been investigated, and it has not been observed whether females engage in calling behavior and emit a volatile pheromone, as do *B. germanica* females (Liang and Schal 1993a, 1993b); if they do, the identification of this pheromone warrants further study.

Deployed in Lo-line sticky traps, blattellaquinone attracted more male *B. asahinai* in the field than any other life stage. However, trap catch was low, despite numerous *B. asahinai* active at each of the collection sites (YKM, personal observation). Two possible reasons for low catch include (a) low trap efficiency and (b) incomplete pheromone blend. In preliminary studies, we placed sticky traps at various heights on tree trunks, attached them to low stakes vertically and horizontally, and placed them horizontally on top of leaf litter. We also tested other styles of traps baited with blattellaquinone, including plastic food storage containers with sections of the sides removed, and plastic two-liter soda bottles with the top third removed, inverted, and reinserted into the bottle to form funnel traps. Additionally, we tested food attractants such as peanut butter, but no trap and food attractant combination

performed as well as blattellaquinone on Lo-line sticky traps. Higher doses of blattellaquinone, such as 100 μg and 1000 μg were tested in preliminary trials, but did not yield significantly higher trap catches than the 10 μg dose. *Parcoblatta lata* (Brunner) males, which also fly like *B. asahinai* males, were attracted to a similar formulation of parcoblattalactone, the female sex pheromone, in sticky traps fastened vertically to tree trunks (Eliyahu et al. 2012). However, it is possible that another trap design or trap placement would be more efficacious at capturing *B. asahinai* males.

Low trap catch could more likely be attributed to an incomplete pheromone blend. In Lepidoptera, closely related moth species often share major sex pheromone components, which alone fail to attract males, and species specificity is encoded in a multi-component sex pheromone blend (e.g., Sasaerila et al. 2000, Lelito et al. 2008). In contrast, for some cockroach species, including the German cockroach, brownbanded cockroach (*Supella longipalpa* F.), broad wood cockroach (*P. lata*), and American cockroach (*Periplaneta americana* L.), a single compound in the pheromone blend is sufficient to elicit the full behavioral response from males (Seelinger and Gagel 1985, Charlton et al. 1993, Nojima et al. 2005, Eliyahu et al. 2012, reviewed in Gemeno and Schal 2004). Nevertheless, in some of these cockroach species males respond better to a blend of multiple sex pheromone components. For example, a complete sex pheromone blend of major component periplanone-B and minor component periplanone-A elicits optimal odor source localization responses in *P. americana* males (Seelinger and Gagel 1985). It is possible that female *B. asahinai* produce more than one behaviorally active pheromone compound. That blend may

be necessary to achieve optimal attraction of males. Additionally, EAG alone cannot inform us whether blattellaquinone could be a major or minor component of the *B. asahinai* female sex pheromone. For example, *P. americana* males showed similar amplitude EAG responses to major component periplanone-B and minor component periplanone-A (Nishino and Manabe 1983, Yang et al. 1992). Further studies running female pygidium extracts on gas chromatography coupled to electroantennographic detection (GC-EAD), and behavioral assays could elucidate the presence and role of minor components in the *B. asahinai* female sex pheromone.

Cockroach sex pheromones have not been as extensively studied as sex pheromones from other insect taxa. Interestingly, sex pheromones identified to date represent unique chemical structures across different cockroach taxa (review: Gemeno and Schal 2004). However, in the genus *Periplaneta*, several variations on the compound periplanone have been identified as volatile sex pheromones in several species (Gemeno and Schal 2004). No chemical ecology studies exist on olfactory sexual communication in *Blattella* species other than *B. germanica*, so it is possible that the major component of the *B. asahinai* female sex pheromone is closely related to blattellaquinone, such that blattellaquinone is sufficient to attract males in the field, but does not perform as well as the authentic *B. asahinai* female sex pheromone.

Our results indicate that *B. asahinai* males respond to blattellaquinone, the female sex pheromone of *B. germanica*. These results suggest that *B. asahinai* females might emit a similar sex pheromone and its proper identification could provide an attractive lure for this

species and prove useful for detection and monitoring Asian cockroach populations. *Blattella* is a diverse genus with 51 species (Roth 1985, 1997) that include pest and non-pest species with diverse ecologies and reproductive biology traits. Elucidating their sex pheromones will facilitate a deeper understanding of species relationships within this important genus.

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Table 1. Electroantennogram (EAG) responses of adult male *B. asahinai* and *B. germanica* antennae to blattellaquinone.

Species	<i>n</i>	Slope \pm SE (95% CL)	ED ₅₀ μ g (95% CL)	<i>F</i>	<i>P</i>
<i>B. asahinai</i>	10	1.02 \pm 0.51 (0.003, 2.045)	0.94 (0.118, 1.755)	39.83	< 0.0001
<i>B. germanica</i>	10	1.33 \pm 0.50 (0.325, 2.332)	1.27 (0.748, 1.789)	132.80	< 0.0001

EAG responses were normalized relative to the hexane control response for each antenna and four-parameter logistic models were fit to the data (PROC NLIN; SAS 2013).

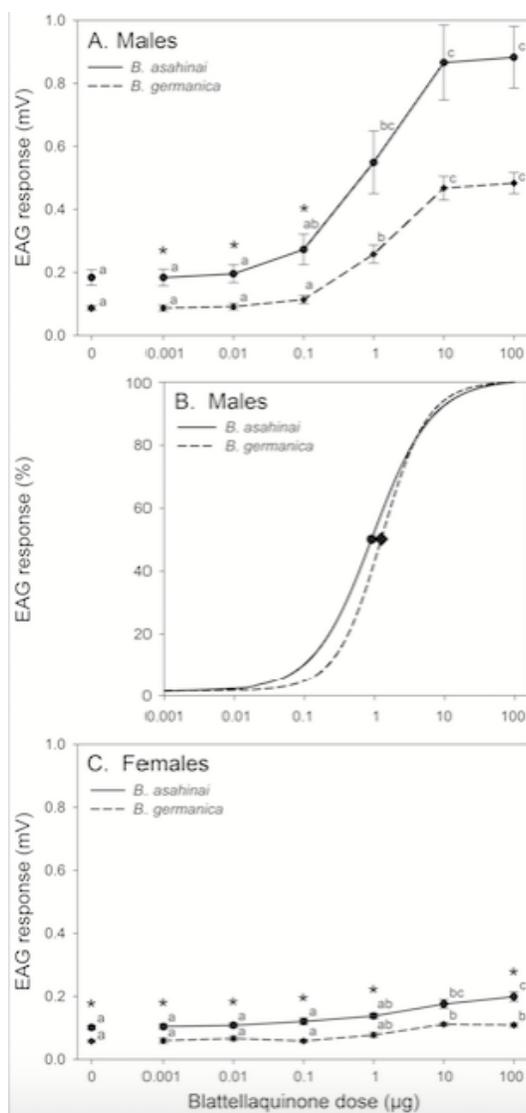


Fig. 1. Mean (\pm SE) electroantennogram (EAG) responses of *B. asahinai* and *B. germanica* to blattellaquinone applied to filter paper. (A) Absolute male response; (B) Normalized EAG responses for males; (C) Absolute female response. In A and C, the peak EAG amplitude is shown in millivolts. Doses with the same letter are not significantly different within each species for each sex. Asterisks denote a significant difference at a given dose between species for each sex. Sample size was 10 antennae for each species and sex. In (B), responses

were normalized as percentages relative to the hexane control for each antenna. The ED_{50} values, denoted by symbols, and the slope of the curve for each species are not significantly different between species (see Table 1). Curves were generated with four-parameter logistic models.



Fig. 2. Blattellaquinone baited Lo-line traps, low position (50 cm) and high position (135 cm) above the ground, in the field site at North Carolina State University, Raleigh, NC, August 2011.

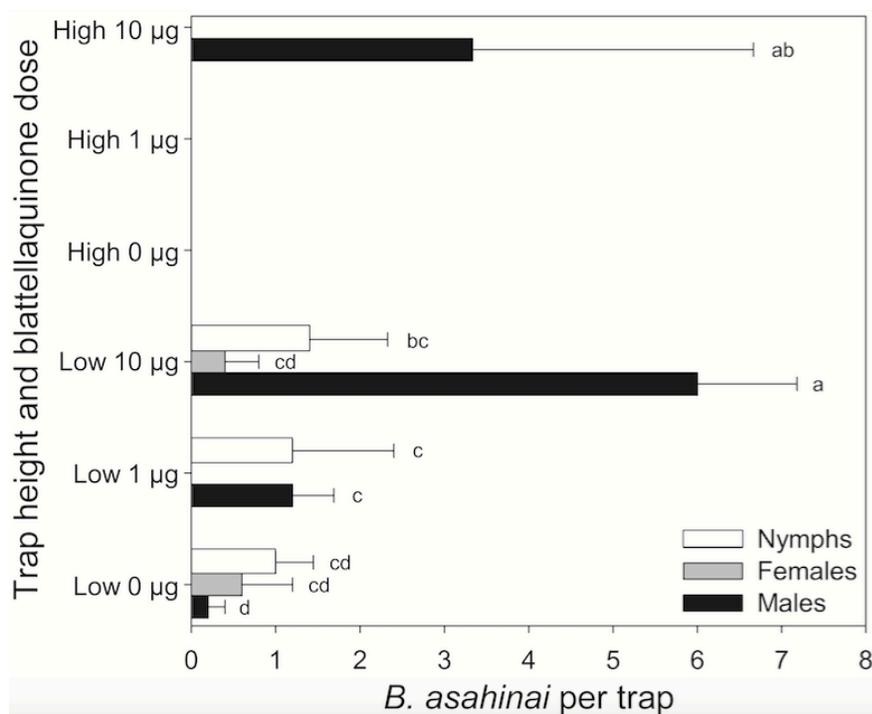


Fig. 3. Mean (\pm SE) *B. asahinai* captured in high (135 cm) and low (50 cm) positioned Lo-line traps, August 2011. Sample size is three for high traps and five for low traps. Bars indicate trap catches for nymphs, adult females, and adult males. Bars with the same letters are not significantly different. A generalized linear model with Poisson distribution was fit to the data.

CHAPTER 5.**Laboratory and Field Evaluation of Zyrox Fly Granular Bait Against Asian and German Cockroaches (Dictyoptera: Blattellidae)**

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ABSTRACT The Asian cockroach (*Blattella asahinai* Mizukubo) was introduced to Florida in 1986 and has since spread throughout the Southeastern U.S. *B. asahinai* is a peridomestic pest and high population densities in residential areas can become a nuisance, especially when adults fly into homes. Few studies to date have been conducted on Asian cockroach control, and we evaluated the efficacy of Zyrox Fly Granular Bait and Maxforce Complete Granular Insect Bait against this species in the laboratory compared to the closely related German cockroach (*Blattella germanica* L.). In no-choice and two-choice assays with both species, Zyrox bait and Maxforce bait achieved nearly 100% mortality within two and five days, respectively. We also tested Zyrox bait against *B. asahinai* in an invasive field population in North Carolina at the label rate (2 g/m²) and at approximately three times the label rate (6.9 g/m²), and found that broadcast applications at both rates reduced populations by an average of 64% and 92%, respectively, for 35 days after the initial application. Zyrox Fly Bait appears to be effective against the Asian and German cockroaches, and could be another tool in an IPM program, if its label could be extended or the active ingredient (cyantraniliprole) formulated into a cockroach bait.

KEY WORDS *Blattella asahinai*, *Blattella germanica*, Zyrox Fly Granular Bait, cyantraniliprole, Maxforce Complete Granular Insect Bait, hydramethylnon

The Asian cockroach (*Blattella asahinai* Mizukubo), first described in 1981 in Okinawa, Japan (Mizukubo 1981), was introduced into Florida in 1986 (Roth 1986) and has been spreading throughout Florida and the Southeastern U.S. along major highway routes to Alabama, Georgia, South Carolina, and Texas (Koehler 1999, Sitthicharoenchai 2002, Pfannenstiel et al. 2008, Snoddy and Appel 2008). *Blattella asahinai* frequently inhabits leaf litter and dense grass in shaded areas (Brenner et al. 1988), and mulch with small to medium interstitial spaces (Snoddy and Appel 2013). Populations of the Asian cockroach can reach high densities in residential settings where adults may often fly into homes when attracted to lights, becoming a nuisance (Brenner et al. 1988). Additionally, *B. asahinai* can damage strawberry fruit by creating excavations, and densities of up to 54,000 per acre have been reported in Florida (Price and Nagle 2008). The Asian cockroach has also been found in citrus groves, where it has been observed to prevent the emergence of parasitoids by feeding on parasitized brown citrus aphids (Persad and Hoy 2004). Nevertheless, because *B. asahinai* also reaches exceptionally high densities in soybean fields in Texas and it is an efficient predator of lepidopteran eggs, it may be considered a beneficial insect in this and related crops (Pfannenstiel et al. 2008).

Few studies have been conducted on control of Asian cockroach populations, although they appear to be susceptible to most insecticides (Valles et al. 1999). Recently however, several insecticides readily available to consumers were evaluated against *B. asahinai*, revealing that fipronil granules and β -cyfluthrin EC are effective in achieving significant reduction in field population levels (Snoddy and Appel 2014), and a suggested

IPM program has been developed (Snoddy 2012). A common control method of other peridomestic pest cockroach species, such as the smokybrown cockroach (*Periplaneta fuliginosa* Serville), is to treat a narrow band around the perimeter of a residence with a broad-spectrum insecticide (Appel and Smith 2002). However, these perimeter sprays can cause large populations to build up directly outside the treated band and the insecticide can degrade rapidly, especially during hot summer months when cockroach populations are highest (Appel and Smith 2002).

In the past few decades, baits have become an increasingly popular and effective control method for cockroaches and other urban insect pests. Baits are preferred because they use smaller amounts of active ingredient and are longer lasting than residual sprays, have low mammalian toxicity, are odorless, and can be used in a diverse range of conditions (Reierson 1995). Moreover, effective active ingredients that cannot be labeled in spray formulations or are photolabile have been formulated in baits (Harpaz 1987). One of the newest active ingredients, cyantraniliprole, is a second-generation anthranilic diamide insecticide (Selby et al. 2013). Cyantraniliprole has low mammalian toxicity. It binds to ryanodine receptors causing calcium ions to be depleted, thus interfering with muscle contraction and leading to paralysis and death (Selby et al. 2013). Cyantraniliprole is being used in agricultural crops against lepidopteran, hemipteran, and coleopteran pests (Selby et al. 2013), and has now been incorporated into a new granular bait for use against the house fly (*Musca domestica* L.), which causes significant reduction in lab and field populations (Murillo et al. 2014). A cyantraniliprole-containing bait, Zyrox Fly Granular Bait (Syngenta, EPA Reg # 100-1541)

is registered for use in commercial and residential areas (Syngenta 2015), the same types of areas that *B. asahinai* frequently inhabit.

The aims of this study were to evaluate the efficacy of Zyrox Fly Granular Bait against *B. asahinai* (1) in the laboratory compared to the closely related German cockroach (*Blattella germanica* L.) and (2) in the field. If Zyrox bait is effective in reducing *B. asahinai* populations in the field, it could be used as a novel control method for this nuisance pest in residential and commercial settings and could reduce the use of broad spectrum insecticides.

Materials and Methods

Insects. *Blattella germanica* were from a laboratory colony (Orlando Normal = American Cyanamid strain, collected >60 years ago in Florida, maintained in the Schal lab since 1989) provided ad libitum with rat chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and water. Cockroaches were maintained at 40-70% relative humidity at 27°C in an incubator with a 12:12 LD cycle.

Blattella asahinai were maintained in laboratory colonies (originally field collected from Auburn, AL and Florida) and provided continuously with rat chow and water. Cockroaches were raised in an incubator at 27°C with a 14:10 LD cycle and 45-55% relative humidity.

Laboratory Assays of Mortality. *Insecticides.* In laboratory assays, we tested Zyrox Fly Granular Bait (0.5% cyantraniliprole) (Syngenta Crop Protection, Greensboro, NC, EPA

Reg # 100-1541) and Maxforce Complete Brand Granular Insect Bait (1% hydramethylnon) (Bayer Environmental Science, Research Triangle Park, NC, EPA Reg # 432-1255), an industry standard for cockroach control.

Experimental Design. *Blattella germanica* males ranged in age from 10-30 days. The age was unknown for *B. asahinai* males, but only males with no apparent damage and full-length antennae were used. Males were separated into treatment groups, provided with water, and starved for 24 hours prior to the start of assays. For *B. germanica*, equal numbers of same-aged insects were put into each treatment group. Equal proportions of *B. asahinai* from the Florida and Alabama colonies were put into each treatment group.

No-choice Assay. Assays were set up in 15 cm x 26 mm petri dishes (Thermo Fisher Scientific, Waltham, MA) with the bottom third of an egg section of an egg carton for harborage, a glass test tube (10 x 75 mm disposable culture tube, Thermo Fisher Scientific) filled with water and plugged with cotton, and a cap for a 7 mL glass scintillation vial (Thermo Fisher Scientific) which held approximately 0.5 g of bait or rat chow. The egg carton and vial cap were held in place with labeling tape without leaving any part of the sticky side exposed, and the water was held in place with modeling clay (Crayola, Easton, PA). Treatment groups received Zyrox bait, Maxforce bait, or rat chow crushed to the approximate size of bait granules as a negative control.

Males were anesthetized briefly for 10 minutes in a refrigerator, and released into assay arenas. Thirty *B. asahiani* were assigned to the control and the Zyrox bait treatments, and 23 were assigned to the Maxforce treatment. Fifty *B. germanica* were assigned to each

treatment group. Insects were kept in a walk-in incubator at 27°C with a 12:12 LD cycle and 40% relative humidity. Mortality was monitored every 2 hours for the first 8 hours, and 20-24 hours. After 24 hours, mortality was monitored three times daily. Individuals that could not right themselves or grasp the egg carton harborage were considered dead. Dead individuals were left in the arenas. Assays were terminated when all individuals in the bait treatment groups were dead.

Two-choice Assay. Two-choice assays received the same setup as the no-choice assays, except with two vial caps on opposite ends of the arena equidistant from the egg carton harborage and water. The first treatment group received Zyrox bait and crushed rat chow. The second treatment group received Maxforce bait and crushed rat chow. The final treatment (negative control) received two vials of crushed rat chow. The same number of insects was used as in the no-choice assays, with one exception: one *B. asahinai* rat chow treatment served as the control for both the no-choice and two-choice assays, since they were conducted concurrently.

Ingestion vs. Contact Assay. Assays were conducted to differentiate ingestion of bait from contact using the same setup as the no-choice assay. These assays were conducted only with *B. germanica*, and males were starved for 18 hours before their mouthparts were glued to prevent ingestion. Males were anesthetized briefly using carbon dioxide and placed immediately on ice. Loctite Superglue (Henkel Corporation, Westlake, OH) was applied using the head of an insect pin laterally across the mouthparts and vertically on the ventral side of the mouthparts. Males were placed back on ice and the glue was allowed to dry. A

second layer of glue was applied in the same fashion. Each cockroach was examined and the mouthparts were tapped to ensure they could not move. Treatment groups included Zyrox bait and a crushed rat chow control. An additional sham control was performed with glue placed between the eyes using the procedure described above to ensure that mortality was not caused by the glue itself. Males were isolated in their treatment groups for 6 more hours before the start of the assay to allow for recovery. Sample size was 30 males per treatment. The assay was conducted at 25°C with a 12:12 LD cycle and 40% RH, and terminated when all individuals in both the control and treatment group were dead.

Field Trial. Experiments were conducted on the North Carolina State University campus with a recently established *B. asahinai* population with well-defined boundaries in a wooded area beside railroad tracks (Matos and Schal 2015). Density of the population was determined via timed counts along a transect in a wooded area beside railroad tracks on North Carolina State University's main campus where a *B. asahinai* population had established. Transects were performed on a non-rainy day on August 5, 2014 between (-78.67169667, 35.78528333) and (-78.66710167, 35.78352667). Samples were taken every 20-30 m along the transect in favorable habitats (i.e. leaf litter and trees present). Unfavorable habitats (i.e. rocks, concrete) were not used. At each sampling site a visual scan was performed in an approximately 8 m² area for 3-5 minutes. The location with the highest density of *B. asahinai* was noted, and a 1.5 m by 4.5 m area (9 m²) was marked off within this area. A timed count of total individuals was performed for 5 minutes within this area.

The sites with the highest timed counts were chosen and designated as high, medium, and low density. On a non-rainy day, August 13, 2014, one site of each density group was treated with Zyrox bait and one site was an untreated control. In the treated groups, a 36 m² area was staked off and 250 g Zyrox Fly Granular Bait was applied evenly at a rate of 6.94 g/m² using a Scatter Box applicator (PlantMates LLC, Pasadena, TX). Seven days after the original application, the treatment and control sites were monitored for population density and bait was reapplied as above. The population density was monitored every 7 days for four weeks after the reapplication.

The same procedure described above was performed again in 2015. On a non-rainy day, July 31, 2015, four sites of each density group were treated with Zyrox bait and the other four served as untreated controls. In the treated groups, 72 g Zyrox bait was applied evenly at the label rate of 2 g/m² to a 36 m² area. Seven days after the original application, the treatment and control sites were monitored for population density and bait was reapplied as above. The population density was monitored every 7 days for four weeks after the reapplication.

Data Analysis. For all laboratory assays, Kaplan-Meier survivorship curves (Parmar and Machin 1995) were used to analyze data instead of regression due to the presence of censored responses, and generated using SPSS Version 22 (IBM Corporation 2013). The log rank option was selected in order to identify differences among treatment groups and pairwise comparisons were conducted across treatments.

Field data were \log_{10} -transformed and analyzed using a mixed ANOVA (General linear model with repeated measures by treatment) in SPSS Version 22. Independent *t*-tests between treatments were conducted at all time points.

Results

Laboratory Assays of Mortality. In the laboratory, Zyrox bait acted quickly against both *B. asahinai* and *B. germanica* with little difference between the two species or between the no-choice and two-choice assays. Overall, all the Zyrox treatments caused high mortality sooner than Maxforce treatments. Little mortality occurred in the control group that received rat chow. In the no-choice assays, there was a difference in survivorship times among treatments and species ($\chi^2 = 218.98$, $df = 4$, $P < 0.0001$; Fig. 1A). As expected, significant differences were found in pairwise comparisons of the *B. asahinai* control and all other treatment and species combinations ($P < 0.0001$ for all). Pairwise comparisons of the Maxforce treatments and the Zyrox treatments for both species revealed that differences in survivorship time were highly significant ($P < 0.0001$ for all) and that cockroaches exposed to Maxforce survived longer. Survivorship times for the *B. asahinai* and *B. germanica* Zyrox treatments were not significantly different ($P = 0.644$), but *B. asahinai* in the Maxforce treatment survived longer than *B. germanica* in the Maxforce treatment ($P = 0.024$).

In the two-choice assays, overall comparisons among treatments and species revealed significant differences in survivorship times ($\chi^2 = 269.35$, $df = 4$, $P < 0.0001$; Fig. 1B). As in

the no-choice assay, pairwise comparisons of the *B. asahinai* control and all other treatment and species combinations were highly significant ($P < 0.0001$ for all). Similarly, comparisons of Maxforce and Zyrox treatments for both species revealed that insects in the Maxforce treatments survived longer than those in the Zyrox treatments ($P < 0.0001$ for all).

Survivorship times were not significantly different in *B. asahinai* and *B. germanica* Zyrox treatments ($P = 0.415$), but male *B. asahinai* survived the Maxforce treatment longer than *B. germanica* males ($P < 0.0001$).

Ingestion vs. Contact Assays. Zyrox bait appeared to have minimal contact activity against *B. germanica* with glued mouthparts within four days. An overall comparison revealed a significant difference in survivorship time when cockroaches ingested bait or were prevented from ingesting bait ($\chi^2 = 123.67$, $df = 2$, $P < 0.0001$; Fig. 2). No difference was evident between cockroaches with glued mouthparts (contact only) that were exposed to Zyrox and those exposed to rat chow ($P = 0.567$) and a significant difference was apparent among all other pairs ($P < 0.0001$).

Field Trial. Application of Zyrox bait reduced the *B. asahinai* population in treated plots by over 90%. When the label rate (2 g/m²) of Zyrox was applied, a mixed ANOVA revealed significant differences between treated and control plots ($F = 28.38$; $df = 1$; $P = 0.002$; Fig. 3A). An independent *t*-test conducted between treatments on day 0 indicated the pre-treatment *B. asahinai* populations did not differ between Zyrox and control plots ($t(6) = -0.030$; $P = 0.977$; Fig. 3A). Independent *t*-tests conducted weekly on days 7 ($t(6) = 5.542$; $P = 0.001$), 14 ($t(6) = 5.854$; $P = 0.001$), 21 ($t(6) = 6.688$; $P = 0.001$), 28 ($t(6) = 2.617$; $P =$

0.040), and 35 ($t(6) = 4.479$; $P = 0.004$) indicated significant reductions in the *B. asahinai* populations between treatments, which remained for at least four weeks after re-application of Zyrox bait (Fig. 3A).

In plots treated with 6.9 g/m² Zyrox bait, a similar pattern emerged. A mixed ANOVA indicated differences between treated and control plots ($F = 34.08$; $df = 1$; $P = 0.001$; Fig. 3B). Independent *t*-tests conducted on day 0 revealed no significant differences pre-treatment ($t(7) = 0.008$; $P = 0.994$) and a significant reduction in *B. asahinai* populations on days 7 ($t(7) = 5.924$; $P = 0.001$), 14 ($t(7) = 5.921$; $P = 0.001$), 21 ($t(7) = 4.483$; $P = 0.003$), 28 ($t(7) = 4.663$; $P = 0.002$), and 35 ($t(7) = 3.324$; $P = 0.013$) (Fig. 3B).

Discussion

Overall, Zyrox Fly bait and Maxforce Complete granular bait showed significant toxicity against *B. asahinai* and *B. germanica* in the laboratory. Zyrox bait achieved near 100% mortality within two days, and Maxforce bait within five days in both species. In no-choice and two-choice assays, Zyrox bait killed males of both species more quickly than Maxforce bait. This difference was expected due to the modes of action of the active ingredients – since cyantraniliprole (Zyrox) targets the nerve-muscle interface, it is expected to act more quickly than hydramethylnon (Maxforce), which disrupts the mitochondrial electron transport chain. There was little difference in the survivorship curves between the no-choice and two-choice assays between species and among treatments, suggesting that both

Zyrox and Maxforce are attractive and palatable baits, even when cockroaches were offered their usual food source of rat chow as an alternative food. Since *B. asahinai* survived longer than *B. germanica* in Maxforce treatments, and a few individual *B. asahinai* survived up to four days longer than *B. germanica* in the Zyrox treatments, it is plausible that low levels of resistance exist in the *B. asahinai* population. *Blattella asahinai* might have had more exposure to insecticides in the Alabama and Florida sites where they were collected than our nonresistant *B. germanica* strain, which has had no insecticide exposure since the advent of modern synthetic insecticides. Indeed, Valles et al. (1999) showed that a *B. asahinai* population collected in 1986 (just after its detection in Florida) was less tolerant of carbamate and organophosphate insecticides than the same Orlando Normal colony of *B. germanica* reared in our lab, suggesting that *B. asahinai* populations might have become more resistant to insecticides. As likely, however, is the possibility that *B. asahinai* has some intrinsic protection against xenobiotics (e.g. lower penetration, greater metabolism or excretion) related to its phytophagous habits, or that ingestion of bait was lower than in *B. germanica*.

Our assays to uncouple ingestion-based from contact-based toxicity of Zyrox bait were conducted with *B. germanica* and were limited to four days because males with glued mouthparts started dying within two days. When deprived of food and water, male *B. germanica* live about 8 days at 40% RH (Willis and Lewis 1957). In our assays, cockroaches survived only four days, which could be due to stress induced by having glued mouthparts. However, since cockroaches with glue placed on the head survived throughout the length of the assay, it is unlikely that mortality was caused by the glue. The mouth is also used for

hygienic behavior and grooming (Böröczky et al. 2013), and it is possible that disruption of these behaviors contributed to lower survivorship. We attempted similar experiments with Zyrox bait or crushed rat chow spread across the bottom of a petri dish in order to maximize contact with the bait. However, despite several attempts, high mortality occurred in both the treatment and control groups, even after smaller particles were sifted and eliminated. Upon examination under a microscope, dead cockroaches displayed a significant accumulation of particles on the cuticle (YKM, personal observation). These observations support the idea that the inability of the cockroach to groom because of its glued mouthparts contributed to unexpected low survival.

Within the four day experiment Zyrox bait did not appear to have contact activity against *B. germanica* males. Ingestion, on the other hand, was highly effective at delivering the active ingredient to the target site, as almost all males died within two days.

Zyrox bait was very effective against the *B. asahinai* field population at the NC State University campus, and effects lasted for weeks. We did not include Maxforce bait in these field trials because of the limited availability of field sites. Application of the label rate (2 g/m²) of Zyrox bait maintained an average of 64% population reduction 35 days after the first treatment. Application of approximately three times the label rate (6.9 g/m²) was even more effective, maintaining an average of 92% reduction 35 days after the first treatment. These are likely underestimates of the potential efficacy of Zyrox bait in an area-wide treatment. The overall infested area occupied a small and relatively contiguous wooded area along a railroad track, with no natural or artificial barriers. Our experimental design assigned

treatment plots within an untreated landscape. Therefore, there were no barriers to prevent cockroaches from untreated areas from entering the plots after treatment. This likely occurred, given the small size of each treated plot (9 m²) relative to its perimeter (12 m), resulting in rather conservative estimates of efficacy. Likewise, if Zyrox were repellent, cockroaches from the treated plots could readily move to adjacent untreated areas. We contend that this is unlikely because cockroaches readily consumed Zyrox bait in the presence of rat chow in the two-choice assays.

Cyantraniliprole appears to be an effective active ingredient against *B. asahinai*, *B. germanica*, and likely other cockroaches. Zyrox bait could be used against *B. asahinai* if the label is extended to include this species, and Maxforce Complete may be an effective control option pending field trials. Human tolerance of the Asian cockroach is low when it enters residences (Brenner et al. 1988) and there is a need to reduce populations in the peridomestic environment before they enter homes. This need is reinforced because *B. asahinai* shares human allergens with *B. germanica* which can trigger allergies and exacerbate asthma (Helm et al. 1990). Granular baits, such as Zyrox and Maxforce, are a preferable control option for the Asian cockroach because they can be effective for longer durations, have low mammalian toxicity and thus pose less risk to humans and companion animals, and use less active ingredient than broadcast perimeter sprays, which are the predominant control strategy for peridomestic cockroach pests (Appel and Smith 2002). In future studies, it would be advantageous to conduct Zyrox and Maxforce bait field trials against other peridomestic cockroach pest species, such as the smokybrown cockroach. This species often inhabits

similar habitats to the Asian cockroach (Appel and Smith 2002), and we have shown Zyrox bait to be an attractive and palatable bait for two other pest cockroach species.

Cyantraniliprole could be formulated against the German cockroach, or the Zyrox bait label could be extended, since we have shown it to be effective against this species in the laboratory. It is permissible to use Zyrox bait indoors, as long as it is within bait stations (EPA 2014). Zyrox bait, or a re-formulated cyantraniliprole bait, could be used as an alternate control to rotate with other baits used in *B. germanica* control, which could aid in insecticide resistance management. A word of caution, however, about the use of Zyrox bait against German cockroach populations: house fly baits are often formulated with large amounts of sugar, and specific sugar aversions have arisen independently in multiple *B. germanica* populations (Silverman and Bieman 1993, Wada-Katsumata et al. 2013). Additionally, non-target species such as ants, which are often attracted to sugars produced in extrafloral nectaries (Koptur 1992) may be affected by Zyrox bait.

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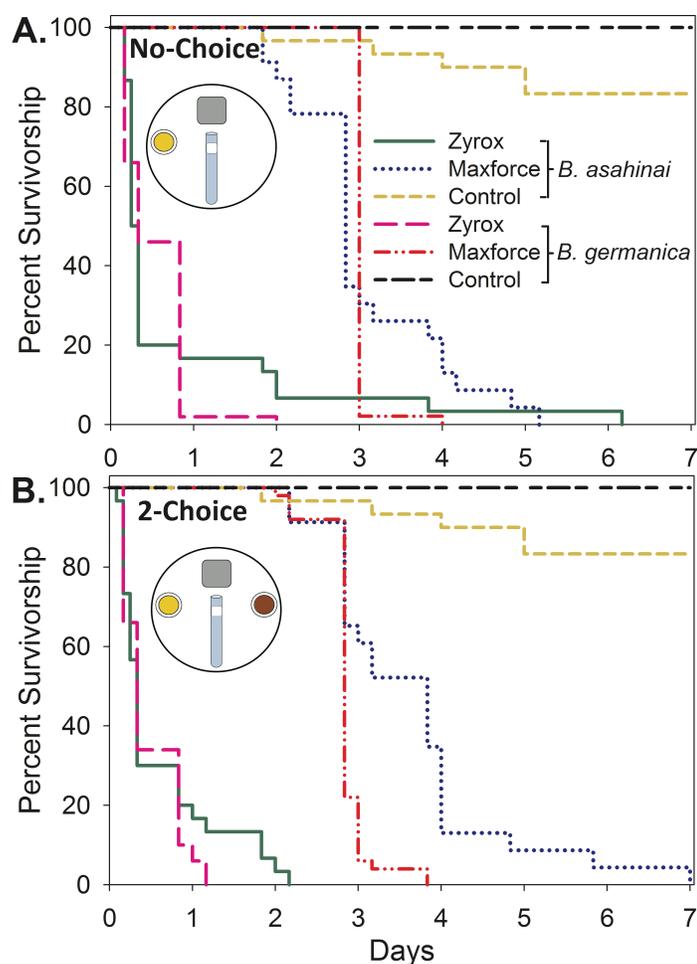


Fig. 1. Kaplan-Meier survivorship curves of *B. asahinai* and *B. germanica* offered Zyrox Fly Granular Bait, Maxforce Complete Granular Insect Bait, and crushed rat chow in no-choice (A) and two-choice (B) assays. Each curve represents an independent species and treatment combination. Sample size was 50 *B. germanica* in all assays, 30 *B. asahinai* in control and Zyrox treatments in all assays, and 23 *B. asahinai* for Maxforce treatments.

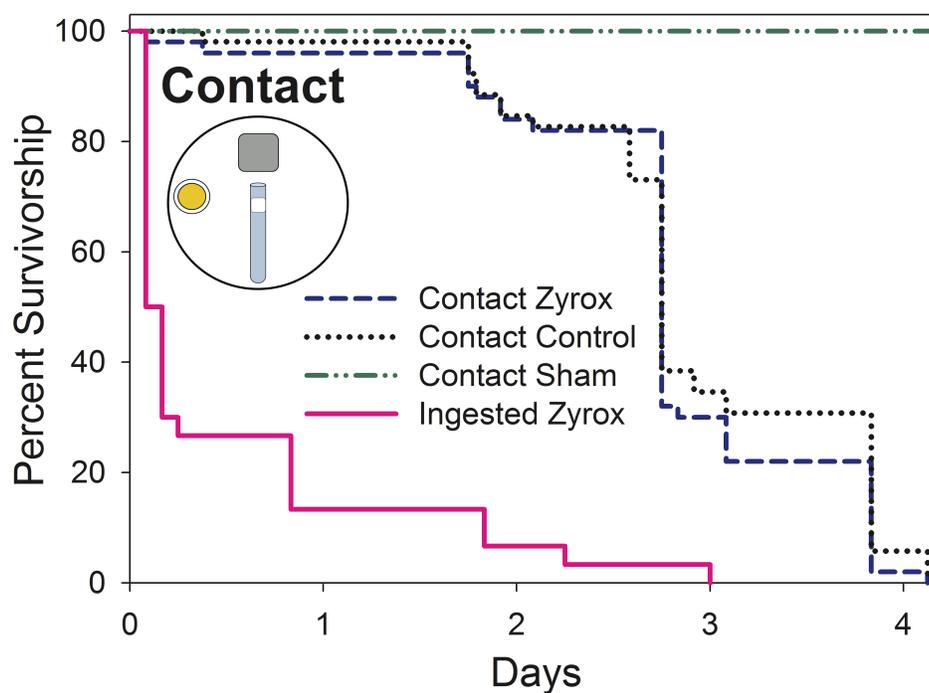


Fig 2. Kaplan-Meier survivorship curves of *B. germanica* offered Zyrox Fly Granular Bait via ingestion or via contact. Each curve represents an independent treatment. In contact-only assays insect mouthparts were glued to prevent ingestion. Glue was applied to the head between the eyes as a sham control. Sample size was 30 insects per treatment.

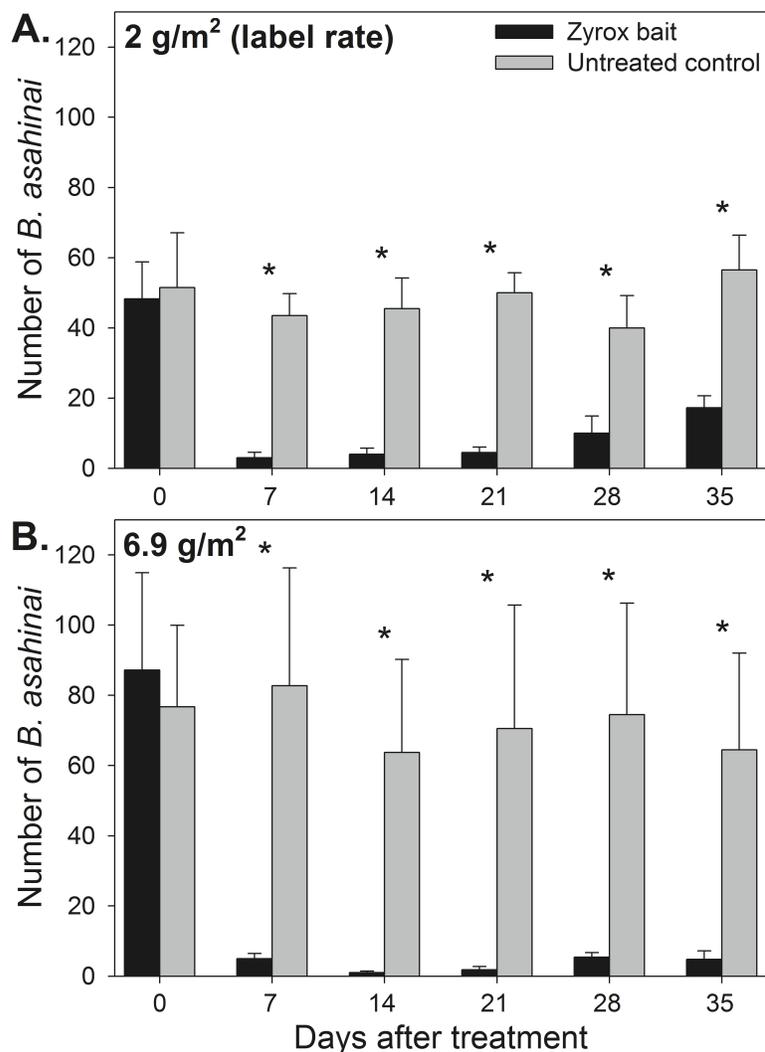


Fig 3. Mean (\pm SE) *B. asahinai* found in timed counts in field sites before and after treatment with Zyrox Fly Granular Bait or an untreated control. (A) Plots treated with 2 g/m² Zyrox bait; (B) Plots treated with 6.9 g/m² Zyrox bait. All plots were treated on day 0 following the initial timed count, and bait was re-applied on day 7. In (A) and (B), asterisks denote significant differences between treatments in mean *B. asahinai* numbers. Sample size was 4 plots each for Zyrox and control (A), 5 plots for Zyrox and 4 plots for control (B). Data were analyzed with a mixed ANOVA