Sensory and chemical basis of off-host aggregation behavior by bed bugs, *Cimex lectularius* (L.)

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DEDICATION

I have a lot of people to thank for supporting this body of work, but this dissertation is simply dedicated to my family. To my husband, Andy, who supported and encouraged me every day. He never let me give up and always made sure I took a break once in awhile to look up and enjoy life. And to my sons, Tanner and Gage, whom I hope to inspire one day to chase their own dreams.

ABSTRACT

After feeding on hosts, bed bugs, *Cimex lectularius* L., aggregate in cracks and crevices near their hosts. Off-host aggregation is mediated by sensory organs on the bed bug antennae and chemical stimuli associated with bug feces. This dissertation examined the sensory bases of bed bug off-host aggregation behavior, and results are presented in four chapters.

Chapter one provides a basic overview of existing literature on the sensory structures located on adult antennae and the chemical stimuli that influence bed bug behavior. The chapter concludes with a discussion of practical applications for bed bug control.

In chapter two, behavioral assays and microscopy were used to study sensilla on the bed bug antenna. A multi-choice behavioral assay using fecal stained filter papers determined which antennal segments mediate off-host aggregation. Both scanning and transmission electron microscopy techniques were used to determine the type and function of sensilla on the pedicel of adults and nymphs. In addition to an abundance of serrated hairs, several smooth hairs with gustatory function were sparsely distributed throughout the segment and a distal patch of sensilla with olfactory function was also described. The identification of sensilla with olfactory and gustatory function on the pedicel suggests off-host aggregation by bed bugs may be mediated by a volatile or semi-volatile compound or compounds.

In chapter three, the chemical stimulus associated with bed bug feces was analyzed, including stimulus volatility, extraction, isolation, and separation of component molecules. Solid phase microextration (SPME) techniques were used to assess the presence of known bed bug pheromones, (E)-2-hexenal (E2H) and (E)-2-octenal (E2O) on fecal stained papers that were heat treated for several days. In addition, multi-choice behavioral assays were used to assess aggregation response to fecal stained papers that were heated, to papers washed in various solvents, and to concentrated methanol extracts and extracts separated by solid phase extraction (SPE) techniques. Results demonstrated that E2H and E2O decrease significantly with heat exposure; however, aggregation response to fecal stained disks remained relatively constant, suggesting that the chemical stimulus is less volatile compared to previous reports. The chemical stimulus was soluble in methanol and water, with bed bug response greatest to concentrations of fecal extracts above 30 mg/ml methanol. Separation of the active components was possible using a C18 SPE cartridge and gas chromatography techniques, which prepared the chemical stimulus for further identification.

In chapter four, crude extracts from bed bug feces were analyzed by a gas chromatograph coupled with an electro-antennogram detector (GC-EAD) and mass spectrometer (GC-MS) to identify essential components of the off-host aggregation pheromone. Adult antennae responded to compounds associated with three elution regions of the crude extract. Several chemical compounds were identified in each of the active regions, and selected groups of compounds were evaluated in multiple choice assays to assess aggregation response. A combination of two compounds, dimethyl trisulfide (DMTS) and methyldiethanolamine (MDEA) resulted in an aggregation response that was equivalent to original extract. This final chapter concludes with a discussion of potential applications of a synthetic aggregation pheromone for surveillance and bed bug control.

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Chapter I. Behavior and chemical ecology of bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae): A literature review

Introduction

The bed bug, *Cimex lectularius* L. is a significant pest of economic importance (Potter et al., 2008; Doggett et al., 2012). Reactions to bed bug bites vary from no response to severe legions depending on previous exposure and individual immunological response (Leverkus, 2006; Doggett et al., 2012). In addition to their bites, bed bugs have been shown to harbor multiple pathogens including HIV and Hepatitis B; however, evidence of transmission from one infected human host to another is currently non-existent (Goddard and DeShazo, 2009; Pascal et al., 2011). Regardless of their vector potential, frequent encounters with bed bugs may cause anemia, lack of sleep, general discomfort and psychological disorders for some individuals (Doggett et al., 2012).

In addition to their medical importance, bed bug infestations are difficult to control. Bed bugs are easily transferred from place to place on personal belongings and infested furniture. If left un-treated, the level of infestation can increase and spread to other areas, making control efforts more difficult. Once an infestation is identified, a combination of chemical and non-chemical methods is usually recommended (Bartley and Harlan, 1974; Doggett, 2013). With insecticide resistance becoming more prevalent (Romero et al. 2007, Potter, 2008), non-chemical control methods (Kells, 2006) are more commonly used. Non-chemical control practices include discard recommendations, laundering, and use of extreme temperatures to treat infested items or areas (Naylor and Boase, 2010; Kells and Goblirsch, 2011; Olson et al., 2013). Although they are effective, non-chemical control methods can be labor intensive, resulting in additional costs for proper control.

Improved methods of monitoring and controlling bed bugs could help minimize the spread of bed bug infestations and the overall cost of current control practices. Current methods of monitoring are limited and only designed to catch host-seeking stages of bed bugs (Weeks et al., 2010; Vaidyanathan and Feldaufer, 2013). More detailed information regarding behavior and chemical ecology of bed bugs could enhance our basic understanding of this economically important pest and help develop more effective monitoring and control programs in the future. This review summarizes the biology, behavior and chemical ecology of bed bugs. Emphasis will be placed on the sensory organs and the chemical stimuli affecting bed bug behavior, in the context of practical applications.

Biology and Behavior

Bed bugs are obligate blood feeders in the family Cimicidae and in the order Hemiptera. Cimicids are ectoparasites of birds and mammals. The Monograph of Cimicidae contains the most recent taxonomic revision of the family with an estimated seventy-four species, arranged into six subfamilies and twenty-two genera (Usinger, 1966). Most are associated with bat or bird hosts, but a few species including the bed bug, *Cimex lectularius* L. and the tropical bed bug, *Cimex hemipterus* Fabricius, prefer a human host (Usinger, 1966). *Cimex lectularius* is distributed world-wide, whereas, the tropical bed bug, *C. hemipterus,* is generally restricted to equatorial regions.

The bed bug life cycle includes an egg stage, five nymphal instars, and adult males and females. Average body size depends on the life stage and frequency of feeding (Fig. 1). Nymphs require a blood meal to develop into the next instar, and adults require additional blood meals to reproduce (Davis, 1955; Usinger, 1966). In the absence of a host, bed bugs can survive several months to a year or more in cooler temperatures (Johnson, 1941). However, in an occupied dwelling at room temperature, bed bugs are likely to feed on a weekly basis and live for about 6-8 months on average (Johnson, 1941; Usinger, 1966).

Bed bugs exhibit many unique and cyclic behaviors that are mediated by a variety of mechanical and chemical factors (Fig. 2). Host-finding, feeding, and mating are typically nocturnal behaviors, while aggregation and arrestment occur throughout the day. Most time is spent assembled into cracks and crevices or aggregation sites near potential hosts (Levinson and Bar Ilan, 1971). More detailed information pertaining to the responses of bed bugs to chemicals will be addressed later in this chapter.

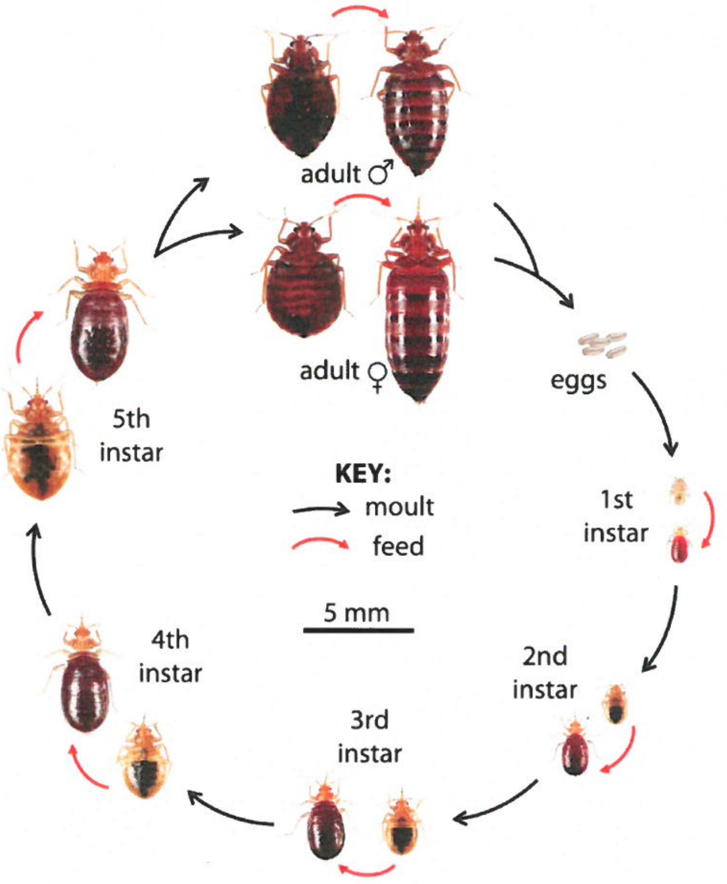

Fig. 1. The complete life cycle of the bed bug, *Cimex lectularius*, includes both fed and unfed stages (Naylor, 2012).

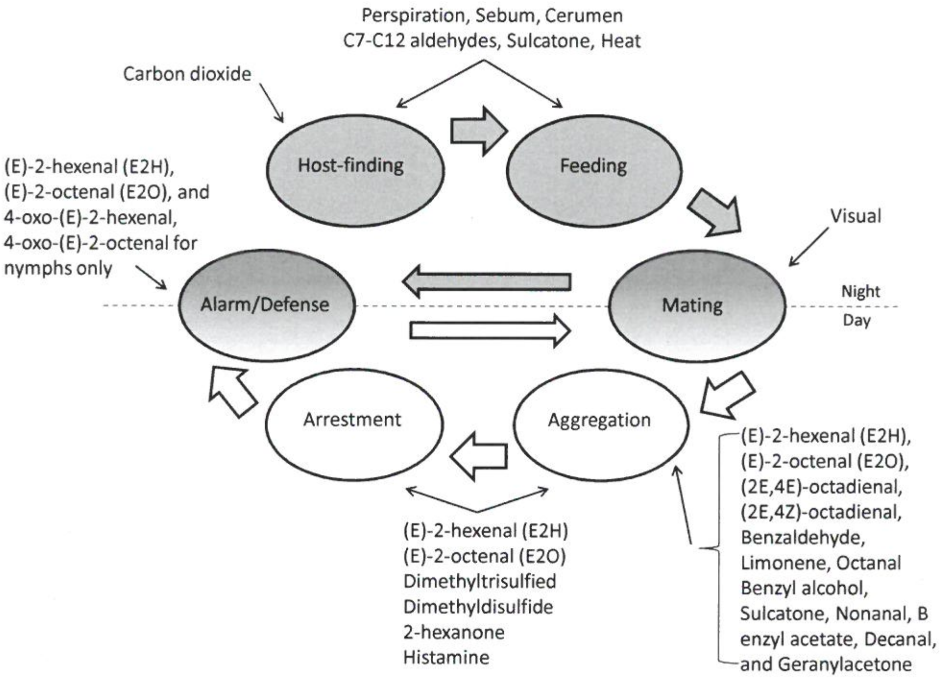

Fig. 2. Stimuli that influences the behavior and chemical ecology of bed bugs, *Cimex lectularius*.

Sensory Organs (Sensilla)

Similar to most insects, bed bugs have a variety of sensory organs or sensilla that respond to specific stimuli or changes in their environment. The distribution and abundance of sensilla on the antennae of *C. lectularius* has been studied extensively by Levinson et al., (1974a) and Steinbrecht and Muller (1976) (Fig. 3). Nine distinct types of sensilla have been identified and described (Table 1). Each of the nine types can be distinguished from one another based on both external and internal structures (Fig. 4). For example, sensilla types A, B, and F have characteristics consistent with mechanical functions, whereas, types C, D, and E have a single or multiple pores and dendrites, representative of olfactory or gustatory sensilla (Hodgson, 1958; Steinbrecht, 1996).

Mechanical sensilla have been identified on all four segments of the bed bug antennae (Levinson et al., 1974a). Evidence suggests types B and F are capable of detecting changes in temperature and humidity, while types A2, A3, and E2 are likely sensitive to tactile or physical manipulation (Steinbrecht and Muller, 1976). Type A1 has characteristics similar to both mechanical reception and gustation. Combined, there are over 100 type A sensilla on the antennae of adult bed bugs (Levinson et al., 1974a). The abundance of type A sensilla may contribute to the bed bugs' sensitivity to tactile stimuli and selection of aggregation sites.

Bed bug olfactory and gustatory sensilla types C, D, and E1 are restricted to the olfactory regions (O_1 and O_2) on the second flagellum (Fig. 3). Single cell recording techniques have demonstrated that sensilla type C responds to ammonia and type D responds to various human kairomones and bed bug pheromones such as hexenal, octenal, ethyl butyrate, sulcatone, dimethyl trisulfide, and pinene (Harraca et al., 2009). The behaviors exhibited by exposure to some of these chemical stimuli are described in more detail in the next section. Currently, it is not clear which compounds are detected by the type E sensilla.

Distribution and abundance of antennal sensilla in *C. lectularius* are comparable to its related species, *C. hemipterus*. The same nine types of sensilla were observed in adults and nymphs of both species (Walpole, 1987; Singh et al., 1996). However, gustatory receptors were identified on the pedicel of adult *C. hemipterus* (Singh et al., 1996), but were not reported for *C. lectularius*. Interestingly, Olson et al., (2009) demonstrated that *C. lectularius* responded to fecal stained disks in the absence of the olfactory regions on the second flagella, suggesting a re-examination of the sensilla on the pedicel is warranted.

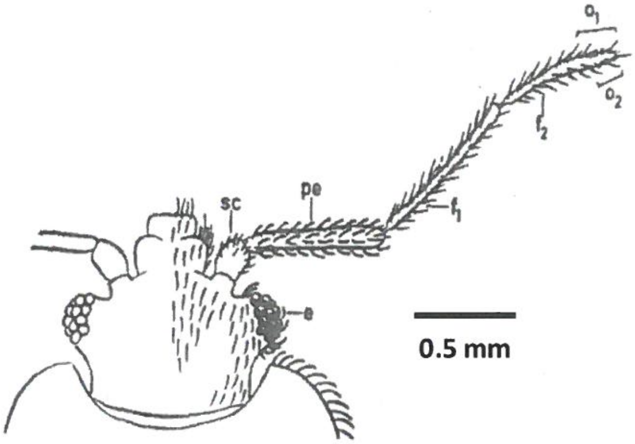

Fig. 3. Bed bug, *Cimex lectularius*, head with eye (e) and antennae with scape (sc), pedicel (pe), first flagellum (f1), and second flagellum (f2) with olfactory regions (O_1 and O_2); (Levinson et al., 1974a).

Table 1. Type, location, and suggested function of antennal sensilla on the adult bed bug, *C. lectularius* (Levinson et al., 1974a; Steinbrecht and Mueller, 1976).

Type	**External Shape**	**Location**	**Function (Stimuli)**
A1	Bristle	F1, F2	mechano, gustatory (tactile/unknown)
A2	Bristle	F1, F2	mechano (tactile)
A3	Serrated bristle	S, P, F1	mechano (tactile)
B	Plate	F2	mechano (temperature/humidity)
C	Grooved peg	F2	olfactory (kairomone)
D	Smooth peg	F2	olfactory (kairomones; pheromones)
E1	Hair	F2	olfactory (pheromones)
E2	Hair	F2	mechano (tactile)
F	Immersed Cone	F2	mechano (temperature/humidity)

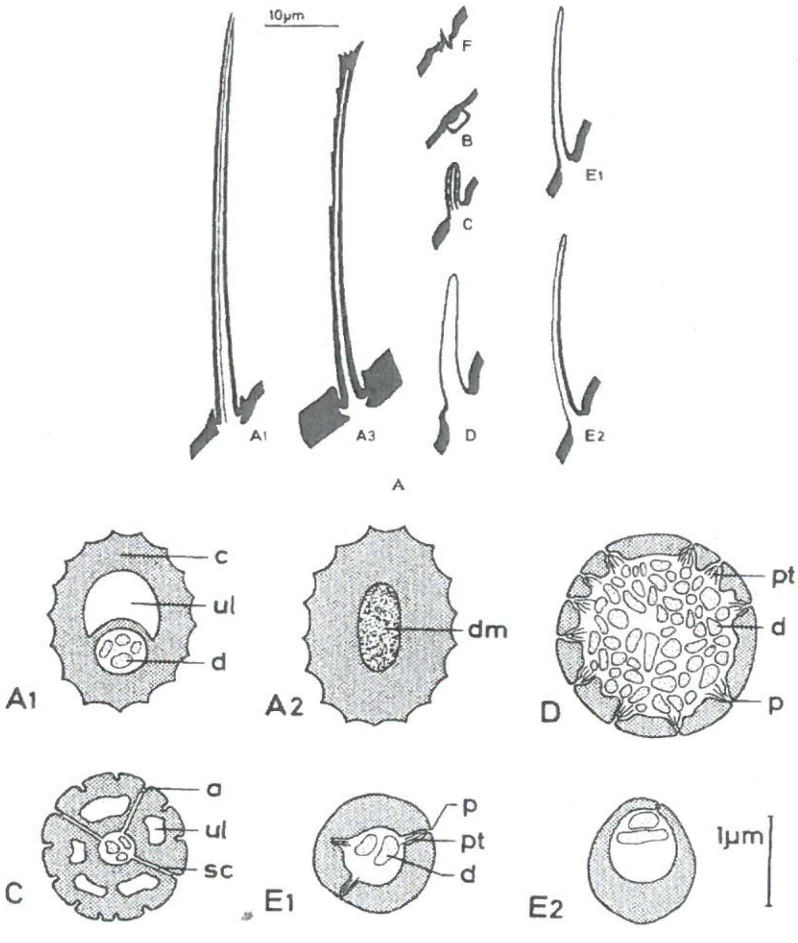

Fig. 4. External and internal structures of the bed bug, *Cimex lectularius*, antennal sensilla (type A1, A2, A3, B, C, D, E1, E2, and F) from the olfactory regions. Structures

shown include cuticle (c), (ul), dentrite (d), dense material (dm), pore tubule (pt), pore (p), and spoke channel (sc) presented; (Levinson et al., 1974a).

Chemical Ecology

A combination of stimuli including visual, mechanical and chemical contributes to the behavioral activity demonstrated by bed bugs (Fig. 2). The following summarizes our current knowledge and understanding of host-finding, feeding, mate selection, alarm or defense, aggregation and arrestment by bed bugs.

Host-finding

Bed bugs must locate a host and acquire a blood meal on a regular basis in order to develop and reproduce (Davis, 1955, Usinger, 1966). Host-finding by bed bugs is influenced by daily activity patterns and chemical stimuli (Fig. 2). The search for a host typically occurs at night following lights off in both experimental and observational studies (Mellanby, 1939), with the highest level of activity recorded shortly before sunrise (Romero et al., 2010; Reis and Miller, 2011). This increase in nocturnal activity continues in the absence of the host (Romero et al., 2010), suggesting circadian rhythms alone may initiate host-finding behavior.

In addition to daily activity patterns, carbon dioxide and several human derived odors also influence host-finding by bed bugs (Fig. 2). Exposure to carbon dioxide increases bed bug movement (Marx, 1955) and studies using time-lapse photography have recorded bed bug attraction to human breath up to 2 ft away (Suchy and Lewis, 2011). In addition to carbon dioxide, heat and other host derived odors influence host-finding by bed bugs (Aboul-Nasr & Erakey, 1968a; Rivnay, 1930; Harraca et al., 2009). Sensilla types C and D in the olfactory regions of adult bed bugs respond to human body secretions (Harraca et al., 2009) (Table 1). The sensory organs responsible for detection of carbon dioxide have not been identified.

Feeding

Once a host has been located, body temperature and chemical stimuli initiate feeding by bed bugs (Fig. 2). They are attracted to and will attempt to feed from various objects heated several degrees above room temperature when placed within a foot of the source (Rivnay, 1932; Aboul-Nasr and Erakey, 1967; Wang et al., 2009). Bed bugs can detect a heat source slightly above ambient temperatures from short distances and prefer temperatures at or near peripheral body temperature when a blood meal is required (Rivnay, 1932; Johnson, 1941; Aboul-Nasr and Erakey, 1967). In contrast, when the temperature of an object exceeds 43 °C, bed bugs are immediately repelled (Rivnay, 1932), suggesting that detection of thermal stimuli is critical in both pre- and post-feeding behaviors.

Host-derived odors also affect bed bug feeding (Fig. 2). Attraction and probing behaviors were observed when bed bugs were placed in close proximity (3-4cm) to a cotton stockinet or glass rod treated with perspiration, sebum, or cercumen (Aboul-Nasr and Erakey, 1968a). Over 200 compounds have been identified in human perspiration (Bernier et al., 2000) and several have been evaluated against bed bugs. Only a few (C7 – C10 aldehydes and sulcatone) have been tested and found to elicit a physiological response from type D sensilla using single cell recording techniques (Harraca et al., 2009). The limited ability to detect chemical stimuli from long distances may help explain the parasitic life style of the bed bug and its close association with its host.

Mate selection/Sexual Behavior

Bed bugs and a few other cimicids mate using a unique process termed traumatic insemination by which the male pierces the female abdomen with his genitalia, and in *C. lectularius,* the sperm is injected into a specific region located on the abdomen of adult females (Davis, 1955; Stutt and Siva-Jothy 2001). Mating attempts typically occur shortly after feeding (Rao, 1972; Reis and Miller, 2011) with males mating up to three females within twenty-four hours of engorgement (Reinhardt et al., 2009). Some reports suggest that multiple pairings are lethal and avoided by females (Stutt and Siva-Jothy, 2001; Reinhardt et al., 2003; Pfiester et al., 2010). However, a female must continue to mate on a regular basis in order to produce viable eggs throughout its life span (Johnson, 1941; Fawziak et al., 1987).

Currently, there are no known sex pheromones produced by bed bugs and mate selection appears to be primarily dependent on visual stimuli (Fig. 2). Adult males do not demonstrate a preference for females during courtship activities. In addition to females, males will attempt to mate with other males, nymphs, as well as inanimate objects similar in shape and size to adult bugs (Rivnay, 1933; Usinger, 1966; Rao, 1972). The close proximity of both sexes during and immediately post-feeding may facilitate sexual encounters.

Alarm and Defense

Bed bugs use defensive secretions derived from specialized scent glands to alert conspecifics and defend against male sexual attention (Fig. 2). The maintenance and use of specialized scent glands for alarm and defense is common among hemipterans (Schuh and Slater, 1995). Cimicid scent glands are located on the dorsal abdominal regions of immature stages and the lateral metathorax of adult stages (Usinger, 1966). The contents of the metathoracic scent gland includes (E)-2-hexenal (E2H), (E)-2-octenal (E2O), butanone, acetaldehyde and two unknown compounds, with E2H and E2O making up 92% of the total composition (Collins, 1968; Levinson et al., 1974b). When irritated, bed bugs will release the contents of its scent gland (Levinson et al., 1974b). Exposure to increased levels of E2H and E2O cause rapid movement and dispersal of bed bug aggregations (Levinson et al., 1974a), suggesting the compounds function as an alarm pheromone.

In addition to dispersal, bed bugs also use their scent gland secretions for defense. Increases in the amount of E2H and E2O were recorded during male-male and male-nymph sexual encounters (Ryne, 2009; Kilpenen et al., 2012). These two compounds were not detected during male-female pairings, suggesting its potential use by males and nymphs for defense purposes. In addition to E2H and E2O, nymphs also emit 4-oxo-(E)-2-hexenal and 4-oxo-(E)-2-octenal during male copulation attempts (Feldlaufer et al., 2010; Harraca et al., 2010). The complex defense compounds produced by nymphs most likely further distinguishes immature stages from adults during courtship activities.

The major components of the bed bug alarm pheromone are detected by sensory organs in the olfactory regions on the second flagellum (Fig. 3). Early research using electroantennogram (EAG) methods concluded that type E sensilla were sensitive to E2H and E2O (Levinson et al., 1974a). However, more recent and precise application of antennal electrodes using single cell recording techniques demonstrated that type D sensilla respond to the two aldehyde compounds, whereas type E sensilla did not respond to any of the compounds evaluated in the study (Harraca et al., 2009). The function of type E sensilla remains unknown.

Aggregation

Shortly after feeding and mating, bed bugs seek harborage within cracks and crevices near a potential host (Usinger, 1966). Refugia include multiple life stages, exuviae and excreta. Although they are not considered social insects, the formation of aggregation sites are common among hemipterans (Schafer and Anizzi, 2000; Millar, 2005; Wertheim et al., 2005).

Aggregation by bed bugs is dependent on time of day. Regardless of whether a blood meal was obtained, assembly into aggregation sites typically occurs within 30 minutes of feeding or by the end of the scotophase (Romero et al., 2010; Reis and Miller 2011). The observed decrease in bed bug activity between night and day (Mellanby, 1939) may also be caused by its preference for dark surfaces (Aboul-Nasr and Erakey, 1968b). Once assembled, aggregation remains relatively constant (Levinson and BarIlan, 1971; Romero et al., 2010) unless alarm pheromones are released (Levinson et al., 1974a) or host-seeking behavior is initiated.

Aggregation by bed bugs is also mediated by chemical stimuli (Fig. 2). An analysis of the air space above groups of bed bugs has revealed several volatile compounds including E2H, E2O, benzaldehyde, sulcatone, octanal, limonene, benzyl alcohol, (2E, 4Z)-Octadienal, nonanal, (2E, 4E)-octadienal, benyl acetate, decanal, and geranylacetone (Siljander et al., 2008). In two-choice behavioral assays, a preference was observed for one side of the arena that had been treated with the chemical blend (Siljander et al., 2008), suggesting its attractive role in bed bug aggregation. However, aggregation to the same compound blend didn't work insitu. The most abundant compounds identified in the blend consisted of E2H and E2O (Siljander et al., 2008), which also make up the two main constituents of the bed bug alarm pheromone (Collins, 1968; Levinson et al., 1974a). Although it is not uncommon for similar compounds to elicit both attraction and repellent behaviors within the order Heteroptera (Millar and Rice, 1998), it is unclear how highly volatile compounds such as E2H and E2O are responsible for establishing and more importantly sustaining aggregation and arrestment between blood meals.

More recent research has demonstrated that aggregation by bed bugs is mediated by a combination of volatile and one less-volatile compound (Gries et al., 2014). Analysis of the bed bug cuticle revealed several compounds including: E2H, E2O, dimethyldisulfied, dimethyltrisulfide, 2-hexanone, and histamine (Gries et al., 2014). With its relatively high boiling point, histamine, would be considered less volatile compared to the others. Exposure to a combination of these compounds resulted in aggregation and arrestment by bed bugs in both laboratory and field settings, suggesting their importance and role in aggregation by bed bugs.

A few components of the airborne aggregation pheromone identified by Siljander et al., (2008) are detected by type D sensilla (Table 1). Type D sensilla responded to low concentrations of E2H, E2O, sulcatone, and benzylaldehyde (Harraca et al., 2009). Limonene was also evaluated, but no response was recorded for type D, C, or E olfactory sensilla (Harraca et al., 2009). Thus, limonene may not be detected by bed bugs and may not be a critical component of the airborne aggregation pheromone. Physiological recordings have not been captured or recorded for octanal, benzyl alcohol, (2E, 4Z)-Octadienal, nonanal, (2E, 4E)-octadienal, benyl acetate, decanal and geranylacetone.

Sensilla responsible for bed bug aggregation are not restricted to the olfactory regions of the antennae. In multiple choice assays, aggregation and arrestment continued after the first and second flagella were removed (Olson et al., 2009). Aggregation was only significantly reduced after removing the whole antennae (Levinson et al., 1974; Olson et al., 2009), suggesting that sensilla responsible for detection of chemical stimuli associated with aggregation may be located on more proximal segments of the antennae.

Arrestment

After assembly into cracks and crevices, arrestment is sustained by physical contact with the substrate and conspecifics (Fig. 2). In choice experiments, bed bugs prefer rough, textured surfaces compared to smooth surfaces and open areas (Aboul-Nasr and Erakey, 1968a). In addition, close contact among individuals within aggregation sites may also contribute and maintain arrestment throughout the day and between blood meals (Usinger, 1966).

For some blood-feeding arthropods, arrestment is also maintained by chemical stimuli associated with fecal material. For example, ticks deposit and use arrestment pheromones to mark areas where hosts are most likely to return (Dusabek et al., 1991; Grenacher et al., 2001; Sonenshine, 2006). Some components of the arrestment pheromone deposited by the black-legged tick, *Ixodes scapularis*, include guanine, xanthine, other purines, and hematin (Sonenshine et al., 2003). The compounds are either produced by the tick or may be a by-product of the blood diet acquired from its host. Based on the blood feeding habits and aggregation demonstrated by the bed bug, an analysis of its fecal material may reveal similar compounds responsible for arrestment.

Bed bugs respond to filter papers that have been stained with feces by conspecifics (Levinson and Bar Ilan, 1971; Siljander et al., 2007; Olson et al., 2009). A fecal extract collected from stained papers caused aggregation and arrestment in multiple choice behavioral assays (Levinson and Bar Ilan 1971). Similar observations were made with *C. hemipterus* to its fecal extracts in choice experiments (Parashar et al., 2003). However, no further attempts have been made to analyze the contents of either extract.

It is not clear whether the arrestment stimuli associated with bed bug fecal extracts is airborne or whether contact with the source is necessary to elicit this behavior. Siljander et al., (2007) suggested that contact with the source of fecal material was required because the response was significantly reduced when the source was placed in a protective barrier. In contrast, Weeks et al., (2010) designed an assay that did not require physical contact, but attraction to the side of the arena positioned above the colony of bed bugs was observed, suggesting contact was not required to elicit attraction. A combination of volatile and non-volatile components may be responsible for arrestment by bed bugs. Further evidence demonstrating the importance of physical contact was confirmed by Olson et al, (2009) who demonstrated that movement towards disks stained by bed bugs was not directional, but activity ceased upon contact with the source. If contact is required, less volatile compounds may be responsible for aggregation and arrestment than what has been previously described.

As stated previously, removal of the olfactory regions on the distal segment of bed bug antennae did not reduce aggregation and arrestment under stained papers (Olson et al., 2009), suggesting that sensilla responsible for detection of chemicals associated with fecal extracts are not olfactory or at least they are not restricted to the olfactory regions on the antenna. Gustatory sensilla have been found on the pedicel of *C. hemipterus* (Singh et al., 1996) and may also be located on the pedicel of the bed bug. Thus, gustatory sensilla located on the pedicel may be responsible for detection of non-volatile compounds that cause arrestment by both species.

A thorough examination of the sensory organs on the pedicel of *C. lectularius* and analysis of its fecal extract is warranted. Examination of the sensory organs by scanning electron microscopy and transmission electron microscopy would provide more information about the presence and distribution of sensory organs on the bed bug antenna. In addition, separation of the fecal extract using analytical techniques similar to those described for identification of the arrestment pheromone of the black legged tick (Sonenshine et al., 2003) may provide more insight into the chemical stimuli that influences bed bug arrestment. Identification of the chemical compounds associated with the bed bug fecal extract may further our understanding of the chemical ecology and provide additional options to monitor and control this economically important pest.

Practical Applications

Insect pheromones and kairomones are often used as part of an insect pest management program (Law and Regnier, 1971; Howse et al., 1998). Chemicals that influence bed bug behavior have been incorporated into traps that can be used to monitor bed bugs, however, the success and use of these devices has been limited.

Host-finding stimuli such as heat, carbon dioxide, and human derived odors have been incorporated into bed bug traps for monitoring purposes. Traps designed to mimic human hosts have been marginally successful at attracting and catching bed bugs (Weeks et al., 1010; Vaidyanathan and Feldaufer, 2013). Traps that release carbon dioxide were found to be equally effective at attracting various life stages of bed bugs compared to traps emitting combinations of heat, CO2, and chemical lures (Anderson et al., 2009; Wang, et al., 2009; Singh et al., 2013), suggesting chemical attractants derived from humans or synthetically produced have a limited affect on bed bugs. In addition, generating and sustaining a production of carbon dioxide is impractical and not cost-effective.

Bed bug alarm pheromones have been recommended to enhance control efforts. The addition of alarm pheromones to desiccant dusts has been shown to increase effectiveness of the dust application in laboratory experiments (Benoit, 2009). However, application of bed bug alarm pheromones to control bed bugs has not been adopted into current pest management practices.

Currently, there are no monitoring or control methods that use bed bug aggregation or arrestment pheromones. Unlike host-derived odors that attract only host-seeking stages, aggregation and arrestment affects all life stages (Siljander et al., 2008; Olson et al., 2009) and use of these compounds could be another option for monitoring and controlling bed bug infestations. More information about the chemical stimuli that elicits aggregation and arrestment by bed bugs would benefit the pest management industry and the general public concerned about this important pest.

Summary

The bed bug, *Cimex lectularius*, is a significant pest of medical and economic importance. If not properly controlled, bed bug infestations can spread and become even more difficult to manage. More information on the basic behavior and chemical ecology about this pest would benefit society.

The distribution and function of the sensilla on the antennae of adult bed bugs has been studied in great detail. Sensilla on the antennae of adult bed bugs have mechanical and olfactory functions. The olfactory regions are restricted to the second flagella of the antenna. The function of type E sensilla remains unknown and further investigation of the types of sensilla present on the proximal segments of the antenna is warranted based on the bed bugs ability to aggregate when the distal segments of the antennae are removed.

We currently have a limited understanding about the chemical stimuli that influence bed bug behavior. Evidence has demonstrated that chemicals elicit host-finding, aggregation, alarm and defense. Current research suggests that aggregation is medicated by a blend of volatile compounds, but it is unclear how the mixture sustains aggregation for longer periods of time and between feedings. Fecal extracts collected in methanol cause aggregation and arrestment by bed bugs and further research is necessary to identify chemical stimuli associated with fecal extracts.

Literature Cited

Aboul-Nasr, A.E., and M.A.S. Erakey. 1967. On the behavior and sensory physiology of the bed bug: Temperature reactions. Bull. Soc. Entomol. Egypte. 43: 43-54.

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968a. Behavior and sensory physiology of the bed-bug, *Cimex lectularius* L., to some environmental factors: chemoreception. Bull. Soc. Entomol. Egypte. 52: 353-362.

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968b. The effect of light reactions upon the bed bug, *Cimex lectularius* L. Bull. Soc. Entomol. Egypte. 52: 338-351.

Anderson, J., F. Ferrandino, S. McKight, J. Nolen, and J. Miller. 2009. A carbon dioxide, heat, and chemical lure trap for the bed bug, *Cimex lectularius*. Med. Vet. Entomol. 23: 99-105.

Bartley, J., and H. Harlan. 1974. Bed bug infestation: its control and management. Mil. Med. 139: 884-886.

Benoit, J.B., S.A. Phillips, T.J. Croxall, B.S. Christensen, J.A. Yoder, and D.L. Denlinger. 2009. Addition of alarm pheromone components improves the effectiveness of desiccant dusts against *Cimex lectularius*. J. Med. Entomol. 46: 572-579.

Bernier, U.R., D.L. Kline, D.R. Barnard, C.E. Schreck, and R.A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). Anal. Chem. 72: 747-756.

Collins, R.P. 1968. Carbonyl compounds produced by the bed bug, *Cimex lectularius*. Ann. Entomol. Soc. Am. 61: 1338-1339.

Davis, N. 1955. The morphology and functional anatomy of the male and female reproductive systems of *Cimex lectularius* L. (Heteroptera, Cimicidae). Ann. Entomol. Soc. Am. 49: 466-478.

Doggett, S.L., D.E. Dwyer, P.F. Penas, and R.C. Russell. 2012. Bed bugs: Clinical relevance and control options. Clin. Microbiol. Rev. 25: 164-192.

Doggett, S. 2013. A code of practice for the control of bed bug infestation in Australia Fourth Edition. (www.bedbug.org.au).

Dusabek, F., P. Simek, A. Jegorov, and J. Triska 1991. Identification of xanthine and hypoxanthine as components of assembly pheromone in excreta of argasid ticks. Exp. Appl. Acarol. 11: 307-316.

Fawziak, K., M. Abdelatif, I. Gaaboub, and E. Helal. 1987. Biological studies on the bed bug (*Cimex lectularius* L.). J. Agric. Res. 32: 359-368.

Feldlaufer, M. F., M. J. Domingue, K.R. Chauhan, and J. R. Alrich. 2010. 4-oxo-aldehydes from the dorsal abdominal glands of the bed bug (Hemiptera: Cimicidae). J Med. Entomol. 47: 140-143.

Goddard, J. and R. DeShazo. 2009. Bed Bugs (*Cimex lectularius*) and clinical consequence of their bites. J. Am. Med. Assoc. 301: 1358-1366.

Grenacher, S., T. Krober, P. Guerin, and M. Vlimant. 2001. Behavioral and chemoreceptor cell responses of the tick, *Ixodes ricinus*, to its own faeces and faecal constituents. Exp. Appl. Acarol. 25: 641-660.

Gries, R., R. Britton, M. Holmes, H. Zhai, J. Draper, and G. Gries. 2014. Bed bug aggregation pheromone finally identified. Angewantde Chemie 54: 1135-1138.

Harraca V., R. Ignell, C. Lofstedt, and C. Ryne. 2009. Characterization of the antennal olfactory system of the bed bug (*Cimex lectularius*). Chem. Senses 35: 195-204.

Harraca, V., C. Ryne, and R. Ignell. 2010. Nymphs of the common bed bug (*Cimex lectularius*) produce anti-aphrodisiac defense against conspecific males. BMC Biol. 8: 121.

Hodgson, E.S. 1958. Chemorecption in arthropods. Ann. Rev. Entomol. 3: 19-36.

Howse, P., I. Stevens, and O. Jones. 1998. Insect pheromones and their use in pest management. London. Chapman and Hall.

Johnson, C. G. 1941. The ecology of the bed bug, *C. lectularius* L. in Britain. J. Hyg. Cambridge 41: 345-461.

Kells, S.A. 2006. Non-chemical control of bed bugs. Am. Entomol. 52: 109-110.

Kells, S. A., and M. J. Goblirsch. 2011. Temperature and time requirements for controlling bed bugs (*Cimex lectularius*) under commercial heat treatment conditions. Insects 2: 412-422.

Kilpinen, O., L. Dezhao, and A.P.S. Adamsen, 2012. Real-time measurement of volatile chemicals released by bed bugs during mating activities. PLOS One 7: 1-9.

Law, J.H. and F.E. Regnier. 1971. Pheromones. Annu. Rev. Biochem. 40:533-548.

Leverkus, M. 2006. Bullous allergic hypersensitivity to bed bug bites mediated by IgE against salivary nitrophorin. J. Invest. Dermatol. 126: 91-96.

Levinson, H. Z., and A. R. Ilan. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. Experientia 27: 102-103.

Levinson, H. Z., A. R. Levinson, B. Muller, and R. A. Steinbrecht. 1974a. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. J. Insect Physiol. 20: 1231-1248.

Levinson, H. Z., A. R. Levinson, and U. Maschwitz. 1974b. Action and composition of the alarm pheromone of the bedbug *Cimex lectularius* L. Naturwissenschaften. 61: 684-685.

Marx, R. 1955. Uber die wirtsfindung und die bedeutung des artspezifischen duftstoffes bei *Cimex lectularius* Linne. Z. Parasitenkd. 17: 41-73.

Mellanby, K. 1939a. The physiology and activity of the bed-bug (*Cimex lectularius*) L. in a natural infestation. Parasitology, 31: 200-211.

Millar, J. 2005. Pheromones of true bugs. Topics in Current Chemistry, 240: 37-84.

Millar, J., and R. Rice. 1998. Sex pheromone of the plant bug *Phytocoris californicus* Knight (Heteroptera: Hemiptera Mirridae). J. Econ. Entomol. 91: 132-137.

Naylor, R.A., and C.J. Boase. 2010. Practical solutions for treating laundry infested with *Cimex lectularius* (Hemiptera: Cimicidae). J. Econ. Entomol. 103: 136-139.

Naylor, R. 2012. December 21. Bed bug biology and the basics. Retrieved from http://cimexstore.co.uk

Olagbemiro, T., and B. Staddon. 1983. Isoprenoids from metathoracic scent gland of cotton seed bug, *Oxycarenus hyalinipennis* (Costa) (Heteroptera: Lygaeidae). J. Chem. Ecol. 9: 1397-1412.

Olson, J. F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect Physiol. 55: 580-587.

Olson, J.F., M. Eaton, S.A. Kells, V. Morin, and C. Wang. 2013. Cold tolerance of bed bugs and practical recommendations for control. J. Econ. Entomol. 106: 2433-2441.

Parashar, B., K. Ganesan, D. Sukumaran,, Y. Rao, V. Vijay, and S. Prakash. 2003. Aggregation activity induced by the excreta extracts in *Cimex hemipterus* (Hemiptera: Cimicidae). Entomon 28: 215-222.

Pascal, D., B. Blanc, P. D. Giudice, A. Levy-Bencheton, O. Chosidow, P. Marty, and P. Brouqui. 2011. Bed bugs and infectious diseases. Clin. Infect. Dis. 52: 200-210.

Pfiester, M., P. G. Koehler, and R.M. Pereiera. 2010. Effect of population structure and size on aggregation behavior of *Cimex lectularius* (Hemiptera: Cimcidae). J. Med. Entomol. 46: 1015-1020.

Potter, M. 2008. The business of bed bugs. Pest Control Technol. 24: 40.

Potter, M.F., A. Romero, K.F. Haynes. 2008. Battling bed bugs in the USA. Proc. Sixth Int. Conf. Urban Entomol. 401-406.

Rao, H. 1972. Abnormal sexual behavior of isolated males of *Cimex lectularius* L. Indian J. Exp. Biol. 10: 295-297.

Reinhardt, K., R. Naylor, M.T. Siva-Jothy. 2003. Reducing a cost of traumatic insemination: female bed bugs evolve a unique organ. Proc. R. Soc. Lond. B22 270: 2371-2375.

Reinhardt, K., R.A., Naylor, M.T. Siva-Jothy, 2009. Situational exploitation: higher male mating success when female resistance is reduced by feeding. Evolution 63: 29-39.

Reis, M.D., and D.M. Miller. 2011. Host searching and aggregation activity of recently fed and unfed bed bugs (*Cimex lectularius* L.). Insects 2: 186-194.

Rivnay, E. 1930. Host selection and cannibalism in the bed bug *Cimex lectularius* L. Ann. Entomol. Soc. Am. 23: 758-764.

Rivnay, E. 1932. Studies in tropisms of the bed bug *Cimex lectularius* L. Parasitol. 24: 121-136.

Rivnay, E. 1933. Tropisms effecting copulation in the bed bug. Psyche 140: 115-120.

Romero, A., M. Potter, D. Potter, and K. Haynes. 2007. Insecticide resistance in the bed bug: a factor in the pest's sudden resurgence? J Med. Entomol. 44: 175-178.

Romero, A., M.F. Potter, K.F. Haynes. 2010. Circadian rhythm of spontaneous locomotor activity in the bed bug, *Cimex lectularius* L. J. Insect Physiol. 56: 1516-1522.

Ryne, C. 2009. Homosexual interactions in the bed bugs alarm pheromones as male recognition signals. Anim. Behav. 78: 1471-1475.

Schaefer, C.W., and A.P. Anizzi. 2000. Heteroptera of Economic Importance. CRC Press; Boca Raton. 828p.

Schuh, R., and J. Slater. 1995. True bugs of the world (Hemiptera: Heteroptera): Classification and natural history. Cornell University Press. 336p.

Siljander E., D. Penman, H. Harlan, and G. Gries. 2007. Evidence of male- and juvenile-specific contact pheromones of the common bed bug *Cimex lectularius*. Entomol. Exp. Appl. 125: 215-219.

Siljander E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708-718.

Singh, R., K. Singh, S. Prakash, M. Mendki, and K. Rao. 1996. Sensory organs on the body parts of the bed-bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) and the anatomy of its central nervous system. Int. J. Insect Morph. 25: 183-204.

Singh, N., C. Wang, and R. Cooper. 2013. Effect of trap design, chemical lure, carbon dioxide release rate, and source of carbon dioxide on efficacy of bed bug monitors. J. Econ. Entomol. 106: 1802-11.See comment in PubMed Commons below

Sonenshine, D., T. Adams, S. Allan, J. McLaughlin, and F. Webster. 2003. Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularius* (Acari: Icodidae) and their use in tick control. J. Med Entomol. 40: 849-859.

Sonenshine, D. 2006. Tick pheromones and their use in tick control. Ann. Rev. Entomol. 51: 557-580.

Steinbrecht, R. 1996. Structure and function of insect olfactory sensilla. Ciba Found Symp. 200: 158-174.

Steinbrecht, R., and B. Muller. 1976. Fine structure of the antennal receptors of the bed bug, *Cimex lectularius* L. Tissue Cell 8: 615-636.

Stutt, A. and T. Siva-Jothy. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. P. Natl. Acad. Sci. U.S.A. 98: 5683-5687.

Suchy, J.T., and V.R. Lewis. 2011. Host-seeking behavior in the bed bug, *Cimex lectularius*. Insects 2: 22-35.

Usinger, R. L. 1966. *Monograph of Cimicidae* (Hemiptera-Heteroptera), The Thomas Say Foundation. Entomological Society of America, Baltimore. M.D

Vaidyanathan, R., and M.F. Feldaufer. 2013. Bed bug detection: current technologies and future directions. Am. J. Trop. Med. Hyg. 88: 619-625.

Walpole, D. 1987. External morphology of the legs of two species of bed bugs (Hemiptera: Cimicidae). J. Ent. Soc. Sth. Afr. 50: 193-201.

Wang, C., T. Gibb, G. Bennett, and S. McKnight. 2009. Bed bug (Heteroptera: Cimicidae) attraction to pitfall traps baited with carbon dioxide, heat, and chemical lure. J. Econ. Entomol. 102: 1580-1585.

Weeks, E.N., M. Birkett, M.M. Cameron, J.A. Pickett, and J.G. Logan, 2010. Semiochemicals of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), and their potential for use in monitoring and control. Pest Manag. Sci. 67: 10-20.

Wertheim, B., E.J. van Baalen, M. Dicke, and L.E.M. Vet. 2005. Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. Annu. Rev. Entomol. 50, 321-346.

Chapter II. Morphology, ultrastructure and functional roles of antennal sensilla that mediate off-host aggregation by the bed bug, *Cimex lectularius*

Introduction

The bed bug, *Cimex lectularius*, is recognized as a pest of significant public health importance by both the Center for Disease Control and Prevention and by the Environmental Protection Agency (CDC and EPA, 2010). Although they have not been shown to transmit human pathogens, bed bug feeding habits can cause severe skin irritations and psychological disorders (Doggett et al., 2012; Goddard and deShazo, 2009). Effective pest management services are expensive and labor intensive and cost the United States an estimated $258 million in 2010 (Alsever, 2010; Nagro, 2012). A better understanding of the basic biology and behavior of this pest could promote more efficient pest management practices.

Immediately after feeding, bed bugs assemble into cracks and crevices within close proximity to a host (Reinhardt and Siva-Jothy, 2007; Usinger, 1966). This off-host aggregation is mediated by multiple factors including circadian rhythms, tactile cues, and chemical stimuli associated with bug feces (Aboul-Nasr and Erakey, 1968; Levinson and Bar Ilan, 1971; Reis and Miller, 2011). Some evidence suggests that volatile cues from bug feces attract bed bugs to off-host aggregation sites (Siljander et al., 2009; Weeks et al., 2010), whereas other reports suggest assembly into aggregation sites is a result of physical contact with the chemical stimuli (Siljander et al., 2007, Olson et al., 2009).

Whether olfactory or gustatory in nature, chemoreceptors are likely responsible for mediating off-host aggregation by bed bugs. Levinson et al. (1974) and Steinbrecht and Mueller (1976) were among the first to describe several distinct types of sensilla located within the olfactory regions of the distal antennal flagellum. Examination and identification of sensilla located on the neighboring pedicel, however, has been limited. Our earlier work has shown that removal of the flagellum (both distal and proximal segments) does not prevent off-host aggregation, but removal of the pedicel does prevent aggrregation (Olson et al., 2009). Thus, investigation of pedicellar sensilla is warranted. Here, we provide further evidence of their role in off-host aggregation and describe the morphological and ultrastructural features of pedicellar sensilla.

Materials and Methods

Insects

Specimens were obtained from stock cultures of the ECL-05 strain of *Cimex lectularius* (Olson et al., 2009) unless noted otherwise. Colonies were maintained under standard conditions of 25° C and 14:10 (L:D) in 473 ml (16 oz) glass jars with folded pieces of filter paper (Fisher 9.0 cm) provided for harborage and egg deposition. Colony jars were covered with a fine mesh fabric (Precision Woven Nylon Mesh 193x193, McMaster Carr, Chicago, IL, USA) with a pore size of 78 µm for ventilation and containment. Colonies were fed weekly using an artificial feeding system adapted from Montes et al. (2002) and soon-to-expire stocks of human blood obtained from the American Red Cross (St. Paul, MN). Field specimens used in the electron microscopy study described below were obtained from a residence for senior citizens in Phoenix, Arizona.

Antennectomy Experiment

A multi-choice assay was performed using 20 cm diameter circular arenas previously described by Olson et al. (2009). Each arena contained one 25 mm stained disk (Whatman™ Grade GF/D), that was conditioned with fecal material by exposure to recently fed bed bugs. The stained disk and seven clean disks were then randomly assigned to eight equally spaced positions around the perimeter of the arena. All disks occupied 12.5 % of the total arena floor space (314 cm^2), and the stained disk occupied 12.5 % of the total disk area. Groups of five males and five females were released into the center of each arena. Treatments were intact bugs (I) or bugs with both antennae severed at A_2, $A_{1.5}$, and A_1 positions (Fig. 1). Replicate arenas ($n = 6$) were housed side-by-side on two shelves in an illuminated growth chamber (Percival E54-U, Boone, IA) maintained at 25 ± 5° C and 40% ± 5% RH. All experiments were initiated approx. 3 h before the end of the bugs' subjective scotophase.

Final positions of bugs were recorded 4 h after release as number under the one stained disk (n_s), number under the remaining seven clean disks (n_c), and number elsewhere on the arena floor (n_f). We calculated arena-level aggregation (P_{arena}) to measure the bugs' propensity to aggregate under any filter paper disk in the arena, regardless of treatment. P_{arena} was indexed by the percentage of released bugs that were under stained and clean disks compared to the total number of bugs in the arena: $P_{arena} = (n_s + n_c)/(n_s + n_c + n_f) \times 100$. We also measured disk-level aggregation (P_{disk}) to assess the bugs' choice among stained and treated disks, which considered only bugs that aggregated under disks of either kind. P_{disk} was calculated as the percentage of bugs that were under the stained disk, compared to the total number of bugs under any disk: $P_{\text{disk}} = n_s/(n_s + n_c) \times 100$. If the final positions of the bugs were independent of stain, then mean values for P_{arena} and P_{disk} would not be significantly different from 12.5%. Values significantly greater than 12.5% would indicate an excess of aggregation at the treated disks, whereas values less than 12.5% would indicate aversion to treated disks.

Electron microscopy

Whole specimens were immersed and dehydrated in an ethanol series consisting of 70 % and 96 % for 24 h each, and 99.5 % for 3 d. Specimens mounted on aluminum stubs with double sided adhesive were air dried and sputter-coated with gold (Polaron E5100, Quorum Technologies, Ringmer, England) prior to imaging with a scanning electron microscope (SEM. S-3400N, Hitachi High-Technologies, Tokyo, Japan). Two bed bugs, one positioned in dorsal view and the other in ventral view, were examined to obtain a total sensillar count for each replicate. Three replicates of the left and right antennae of each stage were examined, making six total sensilla counts per life stage. Six additional antennae from each life stage were positioned in lateral view to count sensilla located in a newly discovered sensory patch (see below).

Because Microwave technology is a relatively new advancement that facilitates tissue penetration of the fixative, the following steps are presented in detail for specimen preparation for transmission electron microscopy (TEM). Antennae were rinsed 3x in 0.1 M phosphate buffer for 20 min, then post-fixed in Dalton's $K_2Cr_2O_7$-OsO_4 mixture (Singh et al., 1996) for 1 min at 0 Watts (W), 80 sec at 120 W, 3 min at 0 W, and 40 sec at 120 W using a PELCO BioWave® Pro Microwave (Ted Pella, Inc., Redding, CA) in a vacuum chamber on a re-circulating water load (PELCO ColdSpot®). Samples were rinsed in 0.1 M phosphate buffer (150 W for 1 min), distilled water (150 W for 1 min) and serially dehydrated in 25%, 50%, 75%, 95%, and 100% (x4) ethanol at 120 W for 1 min each.

Antennae were infiltrated with epoxy resin (Embed-812, Electron Microscopy Sciences, Hatfield, PA) using increasing ethanol:resin ratios: 2:1, 1:1, 1:2, and 100% resin for 3 min at 250 W (x2), 3 min at 0 W, and 3 min at 250 W. Samples were placed on a rotator at room temperature for 8-24 h, embedded in fresh resin, and polymerized for 48 h at 60°C.

Ultra-thin 80-100 nm sections were cut using a diamond knife and collected on carbon-coated copper mesh grids. Sections were post-stained with 3% uranyl acetate followed by Sato's triple-lead stain (Sato 1968) and examined at 60kV with an FEI Phillips CM 12 transmission electron microscope (Hillsboro, Oregon). Images were captured on a SIA L3C digital camera (Duluth, Georgia). Approximately nine individual antennae and 4-40 sensilla of different types were studied by TEM.

Statistical Analysis

Bioassay data were analyzed using Proc NLMIXED in SAS (SAS 2003) to assess effect of antennectomy on arena-level and disk-level aggregation, allowing for contagion among bugs within each arena (Olson et al. 2009). Results were summarized as mean percent ± 95% CI for each treatment group. Sensilla counts by type, life stage, and strain were analyzed using pairwise Wilcoxon rank-sum test, with p-values corrected for multiple comparisons using the Bonferroni-Holm method (Holm 1979).

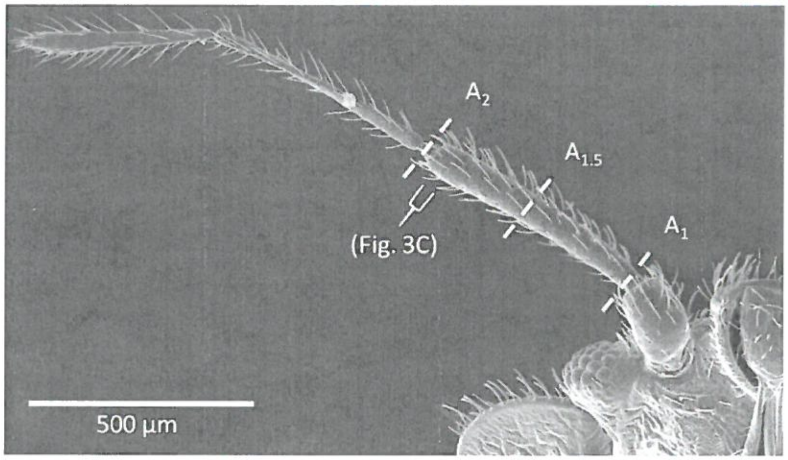

Fig. 1. SEM image of *C. lectularius* antenna in ventral view showing positions (dashed lines) of antennal ablations (A_2, $A_{1.5}$, and A_1) and location of sensory patch on the posterior distal margin of pedicel (see Fig. 3D). Prothorax, compound eye and proboscis are visible, left to right, in lower right corner. The clone tool in Photoshop CS5 was used to remove minor imperfections caused by the adhesive on the mounting stub. No alterations were made to the specimen image.

Results

Bioassay

Arena-level aggregation was unaffected by antennectomy treatments (Fig. 2A). Intact bugs (I) and those with flagella removed (A_2) were equally likely to aggregate under disks of any kind. Within the arena-level response, bed bugs with half or complete removal of the pedicel ($A_{1.5}$ and A_1) were less likely to aggregate under disks, but the means were not statistically different from intact bugs ($P > 0.84$).

In contrast, disk-level aggregation was significantly reduced ($P < 0.01$) by removal of either the distal half ($A_{1.5}$) of the pedicel or the whole pedicel (A_1) (Fig. 2B). Mean disk-level aggregation by bugs with $A_{1.5}$ and A_1 treatments and corresponding 95% confidence intervals were significantly below 12.5%, indicating aversion to the stained disk.

Electron Microscopy

Five distinct types of sensilla were discovered on the pedicel of adult specimens (Fig. 3A–D). The morphological characteristics of four of the sensilla matched those in the olfactory regions on the first and second flagellum previously described by Levinson et al. (1974) and Steinbrecht and Mueller (1976). The four sensilla types included serrated hairs (type A3), grooved pegs (type C), smooth pegs (type D), and immersed cones (type F). The fifth sensillar type on the pedicel most closely resembled the E1 and E2 smooth hairs previously described by Levinson et al., (1974) and Steinbrecht and

Mueller (1976), but the new fifth type lacked cuticular pores. Therefore, we conclude the smooth hairs are different from the previously described ones, and designate them as type E3 sensilla (Fig. 3A–C).

Counts of pedicellar sensilla varied among the five types and the five life stages evaluated (Table 1). There were no significant differences in numbers of sensilla on the pedicels of the left or right antennae of each life stage ($P = 0.87$, data not shown). Hence, positions of antennae were disregarded in subsequent analyses. In addition, there were no significant differences in sensilla counts between males and females ($P > 0.11$) or between lab-reared and field-collected females ($P > 0.19$). Overall, abundance of sensilla increased with developmental stage, however, some types were completely absent on the immature stages. For example, smooth pegs (D) were present in adults, but absent on fifth and first instars. Grooved pegs (C) and immersed cones (F) were also absent on first instars. On the other hand, smooth hairs (E3) were present on all six stages.

The distribution of sensilla on the pedicel of bed bugs also varied by sensillar type. Serrated hairs (A3) were uniformly distributed across the pedicel (Fig 3A), but smooth hairs (E3) were mostly concentrated distally, with only 1-2 sensilla located mid-length on the segment (Fig 3A) and none more basally. Sensillar types C, D, and F on adults were clustered together in a distinctive sensory patch (SP) located distally on the posterior margin of the pedicel (Figs. 1; 3B, D). Sensillar types C and F were also present in the outer lateral regions of the pedicel on fifth instars (not shown).

Serrated hairs (A3) were easily distinguished from other types by TEM based on position, abundance, jagged exterior edge, and absence of dendrites in the lumen (Fig.

4A). Smooth hairs (E3) had a single thick cuticular wall with no evidence of pores (Fig. 4B). In contrast, sensillar types C and D had multiple dendrites and a thin cuticle with numerous spoke channels (Fig. 4C, D). Grooved pegs, type C, also had an inner cuticular wall with uninnervated lumina positioned around the central lumen containing the dendrites that was absent in all other sensilla (Fig. 4C). Images of sectioned immersed cones (type F) could not be obtained.

Table 1. Median (range) numbers of five different types of sensilla on the pedicels of *C. lectularius* of different life stages and genders.

Life stage	Serrated hairs (A3, mech[a])	Smooth hairs (E3, gust)	Grooved pegs (C, olfac)	Smooth pegs (D, olfac)	Immersed cones (F, mech)	Total
1st instar	24.0 (12)a	2.0 (2)a	0.0 (0)a	0.0 (0)a	0.0 (0)a	27.0 (12)a
5th instar	59.0 (16)b	4.0 (4)b	2.0 (2)b	0.0 (0)a	3.0 (1)b	66.5 (17) b
♂	72.0 (8)c	4.5 (3)b	5.5 (5)c	2.5 (3)b	4.0 (0)c	90.0 (12)c
♀	81.5 (18)c	4.0 (2)b	8.0 (3)c	3.0 (2)b	4.0 (1)c	100.5 (18)c
♀(Field)[b]	73.5 (12)c	4.5 (4)b	6.0 (2)c	3.5 (2)b	4.0 (1)c	89.5 (14)c

n = 6, including two antennae from three individual specimens of each life stage. Medians in same column with different letters judged significantly different ($\alpha = 0.05$) by pairwise Wilcoxon rank-sum tests adjusted with Bonferroni-Holm method.

[a] See text for definitions of sensilla and references on function.

[b] Collected in Phoenix, AZ, July 2012

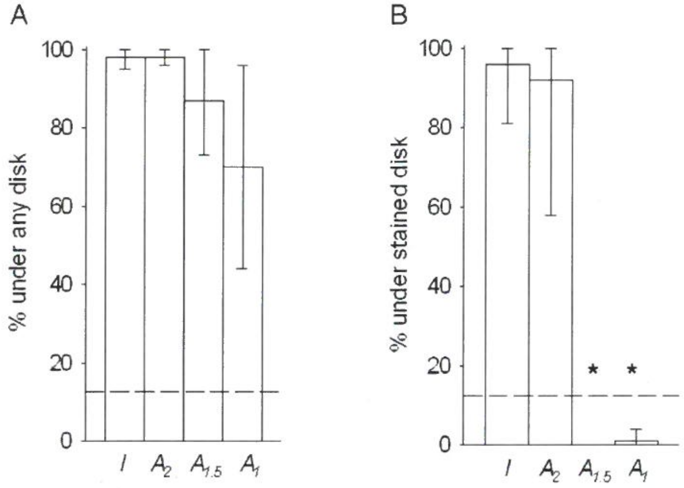

Fig. 2. Effects of antennal ablations on (A) arena-level and (B) disk-level aggregation by adult *C. lectularius*: intact (I), two flagella removed (A_2), two flagella and distal half of pedicel removed ($A_{1.5}$), entire pedicel and two flagella removed (A_1). Error bars represent 95% confidence intervals. Means (n = 6) above 12.5% indicate positive response and means below 12.5% (dashed line) indicate aversion. Treatment effects (*) were tested by significance ($P < 0.05$) of binomial regression coefficients for treatments compared to intact (I) bugs.

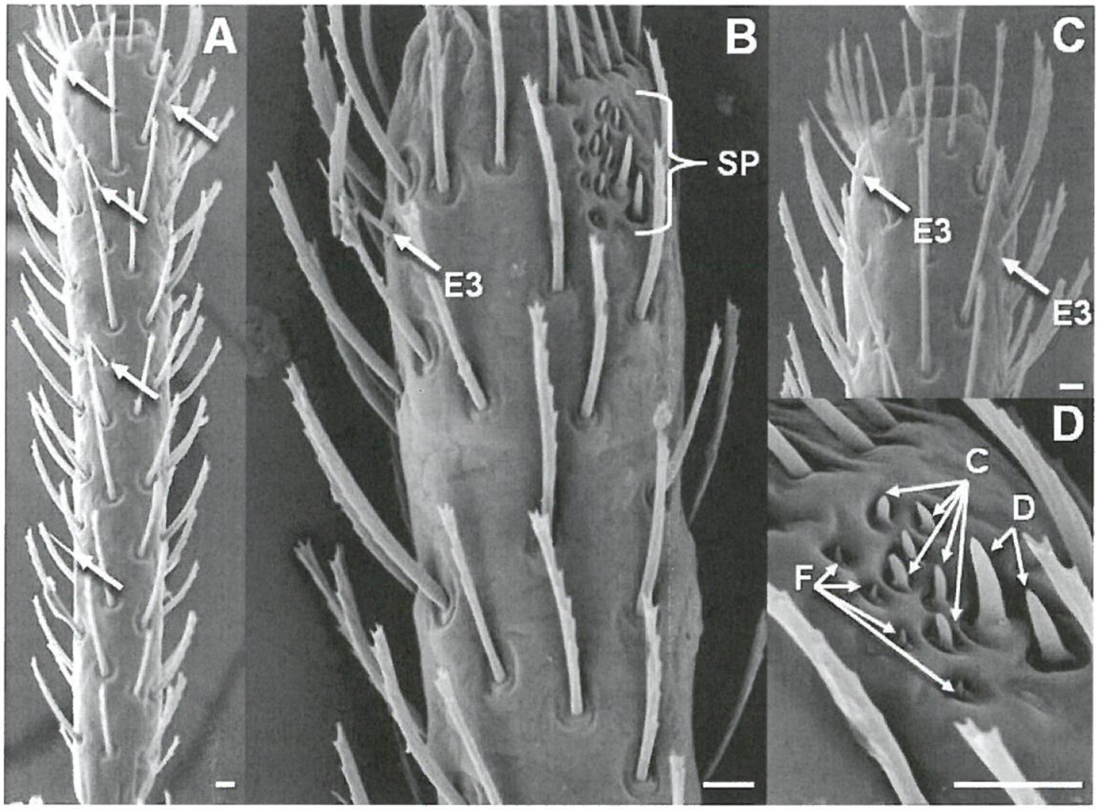

Fig. 3. SEM images of male *C. lectularius* pedicel showing newly discovered smooth hairs (E3) and a distal sensory patch (SP). (A) Dorsal view of left pedicel showing numerous serrated hairs and five smooth hairs (arrows). (B) Posterior view of distal half of left pedicel showing serrated hairs, one smooth hair, and sensory patch. (C) Close-up view of left pedicel showing two E3 hairs (arrows). (D) Close-up view of the sensory patch with sensillar types C, D, and F. Scale bars = 10 μm.

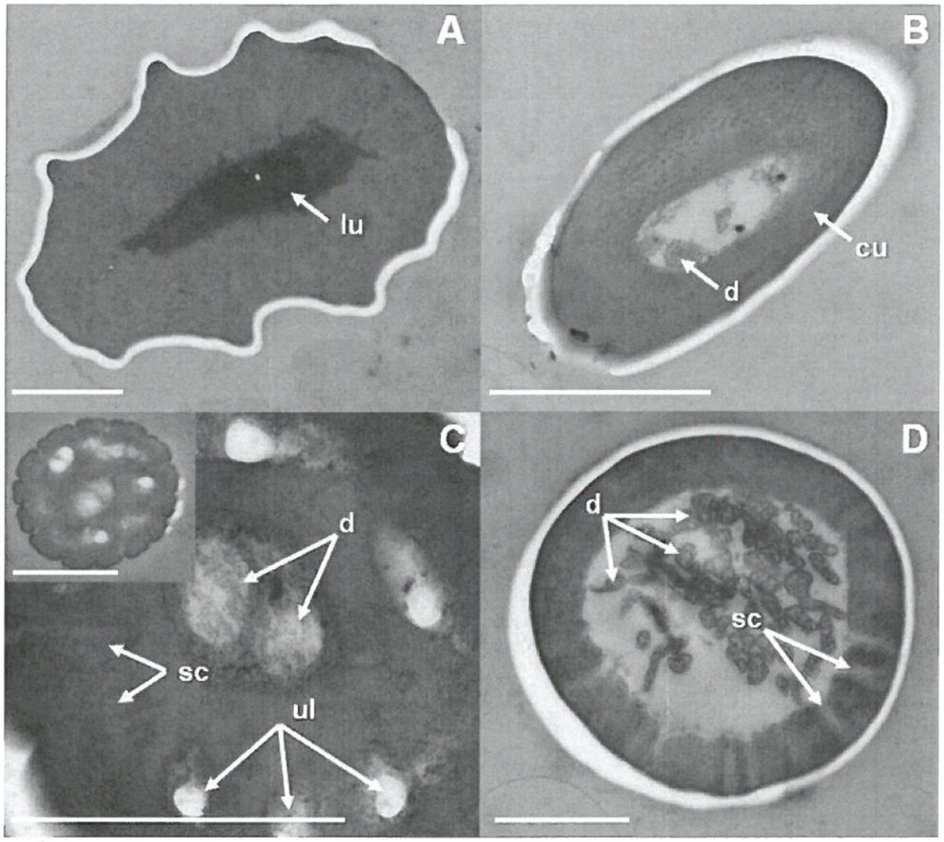

Fig. 4. TEM images of four types of pedicellar sensilla of *C. lectularius*. (A) Mechanosensory serrated hair type A3 from shaft of pedicel, devoid of dendrites in the lumen (lu). (B) Gustatory smooth hair type E3 from shaft, with a single dendrite (d) and thick exterior cuticle lacking pores. (C) Olfactory grooved peg type C from the sensory patch showing multiple grooves along the exterior cuticle (upper left image) and a double walled cuticle (cu), uninnervated luminae (ul), spoke channels (sc) and two dendrites (d) within the center wall (center image). (D) Olfactory cone shaped hair type D from the sensory patch showing multiple pores and spoke channels (sc) and numerous dendrites (d) in lumen. Whitish rings around exterior of all images are artifacts of separation from embedding plastic. Scale bars = 1 µm.

Discussion

Results from our antennectomy experiments (Fig. 2B) indicate that sensilla on the distal half of the pedicel are necessary for off-host aggregation by bed bugs. While it is possible that sensilla sensitive to off-host aggregation stimuli may be present on the first or second antennomere of the flagellum, removal of the flagella did not reduce the aggregation response by bed bugs in the present study or in a previous study (Olson et al., 2009). Thus, pedicellar sensilla are either the primary mediators of off-host aggregation, or they functionally complement those sensilla on regions of the flagellum.

Curiously, extirpation of the pedicel not only resulted in the loss of aggregation, but apparently caused bed bugs to avoid disks that contained bed-bug feces (Fig. 2B). This apparent switch to avoidance may be a consequence of multiple factors, including an unmasked contribution of yet to be identified receptors that are not on the antenna, but that are sensitive to bed-bug fecal material. We speculate that under normal pedicel-intact conditions, the entire collection of sensory inputs results in a neural code mediating aggregation behaviors, but a loss or imbalance of appropriate olfactory-receptor encoding and subsequent activation (or inactivation) of central glomeruli associated with attraction or avoidance behaviors might be shifted. In the fruit fly, for example, specific olfactory glomeruli have been identified that mediate either attraction or avoidance behaviors. In response to the same olfactory cue, but at varying concentrations, shifts between attraction and avoidance can occur (Kreher et al., 2008; Semmelhack and Wang 2009). With the loss of some olfactory projections mediating bed-bug-specific chemical cues, activities in glomeruli that mediate attraction-related behaviors may be shifted to a potential avoidance-mediating glomerulus(i), leading to the fecal-specific avoidance behavior observed here.

This report is the first to identify multiple sensillar types on the pedicel of *C. lectularius*. Sensillar types A3, C, D, and F identified on the pedicel here were similar to those previously described in the olfactory regions on the second flagellum by Levinson et al. (1974) and Steinbrecht and Mueller (1976). The type E3 sensilla described here resemble the single-walled double-lumina sensilla (typical gustatory) identified on the second flagellum of *Cimex hemipterus* F. by Singh et al. (1996). The sensory patch we identified on the posterior margin of the pedicel has not been described previously for either *C. lectularius* or *C. hemipterus* species. However, it may have been overlooked due to its limited visibility in dorsal and ventral views.

There was an increase in abundance of sensillar types and counts during metamorphosis to adult, with several types completely absent in first instars (Table I). An increase in abundance and generation of sensilla with each successive molt is common among insects (Zackaruk and Shields, 1991). Absence of the sensory patch on the pedicel of first and fifth instars is noteworthy, but similar sensilla types may be present on other segments of the bed bug antennae or body regions of these stages.

Pedicellar sensillar types C and D located in the sensory patch have morphological characteristics consistent with olfactory sensilla (Chapman, 1998) and are likely involved in alarm, aggregation, and host-finding behaviors. Single cell recording techniques have demonstrated that olfactory sensilla type D, located in the olfactory regions of the second flagella, respond to trans-2-hexanal (T2H), trans-2-octanal (T2O), dimethyl trisulfide, dimethyl disulfide, benzaldehyde, sulcatone, indole, and ethyl butyrate (Harraca et al. 2009). The aldehyde compounds, T2H and T2O, are major constituents of the alarm and airborne aggregation pheromone emitted by bed bugs (Levinson et al., 1974; Siljander et al., 2009). Olfactory sensilla of type C, also located in the olfactory regions, respond to ammonia (Harraca et al., 2009), a common host odorant detected by *Aedes aegypti* and other blood feeding insect pests (Friend and Smith, 1977; Geier et al., 1999). The type F sensilla also located in the sensory patch and described previously on the olfactory regions of the second flagella are likely thermo-/hygroreceptors based on comparative morphological characteristics (Steinbrecht and Mueller, 1976; Steinbrecht, 1998). However, the function of sensillar type F in *C. lectularius* remains unknown.

Type E3 sensilla were present on the distal half of the pedicel in all life stages evaluated. The type E3 sensilla externally resembled types E1 and E2 in the olfactory regions of bed bug antennae (Levinson et al., 1974), but unlike E1 and E2, type E3 appear to lack olfactory pores. Internal structures of type E3 are consistent with gustatory or contact chemoreceptors described by Chapman (1998). Initial electroantennogram studies suggested that sensillar types E1 and E2 were related to bed bug alarm pheromones (Levinson et al., 1974), but a more precise follow-up study using single cell recording techniques failed to confirm this function (Harraca et al, 2009). Distinction between type E hairs can only be determined by internal examinations of individual sensilla. Thus, the behavioral function and overall abundance of type E1, E2, and E3 sensilla on the antennae of *C. lectularius* remain unknown.

Off-host aggregation by bed bugs may be mediated by a combination of volatile and non-volatile cues. Both olfactory and gustatory sensilla exist on the distal half of the pedicel, making this region capable of detecting a variety of volatile and non-volatile stimuli. Airborne attractants have been identified in a headspace analysis of bed bugs (Siljander et al., 2008). However, range of attractiveness and stability of the volatiles are limited, suggesting some components of the bed bug aggregation pheromone remain to be described. Establishment of aggregation sites by triatomid bugs, a blood feeding pest of vertebrates in tropical regions, is driven by both volatile and non-volatile contact pheromones found in fecal and cuticular extracts (Guerenstein and Lazzari, 2009). Similar extracts from bed bugs should be analyzed further for evidence of non-volatile stimuli related to off-host aggregation.

In summary, the present study demonstrates that the pedicel of *C. lectularius* is essential to off-host aggregation. Five types of sensilla exist on the pedicel including both olfactory and gustatory chemoreceptors. These findings will allow subsequent researchers interested in the chemosensory basis of off-host aggregation to identify the physiological sensitivities and tuning properties of each of these sensilla.

Literature Cited

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968. Behaviour and sensory physiology of the bed-bug, *Cimex lectularius* L., to some environmental factors: chemoreception. Bull. Soc. Entomol. Egypt 52: 353-362.

Alsever, J. 2010. Bedbug invasion is turning into big business. U.S. Business on NBCnews.com; Updated: 10/11/2010.http://www.nbcnews.com/id/39526413/ns/business-us_business/

CDC and EPA. 2010. Joint statement on bed bug control in the United States from the U.S. Centers for Disease Control and Prevention (CDC) and the U.S. Environmental Protection Agency (EPA). Atlanta: U.S. Department of Health and Human Services; Updated: 5/8/2013. http://www.cdc.gov/nceh/ehs/publications/bed_bugs_cdc-epa_statement.htm.

Chapman, R.F. 1998. The insects. Structure and function. Cambridge: Cambridge University Press.

Chapman, R.F. 2003. Contact chemoreception in feeding by phytophagous insects. Ann. Rev. Entomol. 48: 455-484.

Doggett, S. L., D. E. Dwyer, P. Penas, and R. Russell. 2012. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25: 164-192.

Friend, W.G., and J. J. Smith. 1977. Factors affecting feeding by bloodsucking insects. Ann. Rev. Entomol. 22: 309-331.

Geier, M., J. O. Bosch, and J. Boeckh. 1999. Ammonia as an attractive component of host odour for the yellow fever mosquito, *Aedes Aegypti*. Chem. Senses 24: 647-653.

Goddard, J., and R. DeShazo. 2009. Bed bugs (*Cimex lectularius*) and the clinical consequences of their bites. J. Am. Med. Assoc. 301: 1358-1366.

Guerenstein, P.G., and C. R. Lazzari. 2009. Host-seeking: how triatomines acquire and make use of information to find blood. Act Tropica 110: 148-158.

Harraca, V., R. Ignell, C. Lofstedt, and C Ryne. 2009. Characterization of the antennal olfactory system of the bed bug (*Cimex lectularius*). Chem. Senses 35: 195-24.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6: 65-70.

Johnson, C.G. 1941. The ecology of the bedbug, *Cimex lectularius* L., in Britain. J. Hyg. Cambridge 41: 345-361.

Kreher, S. A., D. Mathew, J. Kim, and J. R. Carlson. 2008. Translation of sensory input into behavioral output via an olfactory system. Neuron 59: 110–124.

Levinson, H. Z., and A. R. Bar Ilan. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. Experientia 27: 102-103.

Levinson, H. Z., A. R. Levinson, B. Mueller, and R. A. Steinbrecht, 1974. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. J. Insect Physiol. 20: 1231-1248.

Montes, C., C. Cuadrillero, and D. Vilella. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera Cimicidae) using an artificial feeding technique. J. of Med. Entomol. 39: 675-679.

Nagro, A. 2012. Bed bug supplement: game changer or gimmick? Pest Control Technol. 40: 78-85.

Olson, J.F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect Physiol. 55: 580-587.

Reinhardt, K., and M. T. Siva-Jothy. 2007. Biology of the bed bugs. Ann. Rev. Entomol. 52: 351-374.

Reis, M.D., and D. M. Miller. 2011. Host-searching and aggregation activity of recently fed and unfed bed bugs (*Cimex lectularius* L.). Insects: 186-194.

Sato, T. 1968. A modified method for lead staining of thin sections. J. Electron Microsc. 17: 158–159.

Semmelhack, J.L., and J. W. Wang. 2009. Select drosophila glomeruli mediate innate olfactory attraction and aversion. Nature 459: 218-224.

Siljander E., D. Penman, H. Harlan, and G. Gries. 2007. Evidence of male- and juvenile-specific contact pheromones of the common bed bug *Cimex lectularius*. Entomol. Exp. Exp. Appl. 125: 215-219.

Siljander, E., R. Gries, G. Khaskin, G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708-718.

Singh, R.N., K. Singh, S. Prakash, M. J. Mendki, and K. M. Rao. 1996. Sensory organs on the body parts of the bed-bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) and the anatomy of its central nervous system. Int. Insect Morph. 25: 183-204.

Steinbrecht, R.A., and B. Muller. 1976. Fine structure of the antennal receptors of the bed bug, *Cimex lectularius* L. Tissue Cell 8: 615-636.

Steinbrecht, R. A. 1998. Bimodal thermo- and hygrosensitive sensilla. Pp. 405 – 422. – *In*: F. W. Harrison & M. Locke (eds), Microscopic Anatomy of Invertebrates, vol. 11 B, Insecta. Wiley-Liss, Inc., New York.

Usinger, R. L. 1966. *Monograph of Cimicidae* (Hemiptera-Heteroptera), The Thomas Say Foundation. Entomological Society of America, Baltimore. M.D

Weeks, E. N. I., J. G. Logan, S. A. Gezan, C. M. Woodcock, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2011. A bioassay for studying behavioral responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimcidae) to bed bug-derived volatiles. Bull. Entomol. Res. 101: 1-8.

Zackaruk, R. Y., and V. D. Shields. 1991. Sensilla of immature insects. Ann. Rev. Entomol. 36: 331-354.

Chapter III. Investigation, extraction, and isolation of contact pheromones responsible for off-host aggregation by bed bugs, *Cimex lectularius*

Introduction

The bed bug, *Cimex lectularius*, is a significant pest of economic importance. Although transmission of human pathogens by bed bugs has not been demonstrated, some humans exposed to bed bug attacks develop cutaneous reactions, anemia, insomnia, and psychological disorders (Feingold et al., 1968; Reinhardt et al., 2009; Goddard and DeShazo, 2009). In addition to their medical importance, bed bug infestations are difficult to control and management practices can be expensive, with costs estimated to be $3 billion since early 2000 in the United States (Vaidynanthan, 2012). Research aimed at improving our understanding of the behavior of this important economic pest would greatly improve prevention and control methods.

After feeding, bed bugs form aggregations in cracks and crevices near their host. Aggregation is established and sustained by chemical stimuli associated with bed bug feces (Aboul-Nasr and Erakey, 1968; Levinson and BarIlan, 1971). Some reports have shown that aggregation is mediated by volatile compounds (Siljander et al., 2008; Weeks, et al., 2010), whereas others have demonstrated that contact with the source is necessary in order to elicit aggregation and arrestment by bed bugs (Siljander et al., 2007; Olson et al., 2009). If contact is required, then the stimuli or pheromones responsible for aggregation and arrestment would likely be comprised of non-volatile components. Examination of non-volatile stimuli or contact pheromones associated with bed bug feces should be investigated further.

The airborne aggregation pheromone consists of a complex blend of volatile components consisting of (E)-2-hexenal (E2H), (E)-2-Octenal (E2O), benzaldehyde, sulcatone, octanal, limonene, benzyl alcohol, (2E, 4E)-Octadienal, nonanal, (2E, 4E)-octadienal, benyl acetate, decanal, and geranylacetone (Siljander et al., 2008). The airborne pheromone was collected by extracting the volatiles collected in the air space above colonies of bed bugs. The two most abundant compounds of the blend (>70% of the total composition) include E2H and E2O, which also make up the two main constituents of the bed bug alarm pheromone (Collins, 1968; Levinson et al., 1974). Although it is not uncommon for the same compound(s) to elicit both attraction and repellent behaviors in heteropterans (Moraes et al., 2008), it is unclear how volatile compounds such as E2H and E2O are responsible for establishing and more importantly, sustaining aggregations of bed bugs.

The volatile components of the airborne pheromone may summon aggregation, while less volatile or contact pheromones from feces may be responsible for sustaining aggregation or arrestment. Here, we evaluated the importance of volatile and non-volatile components of the bed bug aggregation pheromone. We also extracted, isolated, and separated the active components of the bed bug feces using analytical techniques.

Methods and Materials

Source of Insects

The bed bugs used in the following assays were obtained from stock cultures of the ECL-05 strain of *Cimex lectularius* (Olson et al., 2009). Colonies were maintained under standard conditions of 25 °C and 14:10 (L:D) in 473 ml (16 oz) glass jars with folded pieces of filter papers (Fisher 9.0 cm) provided for harborage and egg deposition. Colony jars were covered with a fine mesh fabric (Precision Woven Nylon Mesh, McMaster Carr, Chicago, IL, USA) with a pore size of 78 μm for ventilation and containment. Colonies were fed weekly using an artificial feeding system adapted from Montes et al. (2002), which included a stretched Parafilm feeding membrane and soon-to-expire stocks of human blood (type A+) obtained from the American Red Cross (St. Paul, MN). Bugs were fed to repletion 48 h prior to each assay.

Preparation of Fecal Stained Disks

Groups of 24 bed bugs (12 male: 12 female) were used to stain disks. After feeding, each group was transferred onto a clean disk of 25 mm diam. (Whatman™ Grade GF/A) glass filter paper, housed in a 60 mm dia. x 15 mm tall glass Petri dish (VWR, Radnor, PA). Bed bugs remained in contact with the disks for 48 h, after which they were removed. The disks were visibly stained with excrement.

Behavioral Assays & Statistical Analysis

A standard arena assay was used to assess bed bug preference for different filter paper treatments using 20 cm diameter circular arenas previously described by Olson et al. (2009). Each arena contained one treated disk and seven untreated (control) disks that were randomly assigned to eight equally spaced positions approx. 5 mm from the interior perimeter of the arena. All disks were adhered to a paper floor (Boise X-9®, Boise, Idaho) with a spot of hot glue (Surebonder, FPC Corp., Wauconda, IL), which provided a tactile stimulus and allowed for bug entry under the disk. The eight disks occupied 12.5 % of the total arena floor space (314 cm^2). Groups of ten bed bugs (5 male: 5 female) were collected from the larger colony, maintained under an inverted 20 ml glass vial (Fisher Scientific, Waltham, MA) for five minutes in the center of each arena, and simultaneously released by removing the vial at the start of the experiment. Replicate arenas with different treatments were housed side-by-side under incandescent lighting and standard conditions (25 °C ± 5 °C and 40% ± 10% RH). All experiments were started approximately 3 h prior to the end of the bugs' accustomed scotophase.

Final positions of the bed bugs were recorded 4 h after release as number under the treated disk (n_t), number under the remaining seven control disks (n_c), and number elsewhere on the arena floor (n_f). Arena-level aggregation (P_{arena}) evaluated the bugs' natural propensity to aggregate under any filter paper disk, rather than remain out on the arena floor. P_{arena} was calculated as the percentage of released bed bugs that were under the treated and clean disks compared to the total number of bugs in the arena:

$$P_{\text{arena}} = (n_t + n_c)/(n_t + n_c + n_f) \times 100$$

Disk-level aggregation (P_{disk}) determined the bed bugs preference for the treated disk compared to the control disks, and P_{disk} was calculated as the percentage of bed bugs that were under the treated disk, compared to the total number of bed bugs under any disk in the arena:

$$P_{\text{disk}} = n_t/(n_t + n_c) \times 100$$

If the final positions of the bugs were independent of treatment, then mean values for P_{arena} and P_{disk} would not be significantly different from 12.5%. Values greater than 12.5% for Arena- and Disk-level aggregation would indicate aggregation and attraction to the treated disk, whereas values less than 12.5% would indicate aversion. P_{arena} and P_{disk} were analyzed as bionmial responses with Proc NLMIXED in SAS (SAS, 2004) to assess main treatment effects and interactions, and to allow for contagion among bugs within the arena. Results were summarized as mean percent aggregation, with 95% confidence intervals, for each treatment. The treatment effects were tested for significance ($\alpha < 0.05$) of coefficients against the designated control treatment in each assay.

Volatile Removal and Test for E2H and E2O from Fecal Stained Disks

To remove volatile components from bed bug stained disks and asses its affect on aggregation, groups of stained disks were heated in an oven (Fisher Scientific 390; H&C Thermal Systems, Columbia, MD) set at 50 ± 5.0 °C for 0, 3, 7, and 14 days (n = 15). Aggregation to heat-treated disks was compared to non-treated stained disks using the arena assay.

To test for the presence of volatiles emanating from the stained disks, solid phase micro-extraction (SPME) was used to measure the presence of trans-2-hexenal (T2H) and trans-2-octenal (T2O) in the headspaces over a second set of heat treated disks (n = 5). The heat-treated disks were placed individually in 20 ml glass vials with magnetic polymer screw caps (Gerstel, Linthicum, MD). A carboxen/polydimethylsiloxane fiber (CAR/PDMS SPME light blue; 85 µm dia.; Supelco, St. Louis, MO) was inserted in the headspaces for ten minutes to absorb the volatile components while vials were held at 50 °C. Volatiles were collected in this manner for heat treated disks at 0. 3, 7 and 14 days, and the E2H/E2O standards. After exposure, each SPME fiber was inserted in an Agilent 6890 gas chromatograph and Agilent 5975 mass spectrometer (GC-MS) for 90 sec at 250 °C and analyzed by an with a Rtx-5MS column (30.0 m x 250.0 µm; 0.50 µm film thickness; Restek, Bellefonte, PA, USA) with helium as the carrier gas. The temperature profile of the GC oven was initially 35 °C, and increased 15 °C/min to a final temperature of 300 °C. Detection limit for each aldehyde was 0.01 µg per fiber. Differences in presence of the two aldehydes were analyzed statistically with binomial regression. Numbers of samples above the detection limit (≥ 0.01 µg) was a binomial response (n = 5 per treatment combination), and predictors were type of aldehyde and days in oven. Computations were done with the glm package in R (R Core Team 2014), with binomial errors.

Extraction, Restoration and Concentration of the Contact Pheromone

To identify the optimal solvent for extraction of the contact pheromone, groups of four stained disks were submerged in 50 ml of different solvents in 250 ml Erlenmyer flasks and shaken for one hour using a Model C24 Incubator Shaker (New Brunswick Scientific, Edison, NJ) followed by two additional washes each consisting of 50 ml additional solvent. Solvent treatments included hexane, methylene chloride, methanol, and water (n = 16). Solvent washed disks were allowed to dry for 24 h before assay. Blank disks used in each arena were also washed with its corresponding solvent treatment and allowed to dry for 24 h. Aggregation to washed disks was compared to un-washed disks.

To demonstrate retention and restoration of the contact pheromone in methanol extract, a group of six stained disks were tripled washed in methanol as previously described. The extracted solution was then concentrated to 1.2 ml using an Oraganovation N-EVAP 112 (CAE, Redwood City, CA) and 200 μl aliquots of the concentrated extract were re-applied to blank disks (n=6). The solvent washed and extract treated disks were allowed to dry 24 h prior to the start of the assay. Similarly, the other seven blank disks in each assay arena were either left blank, washed, or washed and restored with methanol, respectively. Mean percent aggregation under methanol washed and extract treated disks was compared to stained disks.

To determine if aggregation to the methanol extract was dependent on concentration, 150 stained filter papers were removed from colony jars, extracted in methanol, quantified, and assayed as previously described. The methanol extract was filtered (Supelco Isodisk™ 25 mm x 1 µm; Bellefante, PA) and concentrated to 30 mg/ml and then diluted to 3, 0.3, and 0.03 mg/ml, respectively. Aliquots (200µl) of each concentration were applied to clean disks and dried 24 h prior to the start of the assay for a total of four treatments (n = 6). Blank disks for each arena were treated similarly, with 200 µl aliquots of methanol. Mean percent aggregation of each diluted treatment was compared to 30mg/ml of the original extract.

Separation and Clean-up of the Extract

Preliminary examination of the fecal extract in methanol by GC-MS revealed a substantial number of unknown compounds, making further methods of identification difficult. Separating the active components from the crude extract is a critical step in identification of unknown compounds. Therefore, Solid Phase Extraction (SPE) techniques using Sep-Pak® cartridges (Water, Milford, MA) were evaluated to reduce the number of unknown compounds and prepare the sample for further identification (Table 1). A concentrated dried fecal extract (60 – 90 mg) was reconstituted in 4 g of methanol (EMD Millipore Corp. Billerica, MA) or H_2O (Gradient Milli-Q; Millipore Corp.

Bedford, MA). Solutions were then filtered through a Millex-HV, 0.45 µm PVDF (Millipore Corp. Bedford, MA) filter disk. The samples were then added to each cartridge. The un-retained fraction of the sample was collected for further analysis (Fraction #1; Table 2). Reversed phase extraction was used for C18, NH2, Diol, and Cyano cartridges, while Silica was used in normal phase. Creating reversed phase for CN, Diol and NH2 cartridges consisted of pre-wetting each cartridge with the appropriate organic solvent and then applying the aqueous solution. Separation in reverse phase occurred by decreasing the solvent polarity, and by increasing polarity in normal phase for the silica cartridge using a series of solvent ratios applied to each cartridge (Table 2). Each solvent ratio applied in succession to the cartridge yielding Fractions #2 - #4 for further analysis.

To determine which fraction contained active components, each was tested for aggregation using the arena assay described above. Aliquots (200 µl) of each fraction was applied to clean disks and allowed to dry for 24 h prior to the start of the assay. Blank disks were treated similarly with methanol, methyl cyanide, ethyl acetate, or water, respectively. Aggregation to each fraction was then assayed and compared to the original crude extract.

Active fractions were analyzed by GC-MS to estimate the number of unknown compounds in the fraction and compare it to the original extract. A reduction of unknown components would indicate whether separation of the actives occurred. Each active fraction was injected (2µl) at 250 ^{o}C and a 20:1 split ratio. The fractions were analyzed on a Trace DSQ Single Quadrupole GC-MS (Thermo Fisher Scientific, San

Jose, CA). The GC-MS was equipped with a ZB-5MS column (30 m x 0.25 mm; 0.25 µm film thickness, Phenomenex, Torrance, CA.) The oven program included 50 °C for 5 min, then increased by 20 °C/min with a final temperature of 320 °C for 5 min. Helium was the carrier gas at constant flow of 1.0 ml/min and linear velocity of 36 cm/sec. Mass spectra (EI, 70 eV) were recorded from 28-750 amu. The mass spectra and retention times for the un-identified compounds within the active fraction were recorded.

Table 1. Summary of the Solid Phase Extraction (SPE) cartridges used to separate active stimuli associated with bed bug fecal extract.

Type	**Packing Material**	**Phase***	**Packing Weight (g)**	**Application**
C18	octadecyl bonded silica	RP	0.4	For reversed phase extraction of non-polar to moderately polar compounds
NH2	aminopropyl bonded silica	RP	0.4	For reversed phase extraction of moderately polar compounds combining a somewhat normal phase packing with reverse phase solvents.
Diol	Diol bonded silica	RP	0.4	For reversed phase extraction of moderately polar compounds combining a somewhat normal phase packing with reverse phase solvents.
Cyano	Cyano-propyl bonded silica	RP	0.4	For reversed phase extraction of moderately polar compounds combining a somewhat normal phase packing with reverse phase solvents.
Silica	bonded phase	NP	0.8	For normal phase extraction of polar material

* reverse phase (RP); normal phase (NP)

Table 2. Solvent collection method for each Sep-Pak® cartridge and the isolation of active compounds.

		Fraction Collected*				
Type	**Original Sample**	**#1**	**#2**	**#3**	**#4**	**Isolation of actives****
C18	H2O	Un-retained Sample	10/90 (MeOH/H_2O)	20/80 (MeOH/H_20)	100 (MeOH)	Yes
NH2	H2O	Un-retained Sample	10/90 (MeCN/H_20)	20/80 (MeCN/H_2O)	50/50 (MeCN/H_2O)	No
Diol	H2O	Un-retained Sample	100 (H_2O)	25/75 (MeOH/H_2O)	50/50 (MeOH/H_2O)	No
Cyano	H2O	Un-retained Sample	100 (H_2O)	50/50 (MeOH/H_2O)	100 (MeOH)	No
Silica	EA	Un-retained Sample	100 (EA)	50/50 (EA/MeOH)	100 (MeOH)	No

*Solvents: MeOH = methanol, H_2O = water, MeCN = methyl cyanide, and EA = ethyl acetate

** Isolation of actives was determined by an arena assay response > 12.5% to one or more of the fractions collected, see Fig. 6.

Results

Volatile Removal and Test for E2H and E2O from Fecal Stained Disks

Heating the stained disks to 50.0 ºC for several days did not affect arena- or disk-level aggregation by bed bugs ($P > 0.36$) (Fig. 1). An average of 99.4 % ± 1.1 of bed bugs were under disks of either kind, and 81.0 % ± 22.5 of those under disks chose the stained disks. In subsequent assays, arena-level aggregation was ≥ 83.5% and will continue to be represented in each of the figures, but not discussed in the results.

The percentage of samples that contained >0.01 µg of either compound decreased with number of days in the oven (Fig. 2). Detectable amounts of both aldehydes were recorded in almost all samples at day zero (E2H: 80%; E2O: 100%), whereas only 20% of the samples had >0.01 µg of E2H at 14 d and 0% for E2O.

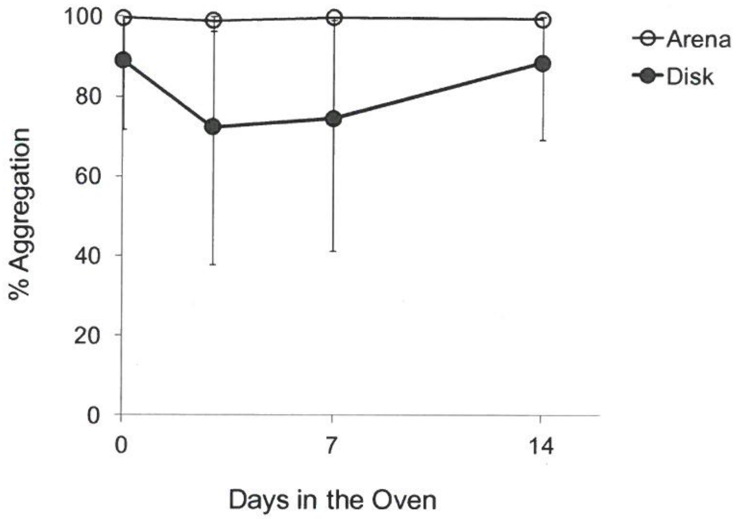

Fig. 1. Mean arena- and disk-level aggregation by adult *C. lectularius* under disks that were heat treated (50 °C ± 5.0 °C) for 0, 3, 7, and 14 days (n = 15). Error bars indicate 95% confidence intervals (CIs). A random response to arena- and disk-level aggregation is represented by the line (---) at 12.5%.

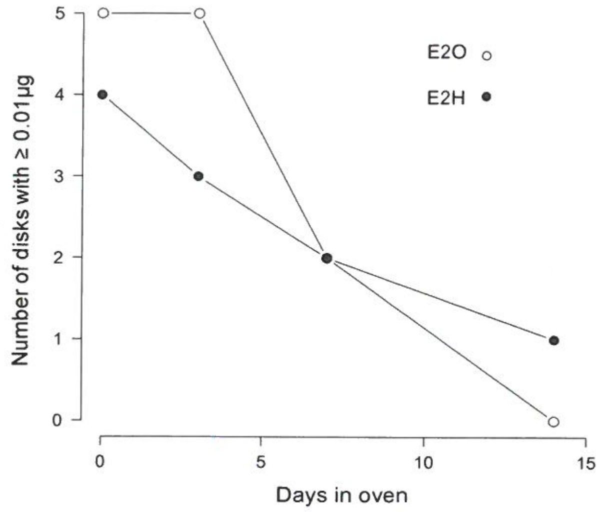

Fig. 2. Number of stained disks containing detectable amounts (0.01 µg) of (E)-2-hexenal (E2H) and (E)-2-octenal (E2O) at 50 °C for 0, 3, 7, and 14 days.

Extraction, Restoration, and Concentration of the Stimulus

Extracting or washing the stained disks in the four solvents did affect disk-level aggregation (Fig. 3). Aggregation under disks washed in MeOH (1.2% ± 2.5) and H_2O (< 1.0% ± 0.1) was significantly lower compared to stained disks (85.7% ± 23.9; $P = 0.04$ and $P < 0.01$, respectively). Thus, washing the disks in MeOH and H_2O removed contact stimuli or pheromones associated with bed bug aggregation.

Extracting and restoring the contact pheromone in methanol also affected disk choice (Fig. 4). Washing the disks in methanol significantly ($P < 0.01$) reduced disk-level aggregation to 0.1% ± 0.1 compared to 99.6% ± 0.2 of bed bugs aggregated under un-washed stained disks. When the concentrated extract was added back to the washed disks, disk-level aggregation by bed bugs was restored with 99.6% ± 2.9 aggregating, which was not significantly different from stained disks ($P = 0.80$), indicating that chemical stimuli associated with bed bug aggregation can be removed and restored in methanol.

Bed bug response was also affected by concentration of the methanol extract (Fig. 5). Mean disk-level aggregation was significantly reduced ($P < 0.04$) to 3.5% ± 11.9 for 300 μg/μl and 0.1% ± 0.4 for 30.0 μg/μl concentrations of extract compared to 3×10^4 μg/μl with 98.3% ± 5.7 disk-level aggregation. Thus, bed bug aggregation under disks treated with methanol extracts was concentration dependent.

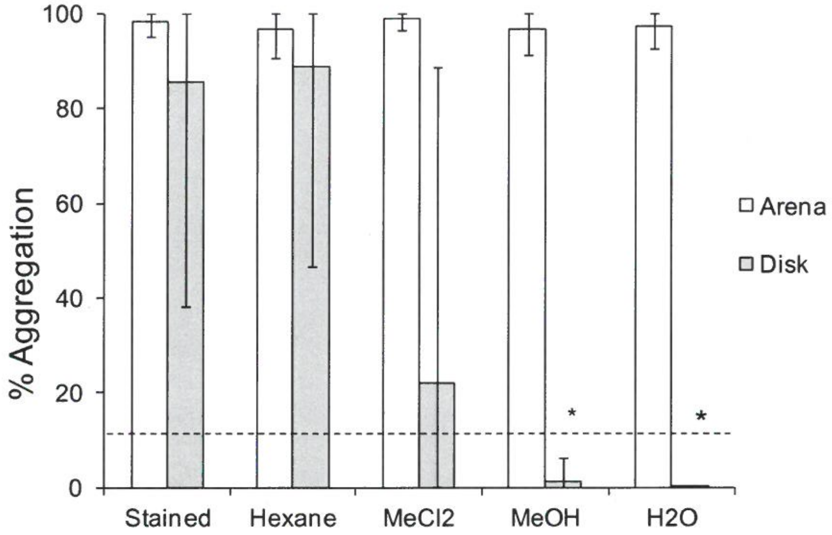

Fig. 3. Arena- and disk-level aggregation by *C. lectularius* under stained disks washed in hexane, methylene chloride (MeCl2), methanol (MeOH), or water (H_2O) compared to aggregation under un-washed stained disks (n = 16). Asterisks indicate significant contrasts ($P < 0.05$) with response to stained papers. Other symbols as in Fig. 1.

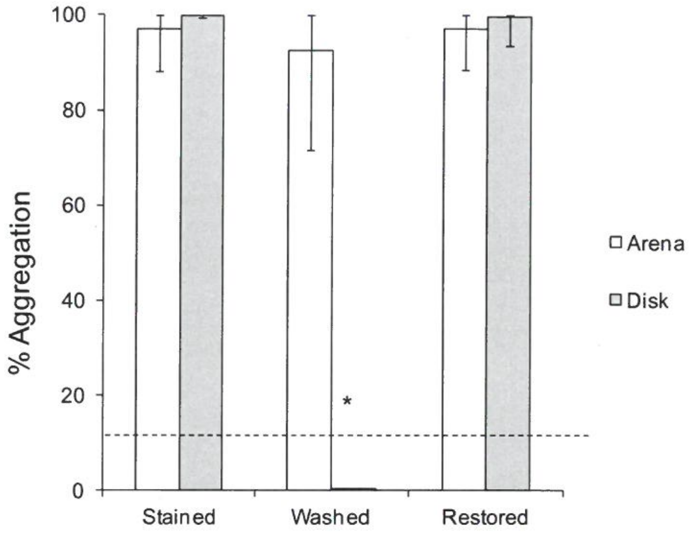

Fig. 4. Arena- and disk-level aggregation by *C. lectularius* under stained disks, stained disks washed in methanol, and blank disks treated with concentrated methanol extract collected from stained disks. Asterisks indicate significant contrasts ($P < 0.05$) with response to stained papers (n =6). Other symbols as in Fig. 1.

Separation and Clean-up of the Extract

Fractions collected from the NH_2, Cyano, and Diol cartridges did not separate the original extract. However, use of C18 cartridges and the solvent method described permitted isolation of active compounds (Table 2). There was a positive disk-level response to Fractions #1 and #2 (Fig. 6). Disk-level aggregation was reduced with each additional solvent wash, with the first fraction (#1) resulting in the highest aggregation response (95.27% ± 21.13). Disk-level response to the original extract collected in methanol was low with only 1.5% ± 8.26 of the bugs under the treated disk.

The GC-MS analysis of Fraction #1 collected from the C18 cartridge indicated that many of the unknown materials from the original extract had been removed from the original extract (Fig. 7). Over 150 unknown compounds were identified within the original extract (Fig. 7A) compared to approx. 25 in Fraction #1 (Fig. 7B). Thus, the total number of unknown materials was reduced by 83%.

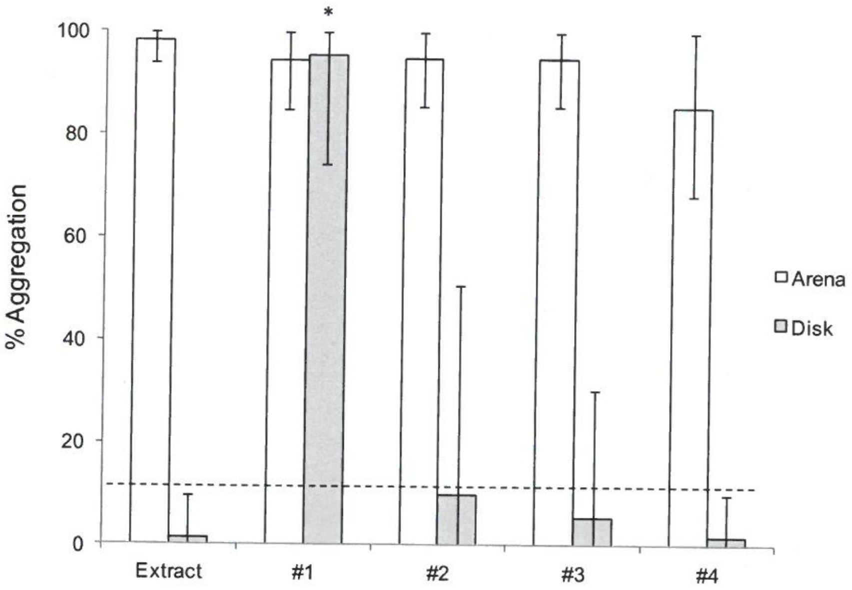

Fig. 6. Mean Arena- and disk-level aggregation by *C. lectularius* under disks treated with aqueous fractions collected by solid phase extraction (SPE) using a C18 SPE cartridge. Fraction #1 included the un-retained sample. Fraction #2 - #4 included solvent washes consistent with the following ratios of methanol/water: 10/90, 20/80, and 100/0 methanol. Asterisks indicate significant contrasts in aggregation levels to the original extract (n = 4). Other symbols as in Fig. 1.

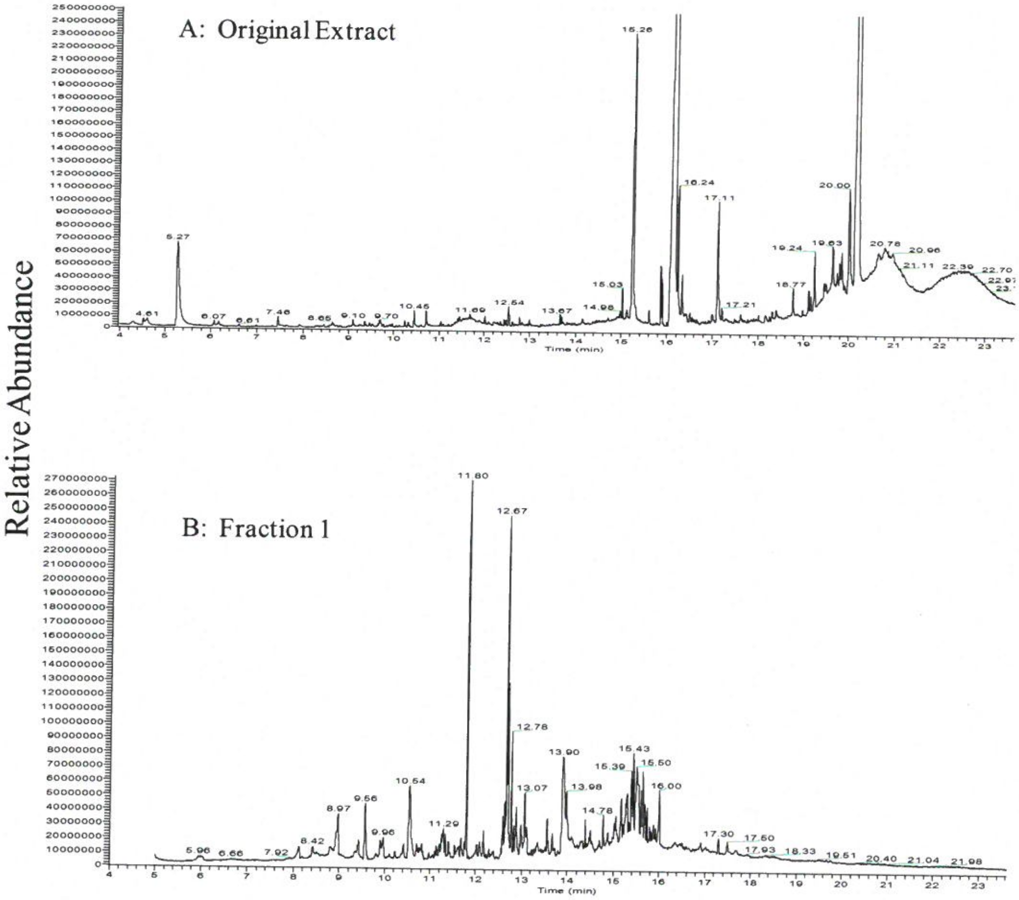

Fig. 7. Gas chromatogram mass spectra including relative abundance versus retention time (min) of un-identified compounds in the (A) original extract and (B) Fraction #1 collected by C18 cartridge.

Discussion

The importance of volatile components as part of the bed bug aggregation pheromone should be re-considered based on the volatile removal experiment presented here. Aggregation by bed bugs remained relatively stable regardless of how many days stained disks were held in the oven. In addition, reduction of E2H and E2O to levels below 0.01 µg did not affect disk-level aggregation under stained disks. While, it is possible that bed bugs are capable of detecting and responding to volatile stimuli such as E2H and E2O at levels below 0.01 µg, this amount was lower than those used and reported by Siljander et al., (2008). In addition, previous reports suggest bed bugs have a very limited range in their ability to detect and respond to volatile stimuli (Usinger, 1966; Aboul-Nasr and Erakey, 1968; Levinson et al., 1974).

Aggregation by bed bugs may be mediated by a combination of volatile and non-volatile simuli or contact pheromones associated with stained disks. The volatile components of the aggregation pheromone identified by Siljander et al., (2008) may be responsible for initiating aggregation or attracting bed bugs into a harborage area, while contact pheromones may be responsible for sustaining aggregation or arrestment over longer periods of time. Similar combinations of sources and compounds have been used to explain aggregation by other insects. For example, Triatoma use a combination of volatile compounds derived from fecal material and non-volatile contact pheromones extracted from the cuticle to elicit arrestment (Guerenstein and Lazzari, 2009). Similarly, a combination of compounds are responsible for German cockroach, *Blattella germanica*, aggregation (Sakuma, 1993; Sreng et al., 1998). Bed bugs may be another example of insects that use multiple stimuli to initiate and sustain aggregation.

Contact pheromones associated with fecal extracts may be deposited and used by bed bugs to identify harborage areas near potential hosts. Ticks deposit non-volatile compounds such as guanine, xanthine, and other purines to indicate areas where hosts are prevalent (Sonenshine, et al., 2003). These nitrogen based compounds cause arrestment by ticks in areas where hosts are most likely to be found. Similar to ticks, bed bugs do not live on the body of their host, but must rely on the frequent availability of a mobile host for survival and reproduction (Radwan et al., 1972; Reinhardt and Siva-Jothy, 2007). Hence, marking areas where hosts are likely to be found would benefit both bed bugs and other blood-feeding ectoparasites.

The extraction, restoration, and concentration assays have provided useful information about the behaviorally active compounds associated with bed bug stained papers. First, solubility of the active material in methanol and water indicate that the components associated with aggregation are moderately to highly polar in chemical structure. Second, demonstration of full restoration of the active components suggests the pheromone is retained and not lost during the extraction process. Finally, concentration of the extract was considered important with aggregation significantly reduced when the extract was diluted (≤ 0.3 mg/ml). Although aggregation was observed at concentrations of 30mg/ml (Fig. 5), concentrations above 60-90mg/ml may be over saturated and may explain the low disk-level aggregation observed in the separation experiment (Fig. 6).

Sep-Pak® cartridges have been successfully used to isolate or remove active compounds or pheromones from crude insect extracts in other studies (Spittler et al., 1988; Kingan et al., 1993). The C18 cartridge used here reduced the amount of unidentified materials in the bed bug fecal extract and provided further insight into the chemical properties of the unknown stimulus. For example, C18 cartridges are used to extract and isolate moderately polar to non-polar materials (Table 1). Finding behavioral activity to the unretained fraction collected from the cartridge suggests the active compounds are moderately polar to polar in structure. In general, polar compounds typically have stronger molecular bonds resulting in a lower vapor pressure of the molecule (Castellan, 1983). Therefore, the active compounds associated with bed bug fecal extract are likely non-volatile or have low volatility.

Further reduction of the unidentified compounds in the bed bug extract would assist in identification of the bed bug contact pheromone. Gas chromatography electroantennogram detection (GC-EAD) can be used to identify active regions of insect extracts (Nojima et al., 2005). However, electroantennogram methods for Cimicidae have not been developed to date. If identification of the active regions or compunds by GC-EAD is not possible, identification of the C18 filtered extract by GC-MS and execution of multiple behavioral assays could also be explored. Similar bioassay guided identification techniques have been used for bed bugs by Siljander et al., (2008). This would be more complicated given the number of unknown compounds presented here, but may be necessary if separation of the material by GC-EAD techniques is not possible.

Literature Cited

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968. Behavior and sensory physiology of the bed-bug, *Cimex lectularius* L., to some environmental factors: chemoreception. Bull. So. Entomol. Egypte 52: 353-362.

Castellan, G. 1983. Physical Chemistry 3rd ed., Reading, MA. Addison-Wesley.

Collins, R.P. 1968. Carbonyl compounds produced by the bed bug, *Cimex lectularius*. Ann. Entomol. Soc. Am. 61: 1338-1339.

Feingold, B., E. Benjamin, and D. Michael. 1968. The allergic responses to insect bites. Annu. Rev. Entomol. 13: 137-158.

Goddard, J., and R. Deshazo. 2009. Bed bugs, *Cimex lectularius*, and the clinical consequences of their bites. J.A.M.A. 301: 1358-1366.

Guerenstein, P., and C. Lazzari. 2009. Host-seeking: How triatomines acquire and make use of information to find blood. Acta Tropica 110: 148-158.

Kingan, T.G., P.A. Thomas-Laemont, and A.K. Raina. 1993. Male accessory gland factor elicit change from 'virgin' to 'mated' behavior in the female corn earworm moth Helicoverpa Zea. J. Exp. Biol. 183: 61-76.

Levinson, H.Z., and A.R. Bar-Ilan. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. Experientia. 27: 102-103.

Levinson, H.Z., A.R. Levinson, B. Muller, and R.A. Stainbrecht. 1974. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. J. Insect Physiol. 20: 1231-1248.

Montes, C., C. Cuadrillero, and D. Vilella. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera Cimicidae) using an artificial feeding technique. J. Med. Entomol. 39: 675-679.

Moraes, M.C.B., M. Pareja, R.A. Laumann, and M. Borges. 2008. The chemical volatiles (semiochemicals) produced by neotropical stink bugs (Hemiptera: Pentatomidae) Neotrop. Entomol. 37: 684-685.

Nojima, S., Schal, C., F.X. Webster, R. G. Santangelo, and W. L. Roelofs. 2005. Identification of the sex pheromone of the German cockroach, *Blattella germanica*. Science 307: 1104-1106.

Olson, J. F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect. Physiol. 55: 580-587.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. URL http://www.R-project.org/.

Radwan, W., A. Guneidy, and A. Soliman. 1972. On the biology of the bed bug, *Cimex lectularius* L. Bull. Soc. Entomol. Egypte. 56: 235-246.

Reinhardt, K. and M.T. Siva-Jothy. 2007. Biology of the bed bugs (Cimicidae). Annu. Rev. Entomol. 52: 351-374.

Reinhardt, K., D. Kempke, R. A. Naylor, and M. T. Siva-Jothy. 2009. Sensitivity to bites by the bed bug, *Cimex lectularius*. Med. Vet. Entomol. 23: 163-166.

Sakuma, M. 1993. Aggregation arrestment pheromone of the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae): isolation and structure elucidation of blattellastanoside –A and –B. J. Chem. Ecol. 19: 2521-2541.

SAS. 2004. SAS/STAT 9.1 Users Guide. SAS Institute Inc., Cary, NC.

Siljander, E., D. Penman, H. Harlan, and G. Gries. 2007. Evidence of male- and juvenile-specific contact pheromones of the common bed bug *Cimex lectularius*. Entomol. Exp. Appl. 125: 215-219.

Siljander, E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708-718.

Sonenshine, D. E., T. Adams, S. A. Allan, J. McLaughlin, and F. X. Webster. 2003. Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularis* (Acari: Ixodidae) and their use in tick control. J. Med. Ent. 40: 849-859.

Spittler, T. D., H. C. Leichtweis, and T. J. Dennehy. 1988. Biorational control of crop pests by mating disruption: residue analyses of Z-9-doecen-1-yl acetate and Z-11-tetradecen-1-yl acetate in grapes. Biotechnology for Crop Protection Chpt. 33, pp. 430-436.

Sreng, L., A. Clorec, and C. Rivault. 1998. Cuticular extracts inducing aggregation in the German cockroach, *Blattella germanica*, (L.). J. Insect Physiol. 44: 909-918.

Usinger, R.L., 1966. *Monograph of Cimicidae* (Hemiptera-Heteroptera), The Thomas Say Foundation. Entomological Society of America, Baltimore. M.D.

Weeks, E. N. I., M. A. Birkett, M. Cameron, J. A. Pickett, and J. G. Logan. 2010. Semiochemicals of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), and their potential for use in monitoring and control. Pest Manag. Sci. 67: 10-20.

Vaidyanathan, R. 2012. Bed bug: the science and costs of infestation. SRI International sri.com/blog/bed-bugs-science-costs.

Chapter IV. Off-host aggregation by bed bugs, *Cimex lectularius*, is mediated by two compounds

Introduction

The bed bug, *Cimex lectularius* L., is a significant pest of economic importance with world-wide distribution (Schaefer and Panizzi, 2000; Usinger, 1966). Bed bugs cause mild to severe cutaneous reactions, asthma, anemia, and mental health issues in some people (Doggett et al., 2012; Feingold et al., 1968; Goddard and deShazo, 2009; Reinhardt et al., 2009; Ter Poorten and Prose, 2005). Pest control efforts have been estimated to be $200 million for Australia and $3 billion for the United States since the resurgence was first reported in early 2000 (Doggett et al., 2011; Miller, 2007). Bed bugs are easily transferred from place to place in used furniture or other personal belongings. Once introduced, an infestation can quickly spread, making control efforts more challenging and difficult to manage (Lehnhert et al., 2012; Wang et al., 2010). Further information pertaining to the behavior and chemical ecology of this important pest would benefit the pest control industry and the general public.

After feeding, bed bugs aggregate in cracks and crevices near the host (Usinger, 1966). Once aggregation is established, bed bugs remain arrested for a minimum of eighteen hours (Levinson and Bar Ilan, 1971; Reis and Miller, 2011; Romero et al., 2009). Aggregation and arrestment is mediated by a combination of tactile and chemical stimuli (Aboul-Nasr and Erakey, 1968; Greis et al., 2014; Levinson and Bar Ilan, 1971; Siljander et al., 2008). Contact with filter papers stained with feces resulted in aggregation by bed bugs (Levinson and Bar Ilan, 1971; Olson et al., 2009; Siljander et al., 2007). In addition to fecal stained papers, bed bugs also responded to fecal extracts collected in methanol (Levinson and Bar Ilan, 1971). However, behaviorally active components of this fecal extract were not identified.

For management purposes, there is a significant amount of interest in semiochemicals that elicit bed bug aggregation. Siljander et al. (2008) described a 13-component airborne aggregation pheromone consisting of (E)-2-hexenal (E2H), (E)-2-Octenal (E2O), benzaldehyde, sulcatone, octanal, limonene, benzyl alcohol, (2E, 4E)-Octadienal, nonanal, (2E, 4E)-octadienal, benyl acetate, decanal, and geranylacetone. Identification of the blend was based on collection of headspace volatiles produced by groups of bed bugs, and determined by bug preference for one side of a treated arena. The two most abundant compounds of the volatile blend (>70% of the total composition) were E2H and E2O, which are also the two principal constituents of the bed bug's alarm pheromone (Collins, 1968; Levinson et al., 1974a). Although it is not uncommon for the same compound(s) to elicit both attraction and repellency among heteropterans (Moraes et al., 2008), it is unclear how this compound blend sustains aggregation and arrestment for longer periods of time. Furthermore, extraction and identification efforts using fifth instar exuviae demonstrated that bed bug aggregation was mediated by a combination of five volatile and one less-volatile components, consisting of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), E2H, and E2O, 2-hexanone), and histamine (Gries et al., 2014). The existence of bed bug pheromones associated with the insect cuticle is of interest, however, the putative non-volatile and arrestant chemical compounds associated with the feces remain unknown. Analysis of the bed bug fecal extract may provide more information about semiochemicals that influence bed bug behavior.

Here, we isolated and identified several active components of bed bug feces that were soluble in methanol. We used gas chromatography coupled with electro-antennogram detection (GC-EAD) and mass spectrometry (GC-MS) to identify active elution regions and individual compounds within the extract. A multiple choice behavioral assay was used to determine that two compounds were sufficient to stimulate aggregation. The potential use of the bed bug arrestment pheromone for management purposes is discussed.

Materials and Methods

Insects and Pheromone Extraction

Bed bugs were obtained from stock cultures of the ECL-05 strain of *Cimex lectularius* (Olson et al., 2009). Colonies were maintained under standard conditions of 25 °C and 14:10 (L:D) in 473 ml (16 oz) glass jars with folded pieces of filter papers (Fisher 9.0 cm) for harborage and egg deposition. Colony jars were covered with a fine mesh fabric (Precision Woven Nylon Mesh 193 x 193, McMaster Carr, Chicago, IL, USA) with a pore size of 78 µm for ventilation and containment. Colonies were fed weekly using an artificial feeding system adapted from Montes et al. (2002), which included a stretched Parafilm® feeding membrane (Bemis Flexible Packaging, Neenah, WI) and soon-to-expire stocks of human blood from the American Red Cross (St. Paul, MN).

Approximately 100 fecal-stained filter papers used for colony maintenance were cut into lengthwise1 cm strips and soaked in 80 ml of methanol overnight at room temperature to extract the active compounds. The methanol was decanted into a 100 ml glass jar and evaporated under a gentle stream of nitrogen using an Organomation N-EVAP 112 (CAE, Redwood City, CA). After decanting, an additional 80 ml of methanol was added to the filter paper strips and this process was repeated a total of three times. Approximately 90 mg of dried material was reconstituted in 4 gm of H_2O and filtered through a Millex-HV, 0.45 µm PVDF (Millipore Corp. Bedford, MA) filter disk to remove particulates. The filtrate was then applied to a C18 Sep-Pak (Waters, Milford, MA) to remove inactive materials and other impurities and 2 ml of the filtered extract was retained for further analysis.

GC-EAD

An aliquot (4 µl) of the filtered extract was analyzed by a gas chromatography electro-antennogram detection (GC-EAD) system. The aliquot was injected onto a splitless injector with a 2 min post-injection purge at 15.4 ml/min. The GC (Agilient Technologies 6890, Santa Clara, CA) was equipped with a ZB-5 column, (30 m x 0.32 mm; film thickness: 1.0 µm, Phenomenex, Torrance, CA.) and connected to a flame ionization detector (FID). The oven was programmed at 50 ºC for 5 min, then ramped up by 20 ºC/min with a 5 min hold at 320 ºC. Helium was used as the carrier gas and effluent from the GC oven was split using a 10:1 fused silica capillary splitter (S.G.E., Inc.) allowing 10 parts to the EAD and 1 part to the FID. A heated-conditioning tube delivered the EAD-outflow into a stream of humidified air (17 ml/min; 75 ºF; 75% RH) and directed over a bed bug antenna using a 48 cm long x 0.5 cm I.D. glass tube. A total of 10 individual insects was prepared as follows using 0.5 mm dia. Tungsten probes (insulated, 12 MEG, 8° tip, A-M Systems, Sequim, WA) that replaced the usual saline used in the glass pipette probe (Syntech Equipment and Research; Kirchzarten, Germany). The head of the bed bug and pronotum were excised from the mesothorax. The indifferent probe was inserted into pronotum with the tip positioned at the base of the antenna to be excised. Then, the first and second flagellomeres were removed using neuro-clipper scissors (Fine Science Tools, Foster City, CA) approx. 1-2 mm from the pedicel terminus. The recording probe was inserted into the distal tip of the pedicel, through the intersegmental membrane, and positioned as close to the distal patch as possible (Olson et al., 2014). A Syntech INR-5 micromanipulator and a light microscope (Leica Microsystems, Inc. Buffalo Grove, IL) were used to ensure correct electrode positioning. The recording electrodes and GC signal were connected through a signal interfacing board (IDAC-4; Syntech). FID and EAD signals were monitored simultaneously using GCEAD 2012 v1.2.4 software (Syntech).

Chemical Identification

The filtered extract used for GC-EAD recordings was also analyzed on a Hewlett Packard 6890GC/5973 mass spectrometer (MS) (Atlanta, GA.). A 2 µl aliquot was applied to a split injector at 250 °C with a 20:1 split ratio. The GC-MS was equipped with a ZB-5MS column (30 m x 0.25 mm; film thickness: 0.25 µm, Phenomenex, Torrance, CA.) The oven was programmed at 50 °C for 5 min, then ramped up by 20 °C/min with a 5 min hold at 320 °C. Helium was used as carrier gas (constant flow of 1.0 ml/min, linear velocity: 36 cm/sec). Mass spectra (EI, 70 eV) were recorded from 28-750 amu. Compounds in the extract were identified using reference spectra from the NIST Mass Spectral library (version 8.0), comparison mass spectra and retention times with reference compounds and manual interpretation techniques.

Chemical Standards

To compensate for the differences between flow rates in the the GC-FID and GC-MS equipment and to determine retention times for compounds of interest, three standards, octanoic acid, methyl palmate, and methyl oleate; all ≥ 99% pure, (Sigma-Aldrich, Milwaukee, WI) were used as internal standards.

All chemicals used in behavioral assays were ≥ 98% pure, except Triethanolamine (TEA) which consisted of 85% (TEA) and 15% diethanolamine (DEA; Dow, Midland, MI). Dimethyltrisulfide (DMTS; Sigma-Aldrich, St. Louis, MO) and methyldiethanol amine (MDEA; Alfa Aesar, Ward Hill, MA), bicine (MP Biomedicals LLC, Santa Ana, CA) and TEA were diluted in water. Octamide (TCI, Portland, OR), nonamide (Pfaltz & Bauer, Waterbury, CT) and palmitic acid (Sigma Aldrich, St. Louis, MO) were diluted in 3% methanol in water solution (Sigma Aldrich, St. Louis, MO).

Behavioral Assays

Multi-choice assays were performed using 20 cm diameter open air circular arenas (Olson et al. 2009). Each arena contained one treated and seven control paper disks (25 mm Whatman™ Grade GF/A filter papers) that were randomly assigned to eight equally spaced positions around the perimeter. Placement of the treated disks was ordinally randomized to prevent confounding with potential environmental variation. Treatments consisted of 200 µl aliquots of the fecal extract or a chosen treatment formula. Each of the seven control disks was treated with 200 µl of methanol (Sigma Aldrich, St. Louis, MO). Both treated and control disks were allowed to dry 24 h prior to the start of the assay. All disks were adhered to a paper floor (Boise® X-9® office paper, Boise, Idaho) by a spot of hot glue (Surebonder, FPC Corp., Wauconda, IL), which allowed bugs to aggregate under disks. All disks combined occupied 12.5 % of the total arena floor space (314 cm^2). Groups of five males and five females were released into the center of each arena and replicate arenas were housed side-by-side on a table top under incandescent lighting and standard conditions of 25 ± 5 °C and 40% ± 10% RH. All experiments were started approximately 3 h before the end of the bugs' subjective scotophase.

Positions of bugs were recorded 4 h after release. Response to a treatment was calculated by dividing the number of bed bugs under a treated disk by the number of bugs under any disk in the same arena, and multiplying by 100 to represent percent regions (Fig. 2). The whole extract contained a mixture of mostly amines, amides, and acids (Table 1). Identification of the compounds within each of the active regions directed attention to compounds for consideration in behavioral arenas (Table 2). Selection of candidate materials was based on concentration of the identified compound ($\geq$ 1.0 ppm), except for R3, in which all identified compounds were evaluated (Table 1).

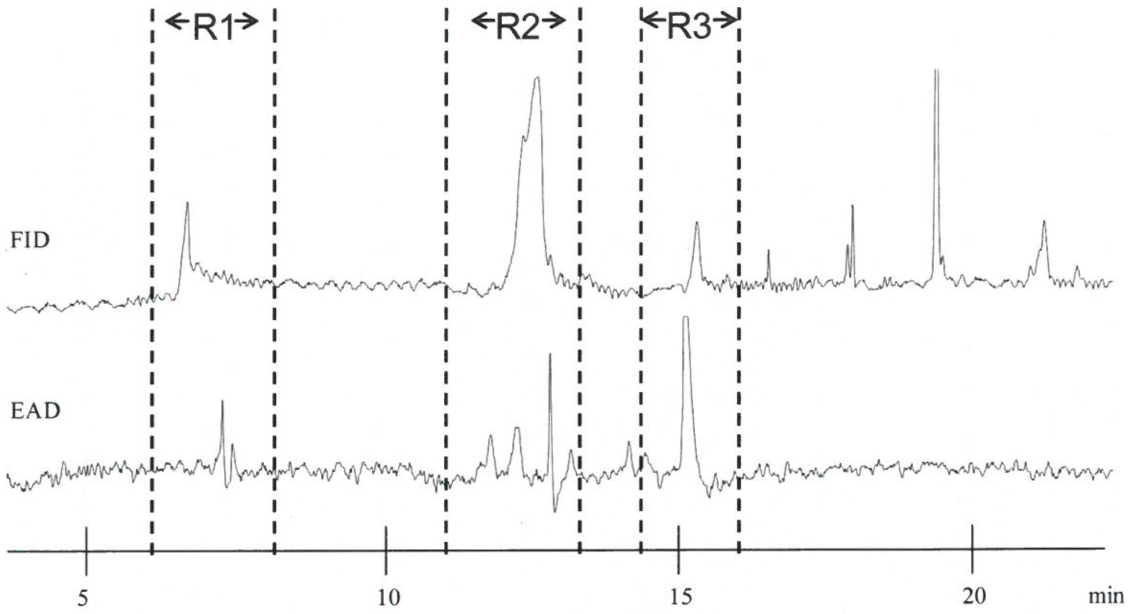

Fig. 1. Gas chromatogram separation of filtered bed bug fecal extract with conventional flame ionization detection (FID) and electroantennogram detection (EAD) using female *Cimex lectularius*. Antennal activity varied slightly among replicates (n = 10) with active regions, R1, R2, and R3 represented between dashed lines.

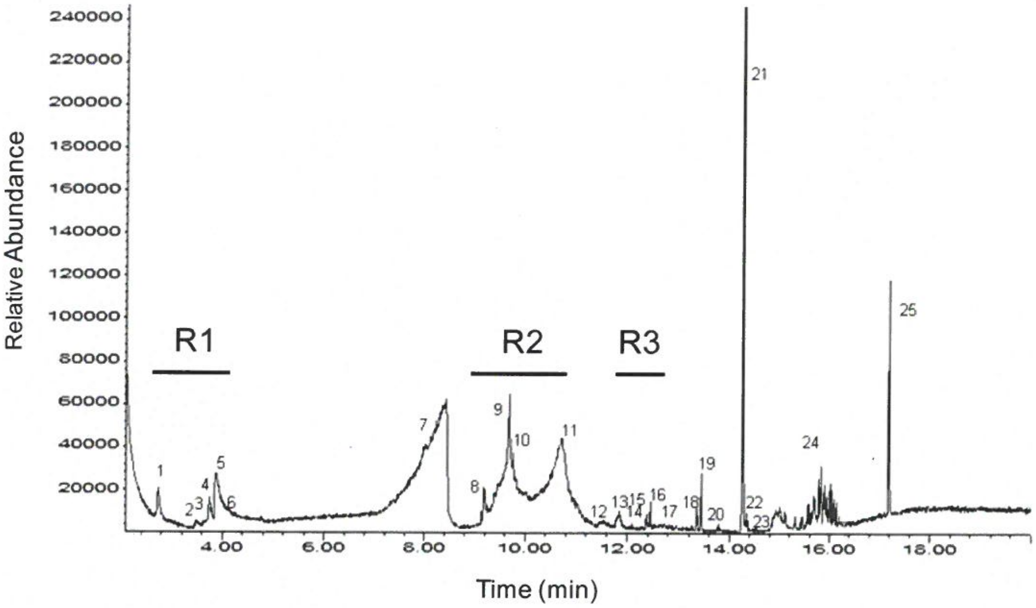

Fig. 2. Total ion chromatogram (TIC) of a filtered fecal extract collected from filter papers stained by *Cimex lectularius*. Active regions determined by GC-EAD are represented by areas under the bars labeled R1, R2, and R3. Numbers indicate compounds that were reliably present and are listed in Table 1.

Table 1. Compounds identified in methanol extracts of filter papers stained with *Cimex lectularius* feces.

EAD Active Region[1]	MSD #	Identification	Method[2]	Conc. (ppm)
R1	1	dimethyltrisulfide (DMTS)	MSL	0.95
R1	2	dimethyl propanamide	MSL, MS	0.20
R1	3	amyl-butyl amine	MSL, MS	0.05
R1	4	methylmethanehiosulfonate	MSL, MS	0.63
R1	5	methyldiethanol amine (MDEA)	MSL, MS	3.75
R1	6	Ethanediamide	MSL, MS, D	0.09
-	7	Urea	MSL, MS, S, D	41.46
R2	8	Bicine	MSL	0.93
R2	9	Triethanolamine (TEA)	MSL, MS, S, D	17.01
R2	10	bis(2hydroxyethyl)formamide	MSL	0.22
R2	11	column artifact	MSL	N/A[3]
-	12	Dimethylpyridinone	MSL	0.60

R3	13	column artifact	MSL	N/A[3]
R3	14	Octamide	MSL, D	0.03
R3	15	plamitic acid	MSL, MS, S, D	0.15
R3	16	Nonamide	MSL, D	0.27
-	17	4-methyl pentanamide	MSL	0.38
-	18	7-nonenamide	MSL, MS, D	0.26
-	19	Lauramide	MSL, MS, D	0.57
-	20	branched amide	MS	0.03
-	21	Oleamide	MSL, MS, S, D	6.91
-	22	Stearamide	MSL, MS	0.10
-	23	phosphorous species	MS	N/A
-	24	Phthalates	MSL, MS, D	N/A
-	25	Lanol	MSL, MS, D	2.50

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[1] Region of the extract (R1, R2, or R3) that stimulated positive GC-EAD activity (Fig. 1).

[2] Methods used for detection: MSL, comparison of mass spectrum fragmentation patterns with the NIST version 8.0 GC-MSD database; MS, mass spectral fragmentation patterns; S, standards analyzed on the

same system; D, an aliquot of the same fraction used for the GC and GC-MSD analyses was derivatized with bis(trimethylsilyl)trifluoroacetamide and analyzed using GC-MSD.

[3] Concentration not estimated as eluting peak was identified as materials unrelated to biological activity (E.g., column artifact).

Table 2. Synthetic formulas used in behavioral assays to evaluate aggregation and arrestment by bed bugs, *Cimex lectularius*.

Formula	**Compounds (ppm)**	**Active Region****
1	methyldiethanol amine (4), bicine (1), triethanolamine (17), octamide (1), palmitic acid (1), nonamide (1)	R1, R2, R3
2	bicine (1), triethanolamine (17), octamide (1), palmitic acid (1), nonamide (1)	R2, R3
3	bicine (1), triethanolamine (17)	R2
4	octamide (1), palmitic acid (1), nonamide (1)	R3
5	methyldiethanol amine (4)	R1

[1] Concentration replicated from GC-MS (Table1) with ppm represented in parentheses. All samples were diluted in water except those containing octamide, nonamide, and palmitic acid which were dissolved in 3% methanol in water.

[2] Active regions R1, R2, and R3 were based on GC-EAD recordings (Fig. 1.)

Behavioral Assays

In a first assay, aggregation by bed bugs to formulas A, B, C, and D (Table 2) were compared to the fecal extract (Fig. 3). There was no significant difference in aggregation ($P = 0.59$) between the fecal extract and formula A, which included compounds from all three active regions (R1, R2, and R3). In contrast, response to formulas B, C, and D, which did not include compounds from R1, was lower than the crude extract (p-values 0.07, 0.04, and 0.03, respectively). The mean percent aggregation for B, C, and D formulas were not significantly different from 12.5%, which indicated the bugs exhibited no preference for disks treated with those formulas.

In a second assay, aggregation to formulas E, F, and G, which included both volatile and one less volatile standard identified in R1, was compared to the original extract (Fig. 4). The mean percent aggregation to formula E and F was significantly lower ($P < 0.05$) than to formula F, and nearly significantly lower for Formula E ($P = 0.06$) compared to the original extract. In contrast, the bed bug response to formula G was not significantly different ($P = 0.85$) from the original extract. These results demonstrate that a combination of dimethyltrisulfide (DMTS) and methyldiethanolamine (MDEA) is sufficient to elicit an aggregation response comparable to the original extract.

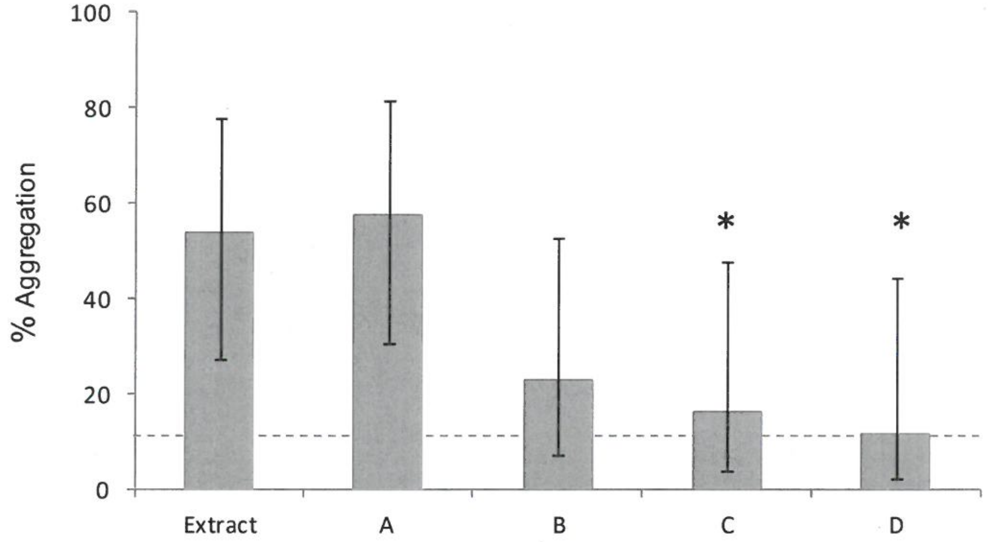

Fig. 3. Mean percent aggregation of 10 adult *Cimex lectularius* that aggregated under filter paper disks treated with fecal extract or synthetic formulas A, B, C, and D (Table 2; n = 17) using a multiple choice behavioral assay. Error bars indicate 95 % confidence intervals (CIs) and asterisks show significant contrasts ($P < 0.05$) between a formula and the extract. A random response is represented by the dashed line at 12.5%.

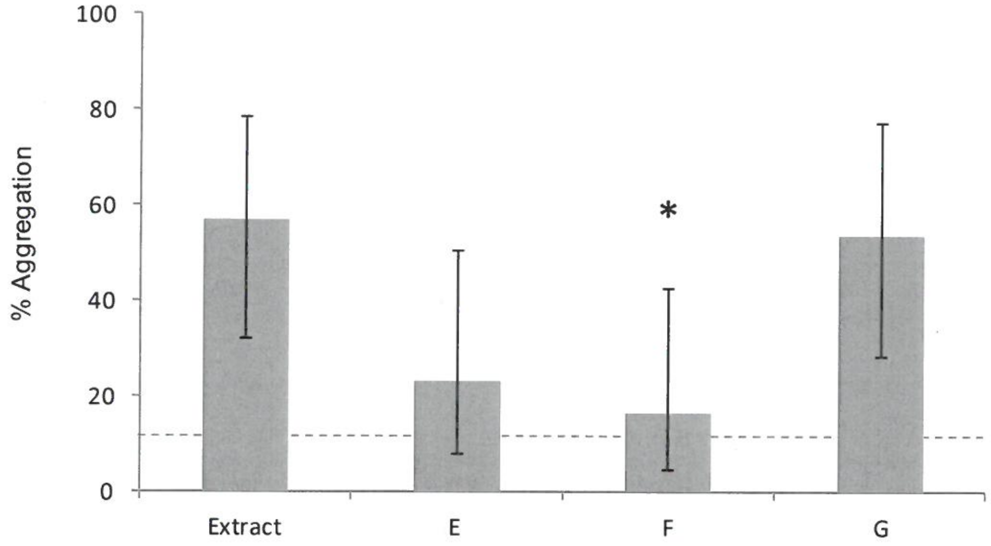

Fig. 4. Mean percent of 10 adult *Cimex lectularius* that aggregated under filter paper disks treated with fecal extract or synthetic formulas E, F, and G (Table 2; *n* = 17) using a multiple choice behavioral assay. Other symbols as in Fig. 3.

Discussion

This is the first published report using GC-EAD techniques to identify chemical compounds responsible for bed bug aggregation and arrestment. Previous research on chemical ecology of bed bugs has relied on electroanntennogram (EAG) detection, single cell recording (SCR) techniques or bioassay-guided fractionation experiments to identify volatile attractants and repellents (Gries et al., 2014; Harraca et al., 2009; Levinson et al., 1974b; Siljander et al., 2008). Because off-host aggregation by bed bugs is mediated by sensory receptors located on the distal half of the pedicel (Olson et al., 2014), removal of the flagellar olfactory regions (Levinson et al., 1974b) prior to GC-EAD recording enabled more precise electrode placement closer to sensory receptors responsible for bed bug aggregation (Olson et al., 2009, 2014).

There have been several attempts to identify the bed bug aggregation pheromone. Previous efforts have identified volatile compounds in the headspace of bed bug colonies (Siljander et al., 2008; Weeks et al., 2012) and on chemicals associated with exuviae (Gries et al., 2014), ignoring earlier reports that fecal extracts collected in methanol also elicit bed bug aggregation (Levinson and Bar Ilan, 1971). Our research focused on identification of less-volatile compounds associated with bed bug feces. We identified numerous components in the fecal extract that were responsible for aggregation, but only two compounds, DMTS and MDEA presented in combination, were necessary to elicit aggregation. MDEA has not been previously identified or described as a semiochemical of bed bugs. In contrast, DMTS has been identified in exuviae extracts (Gries et al., 2014) and described as a critical component of the six compound blend responsible for bed bug aggregation. Although DMTS can be detected in very low concentrations, it has a relatively low vapor pressure (1.1 mm Hg; boiling point ≥170ºC), suggesting the chemical is actually less volatile compared to other compounds described in the blend. The substrate in which the pheromone is applied may also have a factor in its rate of release.

Similar to bed bugs, nitrogenous compounds with arrestment properties have been identified in the fecal material produced by ticks (Sonenshine et al., 2006). For example, guanine, xanthine, hypoxanthine, inosine and 8-azaguanine elicited arrestment by the black legged tick, *Ixodes scapularis*, in multi-choice behavioral assays (Allan and Sonenshine, 2002; Sonenshine et al., 2003). The nitrogenous compounds produced by the tick are likely a by-product of the blood meal ingested by the tick (Soneneshine et al., 2003). The MDEA identified in the bed bug fecal extract here, may also be a by-product of digestion that acts as an arrestment pheromone.

We demonstrated that three regions of the bed bug fecal extract resulted in positive antennal responses using GC-EAD recording techniques (Fig. 1). The positive response to all three regions may be related to the nitrogenous base of the compounds identified in each of the active regions such as MDEA in R1, bicine and TEA in R2 and octamide and nonamide in R3 (Table 1). Due to its high boiling point 246 ºC and low vapor pressure of 0.01 mm Hg at 20 ºC, MDEA would be considered less volatile compared to the other nitrogenouscompounds identified in the fecal extract (Sigma Aldrich; Ternay, 1979). The semi-volatile properties of MDEA may explain why bed bugs were more responsive to this dried residue as compared to the other nitrogen based residues evaluated in the behavioral assays (Fig. 4). Histamine was the only nitrogenous compound identified by Gries et al. (2014) in the exuviae of bed bugs. Interestingly, histamine is also a common compound produced by mammals as a result of repeated exposure from biting insects (Ohtsuka et al., 2009). Presence of histamine in the exuvia of bed bugs may have been a chemical contaminant produced by the host, rather than a compound produced by the bed bugs. We did not identify detectable levels of histamine in our analysis. Further research is warranted to determine bed bug sensitivity to other amine based compounds.

It is essential that bed bugs identify and locate a protected harborage near the host after feeding. Fecal deposits containing DMTS and MDEA may indicate a protected harborage for conspecifics. This fecal marking behavior has also been demonstrated by triatomid bugs (Guerientstein and Lazzari, 2009; Lorenzo and Lazzari, 1996) and cockroaches (Burk and Bell, 1972; Dambach et al., 1993; Miller and Koehler, 2000). Unique aggregation and arrestment pheromones consisting of a mixture of carboxylic acids have been identified in cockroach feces (Scherkenbeck et al., 1999). Deposition and detection of the carboxylic acids likely assist cockroaches in locating safe harborages, finding mates, and locating food sources (Miller and Koehler, 2000). Similarly, marking harborage areas with DMTS and MDEA may provide several benefits for bed bugs.

A semi-volatile aggregation pheromone such as DMTS and MDEA could be used to enhance pest management practices for this important economic pest. Several traps have been developed to monitor bed bug activity (Weeks et al., 2010), but their efficacy is limited (Vaidyanatan and Feldlaufer, 2013). Addition of an aggregation pheromone could improve sensitivity of bed bug monitoring methods. The pheromone could also be used to enhance insecticide applications. Romero et al. (2009) showed that bed bugs spent more time on insecticide treated tents if the tents were also treated with a bed bug fecal extract. Increasing time spent on a treated surface could increase efficacy by increasing the uptake of an insecticide. Further research will be necessary to develop this application.

Literature Cited

Allan S. A., and D. E. Sonenshine. 2002. Evidence of an assembly pheromone in the black-legged deer tick, *Ixodes scapularis*. J. Chem. Ecol. 28: 15-27.

Aboul-Nasr A. E., and M. A. S. Erakey. 1968. Behaviour and sensory physiology of the bed-bug, *Cimex lectularius* L., to some environmental factors: chemoreception. Bull. Soc. Ent. Egypte 52: 353-362.

Burk T., and W. Bell. 1972. Cockroach aggregation pheromone: Inhibility of locomotion (Orthoptera: Blattidae) J. Kas. Entomol. Soc. 46: 36-41.

Collins, R. P. 1968. Carbonyl compounds produced by the bed bug, *Cimex lectularius*. Ann. Entomol. Soc. Am. 61: 1338-1339.

Dambach M., A. Stadler, and J. Heidelbach. 1993. Development of aggregation behavior in the german cockroach, *Blattella germanica* (Dictyoptera: Blattellidae). Entomol. Gener. 19: 129-141.

Dogget, S. L., C. J. Orton, D. Lilly, and R C. Russell. 2011. Bed bugs: the Australian response. Insects 2: 96-111.

Dogget, S. L., E. D. Dominic, P. F. Penas, and R. C. Russell. 2012. Bed bugs: clinical relevance and control options. Clin. Micro. Rev. 25: 164-192.

Feingold, B., E. Benjamini. And D. Michaeli. 1968. The allergic responses to insect bites. Ann. Rev. of Entomol. 13: 137-158.

Harraca, V. R. Ignell, C. Lofstedt, and C. Ryne. 2009. Characterization of the antennal olfactory system of the bed bug (*Cimex lectularius*). Chem. Senses 35: 195-204.

Goddard, J., and R. Deshazo. 2009. Bed bugs, *Cimex lectularius*, and the clinical consequences of their bites. J. Am. Med. Assoc. 301: 1358-1366.

Gries, R., R. Britton, M. Holmes, H. Zhai, J. Draper, and G. Gries. 2014. Bed bug aggregation pheromone finally identified. Angew. Chem. Int. Ed. 54: 1135-1138.

Guerenstein, P. G., and C. R. Lazzari. 2009. Host-seeking: how triatomines acquire and make use of information to find blood. Acta Tropica 110: 148-158.

Lehnert, M. P., E. P., Benson, P. A. Zungoli, and P. D. Gerard. 2012. Assessment of bed bug (*Cimex lectularius* L.) prevalence, control strategies, and challenges facing urban pest management professionals in South Carolina. J. Agri. Urban Entomol. 28: 16-24.

Levinson, H. Z., and A. R. Bar Ilan. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. Experientia 27: 102-103.

Levinson, H. Z., A. R. Levinson, and U. Maschwitz. 1974a. Action and compoostion of alarm pheromone of bed bug, *Cimex lectularius* L. Naturwissenschaften 61: 684-685.

Levinson, H. Z., and A. R. Levinson,B. Muller, and R. A. Steinbrecht. 1974b. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius* in response to its alarm pheromone. J. Insect Physiol. 20: 1231-1248.

Lorenzo, M.G. and C. R. Lazzari. 1996. The spatial pattern of defaecation in *Triatoma infestans* and the role of faeces as a chemical mark of the refuge. J. Insect Physiol. 42: 903-907.

Miller, D., and P. Koehler. 2000. Trail-following behavior in the german cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 93: 1241-1246.

Miller, K. 2007. The cost of bed bugs. *Business Week*. New York. URL: http://www.businessweek.com/.

Montes, C., C. Cuadrillero, and D. Vilella. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera Cimicidae) using an artificial feeding technique. J. Med. Entomol. 39: 675-679.

Moraes, M. C. B., M. Pareja, R. A. Laumann, and M. Borges. 2008. The chemical volatiles (semiochemicals) produced by neotropical stink bugs (Hemiptera: Pentatomidae) Neotrop. Entomol. 37: 684-685.

Ohtsuka, E., S. Kawai, T. Ichikawa, H. Nojima, K. Kitagawa, Y. Shirai, K. Kamimura, and Y. Kuraishi. 2001. Roles of mast cells and histamine in mosquito bite-induced allergic itch-associated responses in mice. Jpn. J. Pharmacol. 86: 97-105.

Olson, J. F., R.D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect Physiol. 55: 580-587.

Olson, J. F., R.D. Moon, S. A. Kells, and K. A. Mesce. 2014. Morphology, ultrastructure and functional role of antennal sensilla in off-host aggregation by the bed bug, *Cimex lectularius*. Arth. Struct. and Dev. 43: 117-122.

Reinhardt, K. and M. Siva-Jothy. 2007. Biology of bed bugs (Cimicidae). Annu. Rev. Entomol. 52: 352-374.

Reinhardt, K., D. Kempke, R. A. Naylor, and M. T. Siva-Jothy. 2009. Sensitivity to bites by the bed bug, *Cimex lectularius*. Med. Vet. Enomol. 23: 163-166.

Reis, M. D., and D. M. Miller. 2011. Host searching and aggregation activity of recently fed and unfed bed bugs (*Cimex lectularius* L.) Insects 2: 186-194.

Romero, A., M. F. Potter, and K. F. Haynes. 2009. Behavioral responses of the bed bug to insecticide residues. J. Med. Entomol. 46: 51-57.

Scherkenbeck, J., G. Nentwig, K. Justus, J. Lenz, D. Gondol, G. Wendler, M. Dambach, F. Nischk, and C. Graff. 1999. Aggregation agents in german cockroach *Blattella germanica*: examination of efficacy. J. Chem. Ecol. 25: 1105-1119.

Schaefer, C. W., and A. R. Panizzi. 2000. Heteroptera of economic importance. CRC Press.

Sigma-Aldrich Company Safety Data Sheet, Version 4.5, Number 471828.

Siljander, E., D. Penman, H. Harlan, and G. Gries. 2007. Evidence for male and juvenile specific contact pheromones of the common bed bug *Cimex lectularius*. Entomol. Exp. Appl. 125: 215-219.

Siljander, E. R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708-718.

Sonenshine, D. E., T. Adams, S. A. Allan, J. McLaughlin, F. X. Webster. 2003. Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularis* (Acari: Ixodidae) and their use in tick control. J. Med. Entomol. 40: 849-859.

Sonenshine, D. E. 2006. Tick pheromone and their use in tick control. Ann. Rev. Entomol. 51: 557-580.

Statheropoulosa, M. A., C. Agapioua, G. Spiliopoulou, C. Pallis, and E. Siano. 2007. Environmental aspects of VOCs evolved in the early stages of human decomposition. Sci. Total. Environ. 385: 221-227.

Ternay, A. 1979. Contemporary Organic Chemistry. Philadelphia: Saunders.

Ter Poorten, M. C., and N. S. Prose. 2005. The return of the common bed bug. Pediatr. Dermatol. 22: 183-187.

Usinger, R. 1966. Monograph of Cimicidae. Thos. Say Foundation, Vol. VII, Entomol. Soc. Amer. Lanham, MD.

Vaidyanatan, R., and M. Feldlaufer. 2013. Bed bug detection: current technologies and future directions. Am. J. Trop. Med. Hyg. 88: 619-625.

Wang, C., K. Saltzmann, E. Chin, G. W. Bennett, and T. Gibb. 2010. Understanding bed bug infestation and dispersal patterns. Pest Control Tech. 38: 94-96.

Weeks, E. N. I., M. A. Birkett, M. Cameron, J. A. Pickett, and J. G. Logan. 2010. Semiochemicals of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), and their potential for use in monitoring and control. Pest Manag. Sci. 67: 10-20.

Weeks, E. N. I., J. G. Logan, S. A. Gezan, C. M. Woodcock, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2011. A bioassay for studying behavior responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) to bed bug derived volatiles. Bull. Entomol. Res. 101: 1-8.

Weeks, E. N., J.G. Logan, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2012. Tracking bed bug (*Cimex lectularius*): a study of the effect of physiological and extrinsic factors on the response to bed bug-derived volatiles. J. Exp. Biol. 216: 460-469.

REFERENCES

Allan S. A., and D. E. Sonenshine. 2002. Evidence of an assembly pheromone in the black-legged deer tick, *Ixodes scapularis*. J. Chem. Ecol. 28(1): 15-27.

Aboul-Nasr, A.E., and M.A.S. Erakey. 1967. On the behavior and sensory physiology of the bed bug: Temperature reactions. Bull. Soc. Entomol. Egypte. 43: 43-54.

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968. Behavior and sensory physiology of the bed-bug, *Cimex lectularius* L., to some environmental factors: chemoreception. Bull. Soc. Entomol. Egypte. 52: 353-362.

Aboul-Nasr, A.E., and M.A.S. Erakey. 1968. The effect of light reactions upon the bed bug, *Cimex lectularius* L. Bull. Soc. Entomol. Egypte. 52: 338-351.

Alsever, J. 2010. Bedbug invasion is turning into big business. U.S. Business on NBCnews.com; Updated: 10/11/2010.http://www.nbcnews.com/id/39526413/ns/business-us_business/

Burk T., and W. Bell. 1972. Cockroach aggregation pheromone: Inhibility of locomotion (Orthoptera: Blattidae) J. Kas. Entomol. Soc. 46 (1): 36-41.

Castellan, G. 1983. Physical Chemistry 3rd ed., Reading, MA. Addison-Wesley.

CDC and EPA. 2010. Joint statement on bed bug control in the United States from the U.S. Centers for Disease Control and Prevention (CDC) and the U.S. Environmental Protection Agency (EPA). Atlanta: U.S. Department of Health and Human Services; Updated: 5/8/2013. http://www.cdc.gov/nceh/ehs/publications/bed_bugs_cdc-epa_statement.htm.

Chapman, R.F. 1998. The insects. Structure and function. Cambridge: Cambridge University Press.

Chapman, R.F. 2003. Contact chemoreception in feeding by phytophagous insects. Ann. Rev. Entomol. 48: 455-484.

Anderson, J., F. Ferrandino, S. McKight, J. Nolen, and J. Miller. 2009. A carbon dioxide, heat, and chemical lure trap for the bed bug, *Cimex lectularius*. Med. Vet. Entomol. 23: 99-105.

Bartley, J., and H. Harlan. 1974. Bed bug infestation: its control and management. Mil. Med. 139: 884-886.

Benoit, J.B., S.A. Phillips, T.J. Croxall, B.S. Christensen, J.A. Yoder, and D.L. Denlinger. 2009. Addition of alarm pheromone components improves the effectiveness of desiccant dusts against *Cimex lectularius*. J. Med. Entomol. 46(3): 572-579.

Bernier, U.R., D.L. Kline, D.R. Barnard, C.E. Schreck, and R.A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). Anal. Chem. 72: 747-756.

Collins, R.P. 1968. Carbonyl compounds produced by the bed bug, *Cimex lectularius*. Ann. Entomol. Soc. Am. 61(5): 1338-1339.

Dambach M., A. Stadler, and J. Heidelbach. 1993. Development of aggregation behavior in the german cockroach, *Blattella germanica* (Dictyoptera: Blattellidae). Entomol. Gener. 19(3): 129-141.

Davis, N. 1955. The morphology and functional anatomy of the male and female reproductive systems of *Cimex lectularius* L. (Heteroptera, Cimicidae). Ann. Entomol. Soc. Am. 49: 466-478.

Dogget, S. L., C. J. Orton, D. Lilly, and R C. Russell. 2011. Bed bugs: the Australian response. Insects 2: 96-111.

Doggett, S.L., D.E. Dwyer, P.F. Penas, and R.C. Russell. 2012. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25(1): 164-192.

Doggett, S. 2013. A code of practice for the control of bed bug infestation in Australia Fourth Edition. (www.bedbug.org.au).

Dusabek, F., P. Simek, A. Jegorov, and J. Triska 1991. Identification of xanthine and hypoxanthine as components of assembly pheromone in excreta of argasid ticks. Exp. Appl. Acarol. 11: 307-316.

Fawziak, K., M. Abdelatif, I. Gaaboub, and E. Helal. 1987. Biological studies on the bed bug (*Cimex lectularius* L.). J. Agric. Res. 32: 359-368.

Feingold, B., E. Benjamin, and D. Michael. 1968. The allergic responses to insect bites. Annu. Rev. Entomol. 13: 137-158.

Feldlaufer, M. F., M. J. Domingue, K.R. Chauhan, and J. R. Alrich. 2010. 4-oxo-aldehydes from the dorsal abdominal glands of the bed bug (Hemiptera: Cimicidae). J. Med. Ent. 47: 140-143.

Friend, W.G., and J. J. Smith. 1977. Factors affecting feeding by bloodsucking insects. Ann. Rev. Entomol. 22: 309-331.

Geier, M., J. O. Bosch, and J. Boeckh. 1999. Ammonia as an attractive component of host odour for the yellow fever mosquito, *Aedes Aegypti*. Chem. Senses 24: 647-653.

Guerenstein, P.G., and C. R. Lazzari. 2009. Host-seeking: how triatomines acquire and make use of information to find blood. Act Tropica 110: 148-158.

Goddard, J. and R. DeShazo. 2009. Bed Bugs (*Cimex lectularius*) and clinical consequence of their bites. J. Am. Med. Assoc. 301: 1358-1366.

Grenacher, S., T. Krober, P. Guerin, and M. Vlimant. 2001. Behavioral and chemoreceptor cell responses of the tick, *Ixodes ricinus*, to its own faeces and faecal constituents. Exp. Appl. Acarol. 25: 641-660.

Gries, R., R. Britton, M. Holmes, H. Zhai, J. Draper, and G. Gries. 2014. Bed bug aggregation pheromone finally identified. Angew. Chem. Int. Ed. 54: 1135-1138.

Harraca V., R. Ignell, C. Lofstedt, and C. Ryne. 2009. Characterization of the antennal olfactory system of the bed bug (*Cimex lectularius*). Chem. Senses 35: 195-204.

Harraca, V., C. Ryne, and R. Ignell. 2010. Nymphs of the common bed bug (*Cimex lectularius*) produce anti-aphrodisiac defense against conspecific males. BMC Biol. 8: 121.

Hodgson, E.S. 1958. Chemorecption in arthropods. Ann. Rev. Entomol. 3: 19-36.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6: 65-70.

Howse, P., I. Stevens, and O. Jones. 1998. Insect pheromones and their use in pest management. London. Chapman and Hall.

Johnson, C. G. 1941. The ecology of the bed bug, *C. lectularius* L. in Britain. J. Hyg. Cambridge 41: 345-461.

Kells, S.A. 2006. Non-chemical control of bed bugs. Am. Entomol. 52(2): 109-110.

Kells, S. A., and M. J. Goblirsch. 2011. Temperature and time requirements for controlling bed bugs (*Cimex lectularius*) under commercial heat treatment conditions. Insects 2: 412-422.

Kilpinen, O., L. Dezhao, and A.P.S. Adamsen, 2012. Real-time measurement of volatile chemicals released by bed bugs during mating activities. PLOS One 7: 1-9.

Kingan, T.G., P.A. Thomas-Laemont, and A.K. Raina. 1993. Male accessory gland factor elicit change from 'virgin' to 'mated' behavior in the female corn earworm moth Helicoverpa Zea. J. Exp. Biol. 183: 61-76.

Kreher, S. A., D. Mathew, J. Kim, and J. R. Carlson. 2008. Translation of sensory input into behavioral output via an olfactory system. Neuron 59: 110–124.

Law, J.H. and F.E. Regnier. 1971. Pheromones. Annu. Rev. Biochem. 40:533-548.

Lehnert, M. P., E. P., Benson, P. A. Zungoli, and P. D. Gerard. 2012. Assessment of bed bug (*Cimex lectularius* L.) prevalence, control strategies, and challenges facing urban pest management professionals in South Carolina. J. Agri. Urban Entomol. 28(1): 16-24.

Leverkus, M. 2006. Bullous allergic hypersensitivity to bed bug bites mediated by IgE against salivary nitrophorin. J. Invest. Dermatol. 126: 91-96.

Levinson, H. Z., and A. R. Ilan. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. Experientia 27: 102-103.

Levinson, H. Z., A. R. Levinson, B. Muller, and R. A. Steinbrecht. 1974. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. J. Insect Physiol. 20: 1231-1248.

Levinson, H. Z., A. R. Levinson, and U. Maschwitz. 1974b. Action and composition of the alarm pheromone of the bedbug *Cimex lectularius* L. Naturwissenschaften. 61: 684-685.

Lorenzo, M.G. and C. R. Lazzari. 1996. The spatial pattern of defaecation in *Triatoma infestans* and the role of faeces as a chemical mark of the refuge. J. Insect Physiol. 42(9): 903-907.

Marx, R. 1955. Uber die wirtsfindung und die bedeutung des artspezifischen duftstoffes bei *Cimex lectularius* Linne. Z. Parasitenkd. 17: 41-73.

Mellanby, K. 1939. The physiology and activity of the bed-bug (*Cimex lectularius*) L. in a natural infestation. Parasitology 31: 200-211.

Millar, J. 2005. Pheromones of true bugs. Topics in Current Chemistry 240: 37-84.

Millar, J., and R. Rice. 1998. Sex pheromone of the plant bug *Phytocoris californicus* Knight (Heteroptera: Hemiptera Mirridae). J. Econ. Entomol. 91: 132-137.

Miller, D., and P. Koehler. 2000. Trail-following behavior in the german cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 93(1): 1241-1246.

Miller, K. 2007. The cost of bed bugs. *Business Week.* New York. URL: http://www.businessweek.com/.

Montes, C., C. Cuadrillero, and D. Vilella. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera Cimicidae) using an artificial feeding technique. J. of Med. Entomol. 39: 675-679.

Moraes, M.C.B., M. Pareja, R.A. Laumann, and M. Borges. 2008. The chemical volatiles (semiochemicals) produced by neotropical stink bugs (Hemiptera: Pentatomidae) Neotrop. Entomol. 37: 684-685.

Nagro, A. 2012. Bed bug supplement: game changer or gimmick? Pest Control Technol. 40: 78-85.

Naylor, R.A., and C.J. Boase. 2010. Practical solutions for treating laundry infested with *Cimex lectularius* (Hemiptera: Cimicidae). J. Econ. Entomol. 103: 136-139.

Naylor, R. 2012. December 21. Bed bug biology and the basics. Retrieved from http://cimexstore.co.uk

Nojima, S., Schal, C., F.X. Webster, R. G. Santangelo, and W. L. Roelofs. 2005. Identification of the sex pheromone of the German cockroach, *Blattella germanica*. Science 307: 1104-1106.

Ohtsuka, E., S. Kawai, T. Ichikawa, H. Nojima, K. Kitagawa, Y. Shirai, K. Kamimura, and Y. Kuraishi. 2001. Roles of mast cells and histamine in mosquito bite-induced allergic itch-associated responses in mice. Jpn. J. Pharmacol. 86(1): 97-105.

Olagbemiro, T., and B. Staddon. 1983. Isoprenoids from metathoracic scent gland of cotton seed bug, *Oxycarenus hyalinipennis* (Costa) (Heteroptera: Lygaeidae). J. Chem. Ecol. 9: 1397-1412.

Olson, J. F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect Physiol. 55: 580-587.

Olson, J.F., M. Eaton, S.A. Kells, V. Morin, and C. Wang. 2013. Cold tolerance of bed bugs and practical recommendations for control. J. Econ. Entomol. 106: 2433-2441.

Olson, J. F., R.D. Moon, S. A. Kells, and K. A. Mesce. 2014. Morphology, ultrastructure and functional role of antennal sensilla in off-host aggregation by the bed bug, *Cimex lectularius*. Arth. Struct. and Dev. 43: 117-122.

Parashar, B., K. Ganesan, D. Sukumaran,, Y. Rao, V. Vijay, and S. Prakash. 2003. Aggregation activity induced by the excreta extracts in *Cimex hemipterus* (Hemiptera: Cimicidae). Entomon 28: 215-222.

Pascal, D., B. Blanc, P. D. Giudice, A. Levy-Bencheton, O. Chosidow, P. Marty, and P. Brouqui. 2011. Bed bugs and infectious diseases. Clin. Infect. Dis. 52: 200-210.

Pfiester, M., P. G. Koehler, and R.M. Pereiera. 2010. Effect of population structure and size on aggregation behavior of *Cimex lectularius* (Hemiptera: Cimcidae). J. Med. Entomol. 46: 1015-1020.

Potter, M. 2008. The business of bed bugs. Pest Control Technol. 24: 40.

Potter, M.F., A. Romero, K.F. Haynes. 2008. Battling bed bugs in the USA. Proc. Sixth Int. Conf. Urban Entomol. 401-406.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. URL http://www.R-project.org/.

Radwan, W., A. Guneidy, and A. Soliman. 1972. On the biology of the bed bug, *Cimex lectularius* L. Bull. Soc. Entomol. Egypte. 56: 235-246.

Rao, H. 1972. Abnormal sexual behavior of isolated males of *Cimex lectularius* L. Indian J. Exp. Biol. 10: 295-297.

Reinhardt, K., R. Naylor, M.T. Siva-Jothy. 2003. Reducing a cost of traumatic insemination: female bed bugs evolve a unique organ. Proc. R. Soc. Lond. B22 270: 2371-2375.

Reinhardt, K., and M. T. Siva-Jothy. 2007. Biology of the bed bugs. Ann. Rev. Entomol. 52: 351-374.

Reinhardt, K., R.A., Naylor, M.T. Siva-Jothy, 2009. Situational exploitation: higher male mating success when female resistance is reduced by feeding. Evolution 63: 29-39.

Reinhardt, K., D. Kempke, R. A. Naylor, and M. T. Siva-Jothy. 2009. Sensitivity to bites by the bed bug, *Cimex lectularius*. Med. Vet. Entomol. 23: 163-166.

Reis, M.D., and D.M. Miller. 2011. Host searching and aggregation activity of recently fed and unfed bed bugs (*Cimex lectularius* L.). Insects 2: 186-194.

Rivnay, E. 1930. Host selection and cannibalism in the bed bug *Cimex lectularius* L. Ann. Entomol. Soc. Am. 23: 758-764.

Rivnay, E. 1932. Studies in tropisms of the bed bug *Cimex lectularius* L. Parasitol. 24: 121-136.

Rivnay, E. 1933. Tropisms effecting copulation in the bed bug. Psyche 140: 115-120.

Romero, A., M. Potter, D. Potter, and K. Haynes. 2007. Insecticide resistance in the bed bug: a factor in the pest's sudden resurgence? J Med. Entomol. 44: 175-178.

Romero, A., M. F. Potter, and K. F. Haynes. 2009. Behavioral responses of the bed bug to insecticide residues. J. Med. Entomol. 46: 51-57.

Romero, A., M.F. Potter, K.F. Haynes. 2010. Circadian rhythm of spontaneous locomotor activity in the bed bug, *Cimex lectularius* L. J. Insect Physiol. 56(11): 1516-1522.

Ryne, C. 2009. Homosexual interactions in the bed bugs alarm pheromones as male recognition signals. Anim. Behav. 78: 1471-1475.

Sakuma, M. 1993. Aggregation arrestment pheromone of the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae): isolation and structure elucidation of blattellastanoside –A and –B. J. Chem. Ecol. 19(11): 2521-2541.

SAS. 2004. SAS/STAT 9.1 Users Guide. SAS Institute Inc., Cary, NC.

Sato, T. 1968. A modified method for lead staining of thin sections. J. Electron Microsc. 17: 158–159.

Schaefer, C.W., and A.P. Anizzi. 2000. Heteroptera of Economic Importance. CRC Press; Boca Raton. 828p.

Scherkenbeck, J., G. Nentwig, K. Justus, J. Lenz, D. Gondol, G. Wendler, M. Dambach, F. Nischk, and C. Graff. 1999. Aggregation agents in german cockroach *Blattella germanica*: examination of efficacy. J. Chem. Ecol. 25(5): 1105-1119.

Schuh, R., and J. Slater. 1995. True bugs of the world (Hemiptera: Heteroptera): Classification and natural history. Cornell University Press. 336p.

Semmelhack, J.L., and J. W. Wang. 2009. Select drosophila glomeruli mediate innate olfactory attraction and aversion. Nature 459: 218-224.

Sigma-Aldrich Company Safety Data Sheet, Version 4.5, Number 471828.

Siljander E., D. Penman, H. Harlan, and G. Gries. 2007. Evidence of male- and juvenile-specific contact pheromones of the common bed bug *Cimex lectularius*. Entomol. Exp. Appl. 125: 215-219.

Siljander E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708-718.

Singh, R., K. Singh, S. Prakash, M. Mendki, and K. Rao. 1996. Sensory organs on the body parts of the bed-bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) and the anatomy of its central nervous system. Int. J. Insect Morph. 25: 183-204.

Singh, N., C. Wang, and R. Cooper. 2013. Effect of trap design, chemical lure, carbon dioxide release rate, and source of carbon dioxide on efficacy of bed bug monitors. J. Econ. Entomol. 106: 1802-11.See comment in PubMed Commons below

Sonenshine, D., T. Adams, S. Allan, J. McLaughlin, and F. Webster. 2003. Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularius* (Acari: Icodidae) and their use in tick control. J. Med Entomol. 40: 849-859.

Sonenshine, D. 2006. Tick pheromones and their use in tick control. Ann. Rev. Entomol. 51: 557-580.

Spittler, T. D., H. C. Leichtweis, and T. J. Dennehy. 1988. Biorational control of crop pests by mating disruption: residue analyses of Z-9-doecen-1-yl acetate and Z-11-tetradecen-1-yl acetate in grapes. Biotechnology for Crop Protection Chpt. 33, pp. 430-436.

Sreng, L., A. Clorec, and C. Rivault. 1998. Cuticular extracts inducing aggregation in the German cockroach, *Blattella germanica*, (L.). J. Insect Physiol. 44: 909-918.

Steinbrecht, R. 1996. Structure and function of insect olfactory sensilla. Ciba Found Symp. 200: 158-174.

Steinbrecht, R., and B. Muller. 1976. Fine structure of the antennal receptors of the bed bug, *Cimex lectularius* L. Tissue Cell 8: 615-636.

Steinbrecht, R. A. 1998. Bimodal thermo- and hygrosensitive sensilla. Pp. 405 – 422. – *In*: F. W. Harrison & M. Locke (eds), Microscopic Anatomy of Invertebrates, vol. 11 B, Insecta. Wiley-Liss, Inc., New York.

Statheropoulosa, M. A., C. Agapioua, G. Spiliopoulou, C. Pallis, and E. Siano. 2007. Environmental aspects of VOCs evolved in the early stages of human decomposition. Sci. Total. Environ. 385: 221-227.

Stutt, A. and T. Siva-Jothy. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. P. Natl. Acad. Sci. U.S.A. 98: 5683-5687.

Suchy, J.T., and V.R. Lewis. 2011. Host-seeking behavior in the bed bug, *Cimex lectularius*. Insects 2: 22-35.

Ternay, A. 1979. Contemporary Organic Chemistry. Philadelphia: Saunders.

Ter Poorten, M. C., and N. S. Prose. 2005. The return of the common bed bug. Pediatr. Dermatol. 22: 183-187.

Usinger, R. L. 1966. *Monograph of Cimicidae* (Hemiptera-Heteroptera), The Thomas Say Foundation. Entomological Society of America, Baltimore. M.D

Vaidyanathan, R. 2012. Bed bug: the science and costs of infestation. SRI International sri.com/blog/bed-bugs-science-costs.

Vaidyanathan, R., and M.F. Feldaufer. 2013. Bed bug detection: current technologies and future directions. Am. J. Trop. Med. Hyg. 88: 619-625.

Walpole, D. 1987. External morphology of the legs of two species of bed bugs (Hemiptera: Cimicidae). J. Ent. Soc. Sth. Afr. 50: 193-201.

Wang, C., T. Gibb, G. Bennett, and S. McKnight. 2009. Bed bug (Heteroptera: Cimicidae) attraction to pitfall traps baited with carbon dioxide, heat, and chemical lure. J. Econ. Entomol. 102: 1580-1585.

Wang, C., K. Saltzmann, E. Chin, G. W. Bennett, and T. Gibb. 2010. Understanding bed bug infestation and dispersal patterns. Pest Control Tech. 38(3): 94-96.

Weeks, E.N., M. Birkett, M.M. Cameron, J.A. Pickett, and J.G. Logan. 2010. Semiochemicals of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), and their potential for use in monitoring and control. Pest Manag. Sci. 67: 10-20.

Weeks, E. N. I., J. G. Logan, S. A. Gezan, C. M. Woodcock, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2011. A bioassay for studying behavior responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) to bed bug derived volatiles. Bull. Entomol. Res. 101: 1-8.

Weeks, E. N., J.G. Logan, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2012. Tracking bed bug (*Cimex lectularius*): a study of the effect of physiological and extrinsic factors on the response to bed bug-derived volatiles. J. Exp. Biol. 216: 460-469.

Wertheim, B., E.J. van Baalen, M. Dicke, and L.E.M. Vet. 2005. Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. Annu. Rev. Entomol. 50: 321-346.

Zackaruk, R. Y., and V. D. Shields. 1991. Sensilla of immature insects. Ann. Rev. Entomol. 36: 331-354.

APPENDIX

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