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Wash-Off Potential of Pyrethroids After Use of Total Release Foggers and the Chemical Ecology of Bed Bugs (Heteroptera: Cimicidae)

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by

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ABSTRACT OF THE DISSERTATION

Wash-Off Potential of Pyrethroids After Use of Total Release Foggers and the Chemical Ecology of Bed Bugs (Heteroptera: Cimicidae)

by

Mark Donald Paul Dery

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2021
Dr. Dong-Hwan Choe, Chairperson

Despite detection of pyrethroids in the influent and effluent of wastewater treatment plants, little is known about the sources and mechanisms responsible for down-the-drain transport of pyrethroids. It was hypothesized that total release foggers may serve as a source of pyrethroids entering wastewater through the deposition of the active ingredients and subsequent transfer from contaminated surfaces into the waste stream through cleaning activities. Experiments were conducted to determine floor deposition characteristics of total release foggers and the transfer from various surfaces and materials. We found that total release foggers can contribute to insecticide loading into the wastewater treatment system via several routes, such as contacting or cleaning exposed surfaces and washing contaminated clothing.

Bed bugs produce volatile aldehydes that have alarm and aggregation functions. Using two synanthropic bed bug species, *Cimex lectularius* L. and *C. hemipterus* (Fabricius), developmental quantity changes were examined for (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal. The quantities and percent

abundances of the aldehydes in the nymphal exuviae and the adults were significantly different between species. The behavioral response of these bed bug species to the conspecific or heterospecific nymphal aldehyde blends (exuviae or a synthetic blend) was examined. In both species, the adults settled preferentially on the treatment side when conspecific volatile aldehyde cues were provided. When tested with heterospecific volatile aldehyde cues, only adult *C. lectularius* preferentially responded to *C. hemipterus* volatile cues. Adult *C. hemipterus* was indifferent to the aldehyde blend of *C. lectularius.*

The use of the entomopathogenic fungus *Beauveria bassiana* for bed bug control is a recent addition to bed bug management. Mortality was monitored following the exposure of bed bugs to commercial products containing *B. bassiana* when in the presence of an aldehyde source. The introduction of bed bug aldehydes significantly reduced the effectiveness of *B. bassiana* as a control method when the product is not formulated specifically for bed bugs. However, the addition of aldehydes only delayed mortality when bed bugs are exposed to a formulation designed for bed bugs. The addition of synthetic bed bug aldehydes delayed and reduced the growth of *B. bassiana* in culture.

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INTRODUCTION

Bed bugs (Hemiptera: Cimicidae) are obligate blood-feeding ectoparasites (Usinger, 1966). Two species of cimicids predominantly utilize humans as a host - the bed bug, *Cimex lectularius* L., and the tropical bed bug, *Cimex hemipterus* (Fabricius). *Cimex lectularius* has a worldwide distribution, while *C. hemipterus* inhabits tropical and subtropical regions (Usinger, 1966). Bed bugs have been associated with human dwellings for thousands of years, with human associations known since antiquity (Usinger, 1966; Panagiotakopulu & Buckland, 1999). After the development and widespread use of synthetic pesticides in the mid-20th century, the status of bed bugs as a major urban pest was significantly reduced in many industrialized countries (Doggett et al., 2004; Romero et al., 2007). However, beginning in the 21st century, a resurgence of bed bugs around the world has once again made them among the most significant urban pests (Potter, 2006; Romero et al., 2007; Doggett et al., 2012).

While bed bugs are not known to vector any diseases, they significantly impact human health and well-being (Doggett et al., 2012). Bed bug bites can cause itchy welts, which can subsequently be infected when scratched (Feingold et al., 1968). Bed bug infestations can also cause severe psychological distress and insomnia (Doggett et al., 2012; Goddard & De Shazo, 2012; Susser et al., 2012). The longstanding misconception that bed bugs only inhabit unclean locations can result in feelings of embarrassment and social stigma by those affected (Doggett et al., 2012).

The resurgence of bed bugs has also resulted in significant economic costs. While bed bugs can affect anyone, bed bugs have a disproportionate effect on low-income

communities and frequently infest densely occupied multi-unit housing (Eddy & Jones, 2011). The control of bed bugs in multi-unit housing can cost hundreds of dollars per unit, with some apartment buildings spending tens of thousands of dollars each year on bed bug control (Cooper et al., 2016). As a result, whether the tenant or landlord is responsible for control costs has been a topic for numerous litigation cases. Several states have responded by enacting laws clarifying the responsibility of each party (Lipman & Miller, 2018). Other economic impacts include the costs of negative publicity and lawsuits against hotels with previous or current bed bug infestations (Potter, 2006; Doggett et al., 2018).

While bed bugs commonly infest homes, apartments, and hotels, they also successfully infest a diverse range of structures and settings. For example, bed bug infestations have been reported in health care facilities, schools, restaurants, movie theaters, college dormitories, and public transportation, such as airplanes, trains, and buses (Hwang et al., 2005; Doggett et al., 2012; Doggett, 2013). Due to the frequent movement of people in these settings, a bed bug infestation in one site can serve as a potential source for the further spreading of bed bugs to new areas.

Current control methods for bed bugs include chemical methods, such as insecticide sprays, total release foggers ("bug bombs"), and inorganic dust (with or without added insecticides), as well as various non-chemical methods (Doggett, 2013). Non-chemical methods include heat treatments, traps, vacuuming, laundering bedding/clothes, bed bug-proof mattress encasements, and steam treatments (Doggett, 2013). Both chemical and non-chemical control methods are often utilized together,

along with an ongoing monitoring program as part of an integrated pest management strategy (Doggett, 2013; Cooper, Wang, & Singh, 2016).

A significant challenge for the chemical control of bed bugs is widespread insecticide resistance among bed bug populations (Romero, 2018). The relatively low number of insecticide active ingredients commonly used for bed bug control further exacerbates the issues with insecticide resistance. For example, despite the widespread distribution of pyrethroid resistance among bed bug field populations, pyrethroids are still among the most commonly used active ingredients in many commercial pesticide products targeting bed bugs (Romero et al., 2007; Lee et al., 2018). In an attempt to address pyrethroid resistance, products containing pyrethroids are increasingly being paired with neonicotinoid insecticides (Lee et al., 2018). However, resistance to neonicotinoids has also been reported in field populations of bed bugs (Gordon et al., 2014; Romero & Anderson, 2016). In an attempt to overcome the issue of insecticide resistance, the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. has been tested against bed bugs, and at least one commercial product, Aprehend (ConidioTec LLC, Centre Hall, PA, USA), is now available for bed bug control.

The objectives of this dissertation are (1) to investigate the potential of total release foggers as a source of municipal wastewater contamination, (2) to compare the quantity of four aldehydes found in *C. lectularius* and *C. hemipterus* in all nymphal instars and adults, (3) to determine if adults of both species preferentially respond to both the conspecific and heterospecific aldehyde blend, and (4) to investigate if the bed bug

aldehyde blend affects the effectiveness of entomopathogenic fungal products for bed bug control.

In Chapter 1, I investigated the potential impacts of total release foggers (TRFs), a chemical product commonly used for bed bugs and other urban pests, as a potential indoor source of insecticide contamination entering the wastewater treatment system. Various pyrethroids have been detected in the influent and effluent of wastewater treatment plants, representing a source of insecticides entering surface waters (Weston & Lydy, 2010; Weston et al., 2013; Markle et al., 2014; Teerlink, 2014). The movement of insecticides applied indoors into the wastewater treatment system is known (Weston et al., 2013); however, the sources and mechanisms through which this occurs are unclear (Teerlink, 2014). The current research determined the floor deposition characteristics of total release foggers and the transfer efficiencies from exposed surfaces such as carpet, tile, vinyl, and wood to filter paper. The extraction of a pyrethroid from filter paper and cotton fabric using water with and without a detergent was also investigated to mimic the washing of contaminated clothing or surfaces.

In Chapter 2, I investigated the ontogenesis (the development from the earliest stage to maturity) of aldehyde pheromone production in two related species of bed bugs, *C. lectularius,* and *C. hemipterus*. Four aldehydes produced by bed bug nymphs [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal] are a part of the bed bug aggregation pheromone (Siljander et al. 2008; Gries et al. 2015; Choe et al. 2016; Ulrich et al. 2016). Each aldehyde was quantified in the five nymphal instars and adult insects (male and female). Even though the importance of these aldehydes in bed

bugs' chemical ecology has been long recognized, the amount of each aldehyde produced at various life stages has not been previously determined, except for *C. lectularius* 5th instar nymphs (Choe et al., 2016).

In Chapter 3, I studied whether adults of two bed bug species (*C. lectularius* and *C. hemipterus*) would respond to the conspecific aldehyde blend as well as the heterospecific aldehyde blend. A still-air olfactometer was used to test two sources of the bed bug aldehydes, the shed exuviae and the synthetic aldehyde blends reconstituted by using the information obtained in Chapter 2. While Choe et al. (2016) found that *C. lectularius* responded to the conspecific blend, my research expanded our understanding of bed bug biology by investigating the responses of these two species to the heterospecific aldehyde blends. The information from this research might help develop a chemical lure that would be effective for both species.

In Chapter 4, I investigated the impact of bed bug aldehydes on the efficacy of the entomopathogenic fungi *B. bassiana*. The fungus *B. bassiana* is currently registered against bed bugs in the United States as a fungal biopesticide. While several studies have investigated the impacts of this entomopathogenic fungus on both bed bug mortality and behavior (Barbarin et al., 2012; Barbarin et al., 2017; Aak et al., 2018), little is known about the effectiveness of these entomopathogenic fungal agents when used in field settings where bed bugs would be typically associated with their natural aggregations. In particular, it was suspected that the presence of many exuviae and other sources of bed bug aldehydes (e.g., production of aldehydes by bed bugs) in natural aggregations might significantly influence the overall efficacy of the fungal biopesticides. To answer this

question, the bed bugs' mortality after fungal exposure was tracked when they were kept with or without an aldehyde source (exuviae or synthetic blend). By mimicking the natural blend of bed bug aldehydes in the exuviae, my research also quantified the potential inhibition of *B. bassiana* in culture when exposed to the volatile aldehydes.

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Chapter 1. Wash-Off Potential of Pyrethroids After Use of Total Release Fogger Products

ABSTRACT

Pyrethroids are frequently detected in urban wastewater. Even though treatment facilities remove most pyrethroids (>90%) in wastewater, residual concentrations can exceed thresholds that are acutely toxic to sensitive aquatic species. Total release foggers (also known as “bug bombs”) are widely available to the general public for insect control. It was hypothesized that these products serve as a potential source of pyrethroids entering the urban wastewater through the deposition of the active ingredients on various surfaces and subsequent transfer from the contaminated surfaces to the waste stream through cleaning activities. Based on experiments conducted in an enclosure, we found that substantial amounts of a pyrethroid (i.e., cypermethrin) were deposited on various surfaces after a total release fogger use. A series of experiments simulating different scenarios indicated that the pyrethroid could be transferred from the contaminated surfaces to other adsorptive materials via physical contact (with or without water as a solvent). The pyrethroid was readily extracted from the adsorptive materials (cotton fabric and filter paper) when water was used as a solvent. Adding a small amount of detergent to the water significantly increased the extraction efficiency compared to water alone. These results indicate that insecticides used in total release foggers can contribute to insecticide loading into the wastewater treatment system via several possible routes,

such as contact with or cleaning of exposed surfaces and washing contaminated clothing after their use within a structure.

INTRODUCTION

Total release foggers (TRFs) (also known as “bug bombs”) are widely available for the general public’s use, commonly utilizing pyrethrins or synthetic pyrethroids (with or without a synergist) as active ingredients (AIs). TRFs function by spraying an insecticide mist into the air, which falls onto exposed surfaces and objects. These products target common indoor flying and crawling pests such as ants, bed bugs, German cockroaches, spiders, flies, and mosquitoes. Despite the widespread use of TRFs, there is limited information available regarding their effectiveness in controlling these pests. However, recent experiments have suggested that these products are ineffective for field populations of bed bugs and German cockroaches due to high pyrethroid resistance in the pest populations and a lack of penetration into pests’ harborage sites (Jones & Bryant, 2012; DeVries et al., 2019a, 2019b). These products can contribute to occupants' inhalation, ingestion, and dermal exposure to insecticidal compounds (Selim & Krieger, 2007; Keenan et al., 2009). Additionally, the misuse of TRFs can have acute impacts on human health (Centers for Disease Control and Prevention, 2008; Forrester & Diebolt-Brown, 2011; Liu et al., 2018).

Insecticides contained in TRFs are frequently detected in surface waters at amounts over regulatory threshold levels, representing a threat to aquatic ecosystems (Stehle & Schulz, 2015). Insecticides are known to enter surface waters via surface runoff

from agricultural (Stehle & Schulz, 2015) and urban sources (Jiang et al., 2012). While much research has focused on the contribution of agricultural insecticides to surface water contamination, less is known about the role that indoor applications of insecticides contribute to pesticide loading in waterways. Pyrethroids have been detected in the influent and effluent of wastewater treatment plants, representing an additional source of insecticides entering surface waters (Weston & Lydy, 2010; Weston et al., 2013; Markle et al., 2014; Teerlink, 2014). While some insecticide contaminants will be removed or reduced by wastewater treatment facilities (e.g., >90% for pyrethroids), residual insecticides that are not removed during water treatment will be discharged into surface waters, threatening aquatic ecosystems (Weston et al., 2013; Teerlink, 2014). Despite frequent detections, the sources and mechanisms of down-the-drain mass loading of pyrethroids are largely unknown (Teerlink, 2014). As insecticides are commonly used in residential homes to control urban pests, many insecticides are found on residential floors and indoor dust (Julien et al., 2008; Stout et al., 2009). Findings by Weston et al. (2013) suggested that pyrethroid inputs from indoor cleaning activities within residential areas are a significant source of pyrethroids in wastewater. This study detected pyrethroids in wastewater influent originating from residential areas at higher concentrations than found in urban runoff, suggesting that down-the-drain transport of pyrethroids from residential homes is a significant source of pyrethroids entering treatment facilities (Weston et al., 2013). The entry mechanisms of insecticides into the waste stream are largely conceptual. For example, Teerlink et al. (2017) found that fipronil from dogs treated with spot-on products could be removed during washing. Additional sources, such as products

containing permethrin used to control head lice and scabies, will enter the water treatment system during normal use. Such sources may be of interest as permethrin has been found in higher amounts than other pyrethroids detected in wastewater treatment influent and effluent in several studies (Weston & Lydy, 2010; Weston et al., 2013; Markle et al., 2014; Teerlink, 2014).

A product survey of California retail stores found that cypermethrin was the most common active ingredient in TRFs available to the general public (Budd & Peters, 2018). In addition, Xie at al. (2021) estimated that TRFs accounted for over 40% of all cypermethrin use by consumers. With an estimated use of 50 million TRFs annually in 2010 (Environmental Protection Agency, 2010), these products represent a significant release of pyrethroids into households. Total release foggers disperse their active ingredients across all exposed surfaces within the range of the product but primarily settle on the floor (Keenan et al., 2009, 2010). The active ingredients deposited on various surfaces can be subsequently transferred to clothing (Ross et al., 1990; Keenan et al., 2009). This provides a possible route for down-the-drain transport when the contaminated surfaces or clothing are washed, representing a potential source of pyrethroids entering the wastewater treatment system.

To understand the potential connection between the use of TRFs and pyrethroids in the wastewater systems, information on potential mechanisms by which an insecticide enters the wastewater treatment system will be helpful. To address this, we first determined the depositional pattern and quantity of a pyrethroid insecticide on various surfaces after releasing a fogger product in an enclosed space. By simulating various

scenarios of transfer and extraction, the current study also investigated the potential for deposited pyrethroids to be transferred from the contaminated surfaces to other adsorptive materials (via physical contact) and subsequently into water (via extraction).

MATERIALS AND METHODS

Tent. A recreational tent (Ozark Trail 6-Person ConnecTent; 3.048 × 3.048 m; 2.49 m center height) was used as a test enclosure. Vents in the top of the tent were sealed with plastic sheets. At the bottom of the tent, interconnecting foam floor tiles (≈ 2.75× 2.75 m; Cap Barbell Inc., Houston, TX, USA) were used to provide a level floor surface. The floor was covered with butchers paper, which was replaced after each trial to prevent cross-contamination. The ambient conditions inside the tent when the fogger was activated was 24.3 ± 1.7 °C (mean ± SE; n = 6) and 33 ± 4.2 % RH (mean ± SE; n = 6).

Fogger. Product choice was based on an online search and a survey of a retail store. The product, Hot Shot® Fogger with Odor Neutralizer (0.05% tetramethrin, 0.75% cypermethrin (wt/wt), United Industries Corporation, St. Louis, MO, USA), was chosen as a representative TRF for this study. In all experiments, the fogger was released on a level platform (0.5 m height) placed in the center of the tent floor. After shaking the can, the release valve was pressed, hooking the catch while the can was stationary on the platform. The orientation of the fogger (release valve oriented towards the bottom right of Figure 1.1) was consistent for all experiments. Following fogger activation, the tent was immediately sealed and left overnight to allow for the settling of the product.

Deposition study on horizontal surfaces – floor. To understand the deposition characteristics (e.g., quantity and spatial pattern) of the active ingredient of the fogger product, the following experiment was conducted. An 11 × 11 square grid with 27.7 cm between points was marked on the tent floor. Numbered filter paper (Whatman #1, Cytiva, Marlborough, MA, USA) squares (5 × 5 cm; n = 120) were placed on each point and secured to the floor of the tent with insect mounting pins. The fogger occupied the central point in the grid. The fogger was activated, and the tent was left sealed overnight.

Each piece of paper was collected approximately 18 h after product activation and placed individually into sealable plastic bags, and stored at -20°C until extraction. All samples were extracted within 14 d of collection. From each square, a 1 × 1 cm square sample was cut out of the same corner and placed into a 2-ml glass vial (Agilent Technologies, Santa Clara, CA, USA). For each sample, 200 µl of hexane was added, and the vial was vortexed for approximately 4 sec. From each sample, 40 µl hexane extract was removed and placed into a new vial containing a glass insert (250 µl, Agilent Technologies) for gas chromatography (GC) analysis (see below). This experiment was replicated three times.

Transfer of insecticide via static or rubbing contact. To examine the transfer of insecticide from one surface to an adsorptive material (i.e., filter paper) with water as the solvent, two methods of extraction (static contact and rubbing contact) were tested. This would simulate a cleaning activity such as mopping of the contaminated surface. Six ceramic tiles (10.5 × 10.5 cm; American Olean, Dallas, TX, USA) with glazed surfaces were arranged along the perimeter of a square (1.66 × 1.66 m) surrounding the fogger at

its center (this corresponds to the 83 cm distance in Figure 1.1). After the activation and settling of the product, the tiles were collected and stored individually in plastic bags at -20°C until extraction. For the static contact extraction, a 1 × 1 cm filter paper square was placed onto a corner of the tile. Twenty microliters of deionized water were added to the filter paper. After 10 sec, the filter paper square was carefully removed from the tile surface by lifting it straight up. The filter paper square was placed into a 2-ml glass vial, and these vials were left uncovered in a fume hood for 24 h to dry. In each vial, 200 µl of hexane was added, and the sample was vortexed thoroughly. Finally, 40 µl of the hexane extract was pipetted out and placed into a new vial with a glass insert for GC analysis.

The second method included rubbing contact between the filter paper and the tile surface. A filter paper square (1 × 1 cm) was placed onto the upper left corner of the tile, and 20 µl deionized water was applied to the filter paper. Subsequently, with a pair of fine forceps, the filter paper was dragged 8 cm along the edge of the tile. This filter paper was then removed and extracted as described above for the static contact extraction.

Transfer of insecticide from vinyl, wood, and tile surfaces. To examine the transfer of insecticide from various surface materials to an adsorptive material (i.e., filter paper) with water as the solvent, the dragging method was repeated on various surfaces. Eight filter paper squares (5 × 5 cm) were arranged along the perimeter of a square (1.66 × 1.66 m) with the fogger located at the center, serving as the reference for pyrethroid quantification. Around each filter paper square (5 cm away), three types of materials were placed. Tiles (10.5 × 10.5 cm) were placed above, vinyl flooring (9.5 × 10 cm;

Invista, Wichita, KS, USA) to the left, and acacia laminate wood flooring (10 × 10 cm; Lowe's Companies, Inc., Mooresville, NC, USA) to the right, relative to the tent entrance.

The filter paper squares were extracted as described for the floor dispersal study. The tiles, vinyl, and wood were sampled by placing a 1 × 1 cm square of filter paper on a corner and adding 20 µl deionized water before dragging the paper over 10 cm along each surface. Each paper was then placed separately into a 2-ml vial, allowed to dry overnight, and subsequently extracted with 200 µl hexane as described for the floor deposition experiment.

Transfer of insecticide from carpet surface (clean vs. dusty and contact only vs. contact with friction). To test the transfer of insecticide from carpet to an adsorptive material without any solvent, transfer from the clean and dusty carpet was tested. This would simulate contact between the contaminated carpet surface and other materials such as socks or pants. Twenty 12 × 12 cm squares of carpet (Shaw reclaim rr textured heirloom interior carpet; polyester) were left outdoors on a rack under building eaves for approximately 4 wk. An additional twenty squares of carpet remained indoors. Twenty filter paper squares (5 × 5 cm) were spaced evenly along the perimeter of a square (1.66 × 1.66 m) with the fogger in the center, serving as the reference for pyrethroid quantification. This trial was done concurrently with a second carpet experiment (see below). The carpet squares were separated from each other by 1 cm and were placed 1 cm from the filter paper.

The carpet was sampled by placing a clean 5 × 5 cm square of filter paper on top of each square. Filter paper squares were divided by placing a large clean tile (12 × 12

cm) on top of each filter paper, completely covering the carpet. Samples were then stacked in this manner, and 9.75 kg of weight was placed on top for 24 h. This weight was chosen to simulate a person (64.3 kg) with an average total foot contact area (81.8 cm^2) standing on a carpet and transferring material to their socks (Birtane & Tuna, 2004). Samples from the clean vs. dusty groups were extracted separately. The filter paper squares were then collected and extracted as described for the floor deposition study.

To determine if the active rubbing influences the amount of insecticide transferred from the carpet surface, two additional 12 × 12 cm squares of clean carpet were placed around each filter paper square during the experiment described above. The carpet squares were placed 1 cm apart from each 5 × 5 cm square of filter paper. One group of carpet squares (n = 20) were extracted with the presence of friction. These carpet squares were placed on a benchtop shaker (Clinical Rotator Model 341; Fisher Scientific International**,** Pittsburgh, PA, USA) with a clean filter paper square (5 × 5 cm) placed in the center of each carpet square. The carpet was then covered with a glass square (12 × 12 cm), and 1 kg of sand in a flask was placed on top. Less weight was used for the friction experiment due to weight limits of the shaker. The shaker was activated and after 10 min, the filter paper square was collected. This would simulate contact between the contaminated carpet surface and other materials such as socks or other clothing with some amount of friction. The second group of carpet squares (n = 20) were sampled in the same manner but with weight alone (static). Each carpet square was extracted individually. The glass squares were cleaned thoroughly with acetone between trials. The filter paper was extracted as described for the floor deposition experiment.

Wash off from filter paper and fabric. To examine how much insecticide can be washed off from filter paper and fabric materials that are exposed to the fogger product, the following experiment was conducted. This experiment would simulate washing or laundering of the contaminated clothes or other fabric materials (e.g., mop). On the tent floor, 24 points were marked evenly along the perimeter of a square (1.66 × 1.66 m) with the fogger located in the center. A 5 × 5 cm piece of filter paper and cotton fabric were placed 1 cm from each side of the marked point. From each fabric and filter paper square, three 1 × 1 cm squares were cut and individually placed into three 2-ml glass vials.

Three different solvent types [hexane, water, and water + detergent (1% Liquinox®; Alconox Inc., White Plains, NY, USA)] were used for initial extraction of each type of material (filter paper and cotton fabric). To extract with hexane, 200 µl of hexane was added into the vial and vortexed. An aliquot (40 µl) of hexane extract was pipetted out and transferred to a new vial with a glass insert. For water extraction, 200 µl of deionized water was first added into the vial and vortexed. The entire water extract was pipetted out from the vial and transferred to a new vial. After adding 200 µl hexane, the vial was vortexed again before removing 40 µl of the hexane layer to a new vial with a glass insert. For the water + detergent extraction, 200 µl of a 1% detergent solution in deionized water was used for the initial extraction. After vortexing, the extract was pipetted out and transferred to a new vial. After adding 200 µl hexane, the vial was vortexed. Since the mixture formed an emulsion, the samples were stored at -20 °C overnight to separate the mixture. From the hexane layer, 40 µl was pipetted out and placed into a new vial with a glass insert for GC analysis.

Extraction from spiked filter paper. Based on the Safety Data Sheet (SDS) of the fogger product (Hot Shot Fogger with Odor Neutralizer, 2016), light aromatic naphtha (2% wt/wt) and petroleum distillates (3.75%) are among the other ingredients of the product, besides the pyrethroids (0.8%) and propellants (30%). The remaining ingredients are either proprietary or non-hazardous and are not identified on the SDS. To determine the effect of these other ingredients on the amount of insecticide removed from a material with water as a solvent, the following experiment was conducted. Crude material from the fogger product was obtained by freezing the fogger can at -20°C overnight and then puncturing the can to release only the propellant. The crude residue was diluted with acetone, and cypermethrin was quantified using an external standard (see Chemical analysis). Based on the quantification, an aliquot (2 µL) of crude material was applied to 1 × 1 cm filter paper squares inside 2-ml glass vials, providing 25 µg of cypermethrin from the fogger crude material applied per filter paper square. For comparison, 25 µg of technical cypermethrin (> 90%; Sigma-Aldrich, St. Louis, MO, USA) dissolved in acetone was applied to 1 × 1 cm filter paper squares inside 2-ml glass vials. The vials were allowed to dry uncapped overnight in a fume hood. These were then extracted using the three solvents (hexane, water, and water + detergent) as described in the previous section. Final hexane extracts were analyzed with a GC. Ten replications were conducted for each combination of cypermethrin source (crude or technical) and solvent (hexane, water, and water + detergent).

Chemical analysis. Between the two active ingredients present in the fogger product used, cypermethrin was chosen for chemical quantification due to its higher abundance in

the product (i.e., 0.05% tetramethrin, 0.75% cypermethrin). The amount of tetramethrin in each sample was not determined. An automatic liquid sampler (ALS) was used to inject 2 µl of the hexane extract onto an Agilent 7890 gas chromatograph equipped with a DB-5 column (30 m × 0.25 mm inner diameter) and a flame ionization detector. Helium was used as the carrier gas, and samples were injected in splitless mode, with a temperature program of 50°C for 1 min and then 10°C min^{-1} to 300°C with a 10-min hold. The integration values of four peaks representing diastereomers of cypermethrin were summed (Liu & Gan, 2004). The amounts of cypermethrin in extracts and samples were determined by comparison to a calibration curve established from samples with various concentrations of technical grade cypermethrin (0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 30, 40, 60, and 70 µg/ml in hexane) analyzed on the same instrument above. In experiments that involved the moving (dragging) of filter paper across a surface (sections 2.4 and 2.5), the total amount of cypermethrin was divided by the area sampled to determine the amount of cypermethrin per square centimeter area.

Statistical analysis. Data were analyzed by conducting Shapiro-Wilk and Levene's tests to determine if there was non-normality or heteroscedasticity, respectively. When present, non-parametric tests were used to compare data. Kruskal–Wallis H test followed by Dunn's Multiple Comparisons were used to analyze the different surface types (vinyl, wood, tile), and the wash-off testing done both in the tent and with spiked filter paper. Two-sample t-tests were used to compare the amount of cypermethrin for the static and friction tile sampling and the extractions of samples spiked with known amounts of cypermethrin. Finally, Wilcoxon rank-sum tests were used to analyze both carpet

experiments. The percent of transfer was calculated for each material based on the amount of cypermethrin recovered from filter paper in each experiment. This provides an estimate for the total amount of cypermethrin that was deposited on each surface. For the extractions using water, hexane extractions were used as an estimate for the total amount of cypermethrin. All statistical analyses were done using R version 4.0.3 (R Core Team, 2020).

RESULTS

Deposition study on horizontal surfaces – floor. An average amount of 6.76 ± 2.23 μg/cm^2 (mean ± SD, n = 3) of cypermethrin was found on the floor of the tent (Figure 1.1). On average, there was a wide range of amounts found, from a maximum of 31.05 μg/cm^2 directly adjacent to the fogger to a minimum of 2.36 μg/cm^2 in one of the tent corners (Figure 1.1). There was a range of 59.04 to 0.003 μg/cm^2 cypermethrin among the total 360 samples collected. Based on the median value (5.77 μg/cm^2; n = 360) applied evenly across the tent floor surface area, we estimate 106% of the product mass on the floor based on the label concentration.

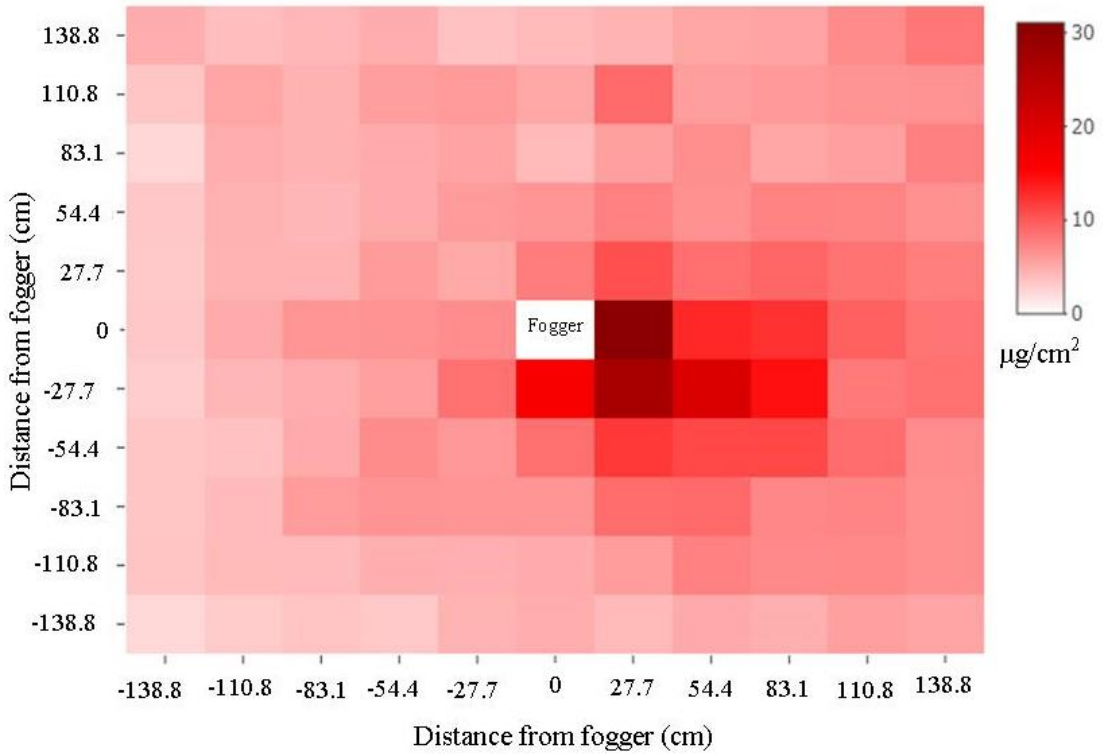

Figure 1.1 Average heat map of cypermethrin deposits (µg/cm^2; *n* = 3) at various distances (cm) from an elevated (0.5 m) centrally placed fogger.

Transfer of insecticide via static or rubbing contact. There was a significant effect of rubbing action on the amount of cypermethrin removed, $T = 4.623$, df = 10, $P < 0.001$, from the tile surface. Significantly more cypermethrin was removed when the filter paper was dragged (1.21 ± 0.17 µg/cm^2; Mean ± SE; *n* = 6) compared to the filter paper that remained static (0.35 ± 0.04 µg/cm^2; *n* = 6) (Figure 1.2). Based on these results, actions such as wiping down a surface while cleaning would remove more cypermethrin per unit of surface area than static contact.

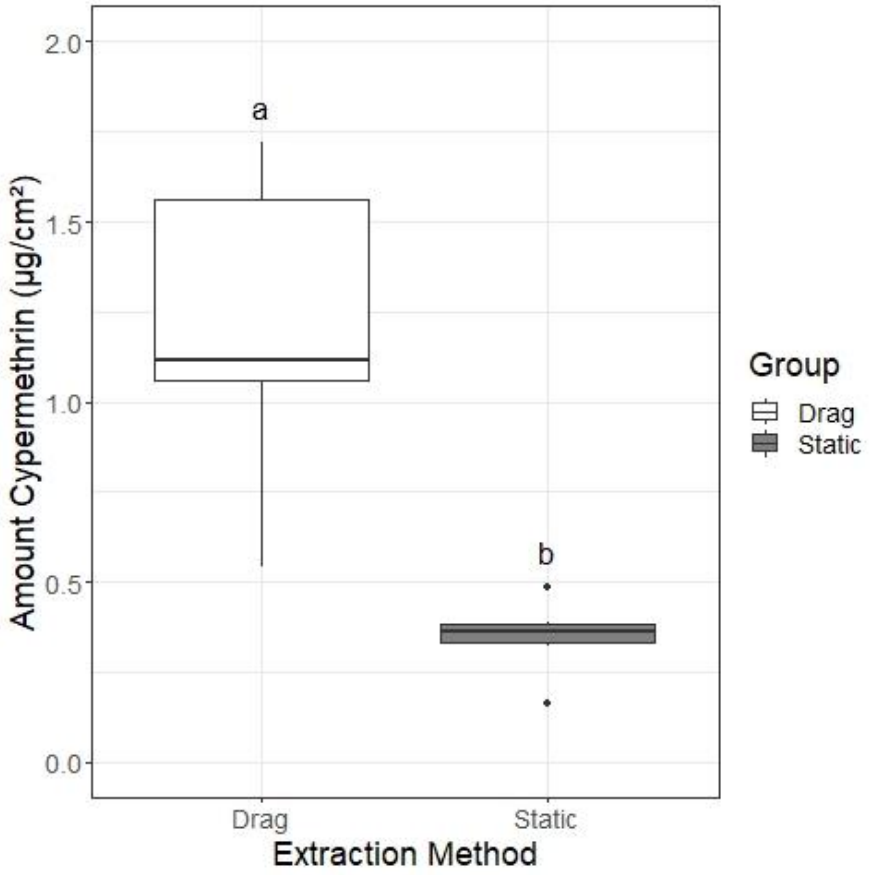

Figure 1.2 Amount of cypermethrin (µg/cm^2) transferred from tile surfaces (n = 6) to filter paper using two methods (static or dragging across tile). Letters indicate significant differences (Two-sample t-test, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

Transfer of insecticide from vinyl, wood, and tile surfaces. The amount of cypermethrin recovered from the reference filter paper (6.29 ± 0.57 µg/cm^2; n = 8) provides an estimate for the total amount deposited onto each surface. The amounts of cypermethrin removed from vinyl, wood, and tiles were significantly different (H = 20.48, df = 2, $P < 0.0010$). The amounts of cypermethrin recovered from tile (3.06 ± 0.32 µg/cm^2; n = 8), vinyl (0.03 ± 0.005 µg/cm^2; n = 8) and wood (0.26 ± 0.04 µg/cm^2; n = 8) were all significantly distinct (Dunn's Multiple Comparisons; $P < 0.05$) (Figure 1.3).

Relative to the reference filter paper, 48.6% of the cypermethrin was recovered from the tile, compared with 0.5% and 4.1% from the vinyl and wood, respectively (Table 1.1). These results show how the amount of cypermethrin that is removed from surfaces is greatly affected by the material type.

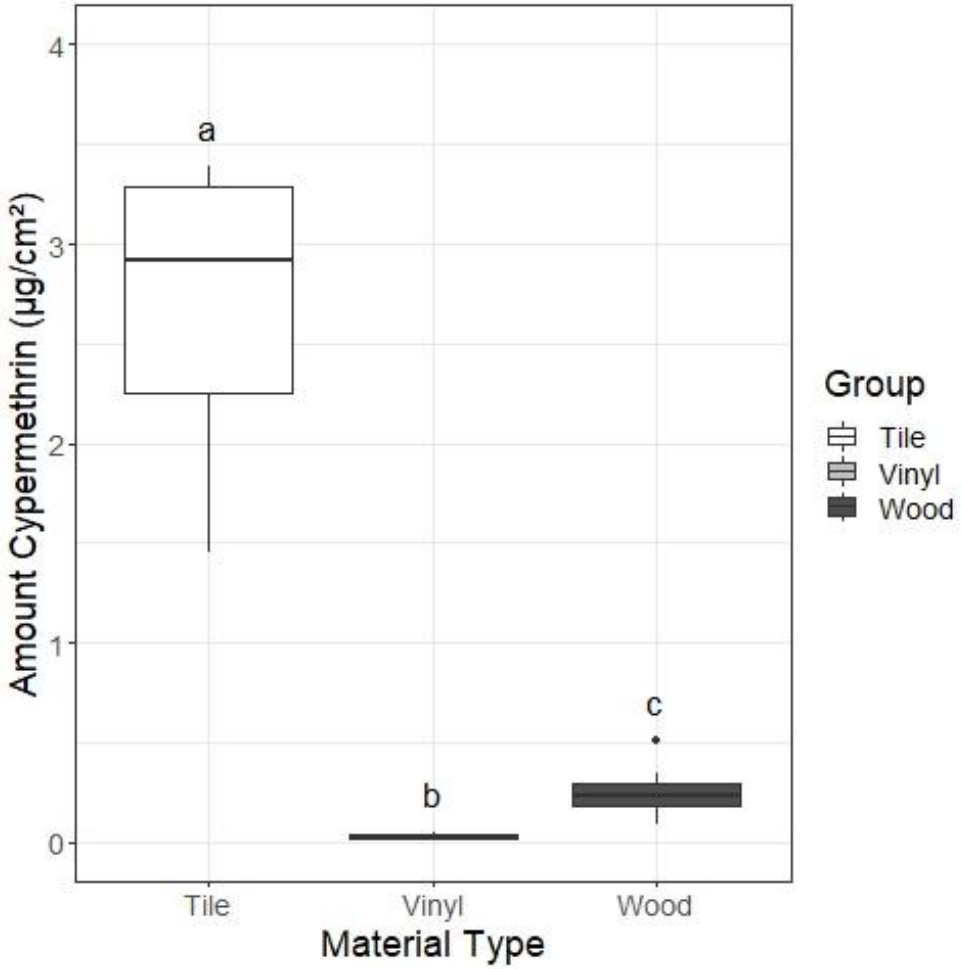

Figure 1.3 Amount of cypermethrin (µg/cm^2) transferred from tile (n = 8), vinyl (n = 8) and wood (n = 8) squares following dragging of a wet filter paper square across each surface. Letters indicate significant differences (Dunn's Multiple Comparisons, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

Transfer of insecticide from carpet surface (clean vs. dusty and contact only vs. contact with friction). The amount of cypermethrin recovered from the reference filter paper (12.79 ± 0.523 μg/cm^2; n = 20) provided an estimate for the total amount deposited onto each surface.

Amounts of cypermethrin transferred by contact with static weight for 24 h from the clean (0.587 ± 0.049 μg/cm^2; n = 20) or dusty carpet (0.567 ± 0.038 μg/cm^2; n = 20) were similar (W = 210, df = 1, P = 0.787) (Figure 1.4). These amounts represent a transfer rate of 4.6 and 4.4% from the clean and dusty carpet, respectively (Table 1.1).

The amount of cypermethrin transferred with friction (0.174 ± 0.015 μg/cm^2; n = 20) was significantly greater than without (0.048 ± 0.004 μg/cm^2; n = 20) (W = 399, df = 1, P < 0.001) (Figure 1.5). With the addition of friction, 1.4% of cypermethrin was transferred, while 0.4% was transferred through static contact alone (Table 1.1).

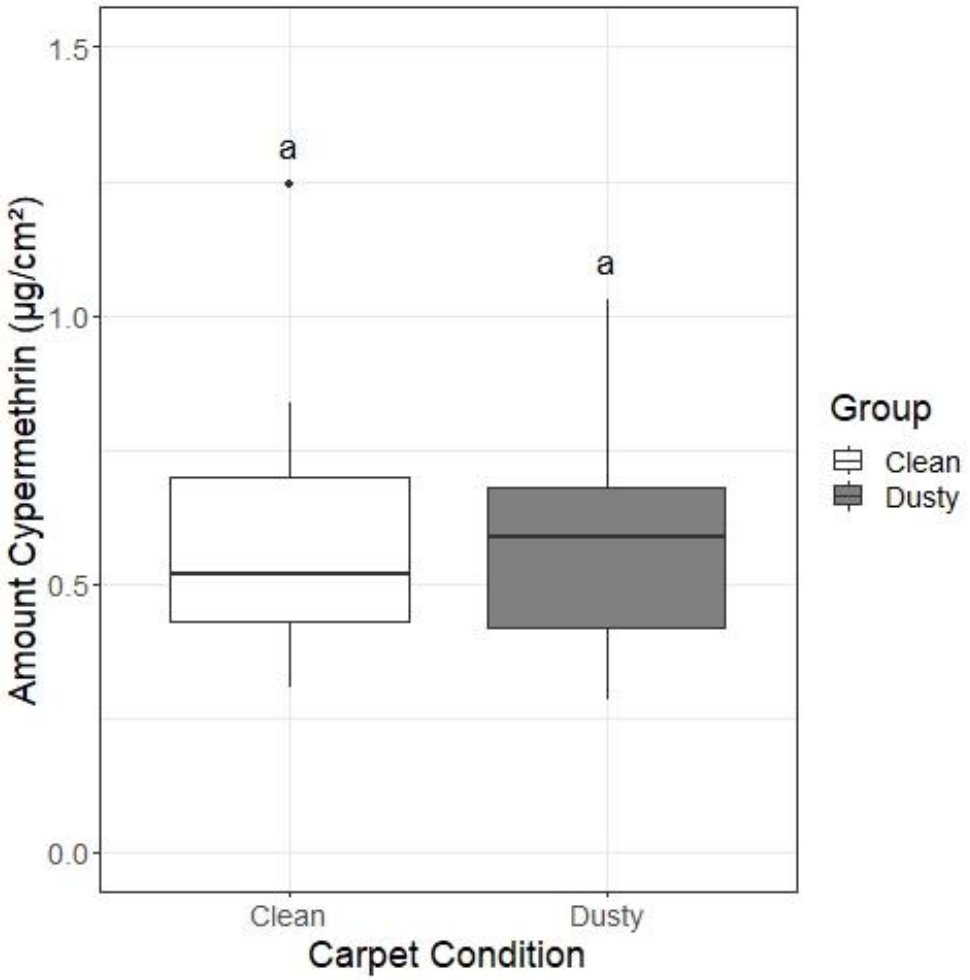

Figure 1.4 Amount of cypermethrin (µg/cm^2) transferred from clean or dusty carpet squares (n = 20) to filter paper. Letters indicate significant differences (Wilcoxon rank-sum test, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

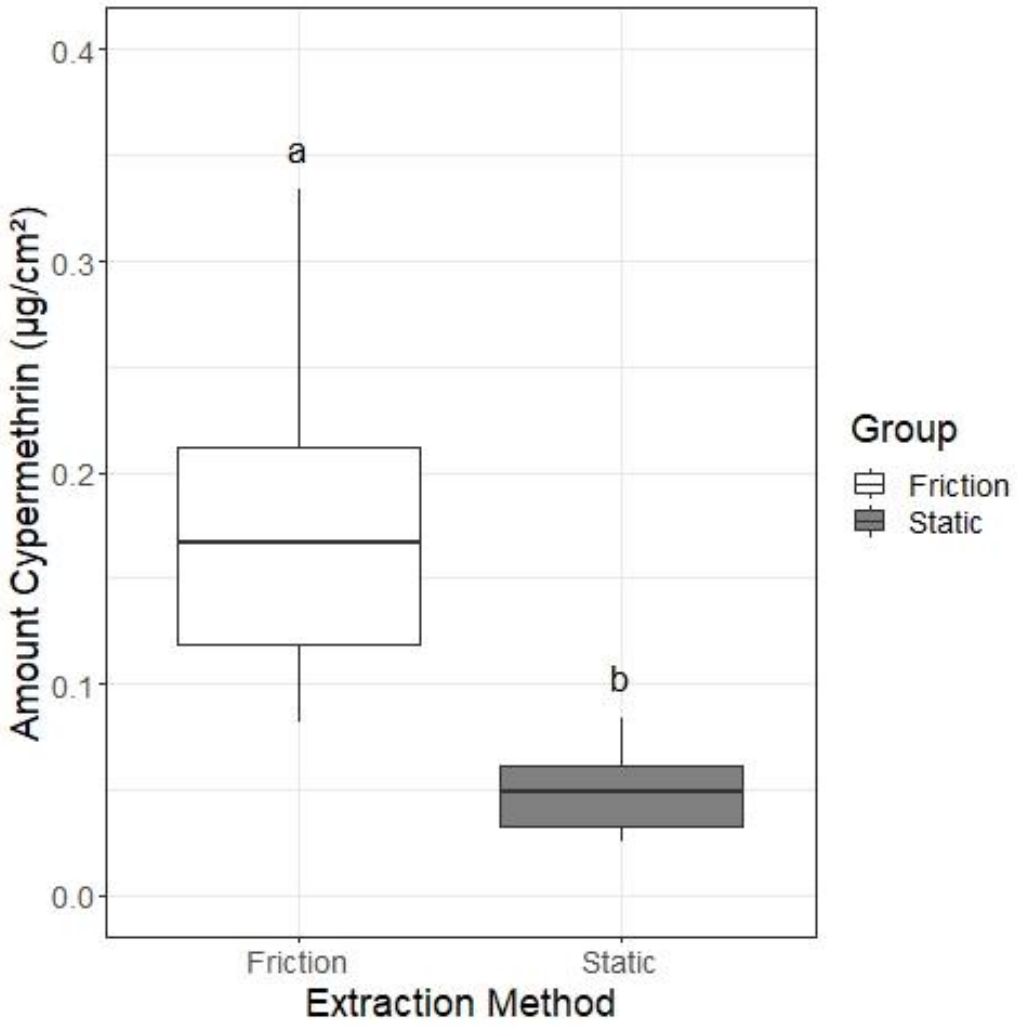

Figure 1.5 Amount of cypermethrin (µg/cm^2) transferred to filter paper from carpet squares using either static weight or weight with friction (n = 20). Letters indicate significant differences (Wilcoxon rank-sum test, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

Wash off from filter paper and fabric. The solvent type had a significant effect on the amount of cypermethrin recovered from filter paper (H = 53.6, df = 2, $P < 0.001$). The amounts of cypermethrin removed from filter paper were significantly different between hexane (16.02 ± 0.94 µg/cm^2; n = 24), water (0.20 ± 0.015 µg/cm^2; n = 24), and water + detergent (11.52 ± 0.498 µg/cm^2; Mean ± SE; n = 24) as extraction solvents (Dunn's

Multiple Comparisons; $P < 0.05$) (Figure 1.6A). Using the data from hexane extraction to estimate the total amount of cypermethrin deposited, 1.2% was removed using water alone, while 71.9% was removed if the water contained a detergent (Table 1.1).

The solvent type likewise had a significant effect when tested on cotton fabric ($H = 54.9$, df = 2, $P < 0.001$). The amounts of cypermethrin removed by hexane (13.13 ± 0.872 μg/cm^2; $n = 24$), water (0.33 ± 0.057 μg/cm^2; Mean ± SE; $n = 24$), and water + detergent (8.32 ± 0.499 μg/cm^2; $n = 24$) were all significantly different (Dunn's Multiple Comparisons; $P < 0.05$) (Figure 1.6B). The transfer rates of cypermethrin into water were 2.5 and 63.4% for water only and water with a detergent, respectively (Table 1.1).

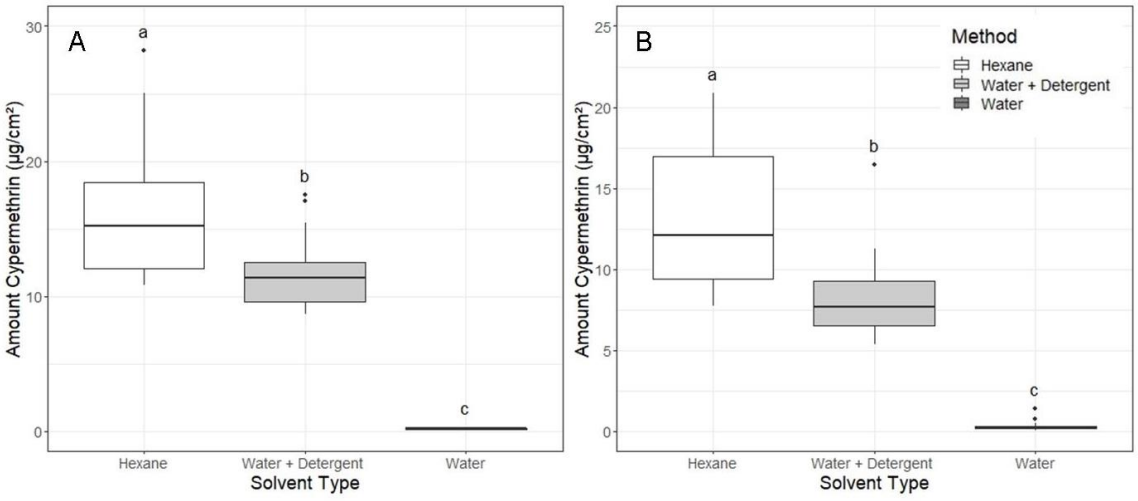

Figure 1.6 Amount of cypermethrin (μg/cm^2) removed from filter paper (a) ($n = 24$) or cotton fabric (b) ($n = 24$) when extracted with hexane, water, or water with detergent following fogger activation. Letters indicate significant differences (Dunn's Multiple Comparisons, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

Table 1.1: Summary of the average amount of cypermethrin transferred from various materials and the percent of recovery based on estimated total deposition.

Material		Extraction method	Estimated total cypermethrin deposited (Mean ± SE; µg/cm^2)	Amount of cypermethrin transferred (Mean ± SE; µg/cm^2)	Transfer rate (%)
From	To				
Tile	Filter paper	Physical contact (rubbing)	6.29 ± 0.57	3.06 ± 0.32	48.6
Vinyl				0.03 ± 0.005	0.5
Wood				0.26 ± 0.04	4.1
Carpet (dusty)		Physical contact (static with weight)	12.79 ± 0.523	0.567 ± 0.038	4.4
Carpet (clean)				0.587 ± 0.049	4.6
		Physical contact (static)		0.048 ± 0.004	0.4
		Physical contact (friction)		0.174 ± 0.015	1.4
Filter paper	Water	Solvent extraction (water)	16.02 ± 0.94	0.20 ± 0.015	1.2
		Solvent extraction (water and detergent)		11.52 ± 0.498	71.9
Cotton fabric		Solvent extraction (water)	13.13 ± 0.872	0.33 ± 0.057	2.5
		Solvent extraction (water and detergent)		8.32 ± 0.499	63.4

Extraction from spiked filter paper. In one sample (water extraction of technical cypermethrin), no cypermethrin was detected and was included as a zero for the analysis. When the filter paper was spiked with 25 µg cypermethrin from the fogger crude material, the solvent type had a significant effect on the amount of cypermethrin extracted (H = 25.8, df = 2, P < 0.001). The amounts of cypermethrin recovered were significantly different between hexane (25.207 ± 0.244 µg/cm^2; n = 10), water (0.015 ± 0.002 µg/cm^2; n = 10), and water + detergent (7.741 ± 0.625 µg/cm^2; n = 10) extractions (Dunn's Multiple Comparisons; P < 0.05) (Figure 1.7A).

Similarly, the solvent type had a significant effect on the amount of cypermethrin recovered from filter paper spiked with 25 µg of technical cypermethrin (H = 25.8, df = 2, P < 0.001). The amounts of cypermethrin recovered were significantly different between hexane (26.038 ± 0.868 µg/cm^2; n = 10), water (0.006 ± 0.001 µg/cm^2; n = 10), and water + detergent (7.169 ± 0.452 µg/cm^2; n = 10) extractions (Dunn's Multiple Comparisons; P < 0.05) (Figure 1.7B).

When comparing between cypermethrin sources (i.e., fogger crude material vs. technical compound) within a solvent type, there was no significant difference in the amount recovered for hexane (T = -0.328, df = 18, P = 0.747), or water + detergent (T = 0.705, df = 18, P = 0.49). However, significantly more cypermethrin was removed from the filter paper treated with crude fogger material (0.015 ± 0.005 µg/cm^2; n = 10) than the filter paper treated with technical cypermethrin (0.006 ± 0.002 µg/cm^2; n = 10) when extracted with water alone (T = 3.651, df = 18, P = 0.002).

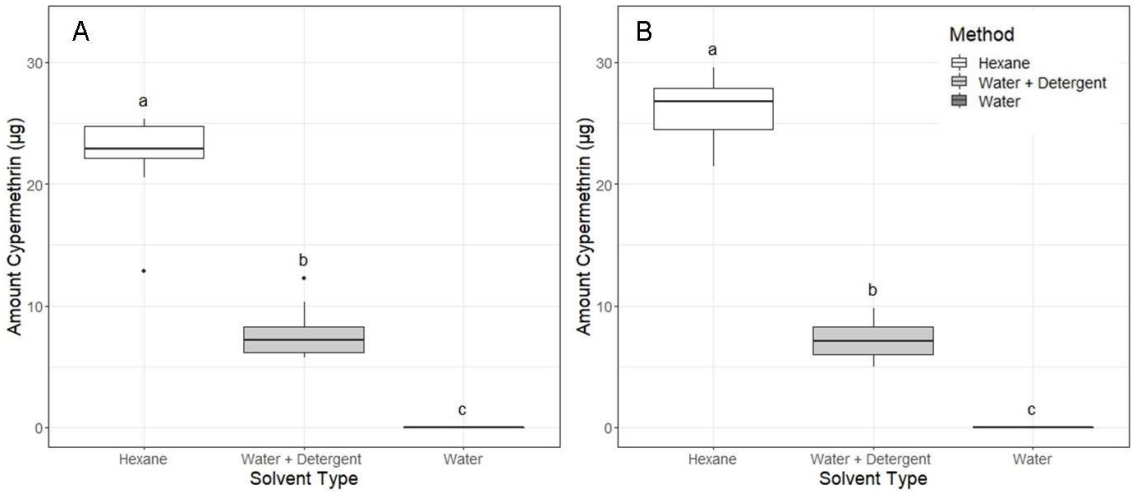

Figure 1.7 Amount of cypermethrin (µg) removed from filter paper (1 cm^2) spiked with 25 µg cypermethrin from crude fogger solution (a) or technical material (b) when extracted with hexane (n = 10), water (n = 10), or water with detergent (n = 10). Letters indicate significant differences (Dunn's Multiple Comparisons, $P < 0.05$). The box represents the interquartile range, with the line representing the median. Whiskers cover values within 1.5 times the interquartile range, with values beyond this graphed individually.

DISCUSSION

The use of TRFs is known to leave a residue on all surfaces and items that are exposed during treatment (Keenan et al., 2009). A product survey of retail stores in Northern California found that cypermethrin was the most common active ingredient in the indoor pest control products that are available to the general public (Budd & Peters, 2018). Using one of the many available TRF products containing cypermethrin as an AI as an example, we found that the active ingredient (cypermethrin) deposited onto such surfaces can be transferred to other adsorptive materials via physical contact. Furthermore, the current findings suggest that cypermethrin can be subsequently extracted into the water in substantial amounts when those adsorptive materials are

washed using a detergent. Our results provide empirical evidence to support one of the possible routes by which common usage of TRFs by the general public can contribute to insecticide loading into wastewater treatment systems.

The deposition pattern of cypermethrin on the floor after a total release fogger use was not homogeneous throughout the test chamber. There was a distinct directionality to product deposition that remained consistent during testing. Interestingly, Selim & Krieger (2007) activated their foggers while set on a rotating surface to achieve even distribution and prevent the semi-directional deposition that we observed in our floor deposition experiments. The exact reason for this directionality observed in the current study is unknown. However, it may occur as a result of the fogger nozzle being slightly bent away from the vertical axis when the release valve is depressed upon fogger activation. During all of our experiments, the release valve was oriented in the same direction (the bottom right of Figure 1.1), which matches the observed directionality in the floor deposition results. The average amount of cypermethrin per unit area was highest directly adjacent to the fogger release point (max: 31.05 $\mu g/cm^2$) and decreased as distance from the fogger increased (min: 2.36 $\mu g/cm^2$). Overall, the average amount of cypermethrin deposited on the floor was 6.76 $\mu g/cm^2$. Our results follow the pattern reported by Selim & Krieger (2007), who found that increased distance from the fogger resulted in a decreased deposition of pyrethrins. The average amount of floor deposition is similar to those reported by Selim & Krieger (2007), who found average amounts of insecticides on carpet ranging from 3.66 – 10.95 $\mu g/cm^2$ after the TRF use. In contrast to our findings that increased distance from the fogger resulted in reduced insecticide deposition, Keenan

et al. (2010) found consistent deposition of cypermethrin following fogger treatment. For example, in a 3.1 m × 3.1 m test room, they found similar concentrations (average of 4.1 ± 1.2 μg/cm^2) of cypermethrin at various distances from a centrally placed TRF. These differences may be a result of the different methods for measuring surface deposition. For example, the current study sampled floor deposition with greater resolution due to more sample locations compared to Selim & Krieger (2007). The use of different TRFs might be an additional variable as different products likely disperse their products differently.

Keenan et al. (2009) measured the transfer rate of cypermethrin from carpeting, tile, wood, carpet, and linoleum after TRF uses. They found the transfer rate from carpeting was 5% of total surface residue. Ross et al. (1991) found that 1 – 3% of chlorpyrifos and allethrin released from TRFs could be transferred from carpeting to cotton cloth after it was rolled over the carpet surface. These results are comparable to our findings, where 4.6 and 4.4% of cypermethrin were transferred from clean or dusty carpets to filter paper, respectively. Additionally, Keenan et al. (2009) reported a 30 and 10% transfer rate of cypermethrin for tile and wood flooring, respectively. In the current study, we found that 48.6% of cypermethrin was transferred from tile to filter paper, but only 4% of cypermethrin was transferred for wood flooring. Despite the use of different extraction methods and various surface materials, these results suggest that the transferability of cypermethrin is largely dependent on the characteristics of the surface material and its interaction with the droplets of TRF formulation.

Insecticides are commonly deposited indoors following total release foggers, perimeter sprays, spot sprays, and crack-and-crevice applications (Keenan et al., 2010)

and are commonly detected indoors (Julien et al., 2008; Stout et al., 2009). DeVries et al. (2019a measured the deposition of several AIs from TRFs on various horizontal locations in residential kitchens and found an average increase of 603 times for insecticide residues 4 – 6 h after TRF discharge relative to the pre-activation baseline. However, the majority (66%) of samples taken one month later were not significantly increased relative to baseline levels, while 50% showed moderate increases. As these homes remained occupied following TRF use, this may be at least in part the result of transfer from and cleaning of these horizontal surfaces, which included the floor, counters, and the top of cabinets. This return to baseline concentrations may be a result of human activities such as cleaning or through normal contact made by residents or as a result of degradation of the insecticides over time.

Cypermethrin deposited on exposed surfaces was readily transferred to adsorptive materials with or without water as a solvent. Ross et al. (1990) found that chlorpyrifos and allethrin from a TRF could be transferred to clothing during a standardized Jazzercise routine. This study highlights the potential for such insecticide transfer by foggers, as every surface or item in the range is exposed, and insecticide application cannot be directed, such as when using methods such as baiting. The overuse of TRFs by consumers disregarding the label rate will result in higher levels of deposition on items/surfaces and likely contributes to an increase of down-the-drain transport of these insecticides. Our results found that 27 – 72% and 30 – 63% of cypermethrin were removed when filter paper and the cotton fabric were extracted with water containing a small amount of detergent, respectively. These findings suggest that the laundering of

clothing/cleaning of surfaces contaminated with pyrethroids likely contribute to down-the-drain disposal of pyrethroids.

The application of insecticides indoors appears to be a likely source of insecticides entering wastewater treatment plants, with various insecticides being detected in wastewater treatment influent and effluent (Weston & Lydy, 2010; Weston et al., 2013; Markle et al., 2014; Teerlink, 2014). Weston et al. (2013) measured the quantity of several common pyrethroids from wastewater samples using interceptors from residential areas and concluded that these were unlikely from stormwater runoff. Weston et al. (2013) concluded that the pyrethroid contamination they found likely occurred as a result of drain disposal from indoor pesticide application due to the separation of the wastewater/stormwater systems and the lack of similarity of the detected insecticides to those previously observed from urban runoff. They further provided an example of how only 2% of the cypermethrin in a TRF entering the drain in 1 out of 700 houses would account for the levels of cypermethrin detected in wastewater samples from residential areas (Weston et al., 2013). Our findings support the possibility that TRFs may be a significant source of the insecticides entering the water treatment system. For a hypothetical estimate based on the present results, consider the effects of mopping a tile floor (1 m^2) contaminated by a cypermethrin-based TFR with an average deposition of 6.76 $\mu g/cm^2$. Assuming the same transferability as found for tile to wet filter paper and the extraction of cotton fabric with water plus detergent, 20.8 mg of cypermethrin would be transferred into the wastewater system, amounting to 5% of the original cypermethrin contents of the TRF. While this calculation makes several assumptions, it demonstrates

that a significant amount of cypermethrin may be deposited into the drain during routine cleaning and laundering after TRF application.

Some caution is warranted when interpreting our results, as we tested only one of the many TRF products available and focused only on cypermethrin transfer. The differing chemistries of other pyrethroids may impact the transferability to the wastewater stream. Additionally, experiments were conducted in semi-field conditions only. While our results strongly suggest that drain disposal of TRF products likely occurs to some extent, we did not directly quantify the down-the-drain movement of insecticides under field conditions. However, this study provides evidence that the movement of insecticides originating from TRFs contributes to the mass loading of insecticides entering water treatment plants, which contributes to entry into surface waters.

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Chapter 2. Ontogenesis of Aldehyde Pheromones in Two Synanthropic Bed Bug Species (Heteroptera: Cimicidae)[1]

ABSTRACT

Bed bugs produce volatile aldehydes that have alarm and aggregation functions. Using two synanthropic bed bug species, *Cimex lectularius* L. and *C. hemipterus* (Fabricius), quantity changes were examined for (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal, the four most abundant aldehydes shared between the two species during different life stages. Quantitative analyses of the aldehydes in the nymphal exuviae indicated that the aldehyde ratio remained similar throughout nymphal development. In general, (*E*)-2-octenal was most abundant, and (*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were least abundant. The fourth aldehyde, 4-oxo-(*E*)-2-hexenal, was present in intermediate quantities. The quantities and percent abundances of the aldehydes in the nymphal exuviae and the adults were significantly different between *C. lectularius* and *C. hemipterus*. The ratio between (*E*)-2-hexenal and (*E*)-2-octenal was determined in adult male and female bed bugs of each species. Adult *C. hemipterus* had a higher proportion of (*E*)-2-hexenal than *C. lectularius*, while no sex differences were found. This work provides the first systematic quantification of four

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aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal and 4-oxo-(*E*)-2-octenal] for all five of the nymphal stages for both *C. lectularius* and *C. hemipterus*.

INTRODUCTION

Bed bugs (Hemiptera: Cimicidae) are ectoparasites that are obligate blood-feeders (Usinger, 1966). The common bed bug, *Cimex lectularius* L., and the tropical bed bug, *Cimex hemipterus* (Fabricius), are considered urban pests, predominantly utilizing humans as hosts. *Cimex lectularius* has a worldwide distribution, while *C. hemipterus* inhabits tropical and subtropical regions (Usinger, 1966). *Cimex lectularius* and *C. hemipterus* diverged from each other ≈ 47 MYA and are descended from the bat and bird-associated lineages, respectively (Roth et al., 2019). *Cimex hemipterus* have been recently reported in Florida for the first time since the bed bug resurgence (Campbell et al. 2016) and for the first time in Hawaii (Lewis et al., 2020). These reports suggest the possibility of *C. hemipterus* further expanding outside of their historical range. While the ranges of *C. lectularius* and *C. hemipterus* overlap (Doggett and Cains, 2018; Lee et al., 2018) and the two species can mate with each other, viable hybrids are not typically produced (Usinger, 1966; Newberry, 1988).

The chemical ecology of bed bugs, particularly *C. lectularius*, has been the subject of many investigations due to its potential use for monitoring and control (Weeks et al., 2011a). *Cimex lectularius* is known to respond to conspecific cuticular hydrocarbons, exuviae, used harborage paper, feces, and their associated volatiles (Siljander et al., 2008; Domingue et al., 2010; Weeks et al., 2011b; Choe et al., 2016). A distinctive odor associated with *C. lectularius* is caused by several aldehydes that they

produce (Schildknecht et al., 1964). *Cimex lectularius* nymphs produce the aldehydes in their dorsal abdominal glands while the adults produce the aldehydes in their metathoracic scent glands (Künckel, 1886; Usinger, 1966; Staddon, 1979; Feldlaufer et al., 2010). Two of these aldehydes, (*E*)-2-hexenal and (*E*)-2-octenal, are released in relatively large quantities when bed bugs are attacked by predators (e.g., bats or ants) or conspecifics (e.g., mating attempts by adult males), serving an alarm/defensive purpose (Usinger, 1966; Levinson et al., 1974; Harraca et al., 2010; Kilpinen et al., 2012). (*E*)-2-hexenal and (*E*)-2-octenal are two of the compounds that comprise the *C. lectularius* aggregation pheromone (Gries et al., 2015). When these aldehydes are present at lower concentrations relative to the amount released during an alarm response, they appear to function as an aggregation pheromone, encouraging bed bugs to form large groups in their harborage sites (Siljander et al., 2008; Gries et al., 2015; Choe et al., 2016; Ulrich et al., 2016).

In addition to (*E*)-2-hexenal and (*E*)-2-octenal, two ketoaldehydes [4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal] have been found in the dorsal abdominal glands of nymphs (Feldlaufer et al., 2010). These ketoaldehydes are not known to occur in adult bed bugs. The ketoaldehydes made up at least 16% of the total aldehydes detected in the dorsal abdominal gland contents of fourth and fifth instars of *C. lectularius* (Feldlaufer et al., 2010). The dorsal abdominal gland reservoirs are shed during molting, and the aldehydes slowly volatilize from the gland reservoirs in the exuviae (Choe et al., 2016). As developing bed bugs would continue to produce a large amount of exuviae, the

accumulated exuviae in the harborage site may contribute to the formation of aggregations (Choe et al., 2016).

Compared to *C. lectularius*, less is known about the chemical ecology of *C. hemipterus*. A headspace collection study with *C. hemipterus* reported the presence of the four aldehydes mentioned above; as in *C. lectularius*, (*E*)-2-hexenal, and (*E*)-2-octenal were emitted by both adults and late instar nymphs. In contrast, the ketoaldehydes were emitted only by nymphs (Liedtke et al., 2011). Liedtke et al. (2011) also found that whole-body extract of late instar *C. hemipterus* nymphs was repellent to conspecific adults and nymphs. In addition, among 33 compounds identified from the excreta of *C. hemipterus*, (*E*)-2-hexenoic acid, hexanal, and (*E*)-2-hexenal elicited aggregation responses in both adults and nymphs (Mendki et al., 2014).

As most of the existing literature on bed bug aldehydes focused on late instar nymphs or adults, information on the aldehyde production in earlier nymphal stages and their quantitative change across development are currently lacking for these two species. To further our knowledge on the production of the bed bug aldehydes, the current study investigates these aldehydes' ontogenesis in two synanthropic bed bugs, *C. lectularius* and *C. hemipterus*. For both species, nymphal exuviae were used to quantify the aldehydes in the first, second, third, fourth, and fifth instar nymphs. Additionally, (*E*)-2-hexenal and (*E*)-2-octenal were quantified for both species' female and male adults.

MATERIALS AND METHODS

Insects. Two species of bed bugs were used throughout the experiments. *Cimex lectularius* originated from colonies started from "Earl" strain individuals obtained from Sierra Research Laboratories (Modesto, CA, USA). The Earl strain was originally collected in Modesto, CA, in 2007. *Cimex hemipterus* were collected between 2005 and 2006 from multiple locations in Kuala Lumpur, Malaysia, imported into the US (CDC PHS Permit No. 03282018-11057, and 20190426-2698A), and reared in the quarantine facility of the University of California, Riverside. Bed bugs were kept in screened vials (9 cm in height, 4.5 cm in diameter) with a corrugated filter paper cylinder as a substrate. Bed bugs were fed using a custom glass feeder (Prism Research Glass, Inc., Raleigh, NC, USA), which allowed defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) to be heated to 37°C using a circulating water bath. Bed bugs were fed through a grafting tape membrane (Aglis & Co., Ltd., Yame City, Fukuoka, Japan) screen approximately every fourteen days. Colonies of *C. lectularius* were maintained at 24–26°C and 15–30% RH, with a photoperiod of 12:12 (L:D) hours. Colonies of *C. hemipterus* were maintained at 22–23°C and 40–60% RH, with a photoperiod of 12:12 (L:D) hours.

Nymphs (Solvent Extraction of Exuviae). Aldehydes were quantified in *C. lectularius* and *C. hemipterus* nymphs by extracting exuviae containing the reservoirs of shed dorsal abdominal glands. Exuviae were used as a substitute for whole nymphs due to the simplicity of extraction and sample clean-up compared with whole insects. As nymphs require a blood meal to molt (Usinger, 1966), the timing of molting was controlled by

feeding. For *C. lectularius*, unfed mixed instars were placed into a new colony vial and fed to repletion. After two days, the fed nymphs were placed individually into the wells (16 mm × 19 mm) of 24-well cell culture dishes (Corning Inc., Corning, NY, USA) lined with filter paper. The nymphal instar of *C. lectularius* was identified using a key to immature stages (Usinger, 1966). Since the nymphal instar key was not available for *C. hemipterus*, nymphs of *C. hemipterus* were raised from first instars to maturity, and the exuviae of each stage were collected. Exuviae were handled by the legs with fine-tipped metal forceps to ensure the shed dorsal abdominal gland reservoirs were not ruptured. Carbon dioxide was not used to anesthetize bed bugs to prevent the release of aldehydes. The bed bugs were observed at least twice daily for the presence of freshly shed exuviae, which were then collected and extracted within 24 hours.

The aldehydes in the exuviae were extracted using the following method adapted from Choe et al. (2016). First, exuviae were crushed in a glass tissue grinder containing 0.5 ml dichloromethane (DCM). Sulcatone (6-methyl-5-hepten-2-one; Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard (200 ng/ml). Due to small quantities of the compounds, pooled samples of five or two exuviae were used per sample for first and second instars, respectively. For other instars, a single exuvia was used per sample. Particulates were removed by filtering the extract through a glass pipette containing a glass wool plug. The filtered extract was collected into a 2-ml vial (Agilent Technologies, Santa Clara, CA, USA) and analyzed on the day of extraction. For both species, a total of 10 samples were obtained for each instar. Using an automatic liquid sampler (ALS) device, 1 µL of the extract was injected onto an Agilent 7890 gas chromatograph

equipped with a DB-5 column (30 m × 0.25 mm inner diameter) and a flame ionization detector (GC-FID). Helium was used as the carrier gas. Samples were injected in splitless mode, with a temperature program of 50°C for 1 min and then 10°C min^{-1} to 280°C with a 10-min hold. The compounds were identified based on a comparison of retention times with authentic standards. Standards of (*E*)-2-hexenal and (*E*)-2-octenal were purchased from Sigma-Aldrich, while 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized using the method described by Moreira and Millar (2005).

Adults. For adult bed bugs, a surface extraction method was used to determine the percent abundance of (*E*)-2-hexenal and (*E*)-2-octenal. The surface extraction method and the use of relative quantity data were advantageous due to (1) the potential loss of the aldehydes during the sample clean-up process for crushed whole body extracts, and (2) the large amount of individual variation in the amount of aldehydes released/collected. Adult bed bugs were collected from a colony vial and placed individually into the wells of 24-well cell culture dishes lined with filter paper. Carbon dioxide was not used during the handling of bed bugs. To reduce aldehyde contamination by other colony members, the adults were kept isolated from the colony for 24 hours before extraction. A single adult bed bug was placed into a 2-ml glass vial and anesthetized with carbon dioxide, causing the aldehydes to be released from the metathoracic scent glands onto the evaporating areas of the bed bug (Usinger, 1966). These released aldehydes were immediately collected by adding 0.5 ml DCM (containing 200 ng/ml sulcatone) into the vial containing the adult bed bug. After gentle swirling for approximately 5 seconds, 40 µl of the extract was removed and transferred into a new vial containing a glass insert

(250 µl, Agilent Technologies, Santa Clara, CA, USA). The samples were analyzed using a GC-FID, as previously described for exuviae. The percent abundance of each compound was calculated based on the quantity of (*E*)-2-hexenal and (*E*)-2-octenal recovered. For each species, a total of 20 adults (10 male: 10 female) were sampled.

Quantification. The quantification of the aldehydes was effected with a calibration curve for each aldehyde (0.059, 0.118, 0.235, 0.469, 0.938, 1.875, 3.75, 7.5, and 15 µg/ml) versus sulcatone (200 ng/ml) as an internal standard, analyzed by GC-FID as described for exuviae. The ketoaldehydes' calibration curves did not include the 0.059 µg/ml standard due to limited detection. For first and second instar exuviae, the resulting quantitative data were divided by the number of exuviae used for each sample (5 and 2 for first and second instar, respectively) to obtain the quantity data per exuvia. The percent abundance of each aldehyde was calculated using the total quantity of the four aldehydes.

Statistical Analysis. Due to heteroscedasticity and non-normality in the quantitative data, nonparametric tests were used for the statistical analysis of aldehydes quantities. Comparisons across different instars within each species were carried out with Kruskal–Wallis H test followed by Dunn's Multiple Comparisons with correction (Dunn, 1964; Benjamini and Hochberg, 1995) using the R package Fisheries Stock Analysis (Ogle et al., 2019). For each instar, Wilcoxon rank-sum tests were used to compare the amounts of each aldehyde between species. Wilcoxon rank-sum tests were used to determine if species or sex had a significant effect on adult aldehyde percent abundance. For

compounds that were not detected in a sample, a zero was used as quantity for the analyses.

A principal components analysis (PCA) was used to visualize differences in the overall aldehyde profile between *C. lectularius* and *C. hemipterus* nymphs. The percent abundance of each aldehyde was calculated based on the total amount of four aldehydes in the sample. PCA was conducted with the relative quantity data for all instars combined using the R package FactoMineR (Lê et al., 2008). All statistical analyses were done using R version 3.6.1 (R Core Team, 2019).

RESULTS

Nymphs (Solvent Extraction of Exuviae). All four aldehydes were consistently detected in the exuviae of all five nymphal instars of *C. lectularius*. In *C. hemipterus*, 4-oxo-(*E*)-2-octenal was not detected in two replications (20%) for the first instar, seven replications (70%) of the second instar, five replications (50%) for the third instar, and one replication (10%) for each of fourth and fifth instars. The other aldehydes were consistently detected in all samples of *C. hemipterus*. For both species, the total amount of aldehydes in the exuviae was generally lowest for the first instar and increased in each successive instar.

Both quantity and percent abundance data from the nymphal exuviae are shown in Table 2.1. In *C. lectularius*, relative amounts of the four aldehydes remained similar across different instars, with (*E*)-2-octenal being most abundant (48-58%), and (*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal least abundant (5-18%). The fourth aldehyde, 4-oxo-(*E*)-2-hexenal, was found in the amounts intermediate of the other three (21-33%). The

percent abundance of (*E*)-2-hexenal was lowest in the first instar (5.6%) but consistently increased in successive instars, eventually reaching 17.5% in the fifth instar. Conversely, the percent abundance of 4-oxo-(*E*)-2-octenal was largest in the first instar (15.8%) but consistently decreased in successive instars, declining to 5.3% in the fifth instar.

In *C. hemipterus*, a similar pattern was found. Relative amounts of the four aldehydes remained similar across different instars of *C. hemipterus*, with (*E*)-2-octenal being most abundant (40-65%), and (*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal least abundant (0.3-24%). 4-oxo-(*E*)-2-hexenal was found in the amounts intermediate of the other three (30-38%) (Table 2.1). As with *C. lectularius*, the percent abundance of (*E*)-2-hexenal consistently increased across the nymphal development of *C. hemipterus* (4% in the first instar to 21% in the fifth instar). However, unlike *C. lectularius*, the amount of 4-oxo-(*E*)-2-octenal remained relatively similar across five instars, comprising 1.2% in the first instar, 0.3% in second, third, and fourth instars, and 0.7% in the fifth instar. Also, unlike *C. lectularius*, the relative abundance of (*E*)-2-octenal decreased over nymphal development of *C. hemipterus* (65% in the first instar to 42% in the fifth instar).

When the quantities of an aldehyde were compared between *C. lectularius* and *C. hemipterus* within the same instar, the majority of comparisons showed significant differences (Table 2.2). For both first and second instars, all four aldehydes were found in greater quantities in *C. lectularius* compared to *C. hemipterus*. In all instars, *C. lectularius* had a significantly greater amount of 4-oxo-(*E*)-2-octenal than *C. hemipterus*. In all instars except the third, quantities of the two ketoaldehydes were significantly different between *C. lectularius* and *C. hemipterus*.

Table 2.1. Mean quantity (µg/exuvia; n = 10) and percent abundance (mean ± SE) of four aldehydes detected in freshly shed exuviae of first through fifth instar *Cimex lectularius* and *Cimex hemipterus* nymphs. Different letters across rows indicate significance differences in each compound across the five instars ($P < 0.05$; Dunn's Multiple Comparisons) (SE = Standard error).

Cimex lectularius										
Compound	**Instar**									
	First		**Second**		**Third**		**Fourth**		**Fifth**	
	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance
(*E*)-2-hexenal	0.018 ± 0.002 a	5.6 ± 0.3	0.074 ± 0.007 ab	7.6 ± 0.8	0.158 ± 0.012 bc	8.7 ± 0.8	0.315 ± 0.049 c	11.6 ± 1.2	1.326 ± 0.292 c	17.5 ± 1.8
4-oxo-(*E*)-2-hexenal	0.083 ± 0.005 a	26.9 ± 0.4	0.219 ± 0.020 a	21.5 ± 1.3	0.628 ± 0.040 b	33.2 ± 1.3	0.690 ± 0.101 b	24.7 ± 1.7	1.472 ± 0.293 b	21.1 ± 0.9
(*E*)-2-octenal	0.159 ± 0.010 a	51.7 ± 0.4	0.586 ± 0.043 ab	57.6 ± 0.9	0.951 ± 0.103 bc	48.2 ± 1.4	1.496 ± 0.154 cd	55.9 ± 1.6	3.624 ± 0.646 d	56.2 ± 1.7
4-oxo-(*E*)-2-octenal	0.048 ± 0.003 a	15.8 ± 0.5	0.137 ± 0.013 b	13.3 ± 0.6	0.198 ± 0.026 bc	9.9 ± 0.6	0.217 ± 0.031 bc	7.8 ± 0.5	0.311 ± 0.053 c	5.3 ± 0.6
Cimex hemipterus										
Compound	**Instar**									
	First		**Second**		**Third**		**Fourth**		**Fifth**	
	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance	Mean ± SE	% abundance
(*E*)-2-hexenal	0.006 ± 0.002 a	4.0 ± 0.6	0.022 ± 0.007 ab	7.7 ± 1.2	0.255 ± 0.054 bc	15.7 ± 1.2	0.854 ± 0.148 cd	24.3 ± 1.4	1.460 ± 0.174 d	20.6 ± 1.4
4-oxo-(*E*)-2-hexenal	0.038 ± 0.008 a	30.0 ± 1.4	0.079 ± 0.019 a	32.6 ± 2.7	0.565 ± 0.083 b	37.9 ± 1.1	1.178 ± 0.133 bc	35.6 ± 1.1	2.639 ± 0.269 c	37.1 ± 1.1
(*E*)-2-octenal	0.075 ± 0.013 a	64.8 ± 1.8	0.119 ± 0.022 a	59.4 ± 3.4	0.658 ± 0.075 b	46.2 ± 1.6	1.297 ± 0.135 bc	39.8 ± 1.0	2.922 ± 0.285 c	41.6 ± 0.9
4-oxo-(*E*)-2-octenal	0.001 ± 0.0003 ab	1.2 ± 0.3	0.001 ± 0.0005 b	0.3 ± 0.2	0.004 ± 0.002 ab	0.3 ± 0.1	0.010 ± 0.002 ac	0.3 ± 0.1	0.049 ± 0.009 c	0.7 ± 0.1

Table 2.2. Quantity (µg/exuvia; $n = 10$) comparisons between nymphal *Cimex lectularius* and *Cimex hemipterus* for each aldehyde compound within an instar. Significant differences (Wilcoxon rank-sum test) between species are indicated in the *C. hemipterus* column (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Instar	Compound	*Cimex lectularius* (Mean ± SE; µg/exuvia)	*Cimex hemipterus* (Mean ± SE; µg/exuvia)
First	(*E*)-2-hexenal	0.018 ± 0.002	0.006 ± 0.002***
	4-oxo-(*E*)-2-hexenal	0.083 ± 0.005	0.038 ± 0.008**
	(*E*)-2-octenal	0.159 ± 0.010	0.075 ± 0.013***
	4-oxo-(*E*)-2-octenal	0.048 ± 0.003	0.001 ± 0.0003***
Second	(*E*)-2-hexenal	0.074 ± 0.007	0.022 ± 0.007**
	4-oxo-(*E*)-2-hexenal	0.219 ± 0.020	0.079 ± 0.019***
	(*E*)-2-octenal	0.586 ± 0.043	0.119 ± 0.022***
	4-oxo-(*E*)-2-octenal	0.137 ± 0.013	0.001 ± 0.0005***
Third	(*E*)-2-hexenal	0.158 ± 0.012	0.255 ± 0.054
	4-oxo-(*E*)-2-hexenal	0.628 ± 0.040	0.565 ± 0.083
	(*E*)-2-octenal	0.951 ± 0.103	0.658 ± 0.075
	4-oxo-(*E*)-2-octenal	0.198 ± 0.026	0.004 ± 0.002***
Fourth	(*E*)-2-hexenal	0.315 ± 0.049	0.854 ± 0.148**
	4-oxo-(*E*)-2-hexenal	0.690 ± 0.101	1.178 ± 0.133*
	(*E*)-2-octenal	1.496 ± 0.154	1.297 ± 0.135
	4-oxo-(*E*)-2-octenal	0.217 ± 0.031	0.010 ± 0.002***
Fifth	(*E*)-2-hexenal	1.326 ± 0.292	1.460 ± 0.174
	4-oxo-(*E*)-2-hexenal	1.472 ± 0.293	2.639 ± 0.269*
	(*E*)-2-octenal	3.624 ± 0.646	2.922 ± 0.285
	4-oxo-(*E*)-2-octenal	0.311 ± 0.053	0.049 ± 0.009***

Principal components analyses (PCA) on the four aldehydes' percent abundances showed clear visual separation between the two species of bed bugs. When the data from all instars were pooled together for PCA, the first two principal components (eigenvalues: PC1 = 2.39; PC2 = 0.84) accounted for 80.9% of the total variation (Figure 2.1).

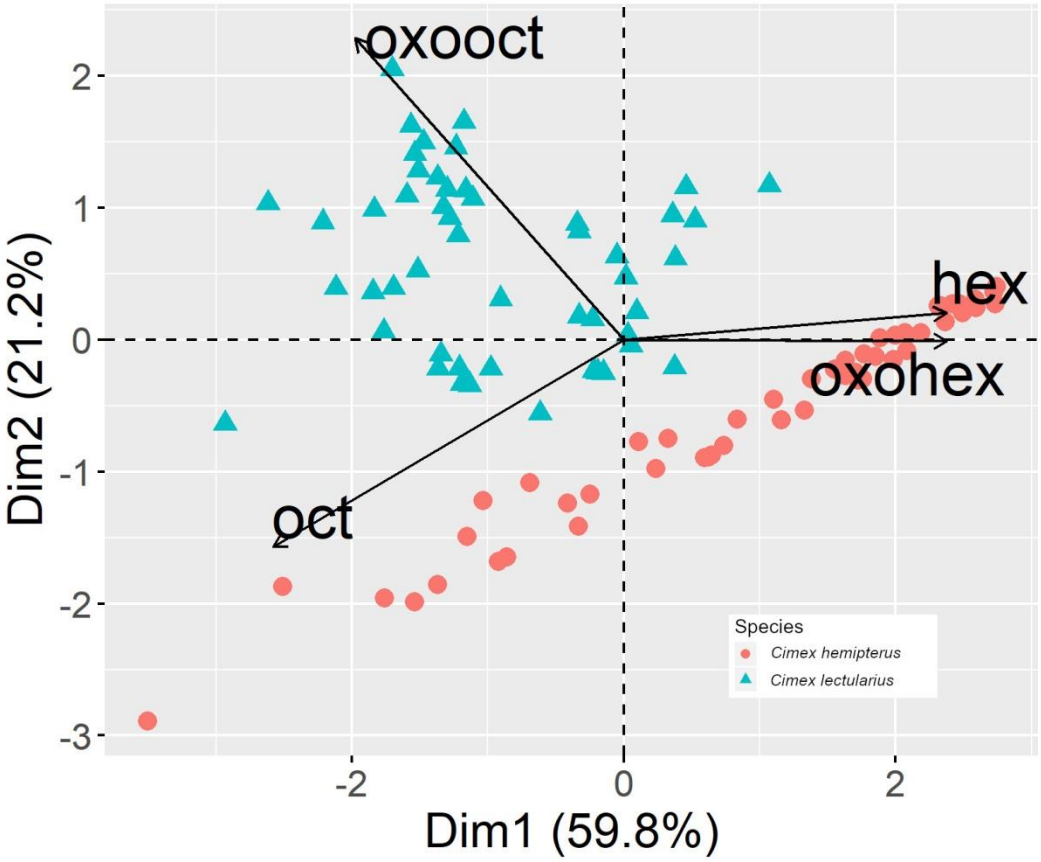

Figure 2.1. Biplot showing the first two principal components of aldehyde percent abundance in *Cimex lectularius* and *Cimex hemipterus* for all instars combined. Arrows show the impact and correlation of variables. Arrows show the contribution of each variable to the first two dimensions. Samples close to the arrows have a higher value for this variable. Variables that create acute angles are positively correlated, while those opposite each other tend to be negatively correlated. [Dim = dimension; hex = (*E*)-2-hexenal; oxohex = 4-oxo-(*E*)-2-hexenal; oct = (*E*)-2-octenal; oxooct = 4-oxo-(*E*)-2-octenal].

Adults. 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were not detected in any of the surface extracts obtained from the adult bed bugs after brief anesthetization with carbon dioxide. Therefore, (*E*)-2-octenal and (*E*)-2-hexenal were the only two aldehydes used for calculating percent abundance. Other compounds present were not identified. In two samples (one male of each species), (*E*)-2-octenal was not detected, and thus (*E*)-2-hexenal had 100% percent abundance in those samples. The percent abundance of (*E*)-2-hexenal is reported, with (*E*)-2-octenal comprising the remaining portion.

There was a significant difference between species ($W = 107.5$, $P = 0.0128$), with adult *C. hemipterus* (57.7 ± 4.0; Mean ± SE; $n = 20$) having a greater percent abundance of (*E*)-2-hexenal than *C. lectularius* (42.1 ± 5.7; Mean ± SE; $n = 20$). There was not a significant difference ($W = 39$, $P = 0.427$) in the percent abundance of (*E*)-2-hexenal between male (52.1 ± 9.7; Mean ± SE; $n = 10$) and female (32.1 ± 4.2; Mean ± SE; $n = 10$) *C. lectularius*. Similarly, there was not a significant difference ($W = 68$, $P = 0.186$) in the percent abundance of (*E*)-2-hexenal between male (55.7 ± 6.8; Mean ± SE; $n = 10$) and female (59.7 ± 4.0; Mean ± SE; $n = 10$) *C. hemipterus*.

DISCUSSION

In all nymphal stages of both species, (*E*)-2-octenal was found in the highest amounts. Among the four aldehydes analyzed, 4-oxo-(*E*)-2-octenal was consistently different between *C. lectularius* and *C. hemipterus*, consistent with findings by Liedtke et al. (2011). We found that the amount of the four aldehydes in the two earliest instars were all significantly different between the two species. Except for third instars, the amounts

of the two ketoaldehydes were always significantly different between species, consistent with results reported by Liedtke et al. (2011). Though Liedtke et al. (2011) found that the amounts of (*E*)-2-hexenal and (*E*)-2-octenal emitted by nymphs did not differ between species, we found significant differences between species in first, second and fourth instars, but not in the third or fifth instars. As Liedtke et al. (2011) collected aldehydes from the headspace of only late-stage instars, this likely explains why they did not see such differences.

The total quantity of aldehydes increased from first to fifth instars of *C. lectularius*, and *C. hemipterus* by approximately 22 and 59 times, respectively. The average total quantities of the four aldehydes found in *C. lectularius* fifth instar exuviae (6.73 μg) were higher than those reported by Choe et al. (2016) (2.11 μg). Though similar methods were used to quantify aldehydes, the discrepancy may be explained by different time intervals between the shedding of exuviae and the chemical extraction. For example, the current study extracted freshly shed exuviae, while the exuviae used by Choe et al. (2016) were aged for at least seven days prior to extraction. If exuviae lose a large fraction of their aldehyde contents shortly after molting, the freshly shed exuviae might serve as significant source of the aldehyde pheromone with significant behavioral implications, such as mediating the formation of aggregations at harborage sites. Choe et al. (2016) estimated that these aldehydes in the fifth instar exuviae (*C. lectularius*) reduced by approximately 1.4-2.3% per day.

Due to the quantification of only one strain for each species, some caution is warranted when interpreting our findings. However, based on other studies investigating

the aldehydes in other strains of these two species, similar aldehyde ratios are apparent. For example, comparing the ratio of the most and least prevalent of the four aldehydes [(*E*)-2-octenal and 4-oxo-(*E*)-2-octenal], we find similarities. Our findings of a ratio of 11.6:1 for fifth instar *C. lectularius* are similar to the 12.6:1 ratio previously reported by Feldlaufer et al. (2010). Choe et al. (2016) and Liedtke et al. (2011) reported ratios of 6.6:1 and 6.2:1, respectively. These discrepancies may be a result of the different methodologies used or differences between bed bug strains. However, these ratios all follow the same pattern of (*E*)-2-octenal being several times the amount of 4-oxo-(*E*)-2-octenal. Our study reports a 60:1 ratio between (*E*)-2-octenal and 4-oxo-(*E*)-2-octenal in *C. hemipterus*, which follows a similar pattern with the 101:1 ratio previously reported by Liedtke et al. (2011).

Based on the percent abundance of 4-oxo-(*E*)-2-octenal in exuviae, the two bed bug species can be readily identified. The differences in both quantity and ratio of these four aldehydes in the exuviae of *C. lectularius* and *C. hemipterus* may provide a method of distinguishing these species based on chemical differences. This approach may be useful when insect specimens are not present or when morphological information is missing due to damaged specimens. The chemical identification approach based on aldehydes can be supplemented by additional information based on cuticular hydrocarbons (CHCs) if the CHC profile of *C. hemipterus* is substantially different from that of *C. lectularius* as reported by Feldlaufer and Blomquist (2011).

(*E*)-2-octenal and (*E*)-2-hexenal were the primary aldehydes in adult bed bug extracts for both species. The ketoaldehydes 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-

octenal were not detected in the surface extracts of adult bed bugs. This finding corroborates previous work reporting that only nymphs produce these ketoaldehydes in the dorsal abdominal glands for both species (Feldlaufer et al., 2010; Liedtke et al., 2011). As adult bed bugs lack the dorsal abdominal glands (Usinger, 1966; Staddon, 1979), this explains the absence of these ketoaldehydes in adult bed bugs. Based on the relative abundance data of (*E*)-2-octenal and (*E*)-2-hexenal, we found significant differences between adults of *C. lectularius* and *C. hemipterus*. *Cimex hemipterus* had a greater proportion of (*E*)-2-hexenal than *C. lectularius* and the proportion of (*E*)-2-octenal was greater in *C. lectularius* compared to *C. hemipterus*. However, Liedtke et al. (2011) did not find significant differences between *C. lectularius* and *C. hemipterus* in the ratio of (*E*)-2-hexenal and (*E*)-2-octenal emitted by the adults based on the sampling of the headspace volatiles. Liedtke et al. (2011) found that the ratio of (*E*)-2-hexenal: (*E*)-2-octenal in *C. hemipterus adult females to be* 59:41 and 45:54 in adult males. In *C. lectularius* adults, Liedtke et al. (2011) found the same ratio to be 52:48 for females and 68:32 for males. We found this ratio for *C. hemipterus* adults to be 60:40 in females and 56:44 in males, while in *C. lectularius* the ratio was 32:68 in females and 52:48 in males. In comparing our findings, we see that this ratio in *C. hemipterus* adult females is nearly identical, while the others differ significantly. This discrepancy may be due to differences in bed bug strains or the different sampling methods used (surface extraction vs. headspace collection).

This work provides the first systematic quantification of four aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal and 4-oxo-(*E*)-2-octenal] for each of the five

nymphal stages for both *C. lectularius* and *C. hemipterus*. Additionally, the percent abundance of (*E*)-2-hexenal and (*E*)-2-octenal was determined for adults of each species. Our findings showed that even though both species produced all of the aldehydes investigated in the current study, aldehyde blends of *C. lectularius* and *C. hemipterus* were quantitatively distinctive. The behavioral study of these synanthropic bed bug species towards these aldehyde mixtures (heterospecific or conspecific blends) might help us further understand the potential significance of the distinctive blend ratio of the intraspecific and/or interspecific communication.

CONCLUSIONS

The amounts of four aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal] differ significantly between *C. lectularius* and *C. hemipterus* in the earliest two instars. The amounts of the two ketoaldehydes vary among the two species, with the exception of third instars. Adult *C. hemipterus* have a higher percent abundance of (*E*)-2-hexenal than *C. lectularius*. We found no differences in aldehyde ratio in adults based on sex for both *C. lectularius* and *C. hemipterus*.

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Chapter 3. Differential Responses to Aldehyde Pheromone Blends in Two Bed Bug Species (Heteroptera: Cimicidae)[2]

ABSTRACT

The behavioral responses of two bed bug species, *Cimex lectularius* L. and *C. hemipterus* (F.), to conspecific or heterospecific nymphal aldehyde blends were examined using a two-choice olfactometer. Volatile cues from exuviae or a synthetic blend containing (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal were tested. In both species, the adults settled preferentially on the olfactometer treatment side when conspecific volatile aldehyde cues were provided. When tested with heterospecific volatile aldehyde cues, only adult *C. lectularius* preferentially responded to *C. hemipterus* volatile cues. Adult *C. hemipterus* were indifferent to the aldehyde blend of *C. lectularius.* Potential implications of the results on bed bug biology and practical pest management are discussed.

INTRODUCTION

Bed bugs (Heteroptera: Cimicidae) are a group of hematophagous external parasites (Usinger, 1966). The common bed bug, *Cimex lectularius* L., and the tropical bed bug *Cimex hemipterus* (F.), are common urban pests. Recent phylogenetic analyses

[2] This chapter is a reprint of material originally published in the journal Chemoecology. Dery M, Lee CY, and Choe DH (2021) Differential responses to aldehyde pheromone blends in two bed bug species (Heteroptera: Cimicidae). Chemoecology, 31:397–403. https://doi.org/10.1007/s00049-021-00359-z

indicated that *C. lectularius* and *C. hemipterus* diverged approximately 47 MYA, descending from bat and bird-associated lineages, respectively (Roth et al., 2019). Their distributions are mostly allopatric; *C. lectularius* is found mainly in cooler environments, and *C. hemipterus* in tropical and subtropical regions (Usinger, 1966). However, these bed bug species can occur in sympatry. For example, both species are found in Australia (Doggett and Cains, 2018), Asia (Lee et al., 2018), Africa (Fourie and Crafford, 2018), and more recently in Florida (Campbell et al., 2016) and Hawaii (Lewis et al., 2020).

Due to bed bugs' status as major urban pests, much research has been done searching for an effective lure for use in a monitor or trap (Weeks et al., 2011a). Investigations on the chemical ecology of bed bugs have found that *C. lectularius* respond to several conspecific chemical cues, such as those associated with cuticles (e.g., cuticular hydrocarbons), exuviae, and feces (Siljander et al., 2008; Domingue et al., 2010; Weeks et al., 2011b; Choe et al., 2016). In particular, like other Heteropterans, bed bugs are known to produce several short-chain aliphatic aldehydes, which are among the major constituents of the typical odors associated with bed bugs (Staddon, 1979). A group of related aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal] are produced by both *C. lectularius* and *C. hemipterus* (Liedtke et al., 2011; Dery et al., 2020). Adult bed bugs produce two of these [(*E*)-2-hexenal and (*E*)-2-octenal] in their metathoracic scent glands, while nymphs have all four in their dorsal abdominal glands (Künckel, 1886; Usinger, 1966; Staddon, 1979; Feldlaufer et al., 2010). In *C. lectularius*, (*E*)-2-hexenal and (*E*)-2-octenal are released in relatively large quantities in response to a predator (Usinger, 1966; Levinson et al., 1974) or unwanted mating

attempts (Harraca et al., 2010b; Kilpinen et al., 2012), causing an alarm response. When released in low quantities, the same aldehydes appeared to function as constituents of an aggregation pheromone (Siljander et al., 2008; Gries et al., 2015; Choe et al., 2016; Ulrich et al., 2016).

Studies on these aldehydes found that while all four aldehydes are produced by *C. lectularius* and *C. hemipterus*, there are significant interspecific differences in the proportions (i.e., the ratio among compounds) produced (Liedtke et al., 2011; Dery et al., 2020). Qualitative similarity but with a distinct quantitative difference of these pheromonal aldehydes brings about an interesting biological question. Despite not producing viable hybrids (Usinger, 1966; Newberry, 1988), "interspecific" mating (in the laboratory as well as in the field) between *C. lectularius* and *C. hemipterus* appears to be common in some of the areas where these species are sympatric (Walpole and Newberry, 1988; Newberry, 1989). Since the mating would occur where the bed bugs congregate (i.e., harborages) (Gershman et al., 2019), *C. lectularius* and *C. hemipterus* might respond to their aldehyde pheromone blends interspecifically as well as intraspecifically. While bed bug aldehydes and their behavioral functions have been previously studied within each species of *C. lectularius* and *C. hemipterus*, their behavioral responses towards heterospecific aldehyde blends have not been investigated. This information may also have important implications for developing novel bed bug chemical lures for commercial traps or monitors. Here, a two-choice olfactometer study was conducted to investigate each bed bug species' behavioral responses to both the conspecific and heterospecific

aldehydes. Two different sources of aldehydes were tested, shed exuviae and a synthetic aldehyde blend.

MATERIALS AND METHODS

Insects. *Cimex lectularius* colonies were started from "Earl" strain individuals collected in Modesto, CA, USA in 2007 and purchased from Sierra Research Laboratories (Modesto, CA, USA). *Cimex hemipterus* were collected from George Town, Penang, Malaysia in 2015 and were reared in the quarantine facility at the University of California, Riverside (CDC PHS Permit No. 03282018-11057 and 20190426-2698A).

Bed bugs were kept in screened vials with a height of 9 cm and a diameter of 4.5 cm containing a corrugated filter paper cylinder. Bed bugs are fed through the screen with a grafting tape membrane (Aglis & Co., Ltd., Yame City, Fukuoka, Japan) feeder containing defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) approximately every fourteen days. Colonies of *C. lectularius* were maintained at 24-26 °C and 15–30% RH, with a photoperiod of 12:12 (L:D) hours. Colonies of *C. hemipterus* were maintained at 22-23 °C and 40–60% RH, with a photoperiod of 12:12 (L:D) hours.

Behavioral Assay. The behavioral responses of adult bed bugs to conspecific or heterospecific aldehydes were examined using two-choice still air olfactometer assays. The experimental design was modified from Choe et al. (2016), using acetone as a solvent to reconstitute the synthetic aldehyde blend. Mixed-sex adult bed bugs were randomly collected from a colony vial and placed individually in the wells (16 mm × 19

mm) of 24-well cell culture dishes (Corning Inc., Corning, NY, USA) lined with filter paper. These bed bugs were kept in the well plate for at least 24 hours before use, at the experimental temperature and humidity. A 15-cm section of transparent flexible polyvinyl chloride tubing (ID: 9.5 mm; OD: 12.7 mm; Superflex Ltd., Elizabeth, New Jersey, USA) was used as the body of the olfactometer. Approximately 50 exuviae (third to fifth instar) were collected from colony vials randomly and placed into a 2 ml glass vial (Agilent Technologies, Santa Clara, CA, USA). Ten vials of exuviae were prepared for each species, and these served as the source of volatiles. One vial with exuviae was inserted into the olfactometer tube with a small mesh screen to separate the vial contents from the inside of the tube. An empty screened vial was inserted on the opposite end of the tube, serving as the control. The position of the vials was randomized. One adult bed bug was introduced into the olfactometer via a slit in the center of the tube. The trials were initiated three hours before the start of scotophase, and the position (control or treatment screen) of the bed bug was observed after 18 hours. All olfactometer assays were conducted at approximately 22-23°C and 20% RH. The mesh screens and olfactometer were used only once for each replicate. For each of the four combinations of bed bug species and exuviae source, 50 replicates were conducted.

To determine if the behavioral response of bed bugs is primarily due to the volatile aldehydes from the exuviae, the olfactometer experiment was repeated using blends of synthetic aldehydes in place of the exuviae. (*E*)-2-hexenal and (*E*)-2-octenal were purchased from Sigma-Aldrich (St. Louis, MO, USA), while 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized using the method described by Moreira and

Millar (2005). A blend of the four aldehydes was prepared in acetone, and 20 µl of this acetone preparation was applied to a small piece of cotton (32.3 ± 7.8 mg; mean ± SD; *n* = 70) inside each treatment vial. The synthetic aldehyde blend of each species mimicked the ratio of the four aldehydes quantified in freshly shed fifth instar exuviae by Dery et al. (2020). The amount of each aldehyde applied for *C. lectularius* consisted of (*E*)-2-hexenal: 66.3 µg, 4-oxo-(*E*)-2-hexenal: 73.6 µg, (*E*)-2-octenal: 181.2 µg, and 4-oxo-(*E*)-2-octenal: 15.55 µg. The *C. hemipterus* synthetic blend was comprised of (*E*)-2-hexenal: 73 µg, 4-oxo-(*E*)-2-hexenal: 131.95 µg, (*E*)-2-octenal: 146.1 µg, and 4-oxo-(*E*)-2-octenal: 2.45 µg. The amount of aldehydes applied per vial was equivalent to that of 50 freshly shed fifth instar exuviae (*C. lectularius*: 336.7 µg, *C. hemipterus*: 353.5 µg). As the amount of aldehydes in shed exuviae volatilize over time (Choe et al., 2016), the total amount of synthetic aldehyde mimicking freshly shed exuviae represents an overall larger aldehydes than were present in the exuviae olfactometer trials. The control vial contained a piece of cotton, to which 20 µl of acetone was applied. The vials remained uncapped for 10 minutes to allow the solvent to evaporate. The remainder of the experimental procedures was otherwise identical with the olfactometer trials with exuviae. Between 50 and 72 replicates were conducted for each combination of bed bugs and aldehyde blend. A total of 464 olfactometer replicates were conducted using either exuviae or synthetic aldehydes. If a bed bug was found nonresponsive (i.e., remaining in the center of the olfactometer or dead) at 18 hours after introduction (*n* = 18), the trial was repeated with a different insect and new olfactometer.

Statistical Analysis. To determine if bed bugs settled on one side of the olfactometer (e.g., the side with a treatment vial) significantly more often than expected from random choice (50% : 50%), the results of the olfactometer experiments were analyzed with chi-square goodness of fit tests using R version 4.0.3 (R Core Team, 2020).

RESULTS

In both bed bugs species, adult insects settled preferentially on the treatment side of the olfactometer when conspecific volatile cues were provided. In *C. lectularius*, adults responded preferentially to both the volatiles of their own exuviae ($\chi^2 = 13.52$, $P < 0.001$, $n = 50$) or the synthetic aldehyde blend mimicking the conspecific ratio ($\chi^2 = 15.68$, $P < 0.001$, $n = 50$) (Figure 3.1). Similarly, adult *C. hemipterus* responded preferentially to the volatiles of their own exuviae ($\chi^2 = 9.68$, $P < 0.005$, $n = 50$) as well as the synthetic aldehyde blend mimicking the conspecific ratio ($\chi^2 = 11.36$, $P < 0.001$, $n = 55$) (Figure 3.1).

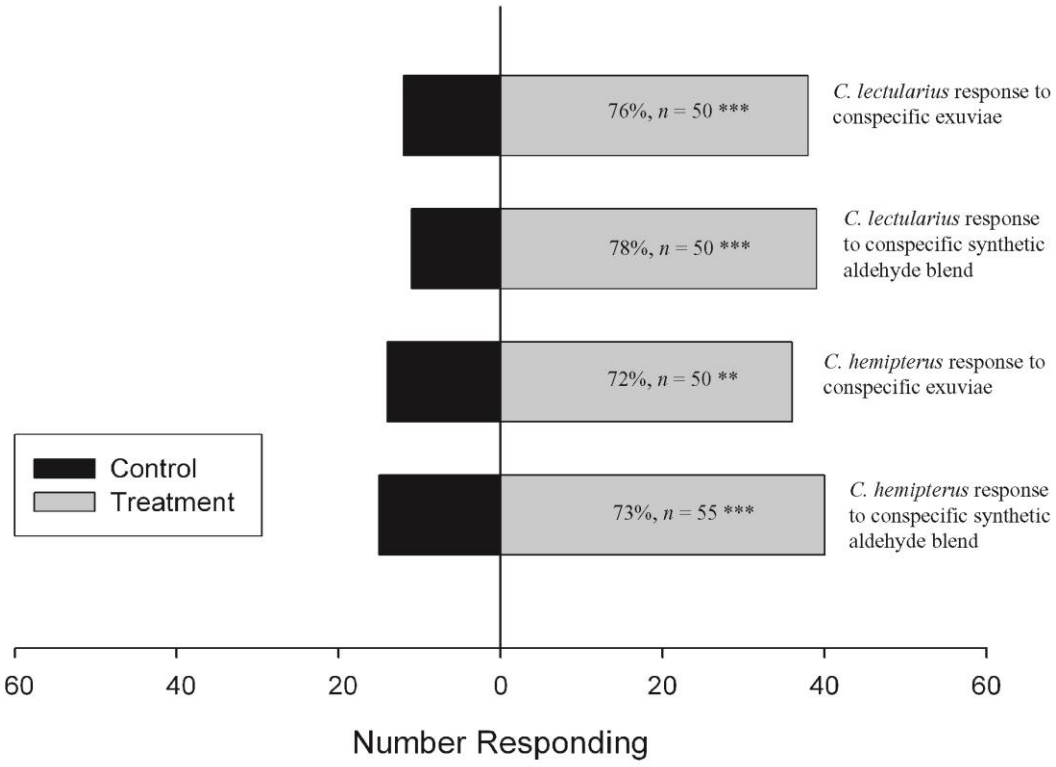

Figure 3.1. Behavioral responses of adult *Cimex lectularius* and *Cimex hemipterus* to conspecific aldehyde sources (exuviae or synthetic blend) in olfactometers. Numbers in bars indicate the percent of bed bugs responding to the volatile cue and sample size (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$; chi-square goodness of fit test).

In stark contrast, the two species of bed bugs responded differently when tested with heterospecific volatile cues. Adult *C. lectularius* still responded preferentially to the volatiles from *C. hemipterus* exuviae ($\chi^2 = 15.68$, $P < 0.001$, $n = 50$) and the synthetic aldehyde blend mimicking the *C. hemipterus* ratio ($\chi^2 = 9.39$, $P < 0.005$, $n = 72$) (Figure 3.2). However, adult *C. hemipterus* preferred neither the volatiles from *C. lectularius* exuviae ($\chi^2 = 0.32$, $P > 0.05$, $n = 50$) nor the synthetic aldehyde blend mimicking the heterospecific ratio ($\chi^2 = 0.01$, $P > 0.05$, $n = 69$) (Figure 3.2).

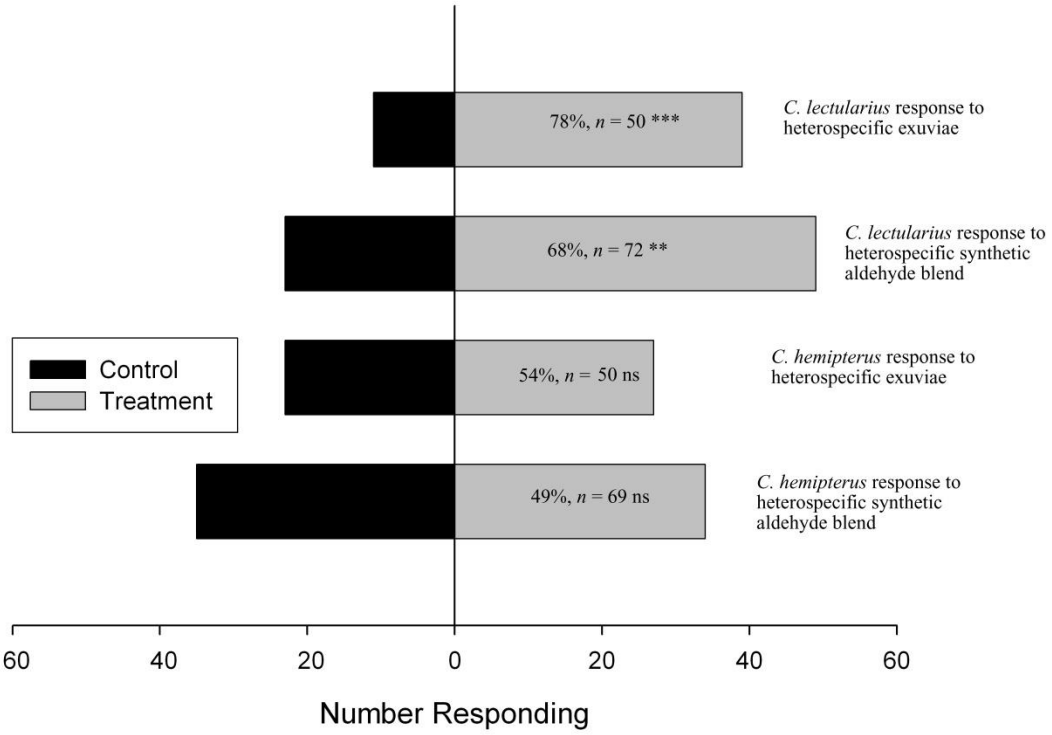

Figure 3.2. Behavioral responses of adult *Cimex lectularius* and *Cimex hemipterus* to heterospecific aldehyde sources (exuviae or synthetic aldehyde blend) in olfactometers. Numbers in bars indicate the percent of bed bugs responding to the volatile cue and sample size (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$; chi-square goodness of fit test).

DISCUSSION

Data from the two-choice olfactometer trials indicated that adult bed bugs of both species preferentially responded not only to volatiles from conspecific late instar exuviae but also a reconstructed synthetic aldehyde blend. These findings are consistent with the observations made by Choe et al. (2016), further supporting the notion that the volatile aldehydes in the exuviae are at least in part responsible for the behavioral response of adult bed bugs towards their exuviae (i.e., attraction and/or arrestment). In contrast,

behavioral responses to heterospecific aldehyde blends were completely different between *C. lectularius* and *C. hemipterus*. While adult *C. lectularius* preferentially responded to *C. hemipterus* volatile cues, *C. hemipterus* were indifferent to the aldehyde blend of *C. lectularius* (i.e., no difference when compared with a blank control). The mechanism underlying the observed differential response to the pheromone blends is unknown. Two types of sensilla on adult bed bug antennae are known to detect these four aldehydes (Harraca et al., 2010a, b). While Liedtke et al. (2011) did not find *C. lectularius* and *C. hemipterus* to differ in the number of antennal sensilla, Singh et al. (1996) reported *C. hemipterus* to have more olfactory and mechanoreceptive sensilla in its antennae than those in *C. lectularius*. Further exploration of how both *C. lectularius* and *C. hemipterus* detect and process the signals from these aldehydes may give more insight into the observed behavior.

Regardless of the mechanism behind this differential response, the current findings that *C. lectularius* and *C. hemipterus* respond differently to the heterospecific aldehyde blend might provide valuable insights for previously observed interspecific interactions of the two *Cimex* species in their habitats. Walpole and Newberry (1988) and Newberry (1989) reported the presence of "interspecific" mating (in the laboratory as well as in the field) between *C. lectularius* and *C. hemipterus*. Interspecific mating between female *C. lectularius* and male *C. hemipterus* has been the focus of several previous investigations. This particular pairing has some serious adverse effects on female *C. lectularius* (e.g., short lifespan, sterility) (Newberry, 1989). Female *C. lectularius* also showed a massive immune response to the heterospecific sperm

(Walpole, 1988). However, female *C. hemipterus* do not seem to suffer that much adverse effect when mated with male *C. lectularius* and they do not show a massive immune response to the heterospecific sperm (Walpole, 1988). Omori (1939) and Newberry (1989) hypothesized that their interspecific mating and its adverse effect on female *C. lectularius* might be playing an important role in determining the abundance and distribution of *C. lectularius* in areas where both species can otherwise coexist.

In many cases, this hypothesis supports the idea that the resident *C. hemipterus* population prevents *C. lectularius* from establishing in new areas. Indeed, Newberry et al. (1987) reported that some houses in South Africa were initially infested with *C. lectularius* and *C. hemipterus*, but a few months later, only with *C. hemipterus*. Similarly, Prisniy (2020) reported that *C. lectularius* were present in dormitories of Belgorod National Research University in Belgorod, Russia in 2010 but was apparently displaced by *C. hemipterus* in 2020. In contrast, Gbakima et al. (2002) reported that both *C. lectularius* and *C. hemipterus* were found in similar abundances (56.1 and 43.9%, respectively) in internally displaced person camps in Freetown, Sierra Leone, in 1999. However, as the research took place in locations where new residents arrived daily from many geographic locations (Gbakima et al., 2002), there is a high likelihood of new introductions of both species to these camps frequently occurring. This aspect, along with the absence of a follow-up study, might explain the apparent discrepancy between Gbakima et al. (2002) and the other two reports mentioned earlier (Newberry et al., 1987; Prisniy, 2020), which found that populations shifted to *C. hemipterus* alone over time.

The aforementioned hypothetical mechanism explaining the observed population shift to *C. hemipterus* will only make sense if *C. lectularius* is always the numerical minority when these species are found in the same habitat. In addition to a possible scenario in which a few *C. lectularius* are introduced in a *C. hemipterus*-dominated habitat, the behavioral observations in the current study might provide some useful insights. In the present study, *C. lectularius* were attracted to their own cues as well as heterospecific cues. However, *C. hemipterus* were only attracted to their own cues, and indifferent to heterospecific cues. In a hypothetical space where small aggregations of these species coexist, these differential behavioral responses would generate the following outcomes. When *C. lectularius* return to aggregations after feeding, they would be attracted to either conspecific aggregations or heterospecific aggregations. However, *C. hemipterus* would only join conspecific aggregations when they come back from feeding bouts. This would almost always make *C. lectularius* numerically "minor" members in the "mixture" aggregations even when relatively large populations of *C. lectularius* are introduced in the common habitat.

Some caution is warranted when interpreting these results. Due to the use of only adults from one strain for each species, the current study could not completely rule out a strain-specific or stage-specific effect for the lack of response from *C. hemipterus* to the *C. lectularius* aldehyde blend. Also, we did not directly test the choice response of bed bugs between conspecific and heterospecific aldehyde blends in one experiment. Instead, we observed the bed bugs' response to the aldehydes against a control (blank or solvent only). Additional work is required to determine the exact differences in the aldehyde

profiles responsible for the observed differential response. Dery et al. (2020) reported that only the ketoaldehydes differed significantly between *C. lectularius* and *C. hemipterus* based on a quantification study with fifth instar exuviae. As such, differences in the quantity or ratio of one or both ketoaldehydes seem a likely cause for the observed lack of response by *C. hemipterus*. For example, a particularly striking difference is that the amount of 4-oxo-(*E*)-2-octenal in the exuviae is much greater in *C. lectularius* than in *C. hemipterus* for all instars (Dery et al., 2020).

Much work has been done investigating the potential use of various attractants in bed bug monitors and traps (Weeks et al., 2011a). Heat and CO_2 have already been successfully incorporated into traps for *C. lectularius* (Anderson et al., 2009; Wang et al., 2009; Weeks et al., 2011a). The potential use of bed bug aldehyde pheromones for bed bug detection and management has been of additional interest. For example, adult *C. lectularius* were attracted to a 1:1 blend of (*E*)-2-hexenal and (*E*)-2-octenal (Ulrich et al., 2016). Benoit et al. (2009) also found that the addition of (*E*)-2-hexenal and (*E*)-2-octenal to desiccants increased their effectiveness against *C. lectularius*.

Behavioral differences have been reported between *C. lectularius* and *C. hemipterus*, and some of them have direct implications for bed bug management. For example, Kim et al. (2017) found that *C. hemipterus* adults are better climbers than *C. lectularius* and can escape from commonly used pitfall-type monitors. The differential responses of these two synanthropic bed bug species to heterospecific aldehydes might add another layer of complexity in incorporating these aldehydes into pest management tools that will be equally valid for both species. Our findings suggest that *Cimex*

hemipterus may not respond to an active monitor that used the *C. lectularius* aldehyde blend as an attractant despite being congeners. Instead, the aldehyde composition of *C. hemipterus* should be investigated for use as a general lure that can be incorporated into the design of active traps and monitors, potentially targeting both *C. lectularius* and *C. hemipterus*. With *C. hemipterus* increasingly being found outside their historical range, for example, in the USA (Campbell et al., 2016; Lewis et al., 2020), Italy (Masini et al., 2020), Russia (Gapon, 2016; Prisniy, 2020), Sweden (Naylor et al., 2018), and Japan (Komatsu et al., 2018), further research on the differences between these two species (e.g., behavioral differences) is required to ensure effective control of bed bugs in these areas.

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Chapter 4. Chemical Ecology of Bed Bugs and Its Implications for the Use of Fungal Biopesticides

ABSTRACT

The use of the entomopathogenic fungus *Beauveria bassiana* is a recent addition to bed bug management. Bed bugs produce a set of aldehydes that are known to affect the growth of some fungi. Mortality was monitored following the exposure of bed bugs to commercial products containing *B. bassiana* when in the presence of an aldehyde source. Bed bug the mortality caused by *B. bassiana* when not formulated for bed bug control was significantly reduced by the presence of aldehydes (62.2% or 61.1%), compared with the no aldehydes (97.7%). However, the addition of synthetic aldehydes only delayed mortality when bed bugs were exposed to a formulation designed for bed bugs. Bed bugs exposed to synthetic aldehydes and fungus reaching 94% mortality at day 15, compared with 100% mortality by day 6 without synthetic aldehydes. When grown in culture, the growth of the fungus with both a low and high amount of aldehydes were significantly reduced at day 7 compared with the control, while only the higher aldehyde group was significantly reduced at day 15.

INTRODUCTION

Bed bugs (Hemiptera: Cimicidae) are among the most difficult to control of the urban pests. A significant challenge for bed bug control is widespread insecticide resistance among bed bug field populations (Romero, 2018). Despite the widespread

distribution of pyrethroid resistance among bed bug populations, pyrethroids remain among the most commonly used active ingredients in many commercial pesticide products against bed bugs (Romero et al., 2007; Lee et al., 2018). In an attempt to address pyrethroid resistance, products containing pyrethroids are increasingly being paired with neonicotinoid insecticides (Lee et al., 2018). However, resistance to neonicotinoids has also been reported in field populations of bed bugs (Gordon et al., 2014; Romero & Anderson, 2016).

Use of entomopathogenic fungi as biopesticides has been considered as one possible solution for the insecticide resistance of bed bugs (Barbarin et al., 2017). Several species of fungi have been investigated for use as control agents against bed bugs, particularly *Beauveria bassiana* (Bals.-Criv.) Vuill. This common soil fungus is currently used against a wide variety of insect pests (Butt et al., 2001; Meyling & Eilenberg, 2007). Upon adhesion to an insect cuticle, *B. bassiana* spores germinate and penetrate the cuticle to utilize nutrients from the insect and produce toxins that negatively impact insect health (Islam et al., 2021). Currently, there is only one commercial biopesticide product containing *B. bassiana* that is registered for bed bug control – Apprehend™ (ConidioTec LLC, Centre Hall, PA, USA). This product is composed of a 2% suspension of *B. bassiana* spores in oil. While this product cannot be applied directly to beds and furniture, it can be applied indoors and is currently registered for use against bed bugs in the United States. This product was found to be effective for both insecticide-resistant strains of *Cimex lectularius* L. (>94% mortality) and a susceptible strain (98-100% mortality) (Barbarin et al., 2017). *Beauveria bassiana* was also found to be highly

virulent to both adult and nymphal stages of *C. lectularius*, usually resulting in 100% mortality by three to five days post exposure, and has the ability to be transferred horizontally from infected to uninfected bed bugs in their harborage sites (Barbarin et al., 2012; Aak et al., 2018).

In addition to *B. bassiana*, several other species of fungi have been investigated as potential biopesticides for bed bug management. Bed bugs exposed to the fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin at 32%, 74%, 98% RH was found to result in high mortality only when relative humidity was 98% (Ulrich et al., 2014). *Aspergillus tubingensis* R. Mosseray and *Trichoderma harzianum* Rifai have also been suggested as possible fungal species to be utilized against the tropical bed bug, *Cimex hemipterus* (Fabricius). Both of these fungi were found to cause up to 90% mortality when *C. hemipterus* were placed on a surface treated with spores (Zahran et al., 2017). However, none of these fungi are currently registered for use against bed bugs.

Various environmental and biological factors impact the effectiveness of entomopathogenic fungi. Suitable conditions for fungal germination such as temperature and humidity, as well as the number of spores an insect is exposed to will impact the effectiveness of entomopathogenic fungi (Islam et al., 2021). The immune system of insects will also fight back against fungal infection, from the physical barrier of the insect cuticle and to antifungal immune response of insects following penetration of the cuticle (Lu & St. Leger, 2016). In addition to these, the presence of aldehydes bed bugs produce might also affect the efficacy of the fungal biopesticides. Four aldehydes produced by bed bugs [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal]

are a part of the bed bug aggregation pheromone (Siljander et al. 2008; Gries et al. 2015; Choe et al. 2016; Ulrich et al. 2016). Previous work has determined that aldehydes can reduce the growth of some fungi. Avissar et al. (1990) found that acetaldehyde inhibited the growth of the fungi *Botrytis cinerea* Pers. and *Rhizopus stolonifer* Vuillemin. Aldehydes produced by bed bugs have also been found to inhibit some fungi. For example, (*E*)-2-hexenal has been investigated for use as an antifungal fumigant for use in agriculture, and was found to inhibit *Botrytis cinerea* from growing on seedless table grapes (*Vitis vinifera* L.) (Archbold et al., 1999). *Botrytis cinerea* has similarly been found to be inhibited by (*E*)-2-hexenal from growing on strawberries after harvesting (Fallik et al., 1998). In addition, synthetic (*E*)-2-hexenal and (*E*)-2-octenal in the headspace of a culture plate were found to inhibit the growth of the fungus *M. anisopliae* (Ulrich et al., 2015). Bed bugs that were exposed to both synthetic (*E*)-2-octenal and the fungal spores simultaneously experienced reduced mortality (10%) compared to bed bugs that were exposed to the fungal conidia only (98.9%) (Ulrich et al., 2015).

To further expand knowledge of the possible interactions between the fungus *B. bassiana* and *C. lectularius*, determining the extent to which the bed bug aldehyde blend may impact the growth and the control efficacy of this fungus will be helpful. Here we expand on prior work by investigating the impact of these aldehydes on *B. bassiana*, currently being used for bed bug control, by determining the impact of all four aldehydes in their natural ratios by using a natural source (exuviae) and synthetic blend based on the ratio found in 5th instar *C. lectularius* nymphs as quantified by Dery et al. (2020). To do this, we used two products containing *B. bassiana* to expose bed bugs to the fungus and

monitored subsequent mortality when a bed bug aldehyde source was present or absent. Additionally, we determined the impact of the bed bug aldehyde blend on the growth of *B. bassiana* in culture.

MATERIALS AND METHODS

Insect. Bed bugs *(C. lectularius*) originated from colonies started from the "Earl" strain obtained from Sierra Research Laboratories (Modesto, CA, USA). The Earl strain was originally collected in Modesto, CA, in 2007. Bed bugs were fed with defibrinated rabbit blood approximately every fourteen days. Colonies were maintained at 24–26°C and 15–30% RH, with a photoperiod of 12:12 (L:D) hours.

Fungal Biopesticides. Aprehend (ConidioTec LLC, Centre Hall, PA, USA) and BotaniGard 22WP (Laverlam International, Butte, MT, USA) were both used as commercial formulations of *B. bassiana* GHA. Aprehend is formulated in a proprietary oil mixture and does not require dilution prior to application. This is currently the only product containing an entomopathogenic fungus that is registered for bed bug control. BotaniGard is a wettable powder that is mixed with water prior to application and is not registered for bed bug control.

Bed Bug Aldehydes. Two different sources of bed bug aldehydes were used: natural blend from exuviae or synthetic blend. The bed bug exuviae retain the reservoir containing aldehydes following molting, and the aldehydes slowly volatilize from the exuviae over time (Choe et al. 2016). Technical grade (*E*)-2-hexenal (98% pure) and (*E*)-2-octenal (94% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 4-

oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized as described by Moreira and Millar (2005).

Mortality Test. To determine if the presence of bed bug aldehydes in the headspace affects the effectiveness of the fungal biopesticides, the following experiment was conducted. Groups of ten adult bed bugs (≈ 10 day's post-blood meal) were collected at random from colony vials. Each group of bed bugs was randomly assigned for one of the following four treatments: (1) fungal exposure only, (2) fungal exposure + exuviae, (3) fungal exposure + synthetic aldehydes blend, and (4) untreated control.

The following process was used to prepare treatment dishes for fungal exposure. Filter paper discs (60 mm diameter) were placed into plastic culture dishes (60 mm diameter; Fisher Scientific, Waltham, MA, USA). BotaniGard 22WP treatments were prepared by adding 0.012 g BotaniGard in 10 ml water. One milliliter of this suspension is used to treat each of the filter paper discs (3.9×10^5 spores/cm^2). This represents a rate that is approximately the middle of the label rate for using this product to control thrips. For fungal exposures conducted with Aprehend, 300 µl was placed on each filter paper square (2.3×10^7 spores/cm^2). This represents approximately 5 times the label rate. As this product is not diluted prior to use, this larger application was used to ensure even coverage of spores on the filter paper disc. The treatment dishes were kept uncovered in a fume hood until they dried (≈ 5 hours). For the untreated control, one milliliter of water was applied to the filter paper disc.

Each group of bed bugs was anesthetized with carbon dioxide (≈ 30 sec) and placed onto the surface of the filter paper in the treatment dish. Each dish was covered

and then sealed with parafilm. After one hour, each group of bed bugs was transferred to a 20-ml scintillation vial containing a piece of clean filter paper (50 × 10 mm) folded in a small tent, serving as a resting platform. For treatment #2 (fungal exposure + exuviae), the vial contained exuviae (≈50) of mixed age and stage (3^{rd} to 5^{th} instar) collected from a *C. lectularius* colony vial. For treatment #3, the vial contained 50 exuviae equivalent aldehyde blend (336.65 µg total) [(*E*)-2-hexenal: 66.3 µg; 4-oxo-(*E*)-2-hexenal: 73.6 µg; (*E*)-2-octenal: 181.2 µg; 4-oxo-(*E*)-2-octenal: 15.55 µg] dissolved in 40 µl acetone (based on the amounts found in freshly shed 5^{th} instar exuviae of *C. lectularius*; Dery et al., 2020). There was likely a larger amount of aldehydes present in the synthetic aldehyde blend compared with the exuviae, as the aldehydes from exuviae volatilize over time (Choe et al., 2016) and exuviae of random age were collected. The synthetic aldehyde blend was applied to absorptive matting (16 mm diameter, laboratory bench and table protector with leak proof moisture barrier, VWR International, Radnor, PA, USA) glued to the underside of the vial cap to prevent direct contact with the bed bugs. All vials remained sealed for the duration of the study. Mortality for each group was recorded daily for 15 days. Mortality was determined visually by counting bed bugs in vials without movement and that could not grasp filter paper upon contact. A total of nine replications were conducted for each of the treatments and control.

Spore Suspension. Pure spore suspension of *B. bassiana* was prepared by the following process. Sterile water with 0.01% (v/v) Tween-80 (Fisher Scientific, Waltham, MA, USA) was added to a microcentrifuge tube containing BotaniGard 22WP (*Beauveria bassiana* GHA) to obtain a spore suspension (9.98×10^8 spores/ml). Ten microliters of

this suspension was used to inoculate a 100 mm petri dish (Fisher Scientific, Waltham, MA, USA) containing potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO, USA). The plate was incubated at 26 °C for two weeks. From this plate, a small piece of mycelium was transferred to new PDA plate. After 14 day incubation, the new PDA plate containing a pure culture of *B. bassiana* is used to obtain a spore suspension. This suspension is made by adding ≈ 4 ml sterile water containing 0.01% (vol/vol) Tween-80 to the plate and scraping the fungus off the media and then filtering through a sterile Kimwipe to remove the fungal hyphae and other debris. The concentration (4.95×10^7 spores/ml) of spores in this suspension was determined using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA).

In Vitro Activity Test. To determine if the rate of fungal growth is affected by the presence of aldehydes in vitro, the rate of hyphal growth was measured using a method adapted from Inglis et al. (2012). PDA plates were inoculated with 1 µl of a 4.95×10^7 spores/mL suspension in 0.01% Tween-80 placed in the center of the plate. These plates were exposed to 100 or 25 exuviae equivalent amount of the *C. lectularius* aldehyde blend dissolved in 40 µl acetone (based on the amount of aldehydes found in 5^{th} instar exuviae; Dery et al. 2020). The aldehyde blend was applied to a sterile filter paper disc (16 mm diameter) placed unattached on the inside of the lid. The dishes were kept inverted during the incubation process to insure there was no physically contact between the aldehyde disc and the PDA. A positive control group received the spore suspension and clean acetone was applied to the filter paper disc, while a negative control group was inoculated with sterile 0.01% Tween-80 and clean acetone was applied to the filter paper

disc. The plates were sealed with parafilm and each group was stored separately at 26 °C in the absence of light. Fugal growth was measured by determining the diameter of the visible fungal growth two times per plate, each done perpendicular to each other and averaging the two measurements. The growth of the fungus from the point of inoculation was measured daily for 15 days. Ten replications were conducted for each of the treatments and controls.

Statistical Analysis. To determine if the addition of aldehydes affected bed bug mortality, survivorship curves were generated by Kaplan-Meier survival analyses with the pooled data (n = 90) and the mortality curves were compared pairwise using the log-rank test with correction (Benjamini & Hochberg, 1995) using the R package survminer (Kassambara et al., 2021). Due to instances of heteroscedasticity, the radial growth of *B. bassiana* in culture was analyzed with Kruskal–Wallis H test followed by Dunn's Multiple Comparisons. All statistical comparisons were conducting using R version 4.0.3 (R Core Team, 2020).

RESULTS

Mortality Test. When bed bugs were exposed to Aprehend, there were significant differences between the four groups (log-rank test; $\chi^2 = 291$; df = 3, $P < 0.001$). The survival curves of exposed bed bugs were significantly different when the bed bugs were exposed to volatiles from the exuviae compared to the "fungal exposure only" (log-rank test; $P = 0.036$). However, both "fungal exposure only" and "fungal exposure + exuviae" groups experienced 100% mortality within a day of each other. The survival curve from "fungal exposure + synthetic aldehyde blend" was significantly different from "fungal exposure only" (log-rank test; $P < 0.001$), despite ultimately reaching 94% mortality by day 15 (Figure 4.1). The median survival time of the "fungal exposure only" group was 3 days (100% mortality on day 6), while the median survival time of "fungal exposure + synthetic aldehyde blend" was 4 days (94% mortality on day 15) (Figure 4.1).

When bed bugs were exposed to BotaniGard, there were significantly differences between survival curves (log-rank test; $\chi^2 = 132$; df = 3 $P < 0.001$). The survival curve from "fungal exposure only" was significantly different from that of "fungal exposure + exuviae" (log-rank test; $P < 0.001$) and "fungal exposure + synthetic aldehydes" (log-rank test; $P < 0.001$). The two groups exposed to aldehydes "fungal exposure + exuviae" and "fungal exposure + synthetic aldehydes" were not significantly different (log-rank test; $P = 0.69$). At day 15, the total mortality of bed bugs with aldehydes was 62.2% or 61.1% when exuviae or synthetic aldehydes were present, respectively, compared with 97.7% without an aldehyde source (Figure 4.2).

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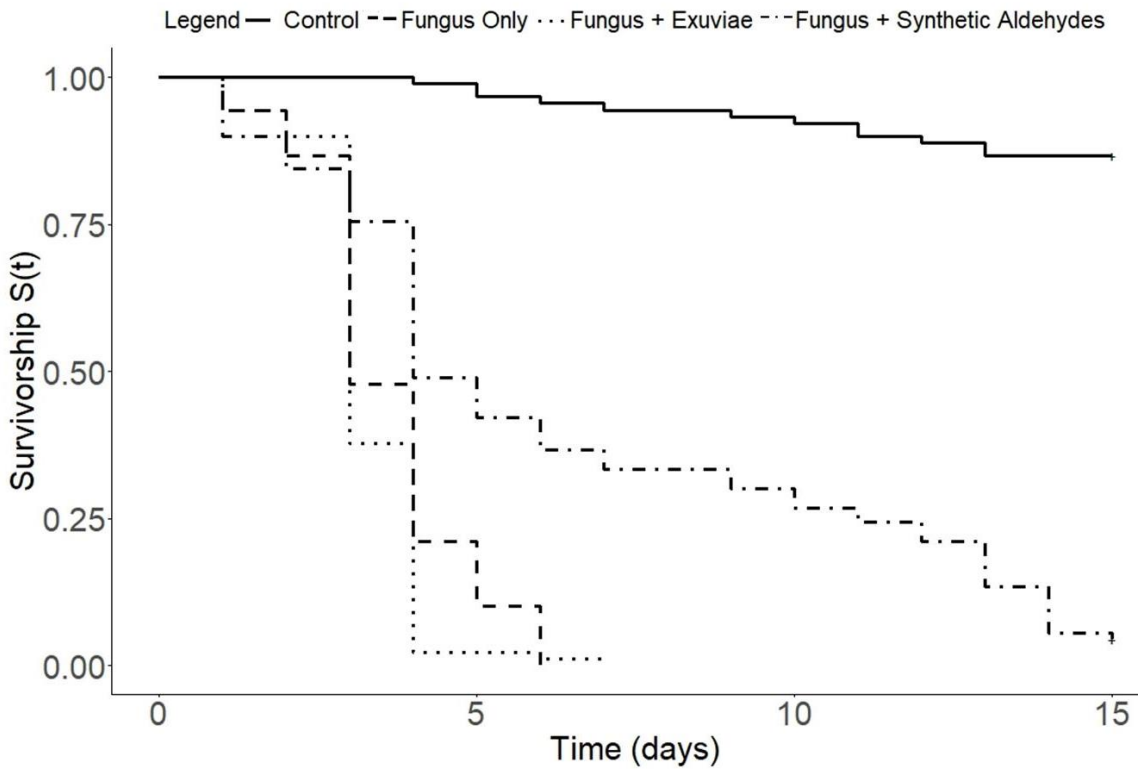

Figure 4.1. Survivorship of *C. lectularius* treatment groups (*n* = 90) following Aprehend exposure.

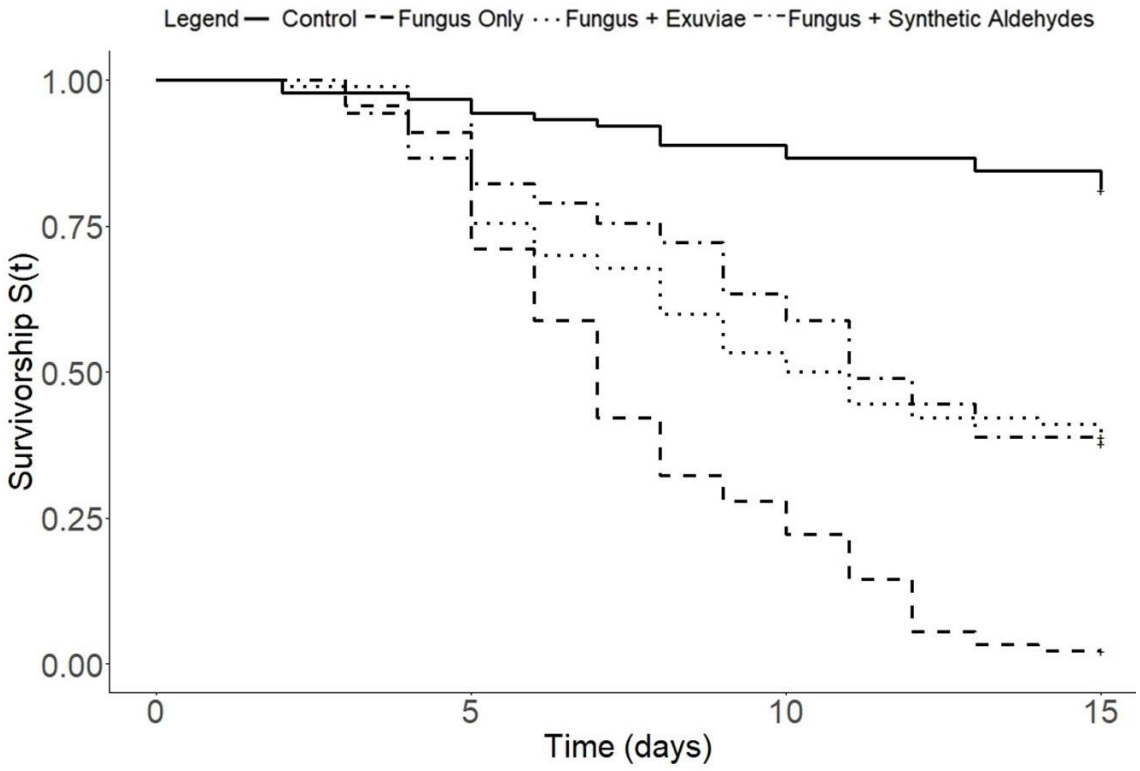

Figure 4.2. Survivorship of *C. lectularius* treatment groups (*n* = 90) following BotaniGard exposure.

In Vitro Activity Test. On one of the ten negative control plates there was an instance of contamination, while no growth was visible on the other nine. There was no obvious contamination on any plates in the other groups. On day seven, the radial growth of the fungus was significantly affected by the presence of aldehydes (Kruskal-Wallis test; H = 25.84, df = 2, $P < 0.001$). The distance of fungal growth for the control (13.38 ± 0.23 mm; mean ± SE; n = 10), 25 exuviae aldehyde equivalent (10.63 ± 0.19 mm; n = 10) and 100 exuviae aldehyde equivalent (5.4 ± 0.90 mm; n = 10) were all significantly distinct (Dunn's Multiple Comparisons; $P < 0.05$) (Figure 4.3).

On day fifteen, the radial growth of the fungus was similarly significantly affected by the presence of aldehydes (H = 9.04, df = 2, $P < 0.05$). The amount of growth for the control (22.88 ± 1.36 mm; n = 10) and 25 exuviae aldehyde equivalent (21.7 ± 0.67 mm; n = 10) were not significantly different (Dunn's Multiple Comparisons; P = 0.559). However, the 100 exuviae equivalent aldehyde group (16.45 ± 1.89 mm; n = 10) was significantly lower (Dunn's Multiple Comparisons; $P < 0.05$) than both (Figure 4.3).

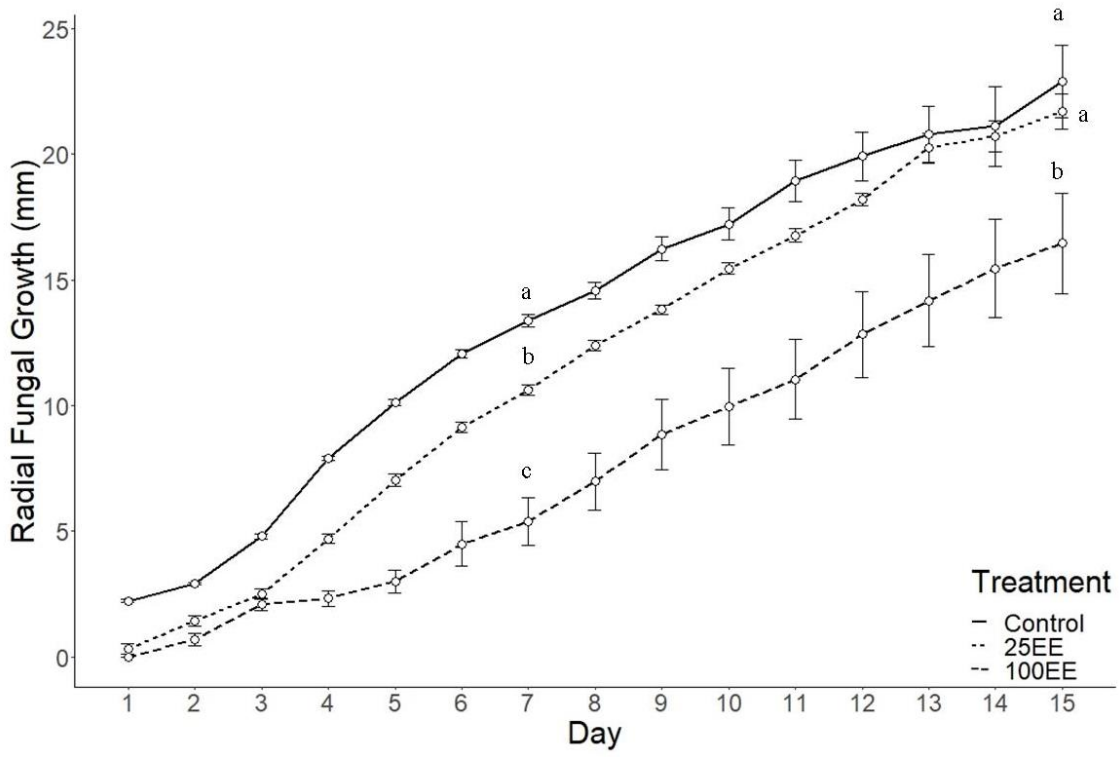

Figure 4.3. Radial growth (mm, mean ± SEM) of *B. bassiana* groups (n = 10) over time when exposed to *C. lectularius* aldehyde blend. Different letters indicate significant differences (Dunn's Multiple Comparisons; $P < 0.05$) (EE: exuvia equivalent).

DISCUSSION

Our results show that aldehydes can impact the growth and resulting mortality of bed bugs following exposure. The results of the mortality study suggest that bed bugs exposed to *B. bassiana* may experience reduced or delayed mortality when aldehydes are present. This effect was especially pronounced when bed bugs were exposed to BotaniGard, a formulation of *B. bassiana* not used for bed bugs. These bed bugs experienced 62.2% or 61.1% mortality when exuviae or synthetic aldehydes were present, respectively, compared with a mortality rate of 97.7% without an added aldehyde source. This contrasts with the results when bed bugs were exposed to the oil based

Aprehend, a product formulated specifically for bed bugs. Bed bugs exposed to this product did not have reduced mortality when exuviae were present and experienced only delayed mortality (see figure 4.1) when a synthetic aldehyde blend was added. While the bed bugs exposed to Aprehend and aldehydes did not experience significantly reduced mortality, exposure did result in a delay. Bed bugs exposed to Aprehend alone reached 100% mortality on day 6, while those exposed to the synthetic aldehyde blend and Aprehend experienced 63% mortality at day 6 before ultimately reaching 94% mortality on day 15, despite the median survival times of the two groups being within a day of each other. While there was a difference in the magnitude of the effect of the aldehydes between the two products, any comparison of these products is difficult due to the application of Aprehend at a greater rate than BotaniGard, causing larger exposure to spores than in bed bugs tested with BotaniGard. Despite the increased exposure to spores, the introduction of synthetic aldehydes resulted in an overall delay in mortality to bed bugs exposed to Aprehend.

Ulrich et al. (2014) exposed bed bugs to the fungus *M. anisopliae* and found high (> 70%) mortality only when relative humidity was 98% (mortality ≤ 25% at 32 or 74% RH) (Ulrich et al., 2014). We found that *B. bassiana* did not require increased humidity to cause high bed bug mortality, as both BotaniGard and Aprehend produced 97.7% and 100% mortality in the fungus exposure only groups, respectively, without requiring additional moisture to be added. Ulrich et al. (2015) found that bed bug mortality when exposed to *M. anisopliae* was reduced to 10% when 0.5 mg (*E*)-2-octenal was present, compared with 98.9% mortality without the aldehyde. Ulrich et al. (2015) found that the

germination rate of *M. anisopliae* in culture was completely or partially reduced in the presence of 0.5 mg (*E*)-2-octenal. In the presence of large amounts (> 0.5 mg) of either (*E*)-2-octenal or (*E*)-2-hexenal, *M. anisopliae* was completely inhibited, while fungal growth occurred at lower aldehyde concentrations (≤ 0.5 mg) (Ulrich et al., 2014). The present results found that the growth of *B. bassiana* was reduced / delayed in culture by the presence of bed bug aldehydes. However, the growth of the fungus in the lower aldehyde group (0.168 mg) had reached an equal level with the control group by day 15, while the higher aldehyde group (0.673 mg) was still significantly reduced relative to the control. The lack of complete inhibition of *B. bassiana* and the only delayed growth may be a result of the aldehydes not preventing germination, as Sosa-Gomez et al. (1997) found that (*E*)-2-decenal did not inhibit the germination of *B. bassiana*.

Aak et al. (2018) found effective horizontal transfer of *B. bassiana* from infected to uninfected adult bed bugs following exposure to Aprehend. However, they did not find efficient horizontal transfer of the fungus between nymphs. In addition, Aak et al. (2018) found reduced mortality (11%) of nymphs 24 days after exposure to Aprehend compared with adults (≈ 95%). This may be a result of nymphs producing all four aldehyde compounds, and may suggest that the two ketoaldehydes produced exclusively by nymphs may play a more significant role in fungal inhibition than the two aldehydes produced by adults.

It is not uncommon to find examples in which insect's semiochemicals have additional protective function against pathogenic fungi. The compounds 2-heptanone, and 4-methyl-3-heptanone are examples of insect pheromones, used particularly as alarm

pheromones, which inhibit the growth of various species of fungi (Blum, 1969; Cole et al., 1975). Bojke et al. (2020) also tested the effects of various insect associated compounds against several fungal species, including *B. bassiana*, and found that heptanal, 2,4-nonadienal, 2-decenal and undecanal were the most effective for inhibiting fungal growth. The red flour beetle, *Tribolium castaneum* (Herbst), is known to produce several quinone-containing compounds that function as defensive compounds against predators (Tschinkel, 1975). These secretions have also been found to inhibit the growth of *B. bassiana* (Pedrini et al., 2015). These defensive compounds, along with a benzoquinone oxidoreductase produced by *B. bassiana* to counteract these compounds, are an example of co-evolution between fungi and insects (Pedrini et al., 2015).

In addition to their role in mediating aggregation and acting as alarm pheromones, the bed bug aldehydes may also function as a defensive adaptation to various fungi that can infect them. The aldehydes that bed bugs produce have been found to inhibit several species of fungi (Fallik et al., 1998; Archbold et al., 1999; Ulrich et al., 2015). The evolution of these compounds within bed bugs may be a result of the ancestor of modern bed bug having been under a larger amount of fungal infection pressure, such as in the nests of birds or caves where bats roost, where temperature and humidity may be elevated relative to indoor environments. For example, the microclimate of the refugia of the bat bug *Afrocimex constrictus* Ferris and Usinger, was determined in two caves in the Mt. Elgon area of Kenya by Reinhardt et al., (2008). Temperature in these locations was variable depending on the location of *A. constrictus* within the cave, with daytime temperatures ranging from ≈ 18 – 25 °C, with relative humidity generally in excess of

70% (Reinhardt et al., 2008). If such cimicids in these environments do experience increased risk of fungal infection, the presence of aldehydes may have a defensive role in reducing infection by fungi.

It is not known how much risk of fungal infection might exist for bed bug aggregations in structural settings (e.g., bedroom). Various pathogens, including bacteria and fungi, are known to be associated with bed bugs (Strand, 1977). One natural fungal pathogen of bed bugs is *Aspergillus flavus* Link, which has been repeatedly found to infect both wild and laboratory colonies of cimicids. For example, *A. flavus* is known to infect the eastern bat bug, *Cimex adjunctus* Barber, in both laboratory and wild colonies in the caves where the bat bugs naturally occur (Reeves, 2001). Usinger (1966) also reported that a laboratory colony of *Paracimex* was infected by *A. flavus*. Further, *A. flavus* was found to infect both adult and second instar nymphs of *C. lectularius* when placed at 30 °C and 90% relative humidity (RH); laboratory colonies placed under these conditions were destroyed by the fungus within 18 days (Cookbain & Hastie, 1961). *Aspergillus flavus* is a common soil fungus and pathogen of some agricultural crops, and is also among the most commonly found indoor fungus (Li et al., 1995; Shelton et al., 2002; Klich, 2007; Hedayati et al., 2010).

Some caution is warranted when interpreting these results. The mortality assay in the current study was conducted in a closed system, whereas bed bugs in a natural harborage will be in an open system where aldehydes from exuviae and bed bugs could constantly diffuse and dissipate. As a result, more realistic concentrations of aldehydes in bed bug aggregations could be lower than the concentrations of the aldehydes tested in

the present study. However, Eom et al. (2012) has reported the presence of detectible amounts of (*E*)-2-hexenal and (*E*)-2-octenal from indoor air of rooms infested with 200–1,000 bed bugs. The compounds were not detected from control samples (no bed bug infestation). Considering live bed bugs can also serve as a source of these aldehydes along with their exuviae, it might not be entirely unreasonable to assume that the considerable amounts of aldehydes would be present in the microhabitats of bed bugs (e.g., cracks and crevices) where the bed bugs aggregate and develop.

Our results show that aldehydes associated with bed bugs can impact the effect of *B. bassiana* on bed bugs. The mortality associated with exposure to the fungus can be prevented or delayed and the growth of the fungus in culture was delayed by aldehyde exposure. The questions of if the effects of bed bug aldehydes on a fungal biopesticide will translate to field conditions should be addressed by field studies investigating the effectiveness of fungal biopesticides in real world infestations. If aldehydes do reduce the effectiveness of fungal based biopesticides, than there are some possible ways to mitigate this effect. Fungal pesticides might work better for bed bug control if applied before large infestations are established, in order to avoid larger aggregations with more aldehydes in the harborage sites. Another possible use would be to use these products as a preventative treatment in situations where an adjacent infestation is known to prevent bed bugs from establishing in a new location. This control method might also work better if all existing exuviae are cleaned before making treatment (i.e., removal of source of antifungal aldehydes) by combining the vacuuming of known harborage sites with this new control tactic.

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CONCLUSION

This dissertation determined that total release foggers (TRFs), commonly used by the general public for controlling indoor pests such as bed bugs, are a likely source of pyrethroids entering the municipal wastewater system. This was determined by examining insecticide floor deposition and the possible mechanisms of transfer from contaminated surfaces and items into the wastewater stream. I next found that the quantities and ratios of four aldehydes found in *Cimex lectularius* and *C. hemipterus* were differed over the course of bed bug development. There are significant differences in the amount of aldehydes in each species for both nymphs and adults. This information was then used to determine that adults of both species preferentially responded to their conspecific aldehyde blend, but not to the heterospecific blend. Finally, I showed that the presence of bed bug aldehydes can reduce or delay the mortality of bed bugs exposed to an entomopathogenic fungus.

Total release foggers. The potential role of pyrethroids dispensed by TRFs entering the municipal wastewater system was investigated. I found that cypermethrin is dispensed unevenly following TRF activation. The ability of cypermethrin to be transferred to filter paper from various surfaces (tile, vinyl, wood, and carpet) was investigated and I found that the type of surface plays a large role in the amount of the insecticide that can be transferred. In experiments simulating the washing of a contaminated cotton fabric, I found that the addition of a small amount of detergent to water greatly increased the amount of cypermethrin than was extracted compared to water alone. These results taken together suggest that the insecticides used in TRFs can be transferred from surfaces or

fabrics during everyday activities such as contacting or cleaning exposed surfaces and washing contaminated clothing, after the use of TRF within a structure. The transfer of such ingredients down-the-drain can thus contribute to the mass loading of pyrethroids into wastewater treatment plants.

While the potential exists for any insecticide application conducted indoors to be transferred down-the-drain, TRFs have characteristics that likely make them a more significant source. Total release foggers differ from other indoor application methods, such as baiting or residual sprays, as the deposition of insecticides from TRFs cannot be directed and will contaminate all items and surfaces in range. When a TRF is activated in a room, every item and surface within range receives the insecticide. While this may be an advantage of TRFs for the control of some pests, it also means that every surface or item that is then contacted or cleaned can transfer the insecticides elsewhere, such as down-the-drain when laundering clothing or bedding. TRFs are a product that is commonly used by the general public for controlling indoor pests, such as bed bugs and German cockroaches. However, due to widespread pyrethroid resistance in field populations and the cryptic nature of these pests, the use of TRFs for this purpose is of questionable value. The continued ineffective use of TRFs will contribute to increased introduction of pyrethroids in indoor settings, which can then serve as a source of entry into municipal wastewater. The pathways of insecticide movement from indoor applications to ultimately entering wastewater treatment plants remain largely unknown. Field studies of homes following total release fogger activation coupled with wastewater monitoring over time would allow for a real world quantification of down-the-drain

insecticide movement to be improved and would provide important information of these mechanisms.

Bed Bug Aldehyde Quantification. Four aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal] are produced by nymphs of two human associated species of bed bugs, *Cimex lectularius,* and *C. hemipterus,* while adults produce only two of these, (*E*)-2-hexenal and (*E*)-2-octenal. The amount of these aldehydes produced these species was determined in all five nymphal instars and in adults (males and females). I found that the amount and ratio of these aldehydes were significantly different between species. For example, the amount of 4-oxo-(*E*)-2-octenal was significantly lower in all five nymphal instar exuviae in *C. hemipterus* compared to *C. lectularius*. When the ratio of aldehydes [(*E*)-2-hexenal to (*E*)-2-octenal] produced by adult bed bugs was determined, adult *C. hemipterus* had a significantly higher proportion of (*E*)-2-hexenal than *C. lectularius*, while no intraspecific differences between male and female adults were found.

This work represents the first systematic quantification of these four aldehydes for all five of the nymphal stages for both *C. lectularius* and *C. hemipterus*. Knowing the ratios of aldehydes for both of these species will be of use for future research regarding the function of these compounds for aggregation, as alarm pheromones, and for defense from fungal infection. These findings were subsequently used in chapters 3 and 4 when using a synthetic aldehyde blend to test bed bug behavioral responses and the impact of bed bug aldehydes on an entomopathogenic fungus. The quantification of bed bug aldehydes also expands on existing knowledge of these compounds, as prior work had not

determined if the early nymphal instars produced these compounds in either species, as studies have used primarily used late instar nymphs as test subjects. Future work could investigate the presence / function of these aldehydes in other species of cimicids, such as in *Cimex* species that are associated with bats, such as *Cimex pilosellus*. Additional investigation of more distantly related species, such as *Afrocimex constrictus*, would also be of great interest. It is not known which compounds or in what amounts are produced by other species of the family Cimicidae that feed on bats and birds. It would be interesting to determine how conserved these compounds and their functions as aggregation and alarm pheromones are across the other cimicid species.

Response of Adult Bed Bugs to Aldehyde Blends. The responses of two species of bed bugs (*C. lectularius* and *C. hemipterus*) when exposed to a conspecific and heterospecific aldehyde source (exuviae or synthetic blend) were determined. Both *C. lectularius* and *C. hemipterus* responded preferentially to their conspecific aldehyde blend. Most interestingly, the two species did not respond in the same manner when exposed to a heterospecific aldehyde source. Only adult *C. lectularius* responded to *C. hemipterus* volatile cues compared to controls. Adult *C. hemipterus* were indifferent to the aldehyde blend of *C. lectularius*.

This information will be of interest if bed bug aldehydes are formulated as an attractant for use in an active monitor or trap. Based on my results, the aldehyde blend of *C. lectularius* would be effective only for its own species whereas the ratio of aldehyde blend of *C. hemipterus* would attract/arrest both species. Future work determining which of the four aldehydes are necessary for this response would be helpful to possibly allow

monitors to use a subset of the aldehydes in the lure, improving the economic viability of the monitors. Determining which component of the blend is required for the lack of response found would also be of interest. As only the quantity of the two ketoaldehydes were significantly different between the two species in 5th instar exuviae, one of these being responsible seems likely. This project investigated only the response of adults to the 5th instar nymph aldehyde blend. Thus, determining the responses of bed bug nymphs to both the conspecific and heterospecific aldehyde blend would be of interest, particularly to see if the same differential response is present in immature bed bugs.

Inhibition of an Entomopathogenic Fungus by Bed Bug Aldehydes. The impact that bed bug aldehydes have on the entomopathogenic fungus *Beauveria bassiana* was investigated. This fungus is currently registered for use against bed bugs, as well as various agricultural pests. The growth of *B. bassiana* in culture was reduced in the presence of a 100 exuviae aldehyde equivalent synthetic aldehyde blend, while growth was delayed at the lower 25 exuviae equivalent amount compared with the control. The effects of the bed bug aldehydes on two commercial products containing *B. bassiana* were tested. While direct comparisons between these different formulations are difficult due to the different rates tested, we found that the aldehydes associated with bed bugs could reduce or delay mortality caused by *B. bassiana*.

Aldehydes in the bed bug harborage sites may function as a defensive mechanism to fungal infection. Both laboratory and field populations of bed bugs have been found infected with various fungi, and the presence of aldehydes may slow or reduce such infection. While I found that the presence of aldehydes may reduce or delay bed bug

mortality when a biopesticide is used to control bed bugs in a closed laboratory system, future studies involving applications of the fungus done under field conditions for infestations of differing sizes will be required to determine if these effects of bed bug aldehydes will translate to field conditions, and if so to what effect. Future work investigating the possible impact of these aldehydes on fungi on other species of bed bugs, such as *A. constrictus*, would be of interest to determine if this antimicrobial role has perhaps evolved in this family in response to past selection pressures by fungal infection.