

ABSTRACT

Title of Document: THE ALARM-DEFENSE SYSTEM OF CIMEX
LECTULARIUS AND ITS IMPLICATIONS
FOR PEST MANAGEMENT

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In this dissertation, I focus on the alarm-defense system of the common bed bug, *Cimex lectularius*, its effectiveness against pathogen attacks, and its role as a mechanism of communication for conspecifics. This dual role of an alarm-defense system is efficient and effective considering that the two functions are typically required simultaneously in times of danger; the same substance should serve both purposes.

First, I surveyed the most common types of commercial bed bug treatments to determine long-term efficacy. Regardless of the treatment, chemical or heat, retreatments for bed bug infestations were required. After 3 years, 20.8% of housing units receiving a chemical treatment required additional treatments; 9.5% of units

receiving heat treatments required a retreatment during the same period. Multifamily units required retreatments significantly more than all other housing types.

Given these findings that emphasize the necessity for a multifaceted IPM program, I investigated whether the entomopathogenic fungus, *Metarhizium anisopliae*, could be used to control bed bugs. Feeding experiments demonstrated that bed bugs were innately susceptible to this fungus. However, regardless of whether bed bugs were sprayed with a fungal solution or contacted a treated surface, only at 98% humidity was mortality high. In addition, the two major aldehydes (*E*)-2-hexenal and (*E*)-2-octenal emitted as defensive secretions by bed bugs inhibited the *in vitro* growth of an isolate of *M. anisopliae*.

The ability to accurately and quickly detect new infestations is a critical element to an IPM-based strategy. This detection requires an understanding of attraction behavior and cues. I show through use of video-tracking software, (*E*)-2-hexenal and (*E*)-2-octenal attract adult bed bugs. Behavioral assays determined both males and females were attracted to 0.04 μg of an aldehyde blend for up to two hours after initial treatment of filter paper disks. Results suggest that these bed bug secretions may be candidates for lures and monitors. Taken together, this research describes the chemical ecology of bed bugs, providing insight into relevant signaling and defensive behavior, which has direct implications on pest management practices.

THE ALARM-DEFENSE SYSTEM OF CIMEX LECTULARIUS AND ITS
IMPLICATIONS FOR PEST MANAGEMENT

By

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Dedication

To Rachel, for her endless support

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Chapter 1: Introduction

Scientific and societal importance of the bed bug

Infestations of the common bed bug, *Cimex lectularius*, have resurged in the last 15 years, impacting people of all ages, races, and socio-economic groups. Explanations for this rebound include increased travel, changes in pest control practices for other urban pests, and insecticide resistance (Romero et al. 2007). The severity and breadth of current outbreaks and the difficulty of eradicating this insect are serious, thus bed bug infestations have received widespread attention in the scientific community and media.

Despite feeding only on blood and requiring a blood meal to molt and reproduce, there is no evidence that bed bugs act as biological vectors of disease pathogens; though mechanical transmission of some viruses remains a possibility (Jupp et al. 1980, Jupp and Lyons 1987, Blow et al. 2001, Silverman et al. 2001, Salazar et al. 2015). Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant bacteria have been recovered from bed bugs in homeless shelters, and it is hypothesized that bed bugs may act as hidden “environmental” reservoirs for drug-resistant bacteria and may promote the spread in impoverished and overcrowded communities (Lowe and Romney 2011). When severe infestations of bed bugs exist, human hosts can exhibit anemia (Pritchard and Hwang 2009).

Additionally, pesticide misuse in treating bed bugs can pose a significant public health risk. People unable to afford bed bug treatments by pest management professionals risk using do-it-yourself pesticide applications with products not labeled for indoor residential use or administered at levels exceeding label regulations. Over the last seven years, the Centers for Disease Control and Prevention reported 111 illnesses and one death associated with bed bug-related insecticide use. Contributing factors included excessive insecticide application, failure to wash or change pesticide-treated bedding, and inadequate notification of pesticide application (CDC 2011).

Although difficult to quantify precisely, economic impacts of bed bug infestations can be substantial, ranging from cost of treatment, need to replace bedding and furniture, to the negative publicity and lawsuits generated when people encounter bed bugs at hotels, retailers, and apartments (Miller 2007, Wenk 2007). While all socio-economic groups are affected by bed bugs, the problem is exacerbated for disadvantaged and low-income people who do not have the financial resources to deal with bed bug infestations (Wong et al. 2013). For example, most detection techniques, with the exception of “self visual inspection,” may be prohibitively expensive for many residents of infested structures. Subsequent chemical and/or heat treatments are also costly, so low-income and disadvantaged groups often lack the resources to treat, thereby enabling infestations to expand and spread within multi-family dwellings and group-housing facilities. Without appropriate treatments, these living arrangements create ideal conditions to facilitate

bed bug movement from one apartment room to the next, thereby placing residents, including the disabled and elderly, disproportionately vulnerable to risk of infestations.

Given the high cost and health risks associated with current protocols, this research will explore new opportunities for control and treatment of bed bugs by investigating alternative and non-chemical control methods. The focus of this dissertation examines the alarm-defense system of the bed bug, its effectiveness against pathogen attack, and its role in intraspecific communication and attraction. I will begin with a historical overview and brief discussion of insect chemical ecology.

Pheromone communication in insects

The term *pheromone*, first introduced by Karlson and Butenandt (1959), identifies particular biologically active substances that are “secreted by an animal to the outside and cause a specific reaction in a receiving individual of the same species.” As such, pheromones are used as an "intraspecific" form of communication. Over the past six decades, extensive research on insect pheromones has resulted in chemical and behavioral discoveries of over 3000 chemical components that influence the behavior of 7000 species of insects (El-Sayed 2012). Chemical signaling is often a more efficient and effective mode of information transfer than sound or visual signals for many animals (Law and Regnier 1971). Suppressing insect populations through pheromones without adversely affecting beneficial organisms has been an important goal of pest population management (Karlson and Butenandt 1959, Carde and Minks 1995, Reddy et al. 2012).

The central role of pheromones in insect societies has been studied and reviewed extensively (Wilson 1965, Blum and Brand 1972, Ali and Morgan 1990). Pheromones are divided into two subgroups, primers and releasers, based on their effect on the receiver. Primers physiologically alter the endocrine and reproductive systems. Releasers produce a response by the nervous system causing immediate behavioral action by the receiving insect. Releasers are categorized into three general behavioral groups: sexual behavior, recruitment and aggregation, and alarm and dispersal (Wilson and Bossert 1963). Some pheromones elicit several different behavioral responses under different situations (Shorey 1973).

Sexual behavior comprises mate attraction, recruitment of a sexual partner, and oviposition. Due to its commercial importance in pest management, researchers have investigated the manipulation of sexual communication.

The second behavioral group of releaser pheromones, recruitment and aggregation, direct receivers' movements toward chemical sources quickly and precisely. Aerial scent trails of honey bees and trail markings of ant and termite species are illustrative of this category (Moore 1966, Tumlinson et al. 1971, Jarau et al. 2004, Mashaly et al. 2011). These types of pheromones can indicate suitable resting sites or locations of food source. While most prominent in colonial behavior and ecology of social insects, aggregation cues are important for semi-social and solitary insects. For example, aggregation of German cockroach nymphs depends largely on olfactory responses to chemical substances (Ishii and Kuwahara 1968).

Alarm and dispersal behaviors are initiated by the release of volatile substances from an individual (Blum 1969, Law and Regnier 1971). Following the discharge, the receiver reacts either by swiftly retreating or aggressively posturing toward the intruder (recruitment). Wilson and Regnier (1971) characterized panic response as primitive and the aggressive attack behavior as derived. Due to their low molecular weight and quick evaporating times, aldehydes and ketones are associated with this group of pheromones.

Chemical defense in arthropods

Allomones are chemical substances produced and released by an individual of one species affecting the behavior of another. Benefiting the producer, allomones are common forms of defense (Brown 1968). Chemical defenses are widespread and varied among insects. Chemical defenses are irritants, toxins, venoms, and deterrents (Schildknecht et al. 1964, Jacobson 1966, Pasteels et al. 1983). The sources of discharge, mechanisms, and effects are dependent on both the evolutionary history of the producer as well as potential predators. These compounds may have broad impacts or be targeted toward specific organisms.

Volatile, nonspecific irritants can repel arthropods and mammal predators (Nishida and Fukami 1989, Hristov and Conner 2005, Noge et al. 2012). Effectiveness of volatiles against birds varies; some evidence shows that even large amounts of irritants discharged accurately only marginally guards the insect (Eisner et al. 1961, Krall et al. 1999). For added protection, social and gregarious insects have

been shown to pool their defensive resources through coordinated behavior (Howard et al. 1983, Lockwood and Story 1986).

As mentioned above it is common for a pheromone to serve multiple functions in intraspecific communication. For example, at low concentrations chemical substances can serve as attractants, while initiating an alarm and attack response at high concentrations (Law and Regnier 1971). Similarly, chemical defenses developed for interspecies behavior (allomones) can also act as both alarm and recruitment pheromones.

Under threat, the ant species *Acanthomyops claviger* expels undecane, which acts as a spreading agent for formic acid. While formic acid destroys enemy tissue, volatile undecane evaporates, acting as a continuous alarm pheromone (Regnier and Wilson 1968). Isobutyric acid in *Rhodnius prolixus* is strongly repulsive and causes paralysis to arthropods. Evidence that isobutyric acid may also act as an alarm pheromone in triatomine bugs was demonstrated by the compound's vapor action to cause rapid arousal and dispersal (Cruz-Lopez et al. 2001). Cornicle secretions in aphids are hypothesized to function as both protection against predators and alarm signaling in aggregated colonies (Mondor et al. 2002).

Chemical mediated behaviors of bed bugs

Carayon described the scent gland system morphology of bed bugs (Usinger 1966). Showing no sexual dimorphism, the metathoracic scent apparatus of *Cimex lectularius* adults predominantly produces the aldehydes (E)-2-hexenal and (E)-2-

octenal (Schildknecht et al. 1964). In adult bed bugs, (E)-2-hexenal is produced at a rate three times higher than the other main aldehyde (Collins 1968, Levinson and Bar Ilan 1971). Possessing no metathoracic glands, nymphs instead have three dorsal abdominal glands. Analysis of nymphs found that (E)-2-octenal was three times more abundant than (E)-2-hexenal (Levinson et al. 1974). In addition, 4-oxo-(E)-2-hexenal and 4-oxo-(E)-2-octenal consisted of 11% and 5% respectively of the total gland secretions in nymphs (Feldlaufer et al. 2010).

C. lectularius often lives in aggregations composed of various life stages, both sexes, and bugs of different feeding and mating status (Johnson 1941, Reinhardt and Siva-Jothy 2007, Pfister et al. 2009). Proposed explanations for this behavior include safe harbor from predators, desiccation resistance, and increased mating opportunities (Benoit et al. 2007, Pinto et al. 2007, Siljander et al. 2008). Marx (1955) was the first to attribute “nest odor” to the aggregation behavior of the species, and aggregation is likely mediated by pheromone cues (Siljander et al. 2007, Olson et al. 2009, Weeks et al. 2011). Siljander et al. (2008) isolated 10 compounds [nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (2E,4E)-octadienal, benzaldehyde, (+)-limonene, (-)-limonene, sulcatone, benzyl alcohol] they believed to be essential to *C. lectularius* aggregation pheromone. A more recent report (Gries et al. 2014), however, indicates only five compounds are necessary for aggregation. Interestingly, (E)-2-hexenal and (E)-2-octenal are common to both reports.

The identical chemicals eliciting aggregation, however, have been implicated in dispersal and alarm reactions. Under laboratory conditions, (E)-2-hexenal and (E)-

2-octenal, in proportions to their presence in the scent gland, caused dispersal of bugs (Levinson and Bar Ilan 1971). Notice that (E)-2-hexenal and (E)-2-octenal, the two major alarm pheromones, are also components of the aggregation blend. Emission of volatiles of the closely related tropical bed bug, *C. hemipterus*, caused a similar dispersal response (Liedtke et al. 2011). Male bed bugs also discharge pheromone to repel other males in an effort to avoid homosexual harassment and mounting (Ryne 2009). An explanation for such conflicting reports may be the result of the quantity of pheromone emitted. For example, in the stinkbug, *Eurydema rugosa*, the alarm pheromone (E)-2-hexenal can function as an aggregation pheromone when gradually discharged in small quantities by first instars (Lockwood and Story 1985).

The same chemical compounds responsible for aggregation and dispersal behavior in *C. lectularius* also have a role in predator avoidance. Individual bed bugs placed in the mouths of bats were spit out. Likewise, bats rejected mealworms smeared with bed bug gland secretions (Usinger 1966). These demonstrations highlight the defensive function of these pheromones.

An additional purpose of bed bug volatiles may be an antimicrobial defense. As previously observed in plants and other insects, aldehydes have the potential to inhibit both bacterial and fungal growth *in vitro* (Roth 1961, Engelhardt et al. 1965, Kubo and Kubo 1995, Bisignano et al. 2001, Cleveland et al. 2009). Currently little is known about how *C. lectularius* combats potential microbial and fungal threats. No research has shown pheromone compounds to have an antimicrobial role in a natural setting. This lack of data represents a potential opportunity for development

of environmentally-sound biological control agents by determining the role of defensive secretions in defense against microbes.

Dissertation overview

For a pest proven adept at evolving ways to resist currently available classes of chemical insecticides, novel approaches must be investigated. Entomopathogenic fungi are logical candidates for bed bug control. There are over 750 species of fungi that infect arthropods (Shah and Pell 2003, St Leger and Wang 2010). In the United States, *Metarhizium anisopliae* has been developed commercially for control of various beetles, termites, flies, and other arthropods (Zimmermann 1993). While broad host range of this fungus offers enormous possibilities, the feasibility of this fungus as a control method may be limited by the natural fungicidal defenses of bed bugs. Because entomopathogens must overcome potential biochemical roadblocks, the defensive (potentially anti-pathogenic) role of bed bug alarm aldehydes requires further study. As described above, the bed bug's chemical secretions may serve multiple purposes. With the ultimate aim of developing a pathogen that is host-specific and highly effective against bed bugs, I will examine relevant behavior and defensive mechanisms of the bed bug alarm-defense system and their implications on pest management strategies.

In Chapter 2, I investigate whether two types of bed bug eradication treatments (heat and chemical) using professional pest management services are equally effective across a variety of housing and building types. My results highlight

how current control practices compare and the requirement for adjusted protocols are discussed. In Chapter 3, I evaluated the susceptibility of bed bugs to the fungus *M. anisopliae* and show the importance of relative humidity towards infection. In Chapter 4, I focus on the role of bed bug defensive secretions in fungal growth *in vitro*. This knowledge of how entomopathogens interact with the alarm-defense system will be a key step toward determining their potential to control bed bug populations in natural situations. Finally, in Chapter 5, I evaluate bed bug behavioral responses to surfaces treated with different amounts of defensive secretions.

Chapter 2: Comparison of two strategies for bed bug control in different housing types

Abstract

Little is known about the long-term efficacy of commercial bed bug treatments. The two most common types of bed bug treatments were analyzed over a 3-year period across a range of housing types. 20.8% of units receiving a chemical treatment during the evaluation period required additional treatments; 9.5% of units receiving heat treatments required a retreatment during the same period. Multifamily units required retreatments significantly more than all other housing types. Regarding time before a retreatment was required, there was no significant difference between heat and chemical applications. Our results emphasize the necessity for a multifaceted IPM program that includes resident education and cooperation when battling bed bug infestations.

Introduction

The common bed bug, *Cimex lectularius*, is a haematophagous ectoparasite of humans. The severity and breadth of current outbreaks and difficulty eradicating this

insect are serious, and bed bug infestations have received much attention in the scientific community and media. Explanations for the bed bug's extensive resurgence over the past 15 years include increased travel, changes in pest control practices addressing other urban pests, and insecticide resistance (Romero et al. 2007, Boase 2008).

Widespread pyrethroid-resistance in field-collected populations (Moore and Miller 2006, Zhu et al. 2013) has shifted use patterns toward products with alternate modes of action. Dual action insecticides containing both pyrethroids and neonicotinoids are now widely used by pest management professionals (Potter et al. 2013). While chemical insecticides continue to be used due to effectiveness and residual activity, the potential for resistance to additional classes of insecticides remains (Gordon et al. 2014), motivating exploration for alternative control methods for bed bug infestations. Extreme temperature, such as sustained high heat (at least 71.5 minutes at 118.4° F), has successfully been used to eliminate bed bugs (Pereira et al. 2009, Kells and Goblirsch 2011). Applications of whole room and building heat treatments for bed bug control were up 25% from 2010 to 2013 (Potter et al. 2013). Use of heat to kill bed bugs offers the advantages of immediate efficacy, the ability to treat household items not amenable to a liquid (chemical pesticide) treatment, and reduction in required cleaning and site preparations by the customer. However, the high cost of heat treatment makes it less feasible for many residents and for multi-unit or large commercial managed properties with limited pest control budgets. Additionally, when heat treatments are not sustained for sufficient lengths of time or heated air is not uniformly distributed, bed bugs may seek localized pockets of cooler

air or move from the heated area into adjacent rooms or units, enabling them to survive and disperse.

While all housing types can harbor bed bugs, multifamily buildings are commonly considered to have the greatest potential for infestations due to frequent turnover of units, high density occupancy, and adjoining walls facilitating active movement of bed bugs through electrical conduits and piping (Wang et al 2010). Some people do not react to bed bug bites, and may not recognize or report a potential infestation. Problems are often exacerbated for low-income residents who may not have the financial resources to effectively deal with bed bug infestations, or tenants who must rely upon a property manager to decide when treatment is necessary. Lack of timely treatments may foster growth and spread of infestations within facilities.

In this study we analyze retreatment data following professional chemical or heat treatments across a variety of housing types and in large commercial structures over a three-year period. This systematic analysis of field treatment method and follow-up information offers insights toward developing long-term, sustainable, and cost-effective bed bug management programs.

Materials and methods

Field sites

Bed bug treatments were conducted from January 2011 to December 2013 in residential and commercial properties in the Washington, DC metropolitan area. Treated residences included detached single-family homes (SFH), attached row homes sharing a common wall with bordering units (RH), and multifamily apartment homes (MF). Commercial properties (COMM) include hotels, medical facilities, stores, etc. Prior to any treatment, bed bug infestations were confirmed by an inspection from either a trained pest management professional (PMP) or by bed bug scent detection canine teams. Types of treatments for individual units were based on client preference following consultation with the pest control company.

Pest management professionals

All inspections, applications, and data records were provided by certified technicians from American Pest, a private company founded in 1925 based in Fulton, Maryland.

Treatment strategies

Chemical Treatment. Specific products used to treat infestations were selected by American Pest based on industry standards and peer-reviewed studies. Products included: Temprid SC (active ingredients: imidacloprid and cyfluthrin; Bayer Environmental Science, Research Triangle Park, NC) with Exponent Insecticide

Synergist (active ingredient: piperonyl butoxide; MGK, Minneapolis, MN) formulated in a B&G spray (1-gal Prime Line 2000, B&G equipment Co. Jackson, GA) for baseboards, floorboards, headboards, and walls; Tempo 1% Dust (active ingredient: cyfluthrin; Bayer Environmental Science, Research Triangle Park, NC) for crack and crevice applications; and Bedlam Plus (active ingredients: imidacloprid and d-phenothrin; MGK, Minneapolis, MN) or Phantoms SC (active ingredient: chlorfenapyr; BASF, Research Triangle Park, NC) for spot treatments along tufts, folds, and seams of mattresses and furniture. Temprid SC and Bedlam Plus are dual-action insecticides containing pyrethroid and neonicotinoid class insecticides. Phantom SC is a single action pyrrole product.

Prior to chemical treatments, residents were instructed to wash and heat dry all clothing and bedding, and place those laundered items in sealed bags. Chemical treatments involved PMPs applying spray and dust formulation insecticides to bedrooms, hallways, and living room areas. PMPs performed two treatments at a 2-week interval. If live bugs were observed by the PMP during the second treatment, an additional treatment was applied in another 2 weeks. Treatments continued at 2-week intervals until the PMP found no live bed bugs. Any treatment conducted after this treatment series was considered a retreatment.

Heat Treatment. Mobile heating units (Thermal Remediation, Temp-Air, Inc., Burnsville, MN) were placed in bed bug-infested areas along with fans to distribute the heat. After the target temperature of 135°F was reached in the residence, it was maintained for an additional four hours. Temperature was monitored throughout the

home using wireless sensors (Thermal Remediation, Temp-Air, Inc., Burnsville, MN) and thermal imaging devices (FLIR Instruments, FLIR Systems, Inc., Wilsonville, OR). Heat remediation typically consisted of one treatment per infested unit.

Treatment efficacy

Following all treatment regimes, callbacks were monitored and additional treatments performed as necessary. A callback received within 30 days after initial treatment was considered part of the original treatment. Periodic canine inspections were part of some properties' bed bug management plan.

Statistical analysis

Treatment type (chemical and heat), building type (multifamily, row house, single family home, and commercial property), and interactions were compared. Counts of treatments and retreatments were analyzed in R (R Core Team 2014) using a generalized linear model where we assumed the sampling distribution was an over-dispersed binomial (quasi-binomial). Estimates and standard errors are on the logit scale. Mean separations were done for building type using the “multcomp” package (Hothorn et al. 2008) in R. We used a nonparametric Kruskal–Wallis rank-sum test to determine whether days between retreatments differed between building type and treatment type.

Results

Assessment of retreatments counts

A total of 4258 units were evaluated over the 3-year period; multifamily housing (MF) accounted for over 50% of the units receiving a heat treatment, and over 88% of the units that were chemically-treated. All building types, including single-family houses, row houses, multifamily apartments, and commercial properties required retreatments, though some types required significantly more than others (Table 2-1). The 3650 properties that received a chemical treatment during the 3-year evaluation period required a total of 759 retreatments (20.8%), while the 608 properties that received a heat treatment to control bed bug infestations during the same period required 58 retreatments (9.5%). No building type receiving an initial heat treatment required more than two retreatments, while five multifamily units that received an initial chemical treatment needed four retreatments. With the exception of these multifamily units, retreatments for other building types that received an initial chemical treatment were typically low (10.4%).

Based on number of retreatments, treatment type (chemical vs. heat) was significantly different ($\chi^2 = 49.0$; $df = 1$; $p < 0.001$), as was the building type ($\chi^2 = 52.6$; $df = 3$; $p < 0.001$). Multiple comparisons among different building types identified only multifamily units as being significantly different from all other housing types. Thus our data suggest two building classes with respect to

differentiating these bed bug treatment protocols: (1) multifamily housing, requiring significantly higher retreatment rates, and (2) row houses; detached, single family houses; and commercial properties, all of which were significantly less likely to need retreatment to control the bed bug infestation. The interaction between building type and treatment type was only borderline significant ($p = 0.054$). The sample size was sufficiently large to justify analyzing the data as if effects were additive.

Time lag analysis

There was no main effect regarding days between retreatments ($\chi^2 = 3.095$; $df = 7$; $p = 0.876$). Chemical treatments averaged 229.0 (SE \pm 7.7) days before a retreatment was required, while a mean of 204.3 (SE \pm 20.9) days elapsed before a retreatment was required for heat retreatments. With the exception of the six multifamily units that required a second retreatment, all heat retreatments were conducted within one year of initial heat treatment. All chemically-treated building types had at least one unit retreated two years after the initial treatment, and 26 multifamily units required a retreatment two years after the initial chemical treatment (Figure 2-1).

Discussion

Bed bugs are found in a wide variety of residences and commercial structures. While all building types required retreatments regardless of whether the initial treatment was chemical or heat, multifamily housing required significantly more

retreatments. We suggest that this is due to multiple bed bug introductions and reintroductions rather than an inadequate initial treatment or insecticide resistance. If either of the latter were responsible for the need for retreatment, we would expect equal retreatment rates across all building types, which was not the case.

A number of factors could contribute to multifamily housing requiring more retreatments. Bed bug infestations are easiest to eradicate when found and treated early. Booth and colleagues (2012) used genetic techniques to document that a single introduction of an inseminated female was capable of establishing and spreading throughout a multi-unit building. Because apartment residents in multifamily units often rely on property managers to schedule treatments, there may be delays in addressing infestations. This contrasts with single-family homeowners, who generally deal directly with a pest management company. Additionally, adjoining walls and halls in apartment buildings allow for active bed bug dispersal through walls, pipes, electrical outlets, and common areas. Wang and colleagues (2010) also observed passive bed bug dispersal, facilitated by residents' social behaviors that included bringing bed bug-infested furniture into the building, resident turnover, visiting friends in other apartments, and use of bed bug-infested items in building common areas. Regardless of possible reasons, managing bed bug infestations in multifamily buildings appear to be more challenging than in other types of living quarters.

Overall, we found heat treatment to be more effective in controlling bed bugs than chemical treatment. Furthermore, heat treatment requires only one home visit compared to a minimum of two initial visits for chemical treatments, and heat

treatments require less advance preparation by occupants. Clothing and loose items do not have to be cleaned and bagged prior to heat treatment. However, cost of a heat treatment to a client is approximately 10 times the cost of a chemical application, thus is not economically feasible for many accounts. During our study period five multifamily units that were chemically treated required an additional four retreatments (five total treatments). Still, from a cost perspective, it is often less expensive to chemically treat a unit even if eliminating the infestation requires multiple treatments. The potential for bed bugs to spread to nearby apartment units between treatments may complicate this reasoning. Likewise, lengthy disruptions and subsequent quality of life issues may outweigh the initial cost advantage of a chemical treatment.

This study has several caveats. (i) We could not determine the number of clients that may have enlisted the services from another pest control company following a reinfestation. Also, it is unknown how many clients received prior treatments from other companies. This limitation likely means actual retreatment rates are higher than stated. (ii) No information was available for initial levels of bed bug infestation, or for the amount of clutter. While these subjective levels would have been useful this analysis of treatments stands independently.

This study evaluates efficacy of controlling bed bugs in units that had either chemical or heat treatment but not both. A combination treatment employing heat to quickly eliminate bugs followed by a residual chemical insecticide may be the most effective. A multifaceted IPM approach including resident education, observations,

and cooperation cannot be understated. Early detection and proactively eliminating sources of introduction is critical in bed bug management.

Tables

Table 2-1. Summary of heat and chemical treatments for bed bug infestations, 2011 – 2013.

	Type of unit ¹	# of units	# of units receiving N retreatments				Total retreatments	% Retreatments
			N=1	N=2	N=3	N=4		
Heat								
	MF	310	31	6	0	0	43	13.9
	RH	109	2	0	0	0	2	1.8
	SFH	123	7	1	0	0	9	7.3
	COMM	66	4	0	0	0	4	6.1
Total		608	44	7	0	0	58	9.5
Chemical								
	MF	3228	460	89	19	5	715	22.1
	RH	163	14	2	0	0	18	11.0
	SFH	116	6	0	0	0	6	5.2
	COMM	143	15	1	1	0	20	14.1
Total		3650	495	92	20	5	759	20.8

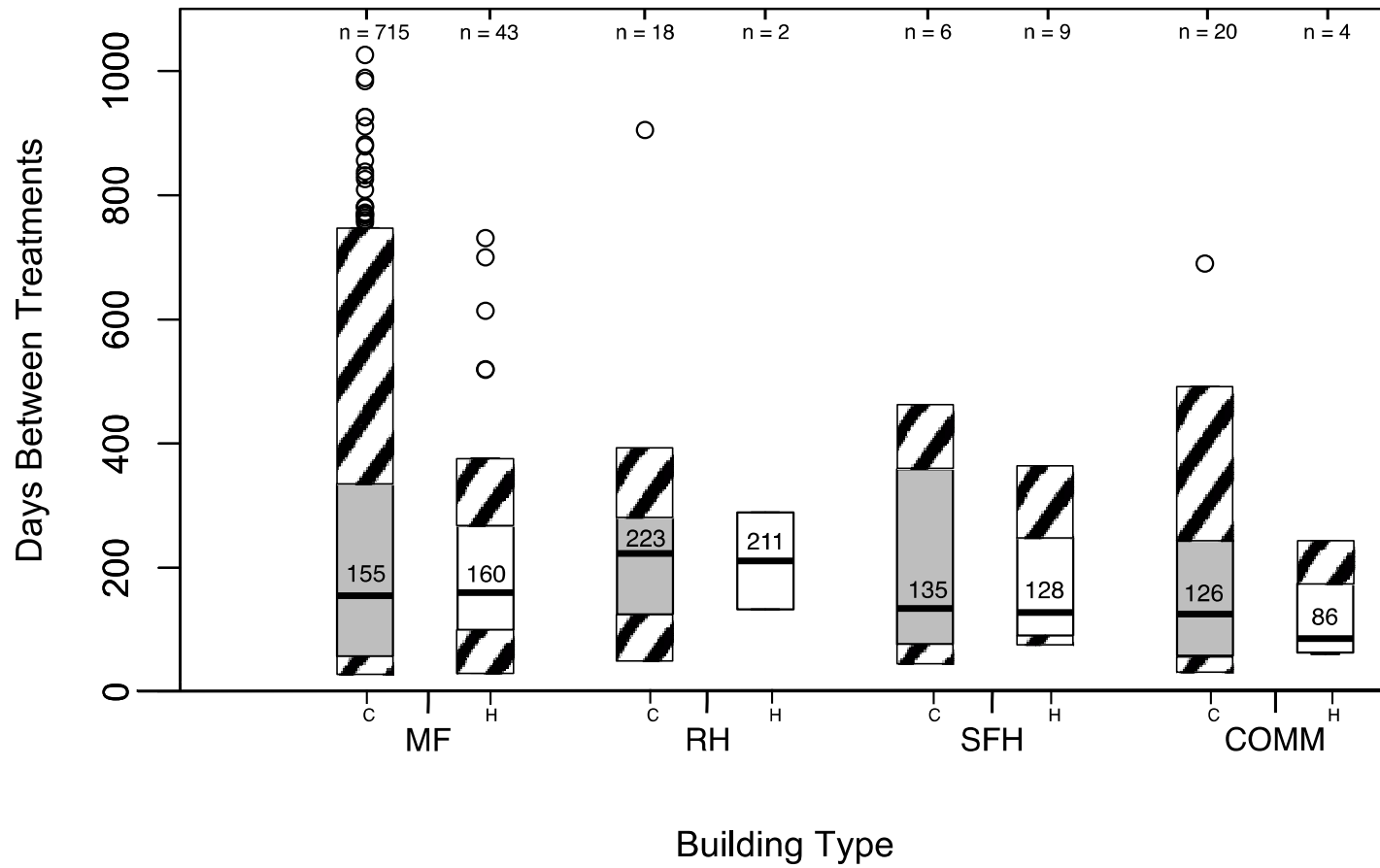
¹MF=multifamily; RH=row house; SFH=detached, single family home; COMM=commercial property

Figure legends

Figure 2-1. Chemical (c) and heat (h) treatment lag time (days) between treatments requiring retreatments. Black bars within each box plot indicate the median; solid boxes encompass 50% of the data while hatched boxes encompass 90% of the data; open circles represent the 5th and 95th percentile outliers; numbers above black bars indicate sample median; sample sizes (i.e. number of retreatments) are indicated above boxes. One chemical treatment includes two repeat treatments at a 2-week interval. A heat treatment consisted of one treatment. All retreatments occurred at least 30 days after the previous treatment was conducted. MF = multifamily; RH = row house; SFH = single-family home; COMM = commercial property.

Figures

Figure 2-



Chapter 3: Exposure of bed bugs to *Metarhizium anisopliae* at different humidities¹

Abstract

Bed bugs, *Cimex lectularius*, were exposed to conidia (spores) of the entomopathogenic fungus *Metarhizium anisopliae* by feeding, aerosol spray, or contact with a treated surface. Feeding experiments demonstrated that bed bugs were innately susceptible to this fungus. However, only at 98% humidity were mortality rates high, regardless of whether bed bugs were sprayed with a fungal solution or contacted a treated surface. Mortality in treated bed bugs at ambient humidity did not increase when these bed bugs were kept in aggregation with other bed bugs that had recently blood fed to repletion. Based on these laboratory studies, we conclude that *M. anisopliae* is a poor pathogen for use in control of bed bugs, particularly at humidities that would likely be encountered under field conditions.

¹ This chapter has been published as: Ulrich K. R., M. F. Feldlaufer, M. Kramer, and R. J. St. Leger. 2014. Exposure of bed bugs to *Metarhizium anisopliae* at different humidities. *Journal of Economic Entomology* 107: 2190-2195.

Introduction

The global resurgence in bed bug *Cimex lectularius* L. populations has demonstrated that controlling this hematophagous insect is challenging (Doggett et al. 2012). Bed bugs have evolved widespread resistance to pyrethroid insecticides (Romero et al. 2007, Zhu et al. 2010, Davies et al. 2012), and while other chemical classes of insecticides have been developed and registered for bed bug control, they have exhibited mixed results (Moore and Miller 2009, Romero et al. 2010, Haynes and Potter 2013, Koganemaru and Miller 2013, Wang et al. 2013). Non-chemical controls have also been proposed as part of integrated pest management strategies (Wang and Cooper 2011), and elimination of bed bugs by exposing infestations to heat (sustained lethal high temperatures) has proven effective (Pereira et al. 2009, Kells and Goblirsch 2011, Puckett et al. 2013).

Recently, the entomopathogenic fungus *Beauveria bassiana* has successfully been shown to be pathogenic to bed bugs (Barbarin et al. 2012). Results demonstrated that the fungus was efficacious, and that due to the gregarious nature of bed bugs, infected individuals could transfer the fungus to uninfected bed bugs. Another entomopathogenic fungus *Metarhizium anisopliae* has been used to target a variety of insect species, including plant-feeding member of the Hemiptera (Shah and Pell 2003, Zimmermann 2007). This fungus has also been genetically modified to increase specificity and efficacy (St. Leger et al. 1996, St. Leger and Wang 2010).

In this laboratory study, we investigate the use of *M. anisopliae* as a potential pathogen against *C. lectularius* in the following ways: (i) by feeding conidia to bed

bugs to bypass the cuticle and determine innate susceptibility to the fungus; and (ii) by treating bed bugs with conidia (aerosol spray and contact) and determining mortality at three different humidities. Additionally, since the feeding status of natural infestations of bed bugs may vary (Reinhardt et al. 2010), we determined mortality of treated, unfed bed bugs that were co-mixed with bed bugs that had fed to repletion.

Materials and methods

Insects

A colony of *C. lectularius* was established from bed bugs originally obtained from Harold Harlan (Crownsville, MD). This pyrethroid-susceptible colony (Feldlaufer et al. 2013) was kept at ambient conditions ($25 \pm 5^\circ\text{C}$ and $30 \pm 5\%$ RH), and fed weekly on expired human red blood cells and plasma using an artificial (*in vitro*) feeding system (Feldlaufer et al. 2014). We assayed adult males, adult females, and nymphs (3rd-5th instar) that had not been fed for eight days prior to fungal treatments. For assays involving blood-fed bed bugs, nymphs and adults were fed to repletion < 2h before use. Bed bugs used in all experiments were kept as groups in 240-ml, wide mouth Mason jars (Jarden Home Brands, Daleville, IN), and provided with fan-folded, filter paper (40 x 140 mm; Whatman 1), which acted as a harborage.

Fungal isolates

Wild-type *Metarhizium anisopliae* (ARSEF 1548) was obtained from the USDA Entomopathogenic Fungus Collection in Ithaca, N.Y. This strain was originally isolated from the rice black bug *Scotinophara coarctata* (F.) (Hemiptera: Pentatomidae). Fungal cultures were maintained in 100 x 15 mm sterile, plastic Petri dishes (VWR International, Radnor PA) on Difco™ potato dextrose agar (PDA; Becton-Dickinson, Sparks MD) incubated at room temperature (Inglis et al. 2012). Virulence was maintained by passage through bed bug hosts every 2 months (Butt and Goettel 2000). Cultures were incubated for 14 days and conidia harvested by scraping colonies with a sterile spatula and were suspended in distilled water containing 0.01% Tween® 80 (Sigma-Aldrich, St. Louis, MO). Spore suspensions were determined using a hemocytometer (Spencer, Buffalo, NY) and adjusted to desired concentrations by diluting in water containing 0.01% Tween® 80.

Ingestion assays

M. anisopliae conidia were added to a blood source and then fed to bed bugs to determine their innate susceptibility. For feeding experiments, 0.5 ml of a spore solution containing 5×10^7 conidia/ml was added to 49.5 ml of a blood-plasma mix to yield a final concentration of 5×10^5 conidia/ml, and fed to mixed stages of bed bugs (n = 729) using an *in vitro* feeding system (Feldlaufer et al. 2014). Concentrations of 1×10^5 conidia/ml, 1×10^4 conidia/ml, and 1×10^3 conidia/ml were also fed to bed bugs (n = 354, 290, and 483, respectively) by first adding 0.5 ml of a spore solution containing 1×10^7 conidia/ml to 49.5 ml of a blood-plasma mix and then making

appropriate dilutions. Control groups consisted of bed bugs ($n = 301$) fed either blood-plasma containing only 0.01% Tween® 80, or bed bugs ($n = 282$) fed *M. anisopliae* spores that had been inactivated by autoclaving 15m at 121°C. In all feeding experiments, any bed bugs that had not fed to repletion (by visual observation) were removed and not included in the analysis. Bed bugs were maintained at room temperature and humidity ($25 \pm 2^\circ\text{C}$ and $30 \pm 5\%$ RH).

Humidity-treatment assays

We examined the effect of humidity on the mortality of bed bugs treated with *M. anisopliae* conidia by either spray application or by contact with a treated surface. Different humidity regimes were achieved by confining bed bugs in glass desiccators (150 mm i.d.; Fischer Scientific, Pittsburgh, PA) over saturated salt solutions (Wexler and Hasegawa 1954). Bed bugs were kept over a saturated solution of calcium chloride ($32 \pm 1\%$ RH) to mimic ambient-room conditions; over a saturated solution of sodium chloride ($74 \pm 1\%$ RH); and over distilled water ($98 \pm 1\%$ RH). In all experiments temperature ($25 \pm 2^\circ\text{C}$) and RH were verified by Traceable™ relative humidity-temperature meters (Fisher Scientific, Pittsburgh, PA).

Bed bugs were treated in one of two ways: For spray applications, bed bugs were placed on filter paper in glass Petri dishes and sprayed with *M. anisopliae* conidia suspended in sterile water containing 0.01% Tween® 80. Using 30-ml amber bottles and a pump applicator (Specialty Bottles, Seattle WA), we calculated that each group of bed bugs received approximately 4.5×10^6 conidia. Treated bed bugs were transferred to Petri dishes (60 x 15 mm) containing untreated (dry), filter paper (47

mm dia.; Whatman 1). Control bugs were sprayed with water containing 0.01% Tween® 80 and maintained the same way as the treated bed bugs.

For contact assays, bed bugs were exposed to *M. anisopliae* by being placed on filter paper previously treated with conidia. In these experiments, conidial suspensions in sterile water containing 0.01% Tween® 80 were applied to 47 mm (dia.) filter paper disks (Whatman No. 1) to yield a final concentration of 1×10^5 conidia/cm². Filter papers were allowed to dry for 120 min before being placed in glass Petri dishes (60 x 15 mm). Bed bugs were then placed on the dried, treated surface. Bed bugs placed on Tween® 80-treated filter paper acted as controls. A total of 90-120 bed bugs (six trials of either 15 or 20 bed bugs/trial) were used for each humidity treatment, including controls.

In all experiments, mortality (see Feldlauer et al. 2013) was assessed daily for one week (168h) post-treatment. Mycoses were confirmed by surface-sterilizing dead bed bugs with 70% ethanol/water followed by distilled water (Lacey and Solter 2012), and by subsequently maintaining dead bed bugs at 98% RH for an additional week. Dead individuals were then examined under a dissecting scope for the presence of fungus (Humber 2012).

Aggregation assays

Because bed bug aggregations can generate humidified boundary layers (Benoit et al. 2007), we conducted an experiment to determine if bed bugs fed to repletion would produce an aggregation microclimate (*i.e.* raise the humidity) that

increased mortality in fungal-treated, unfed bed bugs kept at a low RH. Ten unfed bed bugs were sprayed with conidia of *M. anisopliae*, as described in the humidity-treatment assays, and kept in filter-paper-lined glass Petri dishes with five untreated, bed bugs that had recently (<2h) fed to repletion. Nine trials were run: 90 unfed bed bugs (sprayed with conidia), and 45 untreated, blood-fed bed bugs were used. Controls consisted of two trials (15 bed bugs/trial) of unfed bed bugs sprayed with conidia. Treated and control groups were kept over a saturated solution of calcium chloride ($25 \pm 2^\circ\text{C}$ and $32 \pm 1\%$ RH) to mimic ambient humidity.

Data analyses

We used a generalized linear model logistic regression (R Core Team, 2013) to test for mean differences in treatment combinations. In this model, humidity level, conidia application methods, and their interaction were independent variables; the binomial dependent variable was the number of dead for each trial. An over-dispersion parameter was included in the model to accommodate the large differences among the replicates of the contact filter treatment method, allowing for trial-to-trial variability. Tests for mean differences were performed using the R multcomp package (Hothorn et al. 2008). In the analysis, for each percent relative humidity the controls for aerosol spray and contact were combined; there was negligible mortality in any control group where bed bugs contacted Tween® 80 by spray or by contact. All results were back-transformed to the original scale (either “proportion dead” or “% mortality”) for clarity.

Results

Ingestion assays

After ingesting spores of *M. anisopliae*, bed bugs died in a dose- and time-dependent manner, proving them innately susceptible to this entomopathogenic fungus (Figure 3-1). Concentrations of 5×10^5 and 1×10^5 spores/ml produced 100% mortality in 72h and 96h, respectively. Bed bugs fed lower concentrations of conidia exhibited lower mortalities; a concentration of 1×10^4 spores/ml produced a maximum mortality of 89% after one week (168h), while a concentration of 1×10^3 spores/ml achieved a mortality of only 26% after one week. Mortality in bed bugs fed blood containing 0.01% Tween® 80 (3 dead of 301; < 1%), or inactivated *M. anisopliae* spores (8 dead of 282; < 3%) was negligible.

Humidity-treatment assays

Bed bug mortality at one week post-exposure to *M. anisopliae* conidia varied, depending largely on the humidity at which the bed bugs were kept (Figure 3-2). By both spray and contact, mortality was greatest in bed bugs kept at 98% RH. Mortality in these groups was 71.1 % (64/90) by spray and 97.8% (88/90) by contact, and these differed from each other and from all other treatments. Mortalities at the two lower humidities never exceeded 25% (25/100; aerosol spray at 74% RH), and all but one were not significantly different from the Tween® 80-treated controls. Mycoses were confirmed (noticeable mycelia growth emanating from appendage joints, *etc.*) in all

treated bed bugs that died. Although a few control bed bugs died, no evidence of fungal infection was found in control groups.

Aggregation assays

Because of the high water content of recently-fed bed bugs, we mixed unfed bed bugs sprayed with *M. anisopliae* conidia with untreated bed bugs recently blood-fed to repletion in an effort to determine if mortality in these treated bugs could be increased over what was observed in the previous experiment at low humidity ($32 \pm 1\%$ RH). Mortality in these fungal-treated, unfed bed bugs that were co-mixed with untreated, blood-fed bed bugs was 11.1% (5.3%, 21.7%; lower and upper 95% confidence intervals, respectively). This was not significantly different than the 13.3% mortality (4.1%, 35.4%; lower and upper 95% confidence intervals, respectively), in the fungal-sprayed, unfed bed bugs kept separate from fed bugs, and similar to the 17.8% mortality we observed in fungal-sprayed bed bugs kept at $32 \pm 1\%$ RH in the previous humidity-treatment assays (Figure 3-2).

Discussion

M. anisopliae exhibits a broad invertebrate host range, making this entomopathogenic fungus an attractive biological control candidate (Faria and Wraight 2007). Use of *M. anisopliae* has been investigated for controlling a number of arthropods of medical and veterinary importance including mosquitoes (Blanford et al. 2005, Scholte et al. 2005, 2006, Mnyone et al. 2009), biting flies (Kaaya and

Munyinyi 1995, Ansari et al. 2011), and ticks (Kaaya et al. 1996, Frazzon et al. 2000, Kaaya and Hassan 2000, Benjamin et al. 2002, Kirkland et al. 2004). Use of entomopathogenic fungi to control urban pests such as termites (Rath 2000, Wang and Powell 2004) and cockroaches (Quesada-Moraga et al. 2004, Hernandez-Ramirez et al. 2008) has also been reported, though laboratory studies indicate termites may possess defensive mechanisms to fungal infection (Chouvenc et al. 2009, Chouvenc and Su 2010). The use of the entomopathogenic fungus *B. bassiana* for bed bug control has been demonstrated in the laboratory (Barbarin et al. 2012). Since we do not know the humidity in the *B. bassiana* experiments, direct comparisons with our study cannot be made, though growth and infection of both fungi can be limited by relative humidity (Maximiano et al. 2006).

In general, outdoor field effectiveness of *M. anisopliae* can be limited by unsuitable physical conditions such as heat, humidity, and sunlight (Jaronski 2010). Thus while laboratory bioassays demonstrate that entomopathogenic fungi have the potential to significantly reduce pest populations, results of field trials have been inconsistent. For instance, Benjamin and colleagues (2002) found *M. anisopliae* killed 96% of adult *I. scapularis* (deer or black-legged) ticks in the laboratory, but only 53% of ticks treated in the field. These authors further caution that the 53% mortality in these ticks may be artificially high since the treated ticks were subsequently maintained in the laboratory at relatively high humidity. The need for high humidities for entomopathogenic fungi to be effective killing agents has been previously demonstrated in two species of beetles (Walstad et al. 1970, Doberski

1981). Our laboratory results with bed bugs also demonstrate that high humidity is necessary for mortality.

Because natural infestations of bed bugs usually contain bed bugs that are of mixed feeding status (unfed to fully-fed), we attempted to mimic field infestations by combining fungal-treated, unfed bed bugs with bed bugs that had recently fed to repletion. Our rationale was that the high water content of blood-fed individuals might raise the humidity of the aggregation microenvironment sufficiently to result in increased mortality in the fungal-treated bed bugs. This was not the case as mortality in these treated bed bugs was not significantly different from treated bed bugs kept with other unfed individuals. A caveat to this conclusion is that we cannot say whether true aggregations formed or whether any avoidance was exhibited by either fungal-infected or non-infected bed bugs. For whatever reason, the microenvironment in the individual dishes did not promote fungal growth in treated bed bugs. A detailed review of insect behavior as it applied to fungal pathogens is given by Baverstock and colleagues (2009).

We conclude that mortality in bed bugs treated with the fungal isolate (ARSEF 1548) of *M. anisopliae* used in this study is humidity dependent. While this fungal pathogen can kill bed bugs, the humidity level required to exceed 70% mortality is deemed impractical under natural, indoor conditions, and that low humidity would impede practical use of this strain of *M. anisopliae* in a control program aimed at these indoor, urban pests. Additional studies are needed to identify other strains of *M. anisopliae* that are less impacted by low humidity. Alternatively, the use of *M.*

anisopliae in settings that possess higher relative humidities, such as poultry houses, might prove feasible (see Oliveira et al. 2014).

Figure legends

Figure 3-1. Mortality in bed bugs fed spores of *Metarhizium anisopliae*. Conidia of *M. anisopliae* were added to blood products to achieve the final concentrations indicated, and fed to bed bugs (see Materials and Methods). Mortality (expressed as proportion dead) was recorded from 1h – 168h (one week) post-feeding. Since bed bugs ingest 2-8 μl /insect (see Usinger 1966) the numbers of spores theoretically ingested at the different concentrations were 1000-4000 spores (at 5×10^5 spores; n = 729 bed bugs); 200-800 spores (at 1×10^5 spores; n = 354 bed bugs); 20-80 spores (at 1×10^4 spores; n = 290 bed bugs); and 2-8 spores (at 1×10^3 spores; n = 483 bed bugs). Control bed bugs (n = 301) fed only 0.01% Tween® 80 exhibited <1% mortality after 168h; bed bugs (n= 282) fed inactivated *M. anisopliae* spores (autoclaved 15m at 121°C) had 2.8% mortality at 168h.

Figure 3-2. Mortality in bed bugs treated with spores of *Metarhizium anisopliae* and kept at different humidities. Bed bugs were treated with *M. anisopliae* conidia by either aerosol spray (= ‘Aerosol’) or by placing the bed bugs on a conidia-treated surface (= ‘Contact’), and kept at three different relative humidities. Mortality (expressed as proportion dead) was assessed one week (168h) post-treatment. Control groups consisted of bed bugs that were either sprayed with 0.01% Tween® or placed on filter paper previously treated with 0.01% Tween® 80. Ninety to 120 bed bugs were used for each humidity treatment, including controls. Vertical bars indicate 95% confidence intervals. Means that differ significantly have a different letters.

Figures

Figure 3-1.

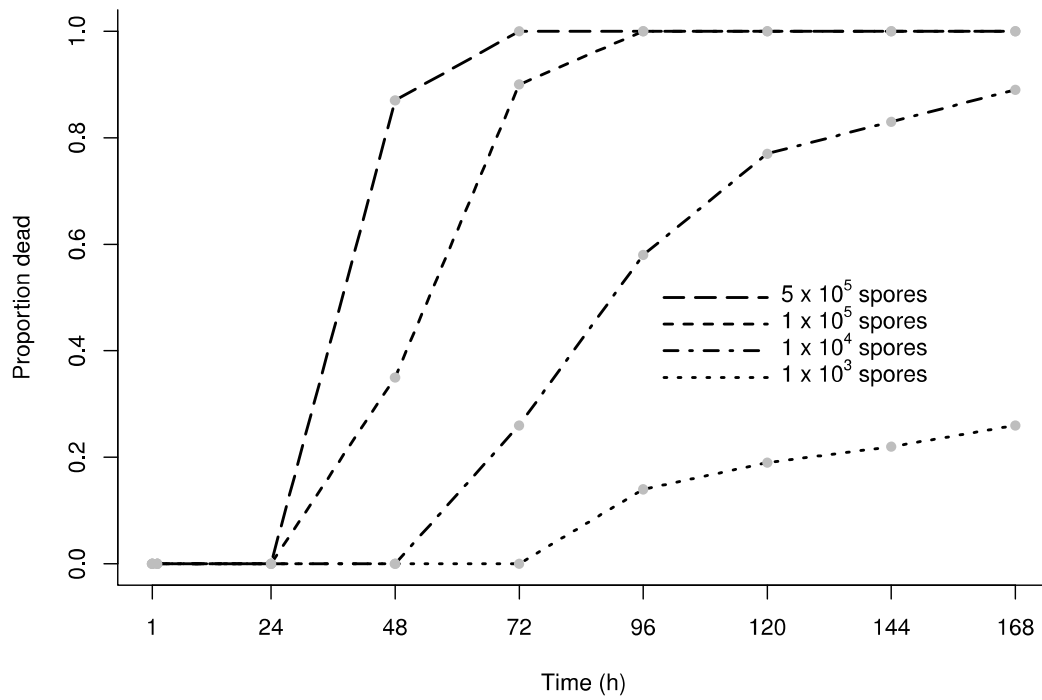
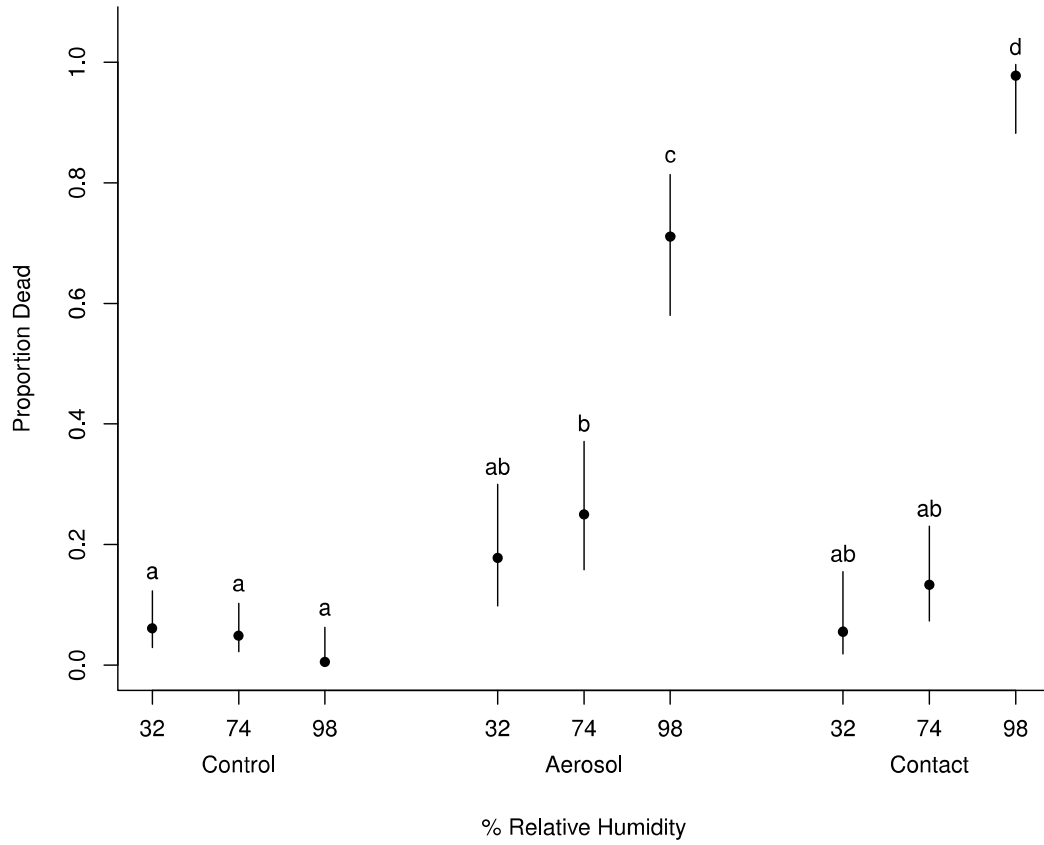


Figure 3-2.



Chapter 4: Inhibition of the entomopathogenic fungus *Metarhizium anisopliae* sensu lato *in vitro* by the bed bug defensive secretions (*E*)-2-hexenal and (*E*)-2-octenal²

Abstract

The two major aldehydes (*E*)-2-hexenal and (*E*)-2-octenal emitted as defensive secretions by bed bugs *Cimex lectularius* L. (Hemiptera: Cimicidae), inhibit the *in vitro* growth of an isolate of *Metarhizium anisopliae* sensu lato (s.l.) (Metsch.) Sokorin (Hypocreales: Clavicipitaceae) (ARSEF 1548). These chemicals inhibit fungal growth by direct contact and via indirect exposure (“fumigation”). Fumigation with 0.5 mg of (*E*)-2-octenal for as little as 0.5h was sufficient to inhibit all fungal growth. Bed bugs placed on filter paper treated with an isolate of *M. anisopliae* s.l. conidia in the absence of (*E*)-2-octenal exhibited 99% mortality after one week. However, bed bugs placed on fungal-treated filter paper and exposed to (*E*)-2-octenal at 1h experienced 10% mortality. The inhibition of fungal growth by bed bug aldehydes is discussed in the context of other biotic and abiotic barriers to infection.

² This chapter has been published as: Ulrich K. R., M. F. Feldlaufer, M. Kramer, and R. J. St. Leger. 2015. Inhibition of the entomopathogenic fungus *Metarhizium anisopliae* sensu lato *in vitro* by the bed bug defensive secretions (*E*)-2-hexenal and (*E*)-2-octenal. *BioControl*. doi:10.1007/s10526-015-9667-2.

Introduction

Entomopathogenic fungi have been used against a variety of insect species (Shah and Pell 2003), with *Beauveria bassiana* (Bals.) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Metsch.) Sokorin (Hypocreales: Clavicipitaceae) representing the active biopesticide in the majority of commercially-developed products for use against most agricultural insect and arthropod pests (Faria and Wraight 2007, Hajek and Delalibera 2009, Vega et al. 2012). In addition to plant pests, entomopathogenic fungi are being developed for control of blood-sucking arthropods, including ticks and mosquitoes (Blanford et al. 2005, Bukhari et al. 2011, Fernandes et al. 2012). *B. bassiana* was shown to be pathogenic to the bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) (Barbarin et al. 2012). Not only was the fungus efficacious, but due to the gregarious nature of bed bugs, horizontal transmission also was observed, as infected individuals could transfer the fungus to uninfected bed bugs.

Various abiotic factors such as temperature, humidity, and sunlight can pose a challenge to the use of entomopathogenic fungi under field conditions (Jaronski 2010). The insect cuticle likewise can pose a physical and chemical barrier to infection by entomopathogenic fungi (Hajek and St. Leger 1994, Wilson et al. 2001, Gołębiowski et al. 2008, Ortiz-Urquara and Keyhani 2013). In this study, we examine another biotic factor, namely chemical defensive secretions, which may impact the ability of an isolate of *M. anisopliae* sensu lato (s.l.) to infect bed bugs. (*E*)-2-hexenal and (*E*)-2-octenal are the major defensive secretions of immature and adult bed bugs

(Schildknecht et al. 1964, Collins 1968, Levinson et al. 1974,). These aldehydes also are considered to act as pheromones, affecting bed bug behavior (Levinson and Bar Ilan 1971, Levinson et al. 1974, Siljander et al. 2008, Benoit et al. 2009, Harraca et al. 2010). Kilpinen and colleagues (2012) reported that one bed bug could release as much as 40 µg of these aldehydes in a single emission. One or both of these aldehydes are found in several other heteropteran species (Aldrich 1988), where they act not only as pheromones, but as kairomones, attracting parasitoids (Vieira et al. 2014). (*E*)-2-hexenal and (*E*)-2-octenal also are found in some plants, where they have been shown to exhibit antibacterial and antifungal activity (Kubo and Kubo 1995, Bisignano et al. 2001, Trombetta et al. 2002, Battinelli et al. 2006, Cleveland et al. 2009).

In this study, we report that (*E*)-2-hexenal and (*E*)-2-octenal, can prevent the *in vitro* growth and development of an isolate of entomopathogenic fungus *M. anisopliae* s.l., and may be a barrier to infection of bed bugs with this fungus.

Materials and methods

Chemicals

(*E*)-2-Hexenal and (*E*)-2-octenal were obtained from Bedoukian Research, Inc. (Danbury CT). Their purity by gas chromatography was 99.7% and 97.6%, respectively. Tween[®] 80 was from Sigma-Aldrich (St. Louis MO), and spectral grade

acetone (Honeywell Burdick & Jackson, Morristown NJ) was used for all dilutions of aldehydes.

Fungal isolates

An isolate of wild-type *M. anisopliae* s.l. (ARSEF 1548) was obtained from the United States Department of Agriculture Entomopathogenic Fungus Collection in Ithaca, N.Y. This strain originally was isolated from the rice black bug *Scotinophara coarctata* (Hemiptera: Pentatomidae). Fungal cultures were maintained on Difco™ potato dextrose agar (PDA; Becton-Dickinson, Sparks MD) and incubated at room temperature for 14 days. Conidia were harvested by scraping colonies with a sterile spatula and suspending in distilled water containing 0.01% Tween® 80. Spore suspensions were determined using a hemocytometer (Spencer, Buffalo NY) and adjusted to a concentration of 1×10^7 conidia/ml. Conidial viability was determined to be above 90% for all bioassay and germination studies.

In vitro fungal growth when exposed to (E)-2-hexenal and (E)-2-octenal

To determine the effect of bed bug aldehydes on fungal growth *in vitro*, sterile 6 mm disks (Whatman, Grade AA) were impregnated with dilutions of either (E)-2-hexenal or (E)-2-octenal in acetone to yield 0.1, 0.25, 0.33, 0.5, 1, 5, 10, or 20 mg of aldehyde/disk. After drying, disks were placed in the center of a PDA plate previously inoculated with 25 μ l of fungal suspension (10^7 conidia/ml). To determine any fumigant action these aldehydes might have, filter paper disks were prepared as above, and then placed on the inner lid that covered fungal-inoculated PDA plates; in

this way, aldehyde-treated disks did not come into direct contact with the inoculated plate. Controls consisted of inoculated plates exposed to untreated (acetone) disks, either by direct contact or by fumigation. All cultures were kept at room temperature and humidity ($25 \pm 2^\circ\text{C}$ and $30 \pm 5\%$ RH) for 72 h at which time fungal growth was visually assessed by measuring any zone of inhibition in the inoculated plates. Each experiment was replicated four times.

To determine the minimum exposure time required for *M. anisopliae* s.l. inhibition by fumigation, sterile 6 mm disks were impregnated with 0.5 mg (*E*)-2-octenal, the minimal amount needed to suppress all *M. anisopliae* s.l. growth (from the preceding experiment). After drying, the treated disks were placed on the inner lid that was used to cover a series of PDA plates that had each been inoculated with 25 μl of fungal suspension (10^7 conidia/ml). Exposure to octenal was terminated at various times (0.25, 0.5, 1, 2, and 3 h and 24 h) post-exposure, by removing the lids containing the treated disks and replacing them with lids containing no disk. Plates were incubated at room temperature for 72 h and fungal growth assessed, as above. Controls consisted of inoculated plates exposed to untreated (acetone) disks. Each experiment was replicated four times, and kept at the same conditions as above.

Plates inoculated with *M. anisopliae* s.l. also were exposed to 0.5 mg octenal at various times post-inoculation, to determine fungal growth, if any, when not exposed to octenal for a prescribed period of time. This was accomplished by inoculating plates with 25 μl of fungal suspension (10^7 conidia/ml), covering these plates with lids containing no disk, and then replacing these lids with lids containing

octenal-treated (0.5 mg) disks at 0.5, 1, 2, 3, 4, 6, 12, 24, and 48 h post-inoculation. Each experiment was repeated four times and plates were kept and assessed as noted above.

Effects of (E)-2-octenal on M. anisopliae s.l. germination

To determine conidial germination in the presence of octenal, plates were inoculated with 2.5×10^4 *M. anisopliae* s.l. conidia and exposed to 0.5 mg octenal at 6 or 12 h post-inoculation by placing lids containing octenal-treated disks (0.5 mg) at either 6 h or 12 h post-inoculation. Germination was observed under 400x magnification at 6, 12, 24, and 48 h from the time the conidia were placed on medium. Conidia with germ tubes larger than twice the diameter of the conidium were considered to have germinated (Inglis et al. 2012). Each treatment was repeated four times. 100 conidia per replicate were counted, and mean germ tube lengths were determined for each treatment. Controls consisted of inoculated plates exposed to untreated (acetone) disks.

Bed bugs

A colony of *C. lectularius* was established from bed bugs originally obtained from Harold Harlan (Crownsville, MD). The colony was kept at ambient conditions ($25 \pm 2^\circ\text{C}$ and $30 \pm 5\%$ RH) and fed weekly on expired, human red blood cells and plasma using an artificial (*in vitro*) feeding system (Feldlaufer et al. 2014). Adult males and females were used for the experiments and had not been fed for eight days prior to use.

Exposure of bed bugs to M. anisopliae s.l. and (E)-2-octenal

Bed bugs were exposed to *M. anisopliae* s.l. by being placed on filter paper previously treated with conidia. In these experiments, conidial suspensions in sterile water containing 0.01% Tween® 80 were applied to 47 mm (dia.) filter paper disks (Whatman No. 1) to yield a final concentration of 1×10^5 conidia/cm². Filter papers were allowed to dry for 120 min before being placed in glass Petri dishes (60 x 15 mm). 15 adult male and female bed bugs were then placed on the dried, treated surface. One treatment group remained as such (fungal-exposed control). Two additional treatment groups of fungal-exposed bed bugs had a disk containing 0.5 mg octenal added to the dish at 1 h and 24 h post-exposure, respectively. Additional control groups consisted of bed bugs placed on 0.01% Tween® 80-treated filter paper (untreated), and another fungal treatment group was removed to an untreated surface 24 h post-exposure. To determine toxicity, if any, to octenal, bed bugs were placed on untreated filter paper to which a disk containing 0.5 mg octenal was added. Each treatment consisted of three replicates and the experiment was repeated twice by preparing new aldehyde dilutions and new fungal suspensions from separate fungal culture plates.

In these experiments, bed bugs were kept in glass desiccators (150 mm i.d.; Fischer Scientific, Pittsburgh, PA) over distilled water ($98 \pm 1\%$ RH) at $25 \pm 2^\circ\text{C}$, and mortality was assessed at one week post-treatment. We had previously shown (Ulrich et al. 2014) that this humidity was necessary to cause a >98% infection rate in fungal-

treated bed bugs. Mycosis was confirmed in all treated bugs that died, by maintaining them at 100% RH for an additional week and viewing them under a stereomicroscope (see Lacey and Solter 2012). Temperature and RH were verified by Traceable relative humidity-temperature meters (Fisher, Pittsburgh, PA).

Statistical analysis

For treatment comparisons for the mortality and germination data, R statistical software (R Core Team 2014) was used to fit a generalized linear model (Bates et al. 2014), where we assumed the sampling distribution was an over-dispersed binomial (quasi-binomial); the over-dispersion scale factor was estimated at 1.48 for the mortality data and 1.93 for the germination data. Germination tube length was approximately Gaussian following log transformation and a linear model was fit, which included a time, treatment, and time by treatment interaction effect. Mean separations were done using the “multcomp” package (Hothorn et al. 2008) using "single-step"; adjusted p -values based on the joint normal or t distribution of the linear function.

Results

In vitro fungal growth when exposed to (E)-2-hexenal and (E)-2-octenal

The two aldehydes inhibited *in vitro* growth of *M. anisopliae* s.l. conidia by both direct contact (when a treated disk was placed on the growth media) and by

fumigation (treated disk placed on the inner cover, not directly contacting the conidia) (Table 4-1). At all exposures, there was either total inhibition of fungal growth (no evidence of hyphal growth) or unrestricted fungal growth (no apparent inhibition or zone of inhibition), when the plates were examined 72 h post-exposure (Figure 4-1a and b). (*E*)-2-hexenal inhibited all fungal growth either by contact or fumigation at amounts of 1.0 mg and above, while (*E*)-2-octenal appeared to be somewhat more active, inhibiting all fungal growth at 0.5 mg and above. Control dishes treated directly or indirectly with acetone-treated disks showed no inhibition of fungal growth.

Using the lowest amount of (*E*)-2-octenal that exhibited total inhibition (0.5 mg/disk) of *M. anisopliae* s.l. by fumigation, we found that all exposure times greater than 0.25 h resulted in total inhibition of fungal growth (Table 4-2). However, when conidia were allowed to propagate prior to the addition of octenal disks (0.5 mg/disk), fungal growth was only observed when the disks were added at 12 h or later. Interestingly, addition of (*E*)-2-octenal to inoculated plates 12 h post-inoculation was the only instance where we observed zones of inhibition (Figure 4-1c). As stated above, in all other experiments, regardless of (*E*)-2-octenal amounts or exposure times, only total inhibition or unrestricted growth was observed.

Effects of (E)-2-octenal on M. anisopliae s.l. germination

We observed that exposure of *M. anisopliae* s.l. conidia to octenal affected germination (Figure 4-2). Germination was significantly delayed (GLM based on quasi-binomial distribution: $\chi^2 = 2551.4$; $df = 6$; $P < 0.001$) (Table 4-3). Conidia

exposed to octenal 6 h after initial inoculation exhibited no germination after 12 h compared to controls that had 80% germination (Table 4-3). When exposed to octenal at 12 h post-inoculation, conidia had significantly less germination at 24 h than controls (85.2% vs 98.0%, respectively). Additionally, aldehyde-exposed conidia had shorter germ tube lengths compared to controls at 24 and 48 h (GLM based on Gaussian distribution: $F = 3534.6$; $df = 4, 495$; $P < 0.001$) (Table 4-4).

Exposure of bed bugs to *M. anisopliae* s.l. in the presence of octenal

There was a significant relationship between mortality of bed bugs exposed by contact to *M. anisopliae* s.l. and the presence (*E*)-2-octenal (GLM based on quasi-binomial distribution: $\chi^2 = 407.0$; $df = 5$; $P < 0.001$). Bed bugs contacting fungal-treated surfaces in the absence of octenal experienced high mortalities, depending on whether they were removed from the treated surfaces at 24 h (79%) or remained on the treated surface for one week (99%) (Table 4-5). However, we observed lower mortalities in bed bugs exposed to *M. anisopliae* s.l. in the presence of (*E*)-2-octenal. Mortality was 10% when an (*E*)-2-octenal-treated disk was added to fungal-exposed bed bugs at 1 h, while mortality rose to about 33% when the octenal-treated disk was added at 24 h. Little or no mortality was observed in bed bugs not exposed to fungal-treated filter paper, even when (*E*)-2-octenal was added to the Petri dish.

Discussion

In addition to the barriers posed by the cuticle and insect immune system (Vilcinskis and Götz 1999, Wang and St. Leger 2005), other biotic factors can limit the effectiveness of pathogens. For instance, termites show an array of behavioral and biochemical defenses to thwart *M. anisopliae* infection (Myles 2002, Chouvenec and Su 2010). Likewise, insect defensive secretions previously have been shown to inhibit both bacterial and fungal growth *in vitro*. Salicylaldehyde, released by larvae of the brassy willow leaf beetle, is toxic to the entomopathogenic bacteria *Bacillus thuringiensis* (Gross et al. 2008), and (*E*)-2-decenal, a primary aldehyde component of the stink bug *Nezara viridula* scent gland, is fungistatic toward *M. anisopliae* (Sosa-Gomez et al. 1997). Our results indicate that these two, structurally-related aldehydes (*E*)-2-hexenal and (*E*)-2-octenal, considered the primary defensive secretions of bed bugs, inhibit the *in vitro* growth of *M. anisopliae* s.l. (ARSEF 1548), and may play a part in disinfecting a bed bug's microenvironment consistent with the action of a defensive secretion. Interestingly, Sosa-Gomez et al. (1997) demonstrated that (*E*)-2-decenal was fungistatic against *M. anisopliae*, but not against *B. bassiana*. This finding may account for the differential susceptibility of bed bugs when comparing our results with those of Barbarin and colleagues (2012).

Our microscopy results indicate that germinated conidia require a period of time to recover from aldehyde exposure before the germination process can resume. Aldehydes are common disinfectants, able to alkylate fungal proteins and DNA (Fernandes et al. 2012). Mutations caused by alkylating agents can induce DNA

repair responses delaying germination, similar to what was reported regarding delayed germination after exposure to UV radiation (Braga et al. 2001). Compared to dormant conidia, metabolically active, germinating conidia may be better able to repair cellular damage more rapidly. This would account for why conidia were able to grow when exposed to octenal 12 h after inoculation but not at 6 h. Our observations on *in vitro* conidial growth of *M. anisopliae* s.l. indicate that the 12-24 h timeframe is critical with regard to exposure to (E)-2-octenal.

While exposure of bed bugs to an isolate of *M. anisopliae* s.l. for 24 h is sufficient to cause 99% mortality under our experimental conditions, we observed significantly lower mortality when octenal-treated disks were added to fungal-exposed bed bugs at 1 h post-exposure. In addition, while mortality in bed bugs increased when the octenal-treated disk was added at 24 h, the level was still significantly lower than in fungal-treated control bed bugs receiving no aldehyde exposure. In research with two species of termites, and four species of beetles, *M. anisopliae* enters the conidial germination phase of host colonization at approximately 12-24 h post-inoculation (McCauley and Zacharuk 1968, Hänel 1982, Moino et al. 2002). Zimmermann (2014) also states that germination of *M. anisopliae* conidia generally takes place about 20 h after contacting the cuticle of a susceptible insect. Whether these observations with other insect species also apply to bed bugs is speculative. We likewise recognize that attempts to correlate germination timing of our *in vitro* results with *in vivo* applications to bed bugs can be problematic, as insect host cuticle can present a very different substrate, as pointed out in aphid studies with entomopathogens (Yeo et al 2003). Regardless, our study demonstrates that exposure

of conidia from an isolate of *M. anisopliae* s.l. (ARSEF 1548) to bed bug defensive secretions inhibits fungal development both *in vitro* and *in vivo*, and these chemicals may play a role in an insects' defense to fungal biopesticides.

Tables

Table 4-1. Inhibition of *M. anisopliae* s.l.¹ (ARSEF 1548) by (E)-2-hexenal and (E)-2-octenal.

Aldehyde	Amount ² (mg)								Control
	20	10	5.0	1.0	0.5	0.33	0.25	0.1	
<i>(E)</i> -2-hexenal									
Contact	- ³	-	-	-	+	+	+	+	+
Fumigation	-	-	-	-	+	+	+	+	+
<i>(E)</i> -2-octenal									
Contact	-	-	-	-	-	+	+	+	+
Fumigation	-	-	-	-	-	+	+	+	+

¹Plates inoculated with 25 µl of a fungal suspension containing 1 x 10⁷ conidia/ml.

²6mm disk treated with either (E)-2-hexenal or (E)-2-octenal in acetone.

³ - = inhibition; + = growth

Table 4-2. Suppression of *M. anisopliae* s.l. (ARSEF 1548)¹ exposed to (E)-2-octenal at various times.

<u>Time (h)</u>	<u>Disk removed at²</u>	<u>Disk added at³</u>
0.0	+ ⁴	nd
0.25	+	nd
0.5	-	-
1	-	-
2	-	-
3	-	-
4	nd	-
6	nd	-
12	nd	± ⁵
24	-	+
48	nd	+

¹Agar plates inoculated with 25 µl of a *M. anisopliae* s.l. suspension containing 10⁷ conidia/ml.

²6mm disks treated with 0.5 mg (E)-2-octenal were placed on the inside lids of agar plates at the time of inoculation (0.0 h), and removed at various times post-exposure by replacing the lid containing the octenal-treated disk with a lid containing no disk.

³6mm Disks treated with 0.5 mg (E)-2-octenal were placed on the inside lids of agar plates at various times post-inoculation.

⁴ + = growth; - = inhibition; nd = not determined

⁵the only time a zone of inhibition was observed (see Figure 4-1).

Table 4-3. Percent mean germination¹ of *M. anisopliae* s.l.² observed after 6, 12, and 24 h of incubation after exposure to (E)-2-octenal at 6 or 12 h after initial inoculation.

Exposure to aldehyde added at time (h) ³	Total hours incubated		
	6h	12h	24h
Control	1.0 (0.2, 3.0) ^a	80.0 (74.2, 85.1) ^b	98.0 (95.5, 99.4) ^c
6h	0	0	1.0 (0.2, 3.0) ^a
12h	0.8 (0.1, 2.6) ^a	80.8 (75.0, 85.7) ^b	85.3 (80.0, 89.6) ^b

¹Each mean percentage represents the average of 4 replicates, with 100 conidia evaluated per replicate. Conidia with germ tubes larger than the diameter of the conidium were considered to have germinated. 95% confidence intervals are shown in parentheses.

²PDA plates inoculated with 25 µl of a *M. anisopliae* s.l. suspension containing 1 x 10⁶ conidia/ml.

³6mm Disks treated with 0.5 mg (E)-2-octenal were placed on the inside lids of agar plates at various times post-inoculation.

Different superscripts indicate means that differ significantly at $p \leq 0.05$.

Table 4-4. Mean germ tube length¹, expressed as log μm , of *M. anisopliae* s.l. (ARSEF 1548)² observed after 12, 24, and 48 h of incubation after exposure to (E)-2-octenal at 6 or 12h after initial inoculation.

Exposure to aldehyde added at time (h) ³	Total hours incubated		
	12h	24h	48h
Control	2.2 (2.1, 2.3) ^a	4.2 (4.1, 4.3) ^c	nd
6h	0	0	0
12h	2.0 (1.9, 2.1) ^a	2.0 (1.9, 2.1) ^a	3.6 (3.5, 3.7) ^b

¹100 germinated conidia evaluated per treatment. Data were subjected to log transformation before statistically analyzed. 95% confidence intervals are shown in parentheses.

²PDA plates inoculated with 25 μl of a *M. anisopliae* s.l. suspension containing 1×10^6 conidia/ml.

³6mm Disks treated with 0.5 mg (E)-2-octenal were placed on the inside lids of agar plates at various times (in hours) post-inoculation.

Different superscripts indicate means that differ significantly at $p \leq 0.05$.

Table 4-5. Mortality (%) of bed bugs exposed to *M. anisopliae* s.l. (ARSEF 1548) by contact in the presence of octenal¹.

	<u>Treatment</u>		
	<u>Fungus (continuous)</u>	<u>Fungus (24h only)</u>	<u>No Fungus</u>
<u>Octenal disk</u>			
Added at 1h	10.0 (4.6, 20.4) ^a	nd	2.2 (0.4, 11.1) ^a
Added at 24h	33.3 (22.7, 46.0) ^b	nd	nd
Not added	98.9 (89.0, 99.9) ^c	78.9 (66.9, 87.4) ^c	1.1 (0.1, 11.0) ^a

¹Bed bugs (n = 15 bugs/group; 6 replicates pooled from two experiments) were placed on filter paper treated with *M. anisopliae* s.l. conidia (1 x 10⁵ conidia/cm²) for either 168 h (one week; ‘continuous’) or 24 h (‘24h only’). Disks containing octenal (0.5 mg octenal/disk) were added at the prescribed times. Mortality assessed at one week post-exposure. All bed bugs kept at 25 ± 2°C and 98 ± 1% RH.

Different superscripts indicate means that differ significantly at p ≤ 0.05. 95% confidence intervals are shown in parentheses.

Figure legends

Figure 4-1. Representative fungal growth on agar plates exposed to either (E)-2-hexenal or (E)-2-octenal. **(a)** unrestricted growth; **(b)** total inhibition of growth. Partial inhibition of growth **(c)** only occurred when an octenal-treated (0.5 mg) disk was added to a plate inoculated with *M. anisopliae* s.l. (ARSEF 1548) conidia 12 h previously.

Figure 4-2. Germination of *M. anisopliae* s.l. (ARSEF 1548) conidia in the presence of (E)-2-octenal. **(a-c)** germination of controls (no aldehyde) at 12, 24 and 48 h respectively; **(d-f)** germination of conidia exposed to aldehyde at 6h post-inoculation at 12, 24 and 48 h after initial inoculation respectively; **(g-i)** germination of conidia exposed to aldehyde at 12 h post-inoculation after 12, 24 and 48 h after initial inoculation respectively. Bar = 25 μ m.

Figures

Figure 4-1.

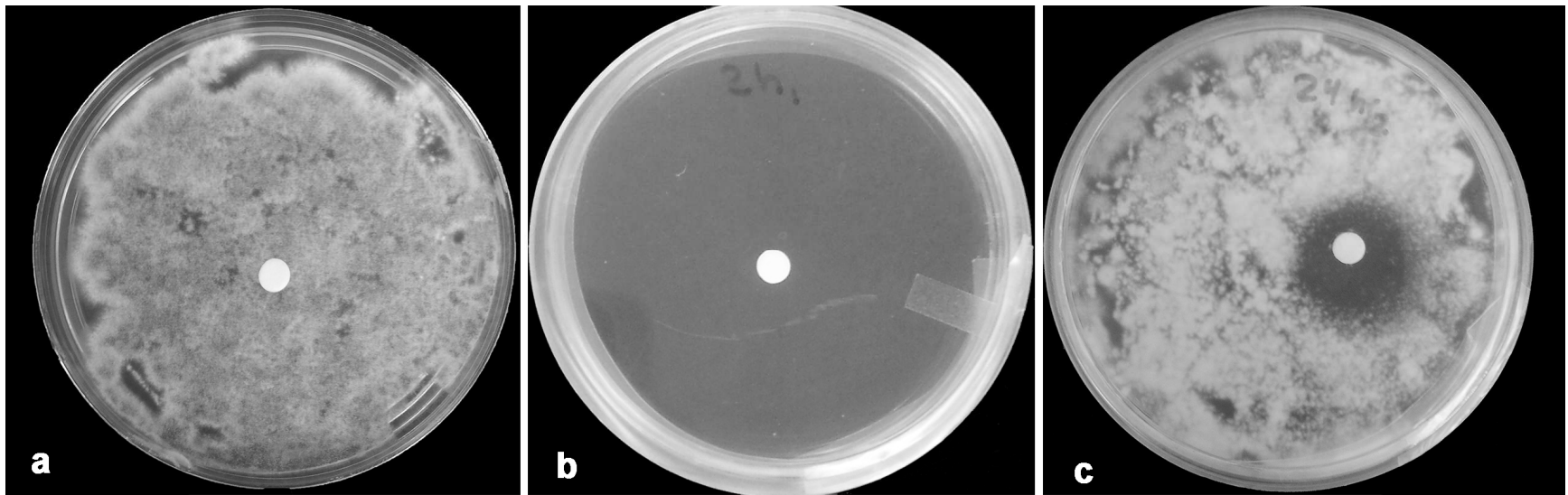
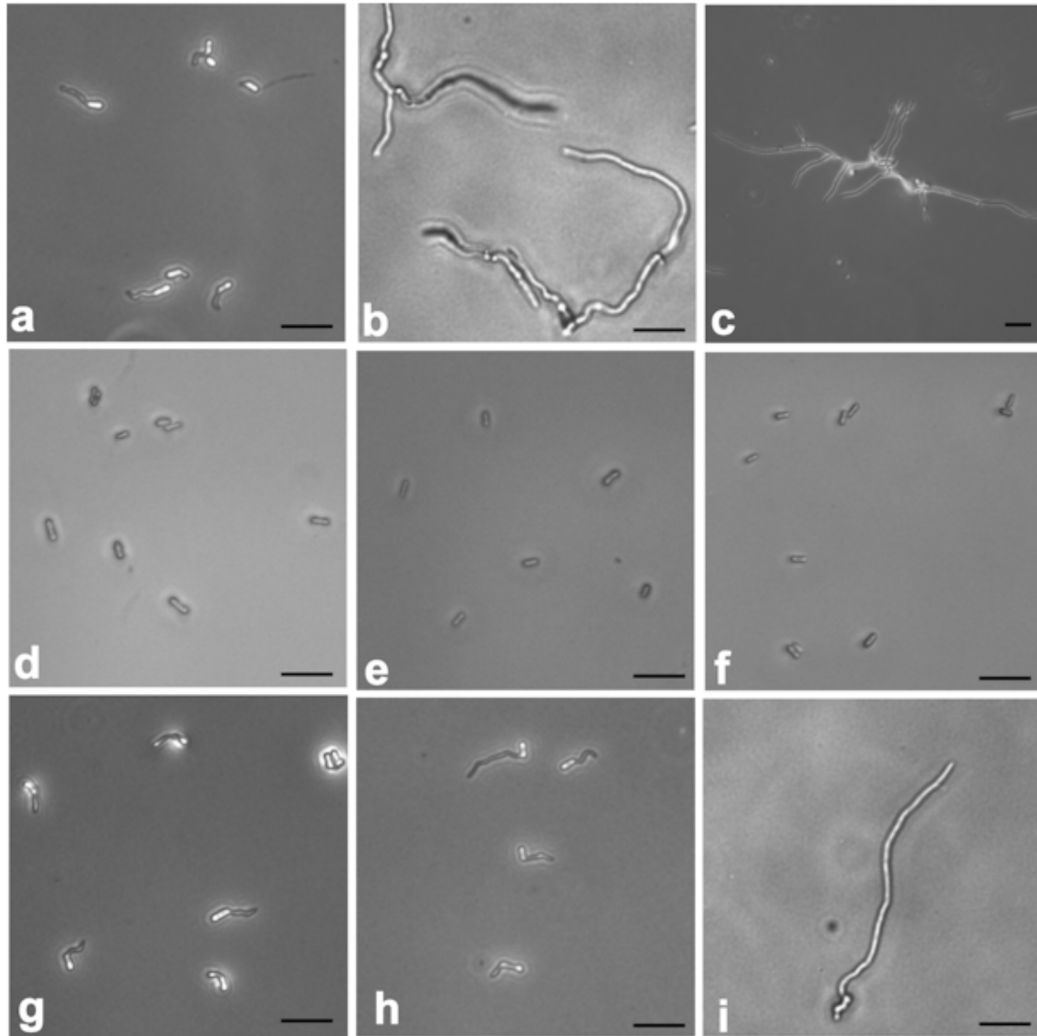


Figure 4-2.



Chapter 5: Effect of the bed bug defensive secretions (E)-2-hexenal and (E)-2-octenal on movement and questing patterns of adult bed bugs

Abstract

Accurate and timely surveillance of bed bug infestations is critical for development of effective control strategies. We show through use of EthoVision® video-tracking software, that the commercially-obtained aldehydes, (E)-2-hexenal and (E)-2-octenal attract adult bed bugs at certain concentrations. Behavioral assays determined both males and females were attracted to 0.04 µg of aldehyde blend for up to two hours after initial treatment of filter paper disks. Males differed from females in their response to intermediate amounts of aldehyde but both sexes showed similar responses at high amounts (400 µg) compared to acetone controls. Results suggest that these bed bug secretions may be candidates for lures and monitors.

Introduction

Cimex lectularius L., the common bed bug, is a nocturnal ectoparasite of humans that feeds solely on blood. The pest's cryptic nature makes it difficult to detect infestations, and renders eradication both challenging and expensive, as

commercial treatments can be prohibitively costly. Do-it-yourself products such as foggers are ineffective (Jones and Bryant 2012), and bed bug-related insecticide misuse has been documented to cause illnesses and at least one death (CDC 2011). Failure to quickly and adequately treat infestations enables bed bugs to spread to new areas of a home or building, thereby exacerbating control efforts (Wang et al. 2010). For this reason, early detection of bed bug populations is critical.

Effective tools and methods to augment visual inspections are valuable for bed bug detection. Canines can be trained to alert to bed bugs (Pfiester et al. 2008, Vaidyanathan and Feldlaufer 2013). However, lack of standardized training and handler inexperience can affect detection accuracy (Cooper et al. 2014). Various passive (without lure or bait) and active (with attractant) monitors can be used to detect bed bugs (Vaidyanathan and Feldlaufer 2013). Wang et al. (2010) concluded passive monitors could more efficiently detect an infestation than visual inspections. Active monitors use either host-specific cues such as carbon dioxide, octenol, and lactic acid (Wang et al. 2011, Singh et al. 2012, Aak et al. 2014) or bed bug-derived components from scent glands and fecal excrement (Olson et al. 2009, Weeks et al. 2013, Gries et al. 2014) to attract bugs. No type of active or passive monitor has gained widespread use, however, due primarily to inconsistencies under field conditions.

Bed bug aggregation is likely mediated by pheromone cues (Marx 1955, Siljander et al. 2007, Weeks et al. 2011). The metathoracic scent glands of adults predominantly produces the aldehydes (E)-2-hexenal and (E)-2-octenal (Schildknecht

et al. 1964) with (E)-2-hexenal produced at a rate three times higher than the other main aldehyde (Collins 1968, Levinson and Bar Ilan 1971). However, in nymphs (E)-2-octenal is three times more abundant than (E)-2-hexenal (Levinson et al. 1974). At high concentrations, these aldehydes apparently induce dispersal and alarm responses (Levinson and Bar Ilan 1971). Those two aldehydes, along with associated 4-oxo-aldehydes (Feldlaufer et al. 2010), also play a role in predator avoidance, defense, and as conspecific cues regarding mating (Usinger 1966, Ryne 2009, Harraca et al. 2010).

Accurate and precise surveillance of bed bug infestation is an essential first step in development of a management strategy. To be commercially viable, a lure must attract bed bugs regardless of sex or life stage, be persistent, and require minimal care and follow up from a technician or applicator between visits. While several compounds have been identified and tested in laboratory and field conditions, there are gaps in our understanding of how bed bugs behave and interact with aldehydes at different concentrations. The purpose of this study was to analyze movement and questing patterns of adult bed bugs to (1) determine a dose response of aldehyde blends that are consistently attractive to bugs and (2) determine the time-frame these volatile compounds remain effective as an attractant.

Materials and methods

Insects

A colony of *C. lectularius* was established from bugs originally obtained from Harold Harlan (Crownsville, MD). The colony was reared at ambient conditions ($25 \pm 2^\circ\text{C}$ and $40 \pm 15\%$ RH) and fed weekly on expired, human red blood cells and plasma using an artificial (*in vitro*) feeding system (Feldlaufer et al. 2014). For the assays described below, individual adults of both sexes were used, and had not been fed for 8 d prior to use.

Chemicals

(*E*)-2-Hexenal and (*E*)-2-octenal were obtained from Bedoukian Research, Inc. (Danbury CT). Their purity was determined by gas chromatography to be 99.7% and 97.6%, respectively. Spectral grade acetone (Honeywell Burdick & Jackson, Morristown NJ) was used for all dilutions of aldehydes.

Behavioral bioassays

Uncovered glass Petri dishes (150 x 20 mm), with bottoms lined with filter paper (Whatman No. 1) were used as assay arenas in all experiments involving bed bug behavior. Each arena was divided into four equal zones, and further delineated into semi-circle subzones (40 mm diameter; Fig. 5-1). One treated and three untreated 6 mm disks (Whatman, Grade AA) were placed at the perimeter of each arena at the

3, 6, 9, and 12 o'clock positions (Figure 5-1). Following a 5 min acclimation period, individual adult bed bugs were released in the center of the arena and their movements were recorded for 1 h. To minimize positional effects, the location of the aldehyde-treated disk was repositioned for each replication. Bugs were used only once. Filter paper bottoms of the arena were replaced and Petri dishes cleaned with acetone after each trial to reduce possible effects of defecation or other cues left by previous occupants.

Treated disks were impregnated with a mixture of (E)-2-hexenal and (E)-2-octenal (1:1) in acetone to yield 0.004, 0.04, 0.4, 40, or 400 μg of aldehyde blend/disk. Sixteen replicates (eight per gender) were conducted for each amount. Acetone was used to treat control disks.

To assess duration that the aldehyde blend remained attractive, 0.04 μg of the aldehyde blend was used. Treated disks were assayed in the arena at 0, 1, 2, 3, or 24 h post-treatment. Twelve replicates (six per gender) were used for each time point. An acetone-treated disk served as a control.

Video analysis

A high-resolution FireWire monochrome camera (Noldus Part No. XVID-002A) with a varifocal lens (Computar model H2Z0414C-MP, 4-8 mm, F 1.4) was used to monitor bed bug movement. The camera was suspended 30 cm above the arena, and light for the recordings was provided by an IR LED illuminator (Axton model AT-8) attached to the camera.

All video tracking was saved to a computer and analyzed using EthoVision® XT automatic tracking software (Version 10, Noldus Information Technology Inc., Leesburg, VA). Bed bugs were tracked at a sampling rate of 15 samples/sec. A threshold movement of 0.25 cm was used as an input filter to eliminate system noise or slight movements not associated with locomotion. A standard calibration of the arena was completed to enable EthoVision® to convert distance between two points from pixels to x, y coordinates. Data was visualized as movement paths (tracks) or as graphical representations of the subject's position where frequency of specific positions are represented as colors ("heat maps"). "Heat maps" facilitated identification of clustered data points.

Thirteen parameters were used as behavioral descriptors including: (1) minimum distance to treatment disk; (2) mean distance to treatment disk; (3) total distance to treatment disk; (4) velocity in treatment zone; (5) duration not moving in the treatment zone; (6) frequency of stopping in the treatment zone; (7) mean time in treatment zone; (8) total time in treatment zone; (9) frequency of entering treatment zone; (10) frequency of movement starts in the treatment subzone; (11) mean time in treatment subzone; (12) total time in treatment subzone; (13) frequency of entering subzone. "Moving" and "not moving" were defined with a threshold at 1 and 0.5 cm/sec. All units were in cm and sec. Additional variables captured by EthoVision® were determined to be highly correlated with the above parameters and were removed from analysis.

Statistical analysis

Data were analyzed as described by Kramer et al. (2009), using canonical discriminant analysis to construct a weighting system for concurrently measured behavioral variables. Optimal weightings of behaviors (*i.e.* those that best differentiate among the treatments) are used to determine composite scores. Bed bugs that behave similarly will produce similar composite scores, while treatment groups inducing different behaviors will produce different composite scores.

First, a stepwise procedure (SAS “proc stepdisc;” SAS Institute Inc., Cary NC) was used to identify a subset of recorded parameters that were useful to separate responses to stimuli. These variables were standardized (“z-score”) so each would contribute evenly to a scale, making interpretation more meaningful. Next, discriminant analysis (SAS “proc candisc” function) was conducted using the subset of variables to obtain weights and create composite scores. Composite scores were produced using only the first canonical discriminant function, which consisted of the sum of these variables with weights from the first axis that best separated the compounds.

For these composite scores, both the Anderson–Darling test for normality ($P > 0.05$) and the Bartlett test of homogeneity of variances ($P > 0.05$) were met for all treatment groups. Thus, we used a parametric ANOVA test in R (R Core Team, 2013) to determine whether compounds differed in their effects. A two-way factorial design was used to compare male and female composite scores. Mean separations were done using the “multcomp” package in R (Hothorn et al. 2008) using “single-step.”

Results

Visualization of bed bug tracks showed differences in movement paths and time spent in zones relative to aldehyde-treated disks (Figure 5-2). The discriminant analyses produced ‘optimal’ weights to best differentiate and order the stimuli based on the bed bugs’ measured behaviors. The discriminant function gave greater weight to behaviors that better separated the responses to different stimuli. The weights of individual bed bug behaviors for composite score construction using all treatment groups are given in Table 5-1.

Of the 13 parameters followed, four were found to be useful for the discriminant function, when examining different amounts of the aldehyde blend (Table 5-1). The most important behavior contributing to the composite score was mean distance to treated disk (partial $R^2 = 0.28$); the next three were velocity in treatment zone (partial $R^2 = 0.12$); mean time in treatment zone (partial $R^2 = 0.09$); and minimum distance to treated disk (partial $R^2 = 0.09$).

Based on composite scores, there was a significant effect of aldehyde amount ($F = 8.05$; $df = 5, 84$; $P < 0.001$) and sex-specific responses ($F = 6.95$; $df = 1, 84$; $P = 0.010$) with no significant interaction between aldehyde amount and sex. The male cohort separated the 0.04 and 0.4 μg aldehyde amounts from the acetone control ($P < 0.05$) (Figure 5-3). The 0.04 μg amount in the female cohort was significantly different ($P < 0.05$) from the acetone control and 40 and 400 μg amounts (Figure 5-4).

The axis appears to order amounts by their attractiveness (i.e. higher composite score).

For the time delay assays, where a bed bug was exposed to a treated disk at different times post-treatment, four variables were used to construct the discriminant function (Table 5-1). While two parameters (velocity in the treatment zone, and mean time in treatment zone) were the same as in the previous bioassay, two others were different. Total time spent in treatment zone contributed most to the score (partial $R^2 = 0.40$); followed by velocity in treatment zone (partial $R^2 = 0.17$); duration not moving in treatment zone (partial $R^2 = 0.14$); and mean time in zone (partial $R^2 = 0.13$). ANOVA showed a difference in score depending on when the treated disk was placed in the arena ($F = 10.23$; $df = 5, 58$; $P < 0.001$). Gender of the bug was not statistically significant ($F = 0.18$; $df = 1, 58$; $P = 0.677$). Only the 0 and 1 h delay differed significantly ($P < 0.05$) from the acetone control (Figure 5-5).

Discussion

Bed bugs in our study were attracted to 0.04 μg of a mixture of the two major bed bug defensive secretions, (E)-2-hexenal and (E)-2-octenal. This response is consistent with aggregation cues produced by low concentrations of bed bug volatiles (Levinson and Bar Ilan 1971, Olson et al. 2009, Weeks et al. 2013). Siljander et al. (2008) reported that a mixture of eight additional compounds in addition to (E)-2-hexenal and (E)-2-octenal were essential to *C. lectularius* aggregation. A more recent

report (Gries et al. 2014), however, found five compounds, including (E)-2-hexenal and (E)-2-octenal, necessary for aggregation. We found a mixture of (E)-2-hexenal and (E)-2-octenal sufficient to induce attraction. We also observed a gender difference in our experiments where we varied the amount of the aldehyde blend. Males did not discriminate between the 0.4 and 0.04 μg amounts. For females, 0.04 μg was the only dose that produced a response significantly different than the acetone control. This tendency for females to be less responsive to volatiles has been observed in other studies (Domingue et al. 2010, Week et al. 2013). The female-specific behavior may be associated with fitness costs. While aggregations offer several advantages to an individual (Benoit et al. 2007, Saenz et al. 2014) strong sexual conflict (Morrow and Arnqvist 2003) may drive females away from high density aggregations (Pfiester et al. 2009).

Under laboratory conditions, high doses of (E)-2-hexenal and (E)-2-octenal cause dispersal of bed bugs (Levinson and Bar Ilan 1971). The emission of volatiles of the closely related tropical bed bug, *C. hemipterus*, caused a similar dispersal response (Liedtke et al. 2011). Our behavioral variables were unable to discriminate separate responses of high aldehyde amounts from the acetone control. Saturation of pheromone receptors could account for this reduced response of aldehyde cues (Benoit et al. 2009). Additionally, evaporation of the volatile aldehydes over the trial period may have allowed the amount to reach a level considered attractive to bed bugs. Regardless, bed bug responses to aldehydes appear dose dependent.

Pheromones could be used for (1) monitoring, (2) mass trapping, or (3) incorporated with insecticides to attract and kill bed bugs (Benoit et al. 2009). Our results show two hours to be the limit of effectiveness of an aldehyde-treated disk. This is significant because it highlights feasibility of practical field use. Commercial lures or baits using these aldehydes would need to be developed as slow-release formulations or dispensers able to steadily release a specific quantity of aldehyde blend over a period of time. It should also be noted that these trials were conducted in a controlled environment; human or household odors might impact attraction behaviors.

Studying the chemical ecology of bed bugs provides insight into relevant signaling cues, with direct implications on bed bug management practices. The present study highlights specific behavioral variables that can be used to determine adult bed bug responses to synthetic aldehydes. An understanding of attraction behavior is necessary to develop an effective monitor that can be used as part of an successful management strategy.

Tables

Table 5-1. Weights for bed bug behaviors used in constructing composite scores for aldehyde blend amount and time delay bioassays.

Behavior, Amount Bioassay	Weight¹
Mean distance to treatment	-0.892
Velocity in treatment zone	-0.238
Minimum distance to treatment	-0.188
Mean time in treatment zone	0.040

Behavior, Time Delay Bioassay	
Total time in treatment zone	1.225
Velocity in treatment zone	-0.449
Mean time in treatment zone	-0.403
Duration not moving in treatment zone	-0.359

¹Weights were determined using the first canonical discriminant function (SAS “proc candisc” function). Behaviors with greater weights better separate the responses to aldehyde stimuli. Weights were used in the discriminant function to create composite scores.

Figure legends

Figure 5-1. Glass Petri-dish arena (15 cm diameter) layout and dimensions for studies assessing response of bed bugs to synthetic aldehydes. Four 6 mm disks were placed at the perimeter of arena at the 3, 6, 9, and 12 o'clock positions. One disk (shaded) was impregnated with a dilution of (E)-2-hexenal and (E)-2-octenal or acetone. Arenas were divided into 4 equal zones (dashed lines): the treated zone containing the impregnated disk and 3 untreated zones. Within each zone, a smaller semi-circle subzone (4 cm diameter) was defined. An individual bed bug was placed in the center of the arena at the start of each experiment.

Figure 5-2. Representative tracks and “heat maps” showing movement of individual bed bugs over a 1-hour period in arena (15 cm diameter). (a-c) recorded bed bug tracks for 0, 0.04, and 400 μg aldehyde blend inoculated disks respectively; (d-f) heat map visualizations for 0, 0.04, and 400 μg aldehyde blend inoculated disks respectively. EthoVision® generated “heat maps” visualize a subject’s frequency at specific positions based on a color gradient. Clustered data points appear near the red end of the gradient scale. Treated disks are at the 3 o'clock position in the arena.

Figure 5-3. Plots of amount of aldehyde blend (in micrograms per treated disk) by composite score using four behaviors of male bed bugs during 1 h confinement in arena. The median scores (circles) and ± 1 SD (horizontal bars) are shown. Amounts with the highest scores most attracted bed bugs. Amounts with the same letters (following the horizontal bars) do not differ significantly ($P > 0.05$) in their effects on male bed bug behavior.

Figure 5-4. Plots of amount of aldehyde blends (in micrograms per treated disk) by composite score using four behaviors of female bed bugs during 1 h confinement in arena. The median scores (circles) and ± 1 SD (horizontal bars) are shown. Amounts with the highest scores most attracted bed bugs. Amounts with the same letters (following the horizontal bars) do not differ significantly ($P > 0.05$) in their effects on female bed bug behavior.

Figure 5-5. Plots of composite scores for aldehyde blend ($0.04 \mu\text{g}$ per disk) added to the arena after a delay of 0, 1, 2, 3, 6, or 24 hours. Scores were determined using four behaviors of bed bugs during 1 h confinement in arena. The median scores (circles) and ± 1 SD (horizontal bars) are shown. Times with the highest scores most attracted bed bugs. Times with the same letter (following the horizontal bars) do not differ significantly ($P > 0.05$) in their effects on bed bug behavior. Blank consisted of acetone-treated disks.

Figures

Figure 5-1.

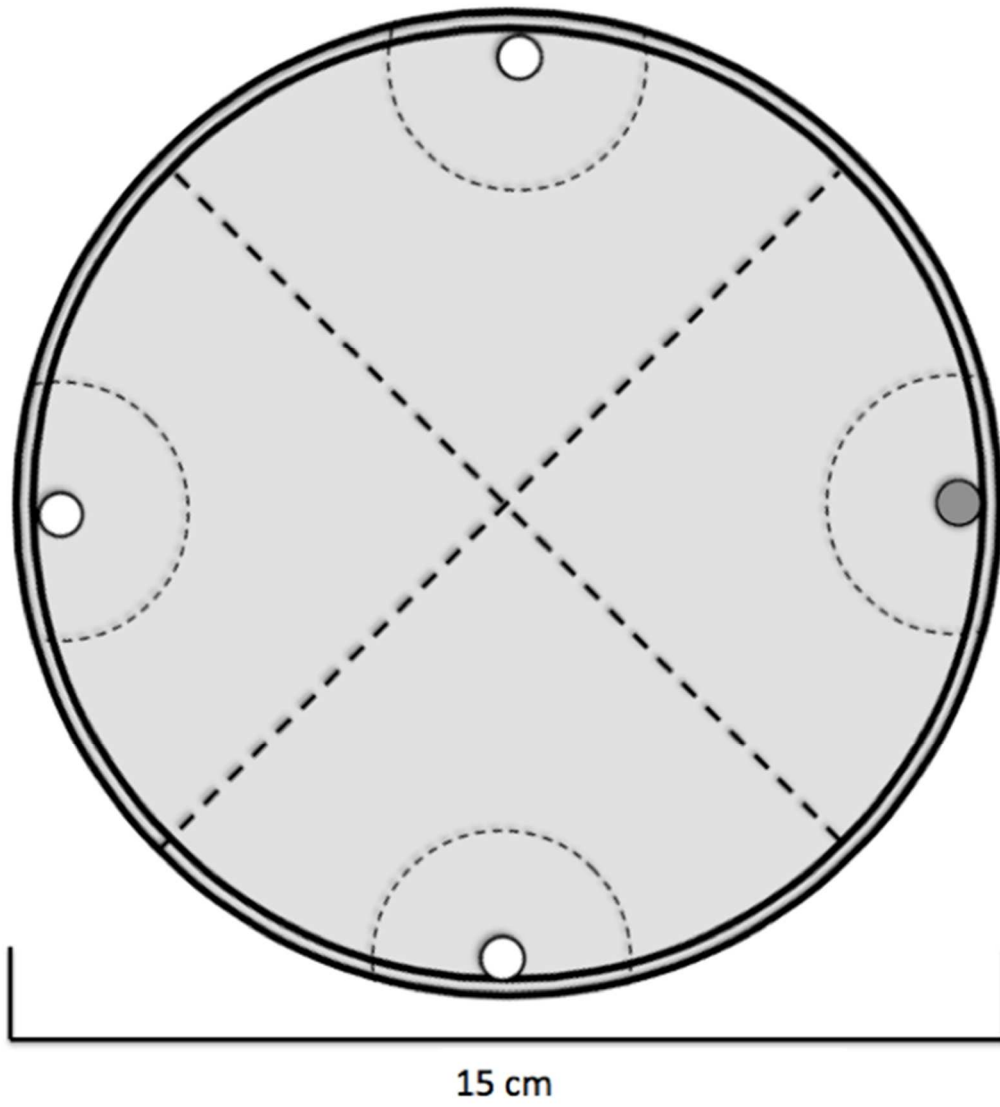


Figure 5-2.

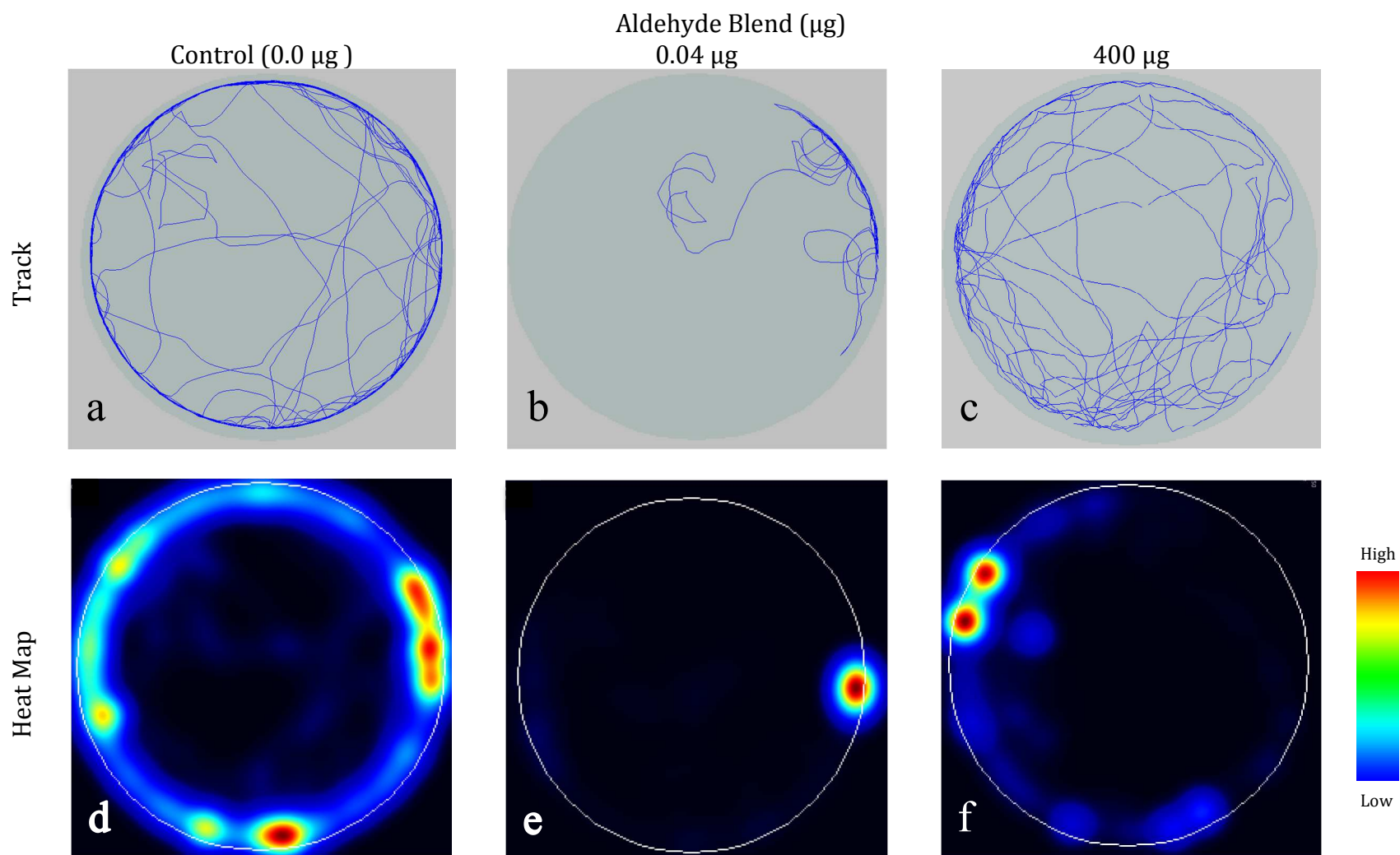


Figure 5-3.

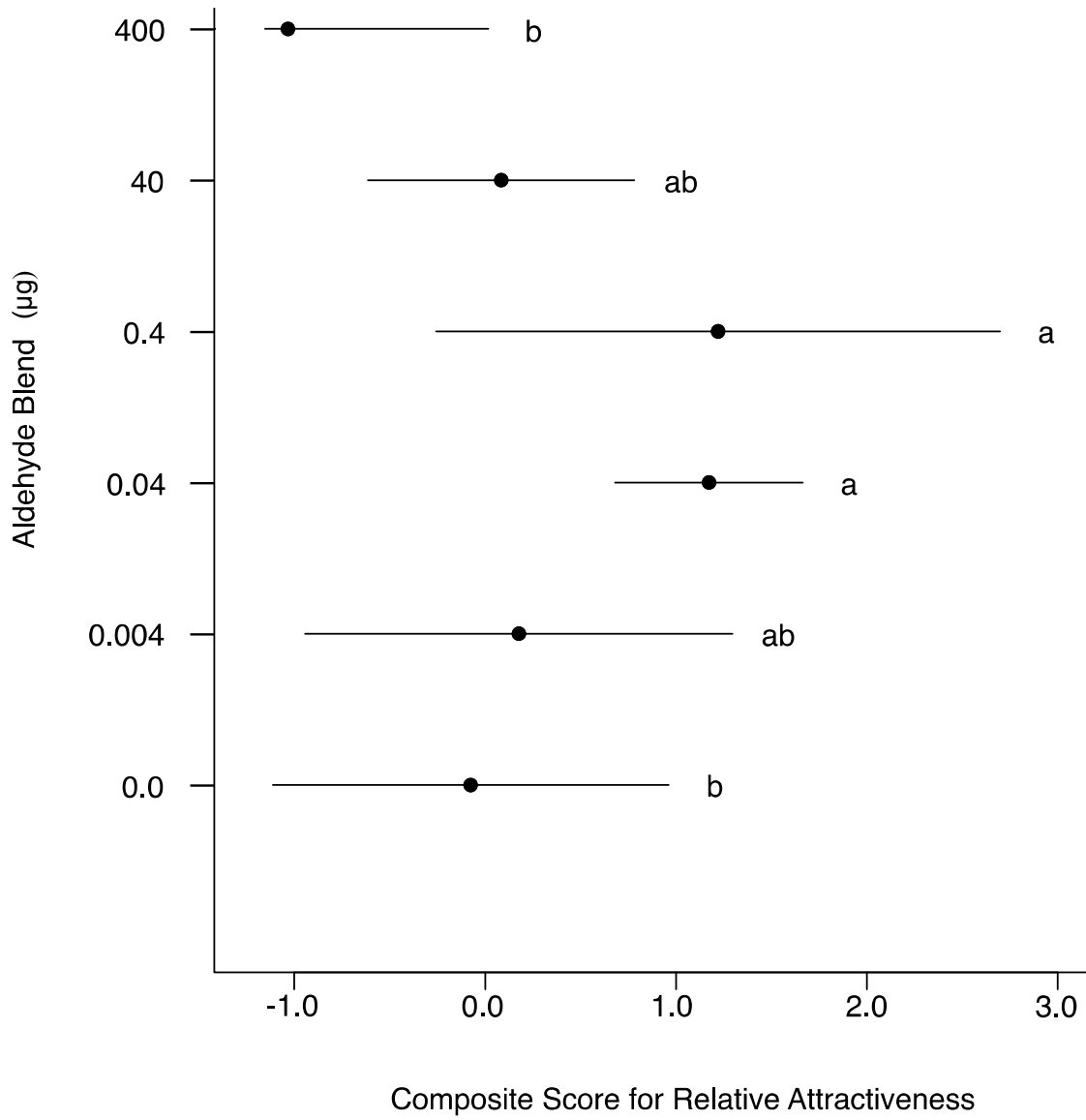


Figure 5-4.

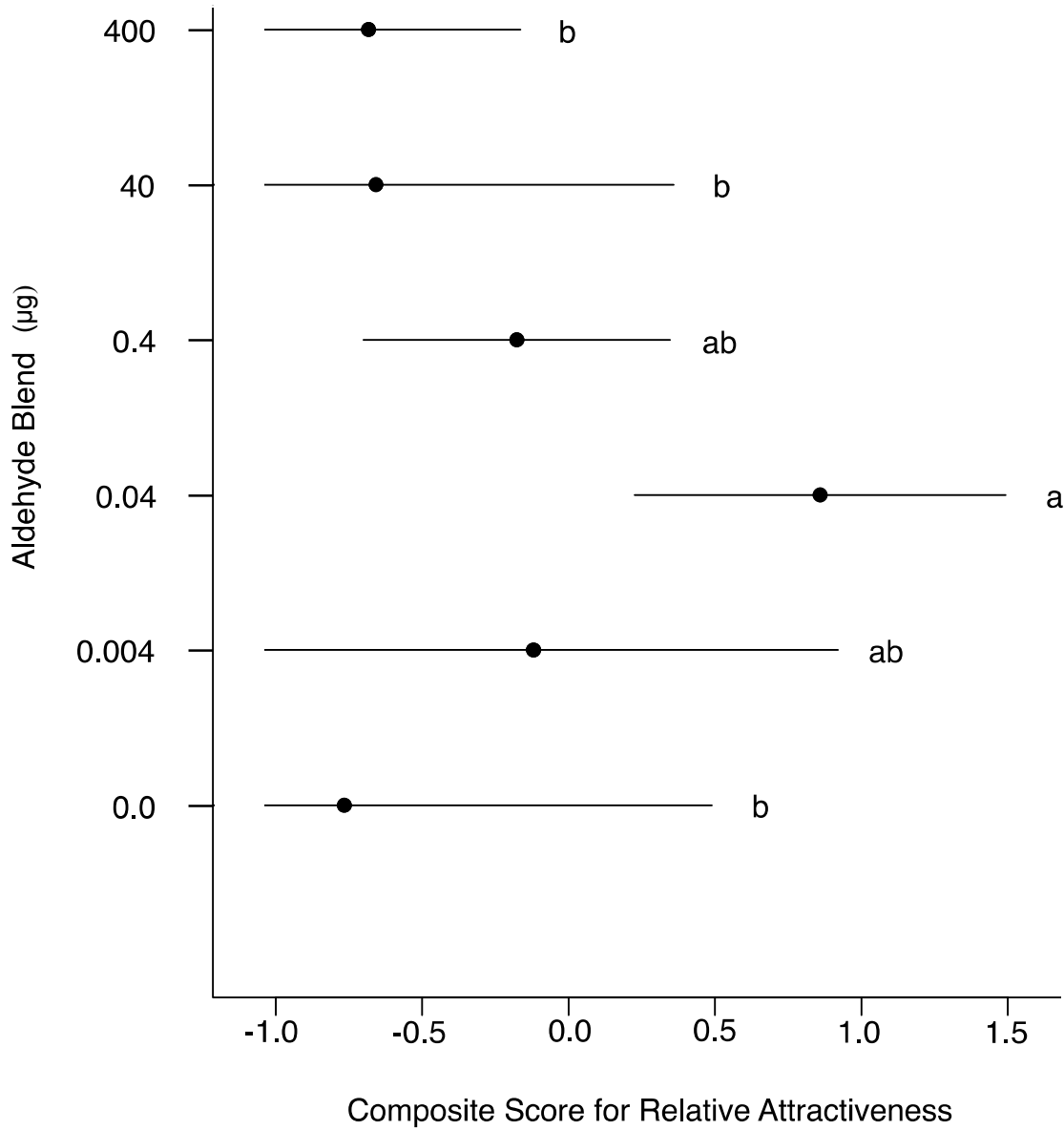
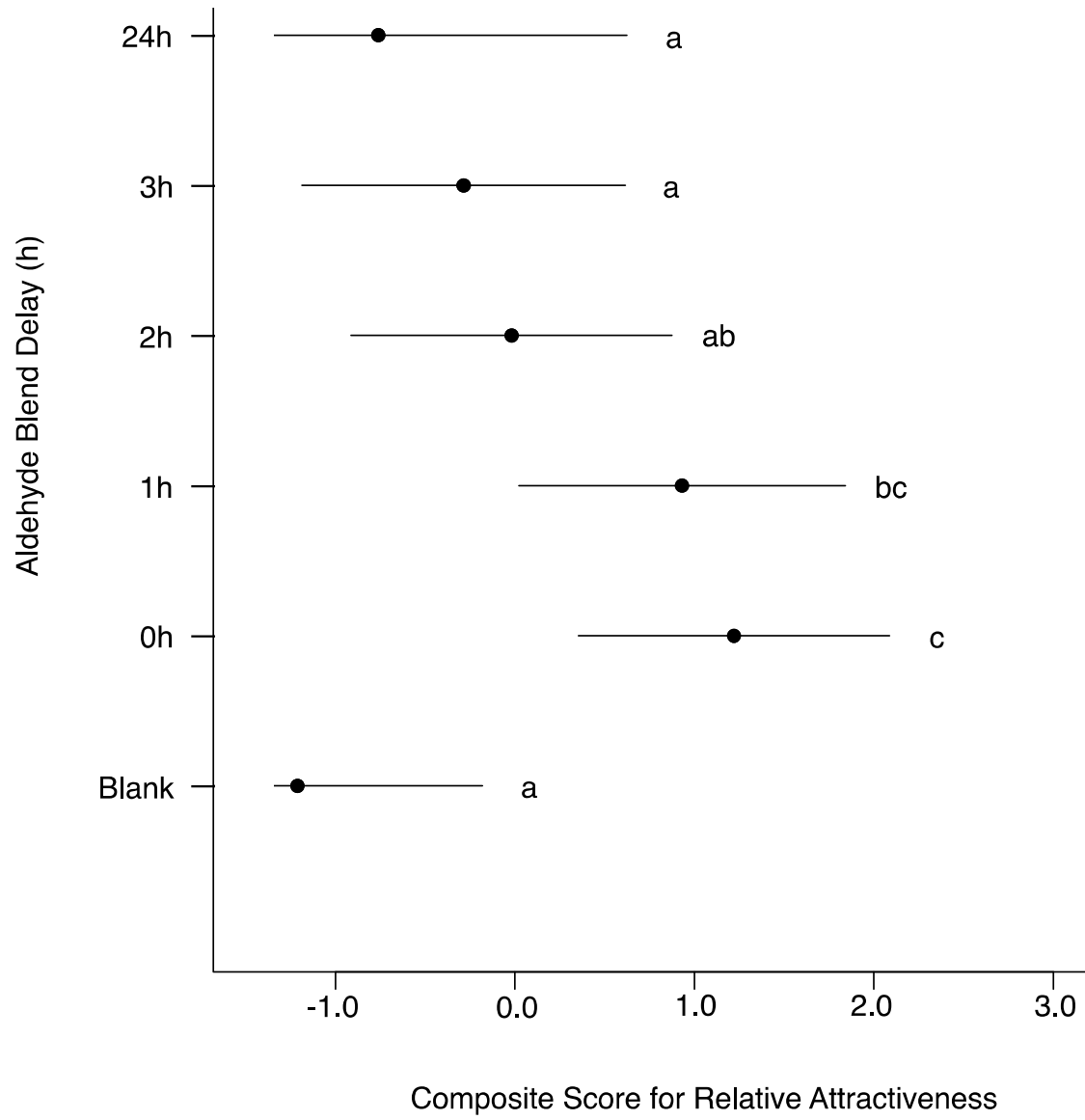


Figure 5-5.



Chapter 6: Conclusion

The chemical ecology of insects has been studied extensively over the last 60 years. The significance of pheromonal communication among social species cannot be understated. Insect societies are organized principally by complex systems of chemical signals (Wilson 1965). While the role of pheromones in solitary insects may initially seem less important, many non-social insects use sophisticated chemical communication mechanisms at various times in their life cycle. Examples include stimulation of behaviors such as mating, aggregation, or dispersion to conspecifics. Some pheromones also serve as defense secretions. The dual role of an alarm-defense system is efficient and effective considering that the two functions are typically required simultaneously in times of danger; the same substance should serve both purposes.

For bed bugs, the function of the metathoracic glands seems to be twofold: one is protection against pathogenic microorganisms. I have demonstrated that exposure of *M. anisopliae* conidia to bed bug defensive secretions inhibit fungal development both *in vitro* and *in vivo*. The second function of the glands is social, as they appear to play an important role in intraspecies attraction. In contrast to their protective role, aldehydes, when emitted at low concentrations, attract adult bed bugs. Taken together this knowledge could provide possible applications for bed bug control.

Bed bugs live in dark crevices, mostly in aggregations, which can accelerate transmission of pathogens (Usinger 1966). The habitat preference and subsocial behavior of bed bugs suggest they need effective protection from pathogenic microorganisms and parasites. Aggregations may constitute a collective chemical defense, because each individual is armed with potent antifungal mechanisms. Individual protection could be increased through synergistic enlargement of an antifungal fumigant cloud. Further studies will be necessary to prove this hypothesis.

The molecular mechanisms by which (E)-2-octenal and (E)-2-hexenal work as antifungal agents are unknown. Research conducted to understand the role these compounds play would provide insight into selection of a suitable fungal strain. Also, recent developments in recombinant DNA techniques could be used to enhance fungal efficacy. For example, transgenic strains of *Metarhizium* exist that have scorpion toxin genes that increase the speed of kill of insect pests. It may be possible to create a hybrid strain of *Metarhizium* that offered a safe and effective control strategy against bed bugs.

My dissertation research focused on the alarm-defense system of *C. lectularius*, its effectiveness against pathogen attacks, and its role as a mechanism of communication for conspecifics. The dual function of glandular compounds is dependent on both context and concentration. Studying the chemical ecology of bed bugs provides insight into relevant signaling and defensive behavior, which has direct implications on pest management practices.

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