ABSTRACT

DEVRIES, ZACHARY CURRAN. Behavior, Health Risks, and Management of Urban Pests (Under the direction of Dr. Coby Schal).

Urban and structural pests cause considerable harm and damage to humans and human structures. Therefore, it is critical that we understand their biology, behavior, health risks, and management. In the following studies, I investigated bed bug (*Cimex lectularius*) evolution, the behavioral responses of bed bugs to heat, and environmental risks associated with bed bugs and cockroaches.

Cimex lectularius was recently found to have two genetically and morphologically distinct host-associated lineages: one associated with humans (HA) and one associated with bats (BA). However, the mechanisms responsible for maintaining genetic separation between lineages remain unclear. In the first study, aggregation behavior was evaluated among host associated lineages of bed bugs (HA, BA) and bat bugs (*Cimex pipistrelli*). Using shelter-choice assays, we found no evidence for aggregation fidelity, with lineages and species aggregating indiscriminately. Thus, we conclude that aggregation fidelity is not a viable mechanism for maintaining genetic differentiation among host-associated populations.

In the second study, reproductive compatibility was evaluated with mating crosses among 6 populations of bed bugs representing different host-associations (HA, BA) and geographic locations (U.S.A., Europe). Results indicated that HA bed bugs had higher fecundity that BA bed bugs, but all inter-lineage crosses produced viable offspring, even between HA and BA bed bugs. We conclude that reproductive barriers are unlikely to prevent gene flow, and thus other mechanisms (behavior, physiology, ecology) likely maintain host-associated differentiation.

Bed bugs are hematophagous pests, feeding only on blood. Hosts are located by the cues they emit, including CO_2, body odors, and heat. Of these cues, heat is not well understood. In the third study, the effects of heat on bed bug activation, orientation, and feeding behavior were evaluated. Bed bugs oriented towards targets above ambient temperature, although positive thermotaxis was limited to short distances (< 3 cm). Furthermore, feeding was found to increase with blood temperature and this response was found to be relative to the ambient temperature. Taken together, these results indicate that heat is important in host location and feeding, but only over short distances.

Urban pests are often best known for the harm they cause and health risks they pose. Bed bugs and cockroaches are two of the most common and problematic indoor pests, each with their own challenges in regards to management and human health. In the fourth study, we evaluated bed bug infested homes for the presence and persistence of histamine. We found that histamine was present at significantly higher levels in bed bug infested homes than in un-infested homes. Furthermore, histamine persisted for three months after bed bugs were eradicated, suggesting it is highly stable in the indoor environment. These findings are the first to report histamine as an indoor environmental contaminant, and suggest bed bugs may pose a major health risk to humans.

In the fifth study, we evaluated the efficacy and health risks associated with total release foggers (TRFs) and compared results to a low-risk management strategy (gel baits). Total Release Foggers were found to be completely ineffective at controlling cockroaches in homes, while simultaneously producing large amounts of pesticide (pyrethroid) residues around the discharge site. Conversely, gel baits were found to be effective at reducing cockroach populations while only depositing small amounts of pesticides in targeted

locations. The ineffectiveness of TRFs at reducing cockroach populations, their similar monetary cost compared to highly effective bait products, and the pesticide exposure risks associated with TRFs, call into question their utility in the marketplace

Behavior, Health Risks, and Management of Urban Pests

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Entomology

Raleigh, North Carolina

2017

APPROVED BY:

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DEDICATION

To my wife, Maryann DeVries, for her love, support, and constant encouragement.

BIOGRAPHY

Zachary Curran DeVries was born in Columbus, Ohio, but moved to Auburn, Alabama before his first birthday. He grew up in Auburn, and later attended Auburn University where he earned both his B.S. in Zoology and his M.S. in Entomology, before moving to Raleigh, NC, to attend North Carolina State University in pursuit of his Ph.D. in Entomology.

ACKNOWLEDGMENTS

I would like to thank and acknowledge my advisor Dr. Coby Schal for his guidance, wisdom, and advice. Additionally, I would like to thank my committee members, Drs. Jay Levine, Michael Reiskind, and Wes Watson, for their support during my doctoral degree. I would also like to thank members of the Schal lab, particularly Rick Santangelo, Russell Mick, and Angela Sierras for their support over the years. I would also like to acknowledge financial support from the Department of Entomology and Plant Pathology at NCSU, the North Carolina Entomological Society, the Foundation for Toxicology and Agromedicine, The Entomological Society of America, Pi Chi Omega, and the W.M. Keck Center for Behavior Biology. Finally, I would like to thank my friends and family for their support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction

Urban Pests

Insects are a significant problem in the indoor environment. Urban pests are responsible for destroying structures, consuming or contaminating food, and causing physical and/or psychological harm to humans (1). Two of the most challenging urban pests in terms of control and human health, are the common bed bug (Hemiptera: Cimicidae, *Cimex lectularius*) and the German cockroach (Blattodea: Blattellidae, *Balttella germanica*).

Bed bugs, *C. lectularius*, are small (< 5 mm) hematophagous ectoparasites of homeothermic species, sharing a close relationship with humans, poultry and sometimes bats (2-4). Bed bugs are entirely dependent on their host for survival, and all life stages require blood meals for growth and development (2). Host are located through a combination of CO_2, host odors, and heat, although how each cue works is unclear and the relative importance of each cue remains unknown. Over the past 20 years, bed bugs have made a resounding resurgence, often attributed to increased travel, insecticide resistance, and changes in pesticide use and regulations (5-7), although it is still unclear which (if any) of these played a role in the resurgence. Bed bugs are not known to produce allergens and their ability to transmit diseases is widely contested (8-11). However, it is clear that bed bugs put tremendous psychological stress upon their hosts (12, 13). Management options for bed bugs are limited. The biggest restraint is the lack of a residual bait, resulting in a reliance on residual insecticides (14). Bed bugs are highly resistant to many commonly used insecticides, particularly those containing pyrethroids, often resulting in control failures (6, 15, 16). Non-chemical control strategies (eg, heat treatments) have become more popular over time, but these strategies are often costly and lack residual efficacy (17, 18).

German cockroaches are omnivorous pests, solely associated with humans and human-built structures (19). German cockroaches are responsible for a number of health problems, including the production of a number of very potent allergens (20) and the potential to vector pathogens (21, 22). Control typically involves the use of sprays in cracks and crevices, along with baits, dusts, and general sanitation (cleaning up food and water) (19). Despite the success of IPM (integrated pest management) in reducing cockroaches and their related allergens (23-27), a recent survey of pest control practices in rural North Carolina revealed sprays to still be the dominant method for controlling German cockroaches (28). This is alarming, because Williams et al. (29) showed that IPM programs involving baits cost about the same as conventional spray-only programs, provide similar levels of control, and ultimately produce only a fraction of the residual insecticides.

The damage from bed bugs and cockroaches can be felt by anyone. However, these pests often persist in lower socio-economic households. The unequal presence of pests such as cockroaches or bed bugs in disadvantaged homes and the propensity for these pests to cause humans harm, creates a situation of social injustice, where poverty can be directly related to a lower quality of life through exposure to pests.

Despite advances in our knowledge of the biology and management of both these species, many questions remain unanswered. This dissertation strives to better understand several aspects of the evolution, behavior, health risks, and management related to both bed bugs and German cockroaches. Background information relating to bed bug evolution and arthropod-related human health risks are provided below.

Evolution and Speciation

The number and diversity of living organisms on Earth is astounding (30, 31). The beauty and breadth of this diversity is a direct result of billions of years of evolution and the continual divergence of taxa (32, 33). Speciation is a complex process that occurs over a range of time scales relative to the generation time of each organism. For speciation to occur, it is critical that gene flow be restricted among populations. Changes in allele frequency which result in speciation can occur through genetic drift, mutation, immigration, emigration, and natural selection and can occur either with (allopatric) or without (sympatric) physical barriers separating populations. While allopatric speciation has been well documented, only recently has sympatric speciation become broadly recognized (34-36). Both processes, however, share a common feature when adaptive radiations are considered – natural selection drives local adaptation. One of the most striking examples of speciation driving natural selection are Darwin's finches on the Galapagos islands, which show remarkable variation in beak morphology and behavior, indicative of the various feeding strategies that have evolved, ultimately resulting in the extant species (37, 38). Other examples include African cichlids (39-41), which display differences in mouth morphology also indicative of different feeding strategies, and the Hawaiian silversword alliance (42, 43), which possess physiologies and morphologies related to the different habitats they occupy, ranging from rainforests to deserts. These and related examples represent the results of thousands to millions of years of adaptive radiations, resulting in well-defined, often co-existing species.

To investigate the process of incipient ecological speciation, host races are often evaluated. As defined by Dres and Mallet (44), host races: (i) use different hosts and exhibit host fidelity, (ii) coexist in sympatry across part of their range, (iii) are genetically differentiated at multiple loci, (iv) show a correlation between host and mate choice while still displaying some gene flow, and (v) have higher fitness on natural hosts and produce less fit hybrids. Most host races described to date are phytophagous insects (44, 45), although there are a growing number of examples from other species, primarily parasites (3, 46-48). Host race formation is driven by a preference among members of a species for different hosts (i.e., host-race association, HRA) (49, 50). After an initial preference for a host develops, many other factors emerge and reinforce the differentiation of populations into host-associated lineages, including oviposition site preference (51), no overlap/interaction during mating times (49), and differential growth/survival on specific hosts (52, 53). These reinforcing behaviors/traits are often intimately tied with host preference (54). Perhaps the best described host-race system is that of the apple maggot fly, *Rhagoletis pomonella*. In *R. pomonella*, there is a clear division among host lineages, with some preferentially attracted to apples and others preferentially attracted to hawthorn (49). These lineages can be distinguished genetically (55, 56), and their isolation appears to stem from odor-based discrimination between the two host plants, possibly driven by only a few genes (57, 58). A similar pattern of incipient speciation can be observed in the yellow fever mosquito, *Aedes aegypti* (59). Two forms of this mosquito have been identified in nature: an ancestral form that is associated with non-human animals (zoophilic) and a derived form associated specifically with humans (anthropophilic) (60). These species are distinguished genetically

and spatially, with the zoophilic form found primarily in Africa, and the anthropophilic form found worldwide in close association with humans (61, 62). Host association in *A. aegypti*, as in *R. pomonella*, is driven by host odor preferences (63, 64), with differential expression of a single odorant receptor playing a key role in driving host associations (65). Thus, the ecological framework underlying speciation is similar in phytophagous and hematophagous species: each species ancestrally specialized on specific hosts, and specialization of an alternate host-adapted lineage lead to host race formation.

Recently, bed bugs were discovered to have two distinct genetically differentiated lineages: one associated with humans (HA = human-associated), and another associated with bats (BA = bat-associated) (66). The BA lineage represents the ancestral zoophilic association, whereas the HA lineage represents a relatively recent (Neolithic) anthropophilic differentiation (3, 66). Notably, changes in construction that brought bats into closer association with humans (e.g., attics, chimneys) have also re-united the two lineages in close sympatry. The two lineages differ morphologically and display differential growth and survival when fed on non-native hosts (52, 66). HA and BA lineages are differentiated genetically (24 microsatellites), with almost no apparent gene flow between them (3). In addition, they appear to be reproductively incompatible, with crosses between HA and BA populations resulting in no oviposition (67).

Arthropod Mediated Indoor Contamination

Risk of exposure to indoor environmental contaminants is a major health concern. This is due in large part to high concentrations of some contaminants indoors and the disproportionate

amount of time spent indoors relative to other locations (68, 69). The most common contaminants that pose risks are often human-made, including lead (70), asbestos (71), pesticides (72, 73), and various volatile organic compounds (VOCs, 74). Health effects from exposure to these contaminants can range from mild irritation to cancer and death, with many of these effects observed through respiratory exposure (75). In recent years, the adverse effects from exposure to indoor contaminants have been reduced, and consequently health outcomes have improved, due to greater awareness of the risks, regulatory changes, and procedures that either prevent exposure altogether (76), or expedite the mitigation of household contaminants (77, 78).

Indoor contaminants can also be derived from biological sources, such as bacteria and fungi, but arthropods are the major etiological agents of indoor allergens. Perhaps the best examples come from house dust mites (HDMs) and cockroaches. House dust mites, represented by at least three different species, produce several potent allergens (79, 80). The importance of these allergens in the development of childhood asthma is still under debate (81, 82), but exposure of atopic individuals to these allergens can trigger severe respiratory symptoms (83). Cockroach allergens are known primarily from two species, the German cockroach (*B. germanica*) and the American cockroach (*Periplaneta americana*), although other species produce cross-reactive allergens (20). Cockroach allergens have been associated with the development of asthma and exacerbation of asthmatic symptoms (84). Despite the dangerous associated with exposure to cockroach allergens, residents are often faced with an impossible decision: use insecticides that may have adverse health effects, or allow cockroaches and cockroach-related health issues to persist.

Additionally, the characteristics of HDM and cockroach allergens suggest that another recently resurged pest, the common bed bug (*Cimex lectularius*), has strong potential to contaminate the indoor environment. Bed bugs were recently shown to defecate histamine as a component of their aggregation pheromone (85). Because histamine is a pivotal modulator in the mammalian immune response (86), its presence indoors, and particularly in beds, could have serious implications for human health, and thus should be further investigated.

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CHAPTER 2

Aggregation behavior and reproductive compatibility in the family Cimicidae

(This work was published in *Scientific Reports*: DeVries ZC, Mick RA, Ondřej B, and Schal C. 2017. Aggregation behavior and reproductive compatibility in the family Cimicidae. *Scientific Reports* 7:13163. doi: 10.1038/s41598-017-12735-3.)

Abstract

Bed bugs (*Cimex lectularius*) provide a unique opportunity to understand speciation and host-associated divergence in parasites. Recently, two sympatric but genetically distinct lineages of *C. lectularius* were identified: one associated with humans and one associated with bats. We investigated two mechanisms that could maintain genetic differentiation in the field: reproductive compatibility (via mating crosses) and aggregation fidelity (via two-choice sheltering assays). Effects were assessed at the intra-lineage level (within human-associated bed bugs), inter-lineage level (between human- and bat-associated bed bugs), and inter-species level (between *C. lectularius* and *Cimex pipistrelli* [bat bug]). Contrary to previous reports, bed bugs were found to be reproductively compatible at both the intra- and inter-lineage levels, but not at the inter-species level (although three hybrids were produced, one of which developed into an adult). Lineage- and species-specific aggregation fidelity was only detected in 8% (4 out of 48) of the aggregation fidelity assays run. These results indicate that under laboratory conditions, host-associated lineages of bed bugs are reproductively compatible, and aggregation pheromones are not capable of preventing gene flow between lineages.

Introduction

The common bed bug, *Cimex lectularius* L., offers a unique system for studies of incipient speciation for several reasons. First, bed bug populations undergo dramatic expansion and contraction cycles, as infestations often start from very small propagules[1-3], rapidly expand within and between apartments with extensive inbreeding, and then undergo repeated population bottlenecks because of human interventions and founder effects[4]. Second, as obligate ectoparasites they rely on their host for nutrition[5], and therefore must adapt to any changes in host behavior and physiology, or alternatively, adapt to a new host. Bed bugs are polyphagous, accepting a broad range of hosts from birds to humans[5], and persistent association with a single host can result in adaptive evolution that makes alternate hosts less acceptable[6,7]. Finally, bed bugs have an ancestral association with bats, predating their more recent association with humans[8]. Balvin et al.[8] recently studied a central European bat-associated (BA) lineage of *C. lectularius* that overlaps geographically with a human-associated (HA) lineage. Despite being found in the same environment, there is little to no apparent gene flow between the two lineages across Europe[8-10]. These factors – small propagule size, extensive inbreeding, recurrent bottlenecks and founder effects, pressure to adapt to changes in host culture, behavior and physiology, and the existence of differentiated host-associated lineages in sympatry – produce a perfect scenario for sympatric speciation[4].

The apparent lack of gene flow between the HA and BA *C. lectularius* lineages suggests that complete or incomplete reproductive isolation might have evolved. Indeed, Wawrocka et al.[11] showed that interbred HA and BA bed bugs were incapable of producing hybrids, suggesting complete reproductive isolation, no gene flow, and that *C. lectularius* has

differentiated into two species. Our preliminary results significantly departed from these findings, however, suggesting differences related to methodology or physiology of the bed bugs.

Incomplete reproductive isolation, with lineage differentiation occurring in allopatry or in sympatry, is expected to result in hybrids with lower fitness, and reinforcement is expected to increase reproductive isolation and reduce inter-lineage hybrids[12]. Mechanisms that minimize gene flow between differentiated lineages may be physiological and/or behavioral, and prezygotic and/or post-zygotic. Determining what factor(s) are responsible for maintaining genetic differences among lineages can be difficult, as many factors can be at play and environmental conditions can modulate their effects.

Bed bugs tend to aggregate, and their fidelity to lineage-specific or even population-specific aggregations might reduce gene flow and cause lineages to differentiate. Aggregations serve a variety of functions in insects, and in bed bugs aggregations have been shown to reduce water loss[13] and accelerate development[14]. Other benefits accrued in aggregations, as shown in the German cockroach, might be nutrient sharing, for example through coprophagy[15], or faster reproduction[16]. Aggregation sites also provide potential arenas for mate location[5]. Since the formation and maintenance of aggregations are guided by aggregation pheromones[17], it is reasonable to expect that aggregation cues should diverge in species and lineages that produce inviable or less fit hybrid offspring. Balvín et al.[18] tested this hypothesis and did not find evidence of aggregation fidelity. However, in these assays only adult males were used to bioassay aggregation preferences, thus it is necessary to assay both females as shelter conditioners and aggregation responders. Additionally, only inter-

lineage preference (HA vs. BA) were evaluated. The extent species-level aggregation fidelity also need to be considered given the co-habitation of BA *C. lectularius* and *Cimex pipistrelli* in bat roosts (O.B. field collection, 2014).

To evaluate the role of lineage- and species-specific aggregation pheromones in guiding fidelity to aggregation sites and possibly in maintaining genetic differentiation of bed bug lineages, bed bugs were tested to determine if they preferred aggregating with their own or other populations. Additionally, reproductive compatibility was assessed among lineages tested for aggregation fidelity to determine if these lineages were reproductively isolated. The results are interpreted in relation to the evolution of HA and BA bed bug lineages and the factors that facilitate the maintenance of these lineages.

Materials and Methods

Experimental Animals

Three populations of *C. lectularius* with different host associations were used: Winston Salem (WS-HA) and Jersey City (JC-HA) were both collected in close association with humans (hence, HA, human associated). The WS-HA population was collected in 2008 from beds and couches in an apartment in Winston Salem, North Carolina, USA. The JC-HA population was collected in 2008 from an apartment in Jersey City, New Jersey, USA. The third population, Moravicany (MO-BA, bat associated), was collected in 2014 living in close association with a bat colony (*Myotis myotis*) in Moravicany, Czech Republic. In addition, a population of bat bugs, *Cimex pipistrelli* (Cp-BA) collected in Duba, Czech Republic in 2014, was also used. Together, these populations allowed for intra- (HA vs. HA) and inter-

lineage (HA vs. BA) comparisons, as well as inter-species comparisons (*C. lectularius* vs. *C. pipistrelli*).

All bed bugs and bat bugs were reared in the laboratory in 168 cm^2 plastic cylinders with cardstock paper substrate at 27°C and ~50% RH on a 12:12 light:dark cycle. Bed bugs and bat bugs were fed defibrinated rabbit blood in an artificial feeding system. This system relied on a heated water bath (B. Braun Biotech Inc., Allentown, PA) to circulate water heated to 37°C through a custom-made water jacketed glass feeder, with bed bugs feeding through an artificial membrane (Nescofilm, Karlan, Cottonwood, AZ, USA). It is important to note that under these conditions all bed bugs and bat bugs experienced the same environmental conditions and blood meal type.

Reproductive Compatibility

Reproductive compatibility was assessed by pairing virgin adults from different populations and looking for offspring. Fifth instars of each population were isolated and allowed to eclose, ensuring newly emerged adults remained unmated. Bed bugs were recombined by sex and population, then fed. One week after feeding, bed bugs were fed again, then individual virgin males and females were combined in 7.5 ml glass vials with a paper shelter and allowed 6 d to lay eggs. Preliminary trials revealed six days as enough time to reflect reproductive output, and no differences were found when comparing results at 6d or 10 d. Only adults that fed to repletion (fully engorged) were used for bioassays. Adults were then removed and vials were monitored for the number of nymphs that hatched after 2 weeks. Sample sizes were 16-18 pairs for intra-lineage assays (WS-HA vs. JC-HA), 9-10 pairs for

inter-lineage assays (WS-HA vs. MO-BA), and 15 pairs for inter-species assays (WS-HA vs. Cp-BA). All assays were female-centric, with comparisons made between individual females of the same population/species mated to males of either the same or a different population/species. In addition, all assays within each level of testing (intra-lineage, inter-lineage, inter-species) were run in parallel.

Aggregation Preference Assays

Aggregation assays were conducted in plastic petri dishes (d = 60 mm; Corning Life Sciences, Durham, NC, USA), with the bottom etched to facilitate bed bug movement and lids on to prevent escape. Two white cardstock paper tents (30 mm x 15 mm, 110 lb weight, Georgia Pacific LLC, Atlanta, GA) were placed adjacent to each other in a symmetrical design in each arena. Each paper tent was conditioned by one fed bug of one of the following groups: male WS-HA, female WS-HA, male JC-HA, female JC-HA, male MO-BA, female MO-BA, male Cp-BA, and female Cp-BA. Bed bugs were fed in large cohorts (50-100 bugs) divided by sex and population, then moved to conditioning vials following feeding. Conditioning occurred in 20 ml glass vials, where the paper tent served as the only shelter for the bugs. Vials had mesh tops to allow for air flow and ventilation. Assay development and validation was run with one population (WS-HA) to establish a conditioning protocol that was sufficient to elicit aggregation. In these assays a conditioned tent was tested against a clean, unconditioned tent (treated identically in all ways to the conditioned tents). The number of bugs and conditioning time were varied to ensure the experimental conditions were sufficient to assess aggregation.

In validated assays, each tent was conditioned for one day, with a new tent conditioned every day for four days following feeding (each bed bug used for conditioning produced four tents over four days after feeding). Two-choice aggregation preference assays were run independently for male and female responders for the following combinations of conditioned tents: WS-HA male vs. JC-HA male, WS-HA female vs. JC-HA female, WS-HA male vs. MO-BA male, WS-HA female vs. MO-BA female, WS-HA male vs. Cp-BA male, and WS-HA female vs. Cp-BA female. After the arena was set up, a single adult of one of the two populations being tested was introduced into the center of the arena. Male and female bed bugs were assayed separately. Bioassayed adults were fed 2 d prior to the start of each experiment to minimize their propensity to seek a host and maximize their tendency to seek a suitable shelter. All assays were set up 4 h after lights-off and responders were then allowed the remaining 8 h of the scotophase to move freely in the dark around the arena and sample both tents. The final position of each bed bug was recorded 3-4 h after lights-on to maximize the number of bugs fully arrested, with non-responders defined as those not located on or under either tent. Assays were always run in blocks, with identical numbers of bed bugs of each population tested for each choice combination.

Male aggregation preference was also assessed on tents conditioned by groups of bed bugs or bat bugs, to see if results differed with increased levels of conditioning. The same protocol used for single-bug conditioned tents was used, with the only difference being that each tent was conditioned by 10 adult male or female bed bugs for 5 d following feeding, allowing us to test substrates that were more heavily conditioned. In addition, and in response to observations from the group-conditioning assays, a final aggregation preference

assay was run comparing aggregation preference of JC-HA and WS-HA males for tents unequally conditioned by ten WS-HA or five JC-HA females for five days. This assays was intended to simulate potential field conditions, where co-habitating populations/species likely have different population sizes. Thus, these assays evaluated quantitative versus qualitative preferences.

Data Analysis

Student's *t*-test was used to compare the number of emergent nymphs between hetero- and homogeneous crosses involving the same female. Aggregation preferences were tested by Chi Square analysis, with the null hypothesis of no aggregation-site preference, or a 1:1 shelter tent choice. All analysis was performed in SAS 9.4[19] (SAS Institute, Cary, NC, USA).

Data Availability

All data and statistical code used in the current study are available from the corresponding author upon reasonable request.

Results

Reproductive Compatibility

The population from which males originated had no effect on the number of viable offspring produced by WS-HA females (t_{31} = -0.87, p = 0.3912, Fig. 1a). However, JC-HA females produced more offspring when mated to WS-HA males than with their own JC-HA males (t_{33} = -2.14, p = 0.0397, Fig. 1b).

Winston Salem (HA) females produced significantly more offspring when mated to their own WS-HA males than to MO-BA males ($t_{18} = 3.28$, $p = 0.0042$, Fig. 1c). However, the number of offspring produced by MO-BA females was unaffected by the male's lineage ($t_{17} = -1.16$, $p = 0.2627$, Fig. 1d).

Bed bugs (*C. lectularius*, WS-HA population) and bat bugs (*C. pipistrelli*, Cp-BA) were not reproductively compatible ($t_{28} = 17.91$, $p < 0.0001$, Fig. 1e; $t_{18} = -8.04$, $p < 0.0001$, Fig. 1f). Only three *C. lectularius*-*C. pipistrelli* hybrid nymphs were observed, one of which was dead during the initial observation. Of the remaining two nymphs, both were offered blood, but only one survived to maturity – an adult male that originated from a female *C. pipistrelli* mated to a male *C. lectularius*. However, the surviving hybrid failed to sire any offspring when maintained separately with unmated females of either parental species. Notably, the bat-associated females (MO-BA, Cp-BA) were significantly less fecund on rabbit blood than the human-associated *C. lectularius* even in conspecific pairings.

Conditioned vs. Control Shelters

To confirm that bed bugs could detect and preferentially arrest under bed bug-conditioned paper shelters, experiments were run comparing WS-HA male-conditioned shelters vs. unconditioned shelters. Bed bugs displayed a strong preference for conditioned shelters, when conditioned by 10 bed bugs for five days ($\chi^2_{1,29} = 18.24$, $p < 0.0001$), or one bed bug for either five days ($\chi^2_{1,17} = 7.12$, $p = 0.0076$), two days ($\chi^2_{1,16} = 4.00$, $p = 0.0455$), or one day ($\chi^2_{1,17} = 17.00$, $p < 0.0001$; Fig. 2). Based on these results, further assays were conducted with shelters conditioned by one bug for one day and 10 bugs for five days.

Intra-lineage Aggregation Preferences

Intra-lineage aggregation preference was not detected in any of the single bed bug conditioning assays, regardless of the sex of the responder or the sex of the bugs used to condition each tent ($p \geq 0.3173$ for all assays, Fig. 3). Assays were also conducted that involved allowing 10 adult males or females to condition each tent for 5 d. These assays indicated some aggregation preference, specifically for the JC-HA males when tested on female conditioned shelters ($\chi^2_{1,13} = 4.55$, $p = 0.0330$, Fig. 4). However, no other groups tested showed significant aggregation site preference ($p \geq 0.1701$, Fig. 4).

Inter-lineage Aggregation Preferences

No evidence for lineage preference was detected in any of the experiments with a single bed bug-conditioned tent, regardless of the sex of the responder or the sex of the bugs used to condition each tent ($p \geq 0.3711$, Fig. 5). For the assays involving tents conditioned by 10 bed bugs for five days, there was a strong preference by WS-HA males for female-conditioned shelters of their own WS-HA population over the MO-BA population ($\chi^2_{1,13} = 11.65$, $p = 0.0006$, Fig. 6). But no other groups tested showed significant aggregation site preferences ($p \geq 0.1824$, Fig. 6).

Inter-Species Aggregation Preferences

Only one of the eight single bed bug conditioning assays (Cp-BA female selecting female conditioned tents) showed significant species-specific aggregation preference ($\chi^2_{1,36} =$

4.00, $p = 0.0455$), with all other assays being non-significant ($p \geq 0.1167$, Fig. 7). For the assays involving tents conditioned by 10 bugs for five days, only the *C. lectularius* WS-HA males showed significant preference for female-conditioned shelters of their own species over the *C. pipistrelli* female conditioned tents ($\chi^2_{1,31} = 5.45$, $p = 0.0196$), with all other assays showing no aggregation preferences ($p \geq 0.3173$, Fig. 8).

Effects of Differential Conditioning on Aggregation Preferences

We suspected that the inconsistent, unidirectional aggregation preferences observed when shelters were conditioned by 10 bed bugs were due to quantitative rather than qualitative differences in aggregation pheromones on conditioned tents. Therefore, we conducted an additional bioassay to assess the effects of differentially conditioned shelters on aggregation preferences. Males of WS-HA and JC-HA both preferentially selected tents conditioned by 10 WS females over tents conditioned by five JC females ($\chi^2_{1,31} = 4.26$, $p = 0.0389$, Fig. 9).

Discussion

Human- and bat-associated lineages of *C. lectularius* were reproductively compatible in our experiments. Although there may be some costs to inter-lineage mating, such as reduced fecundity, the results of our study indicate that gene flow is possible between these two host-associated lineages. These results deviate radically from a previous study that showed no egg production for any crosses between HA and BA bed bugs[11]. These disparate findings could be attributed to several methodological differences between the two studies. A major

difference was that Wawrocka et al.[11] used bugs that were collected in the field as nymphs and tested as same generation adults, whereas bugs in our experiments were acclimated to the laboratory for over a year. Two important changes could have occurred during the >1 year in the laboratory: adaptation to rabbit blood and homogenization of microbiomes between strains. In Wawrocka et al.[11] a diet switch occurred in the 3rd instar or later, whereas in our experiments all the bed bugs had acclimated to rabbit blood over at least 8 generations (estimated based on a development time of 31 d at 27°C[5], and at least one year in culture prior to testing, with some adjustment for slower development at colony inception) . Switching diets during development (even among diets that are readily accepted by a species) has been shown to negatively affect development in phytophagous generalists[20,21]. Human-associated bed bugs have also been shown to develop poorly when switching diets from human blood to bat blood[9], although it is unclear how the timing of this switch (early or late in development) affects growth and reproduction. A common food (rabbit blood) fed to both lineages in our experiments could have also homogenized their gut microbiomes more so than in Wawrocka et al.'s experiments[9]. *Cimex* species harbor *Wolbachia* as an obligatory symbiont, and *Wolbachia* and other endosymbionts have been shown to play significant roles in cytoplasmic incompatibility and reinforcement of speciation[22-25]. It is possible that long-term colonization and feeding on a common food source homogenized the microbial communities and eliminated reproductive incompatibility between the bed bug lineages. Nevertheless, it is important to note that even after such long-term adaptation to rabbit blood, both the BA lineage (represented by MO-BA) and *C. pipistrelli* (Cp-BA) had lower fecundity, even in homogeneous crosses (Fig. 1). The reasons behind the low fecundity

observed in BA bed and bat bugs is unknown, but we speculate it is either due to physiological differences or laboratory conditions.

Previous work has shown that *C. lectularius* and *C. hemipterus* can mate and produce hybrids in the laboratory, although hybrids generally fail to mature[26]. To the best of our knowledge, our results represent the first documented hybridization between *C. lectularius* and *C. pipistrelli*. A single hybrid was reared to an adult male, but was unable to sire offspring with females of either of the two parental species. It is important to investigate this phenomenon with greater sample sizes to determine whether some fertile hybrids might result from this cross. Viable inter-species hybrids could constitute an epidemiological bridge between bats and humans, and significantly affect the public health importance and pest status of both species.

Population genetics studies indicate that the human- and bat-associated lineages are genetically distinct with no apparent gene flow between them[8,10]. Although they may be considered sympatric within human-built structures, it is likely that HA and BA bugs are separated by ecological and behavioral barriers. Ecological separation may be imposed by the divergent niches of the two respective hosts – chimneys and attics for BA bugs and residential rooms, especially bed rooms, for HA bugs. Behavioral barriers may include differential responses to host odors and pheromones, including aggregation pheromones. We hypothesized that the two lineages would exhibit lineage-fidelity in their aggregation responses. To our surprise however, aggregation fidelity was not observed in any of the populations, lineages, or even species tested. These results suggest that there are no

preferences for lineage-specific aggregation pheromones, and that aggregation preferences do not represent a substantial behavioral isolating mechanism.

Our results are consistent with the results of Balvín et al.[18], who documented similar behavioral responses for adult males, and we further extended these findings to females and to lack of preferences between *C. lectularius* and *C. pipistrelli*. The results from the interspecific aggregation assays (*C. lectularius* vs. *C. pipistrelli*) provide strong evidence that in the absence of species-level aggregation fidelity, it is unlikely that lineage-level aggregation preferences alone would be sufficient to maintain genetic isolation of the two lineages. Similar to the reproductive compatibility assays, the results of the aggregation assays might have been affected by the fact that all bugs were reared on the same diet in the same manner and thus likely shared environmental microbes and possibly even endosymbiotic bacteria. Gut microbes have been shown to influence aggregation in cockroaches[27] and firebrats[28]. Such effects likely extend beyond these two insects, and possibly to bed bugs. The homogenization of microbiota within the lab could mask any effects that would have otherwise been observed in the field. The effects of environmental factors, and particularly microbes and diet, should be further evaluated for their roles in bed bug aggregation and host-associated differentiation. It is worth noting that in all experiments involving HA and BA bed bugs, HA bed bugs were found to have higher numbers of non-responders that BA bed or bat bugs. This phenomenon could be indicative of behavioral differences among populations or differential responses to conditioned shelters, although at this time we can only speculate on this observation.

Several aggregation assays deviated considerably from the overall pattern of no population- or lineage-specific aggregation fidelity. For example, JC-HA males preferred shelters that were heavily conditioned (10 bugs for 5 days) by their own JC-HA females over WS-HA females (Fig. 4), but this preference was not evident on lightly conditioned (1 bug for 1 day) shelters (Fig. 3). Likewise, WS-HA males preferred their own females' heavily conditioned shelters over shelters conditioned by either MO-BA females (Fig. 6) or Cp-BA females (but not males) (Fig. 8), but not on lightly conditioned shelters (Figs. 5, 7). We suspected that these results were caused by quantitative differences in shelter conditioning (amount of aggregation pheromones deposited onto the conditioned tents) rather than qualitative differences in pheromone preferences among the populations. We tested this hypothesis by titrating the quantitative effects of the aggregation pheromones and comparing aggregation responses to shelters conditioned by 10 or 5 bugs. The results showed that heavier conditioning of the shelters outweighed qualitative differences in the composition of the aggregation pheromones from different HA populations. These results clearly show that bed bugs are more responsive to quantitative differences rather than qualitative differences. Furthermore, these experiments provide additional evidence against the ability of aggregation pheromones to prevent gene flow among lineages or species.

Overall, these results provide strong evidence that under laboratory conditions, HA and BA bed bug lineages are reproductively compatible, and aggregation preferences do not facilitate behavioral reproductive isolation. This suggests that other factors are responsible for the observed genetic divergence among HA and BA bed bug populations in the field. It is still unclear how environmental factors (diet, microbes) influence reproductive compatibility

and aggregation fidelity and whether ecological barriers prevent contact between divergent host-associated bed bugs. Therefore, future studies should investigate reinforcement mechanisms that sustain genetic differentiation between these two host-associated lineages of *C. lectularius*.

Acknowledgements

We thank Richard Santangelo for assistance with experimental animals during this study, Maryann DeVries for assistance with bioassays, and Tomáš Bartonička for providing the Moravicany bed bug population.

Funding

Funding for this study was provided by the Blanton J. Whitmire Endowment at North Carolina State University, the US Department of Housing and Urban Development Healthy Homes program (NCHHU0017-13), the Alfred P. Sloan Foundation (2013-5-35 MBE), and a National Institute of Environmental Health Sciences grant to the Center for Human Health and the Environment (CHHE, P30ES025128). ZD received the David R. Nimocks Jr. Fellowship and scholarship awards from the Foundation for Agromedicine and Toxicology, Pi Chi Omega and the Entomological Society of America (Monsanto Research Grant Award, MUVE Scholarship).

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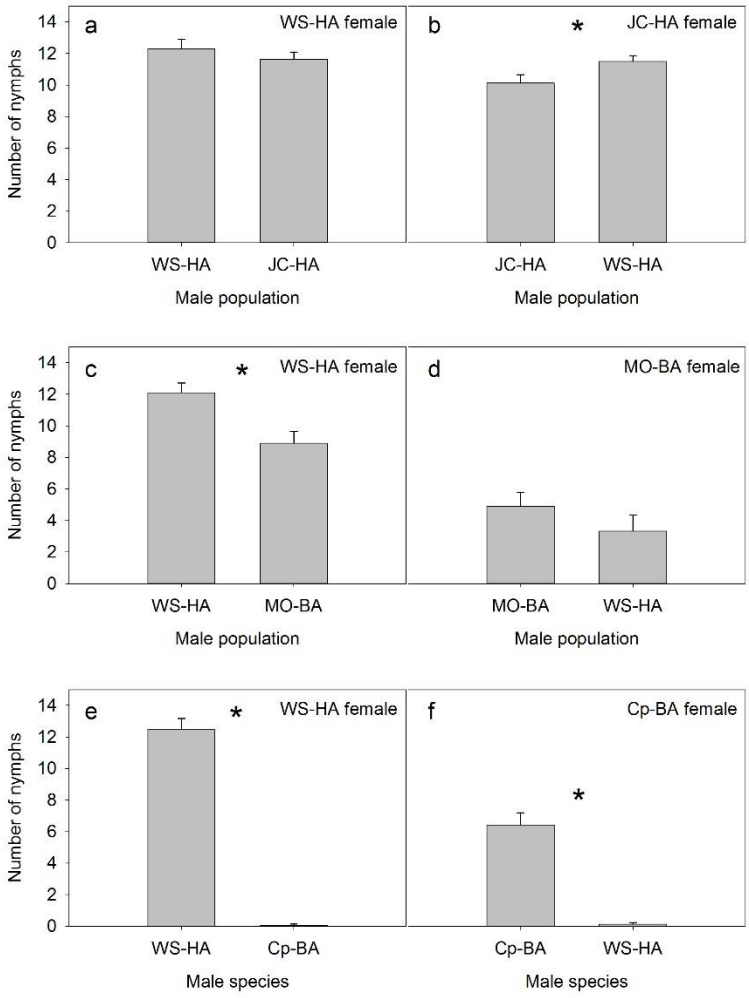

Figure 1. Reproductive compatibility as indicated by the number of nymphs produced between homogeneous and heterogeneous crosses. Comparisons are focused on each female, and are made within a lineage (WS-HA vs. JC-HA; a-b), between lineages (WS-HA vs. MO-BA; c-d), and between species (WS-HA vs. Cp-BA; e-f). Significance is based on Student's *t*-test ($p < 0.05$), and indicated by an asterisk (*).

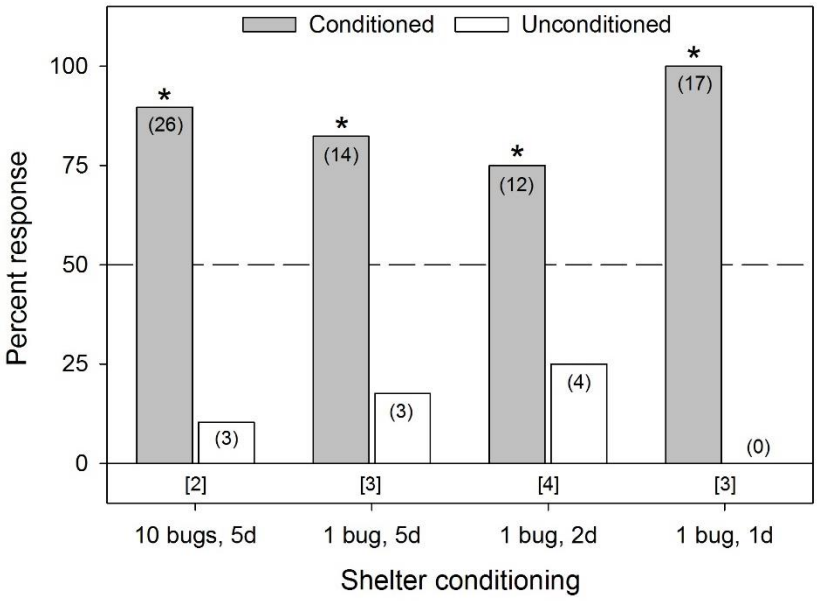

Figure 2 – Aggregation responses of individual male bed bugs (*C. lectularius*) of the human-associated WS strain to tent shelters either conditioned by various numbers of WS males for various amounts of time or unconditioned (clean controls). The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

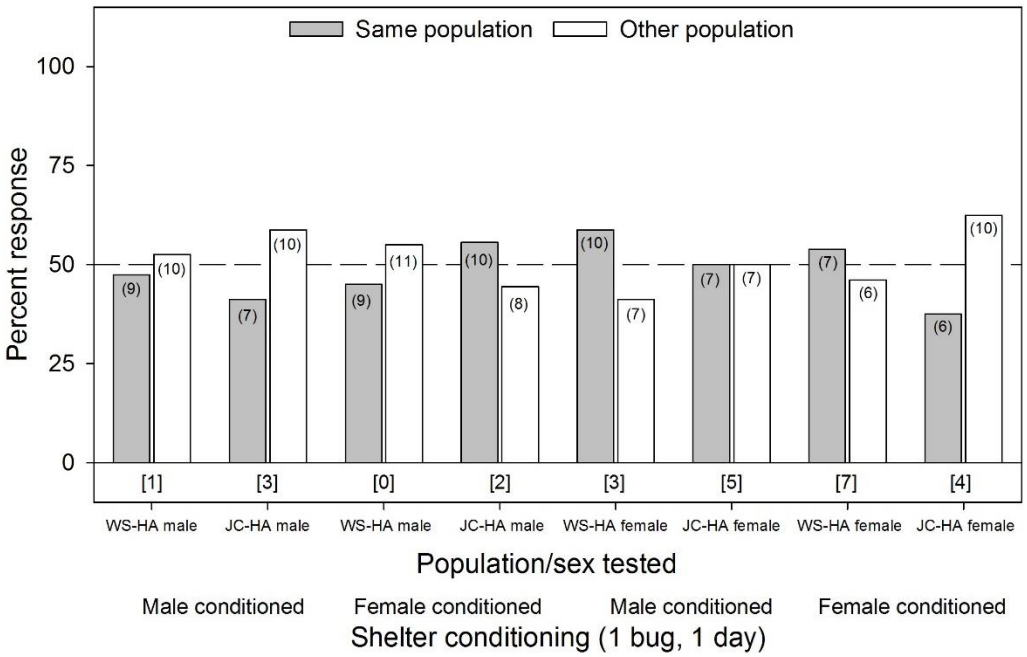

Figure 3 – Aggregation responses of individual bed bugs (*C. lectularius*) of the human-associated WS and JC strains to shelters conditioned for 24 hrs by single members of their own population or the other population. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. All the assays did not show significant differences in the choice of one shelter over the other, based on the Chi-square test ($p \geq 0.3173$ for all assays).

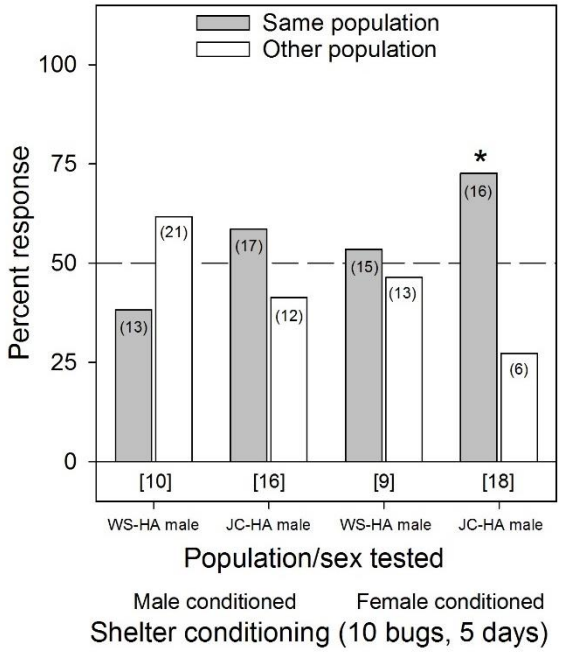

Figure 4 – Aggregation responses of individual male bed bugs (*C. lectularius*) of the human-associated WS and JC strains to shelters conditioned for 5 days by 10 adult male or female bed bugs of their own population or the other population. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

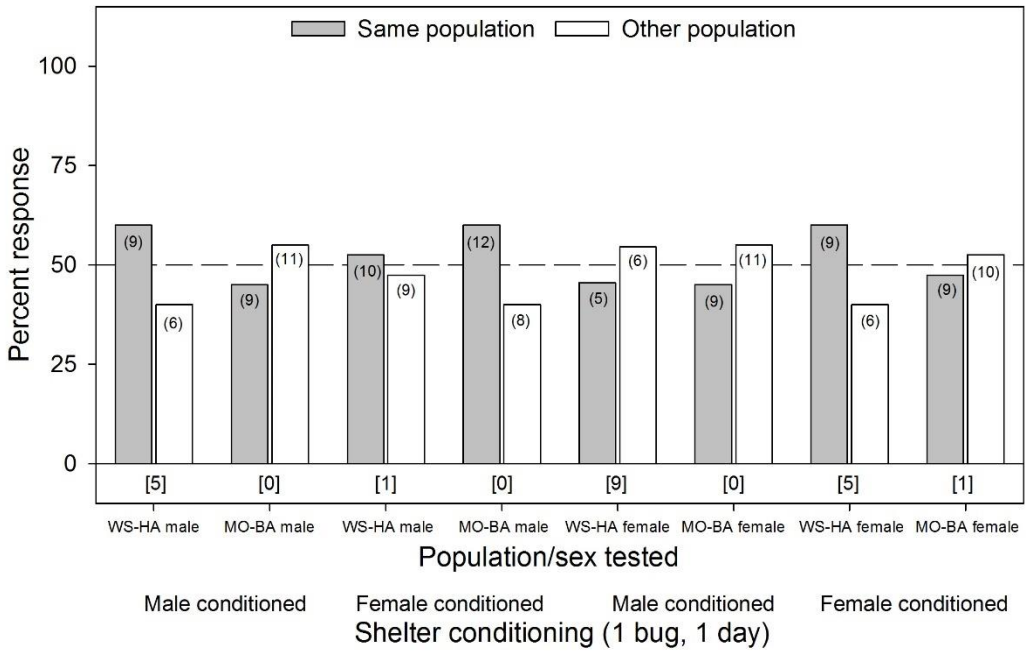

Figure 5 – Aggregation responses of individual bed bugs (*C. lectularius*) of the human-associated WS strain and the bat-associated MO strains to shelters conditioned for 24 hrs by single members of their own lineage or the other lineage. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. All the assays did not show significant differences in the choice of one shelter over the other, based on the Chi-square test ($p \geq 0.3711$ for all assays).

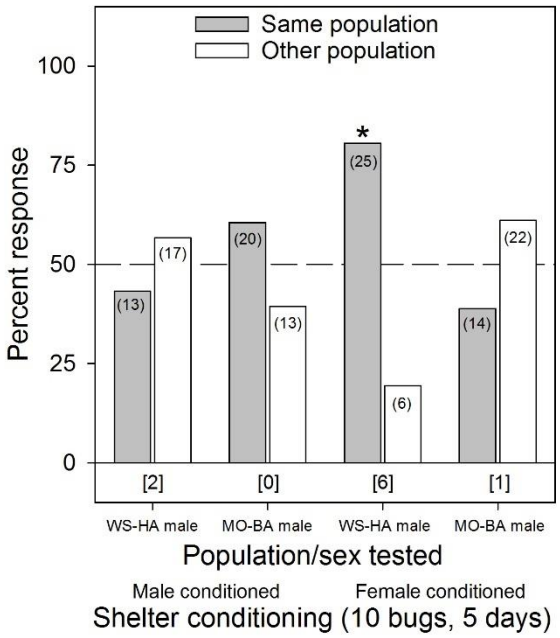

Figure 6 – Aggregation responses of individual bed bugs (*C. lectularius*) of the human-associated WS strain and the bat-associated MO strain to shelters conditioned for 5 days by 10 adult male or female bed bugs of their own lineage or the other lineage. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

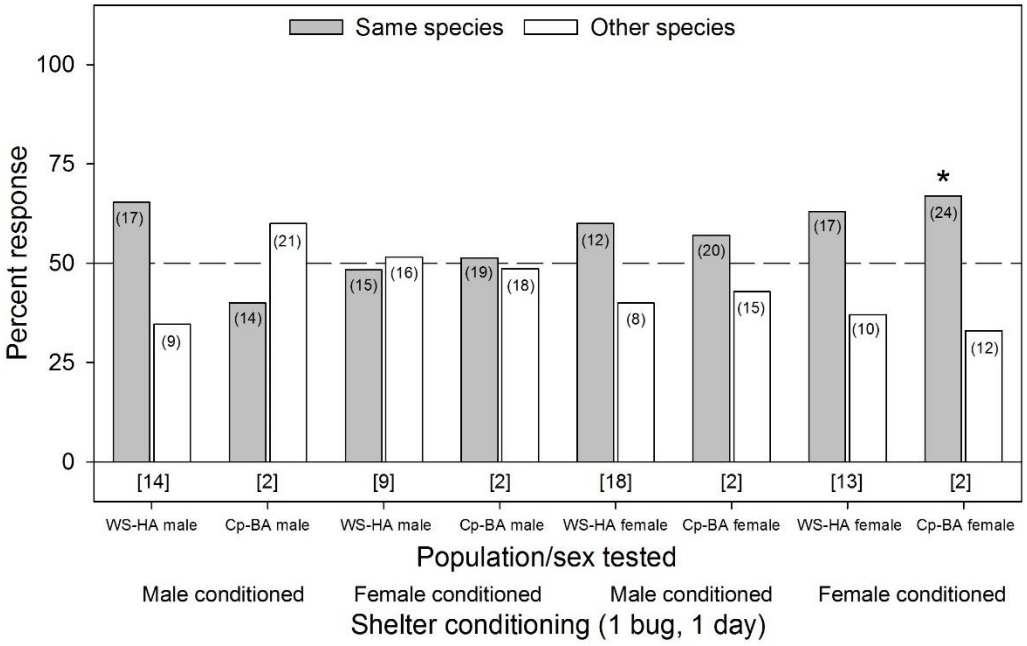

Figure 7 – Aggregation responses of individual bed bugs (*C. lectularius*, WS-HA strain) and bat bugs (*C. pipistrelli*, Cp-BA) to shelters conditioned for 24 hrs by a single member of their own species or the other species. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

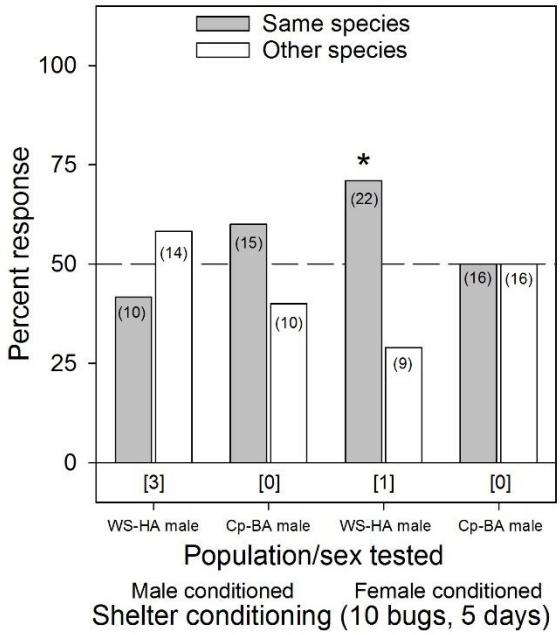

Figure 8 – Aggregation responses of individual bed bugs (*C. lectularius*, WS-HA strain) and bat bugs (*C. pipistrelli*, Cp-BA) to shelters conditioned for 5 days by 10 adult male or female bed bugs of their own species or the other species. The population/sex tested and the shelter conditioning information (male or female conditioned) are listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

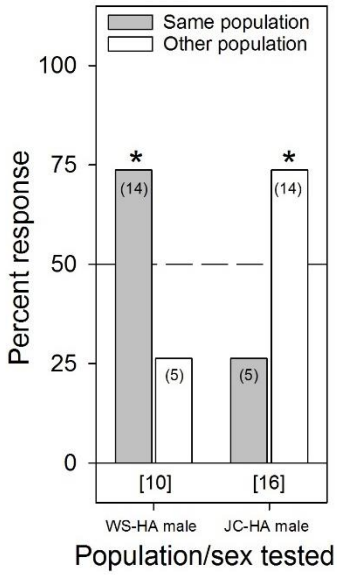

Figure 9 – Aggregation responses of individual bed bugs (*C. lectularius*) of the human-associated WS and JC strains to shelters unequally conditioned for 5 days by either 10 WS males or 5 JC males. The population/sex tested is listed below the figure. The number of individual bugs that responded to each choice is indicated in parenthesis. The number of non-responders is indicated in brackets below each set of choices for each assay. An asterisk (*) indicates significant choice of one shelter over the other, based on the Chi-square test ($p < 0.05$).

CHAPTER 3

Reproductive compatibility among populations and host-associated lineages of the common bed bug, *Cimex lectularius*

Abstract

As populations differentiate across geographic barriers or among hosts, inter-population fertility is a critical measure of the extent of incipient speciation. The bed bug, *Cimex lectularius*, was recently found to form two host-associated lineages: one found with humans (human associated, HA) and one found with bats (bat associated, BA). These lineages have limited gene flow between them; however, it is unclear if they are capable of mating and producing viable offspring. To address these questions and determine the extent of compatibility between host-associated lineages, we set up mating crosses among populations of bed bugs based on their host-association (HA vs. BA) and geographic locations (North America vs. Europe). Within-population fecundity was significantly higher for all HA populations (>1.7 eggs/day) than for BA populations (<1 egg/day). However, all within-population crosses, regardless of host-association, had >92% egg hatch rates. In all inter-lineage crosses, successful mating occurred, fertile eggs were oviposited, and the F_1 "hybrid" generation was found to be viable and produce offspring. These results indicate that while the HA and BA populations of *C. lectularius* represent genetically differentiated host-associated lineages undergoing incipient, possibly sympatric, speciation, they remain reproductively compatible. Other behavioral, physiological, and/or ecological factors likely maintain host-associated differentiation.

Keywords: bed bug, *Cimex*, host-associated differentiation, reproduction, sympatric speciation

Introduction

Understanding the mechanisms responsible for incipient speciation is critical to our understanding of evolution. Recently, the bed bug *Cimex lectularius*, an ectoparasite frequently associated with humans (Usinger 1966), was discovered to have two morphologically and genetically differentiated lineages: one associated with humans (HA = human-associated), and another associated with bats (BA = bat-associated) (Balvín et al. 2012). Despite strong geographic overlap, there is no apparent gene flow between these lineages, as assessed with both mitochondrial and nuclear markers (Booth et al. 2015). Broad geographic overlap of bed bug populations and lack of gene flow among them suggests that the two host-associated lineages have sympatrically differentiated into two host races and are undergoing incipient speciation.

Differentiation of host races has been extensively studied in phytophagous insects. Perhaps the best described host-race system is that of the apple maggot fly, *Rhagoletis pomonella*. In *R. pomonella*, there is a clear division among host lineages, with some preferentially attracted to apples and others to hawthorn (Bush 1969). These lineages can be distinguished genetically (Feder et al. 1988, McPheron et al. 1988), and their isolation appears to be reinforced by odor-based discrimination between the two host plants, possibly driven by only a few genes (Linn et al. 2003, Dambroski et al. 2005). In the case of bed bugs, it is unclear what mechanisms restrict gene flow between host-associated lineages.

Several mechanisms have been evaluated for restricting gene flow between the two host-associated lineages of bed bugs. Wawrocka and Bartonička (2013) showed that *C. lectularius* had lower fecundity and survivorship when reared on non-native host blood (e.g.,

human blood for BA bed bugs, bat blood for HA bed bugs) than on native-host blood. Wawrocka et al. (2015) further showed that HA and BA bed bugs were reproductively incompatible, with no eggs produced from inter-lineage crosses. Complete reproductive incompatibility would support that these host races have evolved to at least two distinct biological species. Behavioral isolating mechanisms however, remain elusive. Bed bugs use aggregation pheromones to orient to and arrest within bed bug-conditioned shelters (Siljander et al. 2008, Gries et al. 2015). Balvín et al. (2017) showed that bed bugs from the HA and BA lineages were incapable of discriminating lineage-specific shelters, and thus might co-aggregate in the wild. We recently extended these observations, demonstrating that *C. lectularius* bed bugs could not discriminate conspecific shelters from *Cimex pipistrelli* (bat bug)-conditioned shelters (DeVries et al. in press). Preliminary studies also revealed that inter-lineage crosses produced viable offspring (DeVries et al. in press), suggesting that disparate results on reproductive compatibility may relate to technical and methodological differences between studies.

To better understand reproductive (in)compatibility between different host-associated bed bug populations, we conducted reproductive crosses that spanned across geographic locations, and both within and between the two host-associated lineages. Our results indicate that bat- and human-associated bed bug populations are reproductively compatible. We discuss potential ecological and chemosensory mechanisms that might maintain the genetic separation of host-associated lineages of bed bugs.

Materials and Methods

Experimental Animals

Six populations of bed bugs were used in this study. Bed bugs were collected from bat roosts and homes, and from the United States and Europe. A full description of each population is provided in Table 1. After collection, bed bugs were maintained in the lab in plastic jars (6 cm diameter x 7 cm high) at 27°C and ~50% RH. All populations were fed defibrinated rabbit blood through an artificial feeding system which utilized a heated water bath (B. Braun Biotech Inc., Allentown, PA) to circulate water at 37°C through custom-designed water-jacketed glass feeders. Blood was retained in the feeders by an artificial membrane (grafting tape; A.M. Leonard, Piqua, OH, U.S.A.) through which bed bugs could feed on warmed blood. Field-collected bed bugs were reared in the lab through at least two generations prior to testing.

Reproductive Compatibility and F_1 Viability

Reproductive compatibility was assessed using the methods described by DeVries et al. (in press). Briefly, fifth instar nymphs were fed, isolated, and allowed to eclose to adults, thus ensuring that all adults used in the experiments had not previously mated. Adults were recombined into same sex groups by population and fed again. After one week, all bugs were fed a second time, then single male and single female pairs were introduced into 7.5 ml glass vials and allowed 6 d to freely mate and lay eggs. A paper insert within the vial served as a ramp to reach the feeder, shelter and oviposition substrate. After 6 d, adults were removed and the number of eggs was recorded in each vial. Eggs were monitored for the

next 14 d and the number of first instars was recorded. Finally, the offspring from each replicate cross were combined and reared under similar conditions as the bed bug colonies to assess their reproductive viability – the ability of the F_1 generation to produce offspring. This measure was not quantitative, so viability is reported only as a Yes/No at the population level.

All assays were female-centric, so each comparison was between females that mated with males from their own population and females that mated with males from a different population. Because the large number of crosses could create substantial within-population and temporal variation. To minimize these sources of variation, all crosses were run in a 2 x 2 matrix design. Thus, in a cross between populations A and B, females of population A were mated to males of populations A and B, and females of population B were mated to males of populations A and B. Although this design resulted in crosses being repeated, it ensured that each cross had concurrent within-population positive controls (e.g., A females x A males), which were used to normalize all crosses involving that population (e.g., A in this example). All assays were conducted between May 2014 and April 2016.

Data Analysis

Fecundity was compared among all within-population crosses using ANOVA, with means compared using the Tukey-Kramer multiple comparison test. Egg hatch rate (percentage of eggs resulting in 1st instars) was compared among all within-population crosses using the Kruskal–Wallis test. Fecundity was compared in crosses between populations using a student's *t*-test because each 2 x 2 design included within-population positive control crosses.

Hatch rate in inter-population crosses was assessed using the Kruskal–Wallis test. All analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, U.S.A.)

Results

Fecundity and Hatch Rate within Populations

Fecundity differed significantly among all populations tested (ANOVA, $F_{5,377} = 96.17$, $p < 0.0001$, Fig. 1). Although there were some differences among the HA populations (BE-HA significantly lower than the other three HA populations), fecundity in the four HA populations was significantly higher than in both BA populations. Hatch rate however was >92% in all six populations and was not significantly different among populations (Kruskal–Wallis test, $H_{5,365} = 7.8037$, $p = 0.1674$) (Fig. 2).

Reproductive Compatibility in Inter-Population Crosses

The inter-population crosses revealed no evidence of reproductive incompatibility, with fecundity ranging between 70.7% and 147.9% of the respective within-population fecundity (Table 2). Out of a total of 30 crosses between populations, only three resulted in significant changes in fecundity compared to the respective within-population crosses. However, we could not detect any patterns in these three crosses. Two crosses resulted in significant increases in fecundity; one involved HA populations (JC-HA x WS-HA) and one was between the two European host-associated lineages (MO-BA x OS-HA). The single significant decline in fecundity was a within-lineage cross between OS-HA females and WS-HA males (Table 2).

Hatch rate in the inter-population crosses ranged from 72.4% to 111.1% of the respective within-population hatch rates (Table 3). Only five out of the 30 inter-population crosses resulted in significant changes in hatch rate, and notably, all five were inter-lineage crosses. All crosses between females from four HA populations and MO-BA males produced eggs with significantly lower hatch rates (72.4 to 83.5% of their respective within-population hatch rate). However, hatch rates for the other BA population (HN-BA) were not affected by intra- or inter-lineage mating, suggesting that lower hatch rate was unique to the MO-BA population and thus represented a population effect and not a host-associated lineage effect.

The F_1 offspring of all inter-population crosses (including inter-lineage crosses) were reared to adults, and examined for fertility. All progeny of inter-population crosses produced offspring, indicating reproductive compatibility among all populations.

Discussion

The common bed bug, *Cimex lectularius*, appears to be an excellent model for host-associated genetic differentiation in sympatry. Its limited mobility (wingless adults), hematophagy and close association of all life stages with the host makes it a particularly attractive model for investigations of incipient speciation. Two alternative host-associated lineages co-exist in Europe: human-associated (HA) and bat-associated (BA) populations (Balvín et al. 2012). These two lineages, or host races, have differentiated morphologically, behaviorally and physiologically, suggesting adaptation to their respective hosts, and have limited gene flow between them (Booth et al. 2015). Moreover, divergent insecticide

selection pressures on HA and BA populations lead to polymorphisms at insecticide target loci of HA, but not BA bugs (Booth et al. 2015). Ultimately, lineage divergence based on host-association is expected to promote host fidelity and reinforce further differentiation. Indeed, Wawrocka et al. (2015) showed compelling evidence that crosses between HA and BA bed bugs failed to produce any eggs, indicating reproductive incompatibility and the possible emergence of separate biological species.

Surprisingly, our results departed radically from those of Wawrocka et al. (2015). Crosses of all HA and BA bugs mated successfully, produced viable eggs that hatched, and their progeny produced viable offspring. The small differences in fecundity among populations appear to be inconsequential and not related to host-differentiation. Interestingly however, the two BA populations had significantly lower fecundity than the four HA populations in within-population crosses. Although previous studies have documented variation in fecundity among HA populations (Barbarin et al. 2014, Gordon et al. 2015), the 2-fold greater fecundity in HA than BA populations is striking. These differences, within our experiments, may be attributed to host blood type and differential adaptations to the lab conditions which are related to time in culture. Diet has been shown to affect fecundity, growth, and development in HA populations (Barbarin et al. 2013) and BA populations (Wawrocka and Bartonička 2013). In our assays however, all populations were reared on the same diet (defibrinated rabbit blood), so the observed differences likely represent physiological adaptations among the populations. The two HA populations we collected in 2008 could be better adapted to lab conditions. Yet, the two HA populations that were collected either concurrently (OS-HA) or after (BE-HA) both BA populations were collected

had significantly greater fecundity than the BA populations. Overall, these observations suggest that time in the lab was not a key factor responsible for inter-lineage differences in fecundity. Furthermore, eggs produced in all crosses, including HA x BA crosses, were viable and had high hatching success (> 92%). Therefore, reproductive barriers do not appear to prevent gene flow between these two host-associated lineages of *C. lectularius*.

The differences between our present and previous preliminary findings (DeVries et al. in press) and those of Wawrocka et al. (2015) could be due to a number of factors. The bugs tested by Wawrocka et al. (2015) were collected as nymphs, reared in the lab to adults, and used in crosses; they likely did not fully adapt to laboratory conditions. Although bed bugs can develop on a range of hosts (Usinger 1966), it is still unclear if they require any time to switch from one host to another. Many phytophagous insects are incapable of switching diets later in life (Scriber 1979, Karowe 1989), so it is not unreasonable to speculate that the switch of diets could negatively affect reproduction and reproductive compatibility. Furthermore, bed bugs maintain a symbiotic relationship with intracellular *Wolbachia* (Hosokawa et al. 2010), although it is unclear if different strains of *Wolbachia* are present in different populations of bed bugs. If *Wolbachia* were found to differ among populations, it could potentially result in reproductive incompatibility, as seen in other arthropods (Werren 1997, Stouthamer et al. 1999, Werren et al. 2008, Sharon et al. 2010). However, this effect could be masked under laboratory conditions where the microbiome would likely be homogenized over time due to communal feeding and mass rearing in close proximity, possibly explaining the differences between the current study and that of Wawrocka et al.

(2015). Despite these possibilities, our results are very clear and show crosses between HA and BA bed bugs can produce viable offspring.

Similar levels of reproductive compatibility have been reported in other host races as in the current study. In the case of the apple maggot fly, which has two genetically distinguishable host races (Feder et al. 1988, McPheron et al. 1988), viable hybrids can be produced between the races, although they are often selected against (Linn et al. 2004). In a similar example, the pea aphid (*Acyrthosiphon pisum*) is also capable of forming less-fit but viable hybrids between two host races found associated with alfalfa and red clover (Via et al. 2000). In sympatric speciation, the difference between host-race formation and true speciation can be linked to an ability of crosses between host-races to produce viable offspring. Our results support a model of host-race (or lineage) formation, not true species formation, because all crosses produced eggs that hatched (F_1 generation), and when reared to adults, produced offspring.

The factor(s) responsible for maintaining genetic separation between HA and BA lineages of bed bugs remain unclear, but in the absence of reproductive incompatibility ecological and behavioral factors likely maintain genetic isolation. Fidelity to lineage- or population-based aggregations was dismissed as a behavioral isolating mechanism (Balvín et al. 2017, DeVries et al. in press). However, a range of other behaviors have not been investigated, and differential host attraction is a primary candidate. Bed bugs are attracted to human odors (Harraca et al. 2012, Liu and Liu 2015), but it is unknown how specialized their odor preferences are and whether host attraction differs in HA and BA bed bugs. It is likely that genetic isolation between host associated lineages is maintained by a combination of

factors working in concert, including host preference, different diets influencing microbes, different strains of *Wolbachia*, and differentiation of morphological adaptations. Further testing is needed to better understand this system and the mechanisms responsible for preventing gene flow between lineages.

In conclusion, reproductive incompatibility does not appear capable of preventing gene flow between host associated lineages of bed bugs. Although there were some differences in fecundity among lineages, all human- and bat-associated populations were fully compatible with each other under laboratory conditions. Future work should focus on ecological factors (diet, microbiome), chemosensory specialization (such as host preferences), and morphological adaptations which may maintain genetic isolation.

Acknowledgements

We thank Russell Mick for assistance with experimental animals during this study. Funding for this study was provided by the Blanton J. Whitmire Endowment at North Carolina State University, the US Department of Housing and Urban Development Healthy Homes program (NCHHU0017-13), the Alfred P. Sloan Foundation (2013-5-35 MBE), and a National Institute of Environmental Health Sciences grant to the Center for Human Health and the Environment (CHHE, P30ES025128). ZD received the David R. Nimocks Jr. Fellowship and scholarship awards from the Foundation for Agromedicine and Toxicology, Pi Chi Omega and the Entomological Society of America (Monsanto Research Grant Award, MUVE Scholarship).

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Table 1. Bed bug populations used in reproductive crosses.

Population	Host	Location	Year collected
JC-HA	Human	Jersey City, NJ, USA	2008
WS-HA	Human	Winston Salem, NC, USA	2008
OS-HA	Human	Oslo, Norway	2014
BE-HA	Human	Beroun, Czech Republic	2015
MO-BA	Bat (*Myotis myotis*)	Moravicany, Czech Republic	2014
HN-BA	Bat (*Myotis myotis*)	Hanusovice, Czech Republic	2014

Table 2. Relative fecundity in inter-population crosses. All percentages are relative to female fecundity in within-population crosses (patterned cells set to 100%), and thus reflect the increase or decrease in fecundity when mated to males from another population. Bat-associated (BA) populations are shaded in blue. Significance according to the Student's *t*-test (2-tailed) is indicated with an *, with the test statistic and sample size listed below each percentage (all tests with 1 *df*).

Female population \ Male population	JC-HA	WS-HA	OS-HA	BE-HA	MO-BA	HN-BA
JC-HA	**100**	116.6* *t* = -2.53 n = 35	95.3 *t* = 0.72 n = 28	92.7 *t* = 0.46 n = 16	87.6 *t* = 1.07 n = 16	86.7 *t* = 1.66 n = 20
WS-HA	94.3 *t* = -0.89 n = 33	**100**	98.7 *t* = 0.18 n = 24	109.3 *t* = -0.96 n = 20	89.7 *t* = 1.03 n = 20	97.5 *t* = 0.26 n = 18
OS-HA	101.6 *t* = 0.24 n = 28	84.9* *t* = -2.26 n = 24	**100**	95.8 *t* = 0.51 n = 26	93.2 *t* = 0.62 n = 26	90.0 *t* = 1.55 n = 32
BE-HA	103.3 *t* = 0.80 n = 16	103.7 *t* = 0.48 n = 20	94.7 *t* = -0.86 n = 30	**100**	103.4 *t* = -0.58 n = 30	93.3 *t* = 1.06 n = 29
MO-BA	80.6 *t* = -0.64 n = 16	70.7 *t* = -1.03 n = 19	147.9* *t* = 2.09 n = 26	119.0 *t* = 1.09 n = 30	**100**	96.1 *t* = 0.17 n = 18
HN-BA	140.7 *t* = 0.96 n = 15	86.5 *t* = -0.45 n = 18	110.8 *t* = 0.21 n = 31	80.0 *t* = -1.09 n = 30	128.8 *t* = 2.20 n = 18	**100**

Table 3. Relative hatch rate in inter-population crosses. All percentages are relative to hatch rate in within-population crosses (patterned cells set to 100%), and thus reflect the increase or decrease in hatch rate when mated to males from another population. Bat-associated (BA) populations are shaded in blue. Significance according to the Kruskal–Wallis test is indicated with an *, with the test statistic and sample size listed below each percentage (all test with 1 *df*).

Female population \ Male population	JC-HA	WS-HA	OS-HA	BE-HA	MO-BA	HN-BA
JC-HA	**100**	97.4 H = 2.71 n = 35	101.5 H = 0.79 n = 28	94.0 H = 2.27 n = 16	83.2* H = 5.42 n = 16	99.9 H = 0.08 n = 20
WS-HA	100.2 H = 0.04 n = 33	**100**	98.0 H = 0.97 n = 24	98.2 H = 0.44 n = 20	83.5* H = 9.88 n = 20	96.3 H = 0.20 n = 18
OS-HA	98.6 H = 0.10 n = 28	98.2 H = 0.41 n = 24	**100**	93.0 H = 1.59 n = 26	72.4* H = 7.20 n = 26	83.5* H = 4.97 n = 32
BE-HA	104.8 H = 0.71 n = 16	98.7 H = 0.62 n = 20	99.8 H = 0.03 n = 30	**100**	82.4* H = 12.95 n = 30	102.8 H = 0.18 n = 29
MO-BA	107.5 H = 1.49 n = 16	95.5 H = 0.83 n = 19	98.2 H = 1.10 n = 26	111.1 H = 0.58 n = 30	**100**	102.1 H = 0.39 n = 18
HN-BA	102.9 H = 0.46 n = 15	104.3 H = 0.88 n = 18	91.9 H = 0.33 n = 31	94.3 H = 1.00 n = 30	87.8 H = 0.14 n = 18	**100**

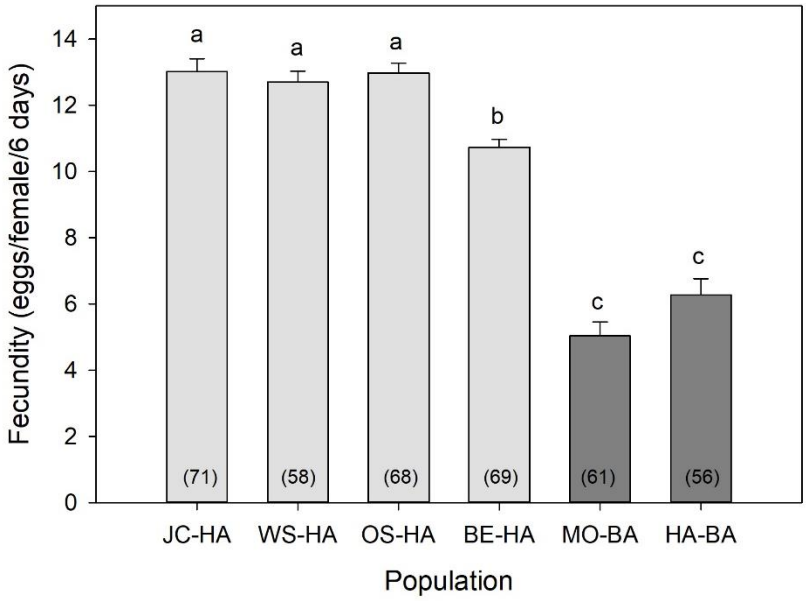

Figure 1. Average fecundity of bed bugs with different host associations (HA – human associated, BA – bat associated) in within-population crosses. Sample size is indicated in parentheses within each bar. Significant differences among populations based on ANOVA and Tukey's post-hoc test ($P < 0.05$) and indicated with different lowercase letters.

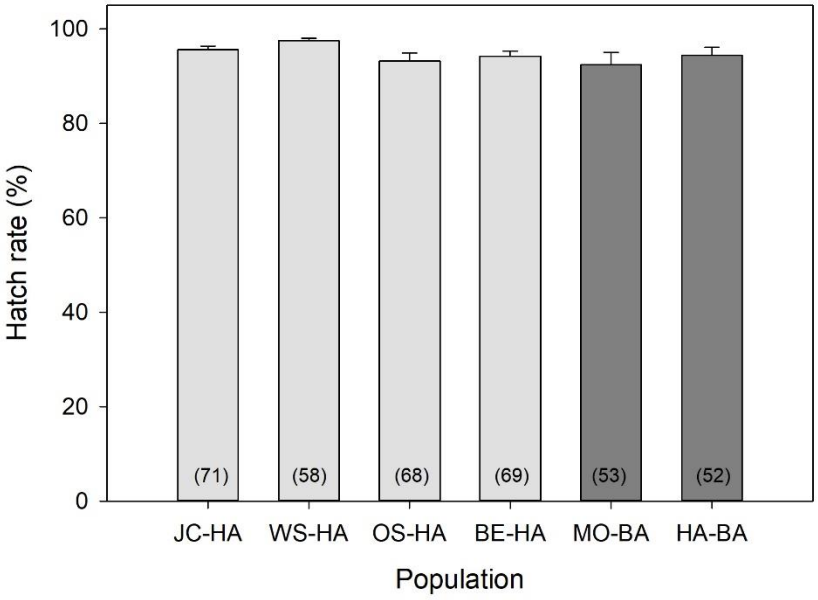

Figure 2. Average hatch rate of bed bugs with different host associations (HA – human associated, BA – bat associated) in within-population crosses. Sample size is indicated in parentheses within each bar. There were no significant differences among populations based on ANOVA ($P = 0.1674$).

CHAPTER 4

Feel the heat: Activation, orientation, and feeding responses of bed bugs to targets at different temperatures

(This work was published in the *Journal of Experimental Biology*: DeVries ZC, Mick RA, and Schal C. 2016. Feel the heat: Activation, orientation, and feeding responses of bed bugs to targets at different temperatures. *Journal of Experimental Biology* 219(23): 3773-3780. doi: 10.1242/jeb.143487)

Abstract

Host location in bed bugs is poorly understood. Of the primary host-associated cues known to attract bed bugs – CO_2, odors, heat – heat has received little attention as an independent stimulus. We evaluated the effects of target temperatures ranging from 23-48°C on bed bug activation, orientation, and feeding. Activation and orientation responses were assessed using a heated target in a circular arena. All targets heated above ambient temperature activated bed bugs (initiated movement) and elicited oriented movement toward the target, with higher temperatures generally resulting in faster activation and orientation. The distance over which bed bugs could orient toward a heat source was measured using a 2-choice T-maze assay. Positive thermotaxis was limited to distances < 3 cm. Bed bug feeding responses on an artificial feeding system increased with feeder temperature up to 38°C and 43°C, and declined precipitously at 48°C. In addition, bed bugs responded to the relative difference between ambient and feeder temperatures. These results highlight the wide range of temperatures which elicit activation, orientation, and feeding responses in bed bugs. In contrast, the ability of bed bugs to correctly orient towards a heated target, independently of other cues, is limited to very short distances (< 3 cm). Finally, bed bug feeding is shown to be relative to ambient temperature, not an absolute response to feeder-blood temperature.

Introduction

Heat is a common sensory cue used across diverse taxa, ranging from microscopic organisms such as bacteria (Paster and Ryu, 2008) and protozoans (Poff and Skokut, 1977) to a wide range of animals including insects (Dillon et al., 2009), fish (Reynolds, 1977), reptiles (Buning, 1983), and mammals (Leonard, 1974). Heat cues serve a multitude of functions, such as indicating the presence of appropriate habitats (Graham, 1958; Holsapple and Florentine, 1972; Leonard, 1974), signaling the need to initiate aestivation (Finch and Collier, 1985), and mediating orientation to hosts (Bullock and Cowles, 1952; Lazzari and Núñez, 1989b; Lees, 1948; Peterson and Brown, 1951), prey (Buning, 1983) and thermogenic flowers (Ivancic et al., 2008; Seymour and Schultze-Motel, 1997; Wang and Zhang, 2015).

Many arthropods can detect heat to locate hosts and flowers, including hematophagous mosquitoes (Peterson and Brown, 1951), kissing bugs (Lazzari and Núñez, 1989b), ticks (Lees, 1948), fleas (Osbrink and Rust, 1985), bed bugs (Rivnay, 1932) and some pollinators (Seymour and Schultze-Motel, 1997). However, the ability to perceive heat and the manner in which heat is used vary widely among species. Most species orient to heat over short distances (Lazzari and Núñez, 1989b), with only the buprestid *Melanophila acuminate* known to be capable of positive thermotaxis over long distances of several km, through the use of specialized infra-red receptors (Evans, 1964; Schmitz and Bleckmann, 1998). Mosquitoes typically use CO_2 and visual cues at long distances, which subsequently guide them close enough to the host to utilize odors, humidity, and heat to locate a landing site (van Breugel et al., 2015). Lazzari and Núñez (1989b) found the kissing bug, *Triatoma*

infestans, capable of infrared heat detection, quite different from other arthropods which primarily rely on conduction and convection (Khan et al., 1966; Khan et al., 1968). When orienting toward a heat source, kissing bugs were observed to use both telotaxis (orientation using a single sensory structure over longer distances) and tropotaxis (orientation using dual sensory structures over short distances) (Lazzari, 2009). Although thermotaxis for host location has received some attention in hematophagous insects, it has traditionally been evaluated in combination with other host cues (Grossman and Pappas, 1991; Khan et al., 1968; Takken and Verhulst, 2013). Only recently have researchers elucidated the integration of heat with CO_2, odors, and vision in host location by a mosquito (van Breugel et al., 2015).

Bed bugs (*Cimex lectularius* Linnaeus) are hematophagous ectoparasites that feed on endothermic hosts (Usinger, 1966). They require a blood meal at each of their five nymphal instars to molt and as adults to survive and reproduce (Usinger, 1966). Bed bugs are typically found living in close proximity to their host, but not directly on their host (i.e., unlike lice, and some fleas). Host seeking appears to be highly mediated by a circadian rhythm, with unfed bed bugs showing increased activity levels at night (Reis and Miller, 2011; Romero et al., 2010). When searching for a host, bed bugs actively orient to three host-related stimuli: body odors (Harraca et al., 2012), CO_2 (Anderson et al., 2009), and heat (Rivnay, 1932). Since its discovery as an attractant, heat has been evaluated through trap catch assays designed to understand bed bug orientation and improve detection and monitoring of infestations (Anderson et al., 2009; Singh et al., 2012; Wang et al., 2009). However, such endpoint assays that measure the accumulation of insects at a site inform us

little about thermotactic strategies and the direct role of heat alone in bed bug host searching and feeding behavior.

The purpose of this study was to evaluate the effects of heat on bed bug activation, orientation, and feeding. In addition, we aimed to empirically assess the distance over which bed bugs detect a heated target and orient toward it, and how changes in ambient temperature affect bed bug feeding behavior. We used behavioral assays and an artificial feeding system to evaluate the effects of different temperatures on bed bug behavior.

Materials and Methods

Experimental Animals

A laboratory colony of bed bugs was used for all experiments. This strain, Harold Harlan (HH), was collected in 1973 in Ft. Dix, NJ, USA. Another strain, Winston Salem (WS), originally collected from Winston Salem, North Carolina, USA, in 2008 was also used for some feeding experiments.

Bed Bugs were reared on a 12:12 light:dark cycle at 27°C and 50±5% relative humidity. All bed bugs were fed defibrinated rabbit blood in an artificial feeding system similar to that described by Montes et al. (2002), which used a circulating water bath (B. Braun Biotech Inc., Allentown, PA) to warm the blood to 38°C. Bed bugs fed through an artificial membrane (Nescofilm, Karlan, Cottonwood, AZ, USA). All experiments used only adult males that were 7-10 d post-feeding and hence likely motivated to host-seek. Additionally, all experiments were conducted during the scotophase (under red-light) and at 25°C and 50±5% relative humidity, unless otherwise stated. All temperatures (including

experimental apparatuses) were checked using a BAT-12 microprobe thermometer (Physitemp Instruments, Inc., Clifton, NJ, USA).

Activation in Response to Heat

A single HH bed bug was allowed to acclimate for 3 d in a plastic Petri dish (90 mm diameter, Fisher Scientific, Waltham, MA, USA) lined with a filter paper floor and a paper tent (8 mm x 30 mm) at the edge of the dish. This period was long enough for most bugs to shelter in the tent, although longer times were granted if after 3 d bugs were found moving during the scotophase. During the scotophase, a copper coil (i.d. = 1.5 mm, coiled diameter = 3 cm) heated or cooled via a circulating water bath (RM6 Thermostat, Brinkmann Instruments Inc., Delran, NJ, USA) to 23, 28, 33, 38, 43, or 48°C was carefully introduced into the arena, in line with the opening of the paper tent (where a single bed bug was quiescent) at a distance of 40 mm from the bug. The bed bug was observed and time to first movement was recorded. Each trial lasted for up to 5 min; after this time, bugs that did not move were scored as unresponsive. At least 12 replicates were performed for each coil temperature.

Orientation in Response to Heat

HH bed bugs were placed into individual glass vials (7.5 ml) 24 h prior to the start of the experiment. Next, each bed bug was introduced into a large plastic Petri dish arena (141 mm diameter) by inverting the glass vial along the edge of the arena wall. The arena was lined with a filter paper floor and contained the same copper coil as before in the center of

the arena (45 mm from the glass vial). The coil was set to one of six temperatures: 23, 28, 33, 38, 43, or 48°C. Each bed bug was allowed 5 min to acclimate to the arena within the glass vial. The glass vial was then carefully removed and bed bug behavior was recorded using a Sony Handycam video recorder (HDR-XR260, Sony Corporation, Minato, Tokyo, Japan) until the bed bug contacted the copper coil (5 min maximum time limit). The time taken to reach the coil was recorded. Digital videos were analyzed for a suite of behaviors using the video analysis software Ctrax (Branson et al., 2009), with corrections to videos made with Matlab (The Mathworks, Natick, Massachusetts, USA). The following parameters were calculated for each video: average velocity, angular speed, average distance to the wall, total distance traveled, and angle to arena center. At least 14 replicates were performed at each coil temperature.

Effect of Distance on Orientation to Heat

The distance over which bed bugs were capable of detecting heat was evaluated with two-choice assays. Each HH bed bug was placed in a glass vial (7.5 ml) 24 h prior to the start of the experiment. A bed bug was introduced at the base of a vertically oriented T-shaped two-choice arena constructed by adhering a paper substrate onto a Plexiglas backing. The dimension of the T-maze were as follows: 20 mm wide base which narrowed to a width of 3 mm over 50 mm. The two side arms of the T-maze were each 75 mm long and 20 mm wide. The copper coil was heated to 38°C and placed at one side of the "T" 10, 30, or 50 mm from the choice point. The heated side was alternated left and right to account for any position biases and a control assay was run with the coil set to room temperature (25°C) to

account for vibrational effects. A choice was recorded when the bed bug moved 10 mm in one direction at the top of the "T". All assays were conducted under red light and at least 30 replicates were performed for each distance.

Feeding in Response to Heat

The effects of both feeder temperature and ambient temperature on bed bug feeding were evaluated. Each HH or WS bed bug was placed individually into a glass vial (7.5 ml) ~ 24 h prior to the start of the experiment. The vial contained a paper ramp leading up to a nylon mesh lid (0.3 mm mesh size; BioQuip Products, Rancho Dominguez, CA, USA), which permitted feeding but retained the bug in the vial. The vial was placed under the artificial feeding system for 30 min at one of six feeder temperatures (measured by a thermocouple inserted directly into the blood): 23, 28, 33, 38, 43, or 48°C. The number of fully engorged bed bugs was recorded at each temperature. A sample size of 36 bugs was used for each temperature, with bugs fed individually.

Bed bugs from both populations fed at feeder temperatures below the ambient temperature. To confirm this observation and control for any olfactory or gustatory cues the blood may have provided, we repeated the above feeding experiment at 23 and 38°C using only a solution of 1 mmol l^{-1} ATP in PBS, previously confirmed by Romero and Schal (2014) to be sufficient to elicit feeding in bed bugs. In addition, all surfaces were sterilized using ethanol prior to feeding.

To evaluate the interaction between ambient temperature and feeder temperature, the same feeding methods previously described were used, but the room was either maintained at

25°C, heated to 30°C or cooled to 20°C. In addition, only feeder temperatures that produced intermediate levels of feeding at an ambient temperature of 25°C were used, i.e., 23, 28 and 33°C. The number of fully engorged bugs was recorded for each ambient-feeder temperature combination. At least 36 bugs were used for each ambient-feeder temperature combination, with bugs fed individually.

Data Analysis

The proportion of bugs that activated and oriented were analyzed using logistic regression. Only the bugs that responded were analyzed further. Regression analysis was used to understand the effects of target temperature on activation, time to reach the coil, and all additional orientation parameters (velocity, angular speed, distance to the wall, total distance traveled). Angular data were evaluated using circular statistics. At each temperature, the length and angle of the mean vector of orientation were calculated for the bed bugs which reached the coil. Significance of mean vectors were determined at each temperature using the Rayleigh test (Batschelet, 1981). Chi-square tests were used to evaluate the effects of distance on bed bug orientation. Logistic regression was used to evaluate all feeding data. In addition, a Chi-square test of independence was used to determine the effects of ambient temperature and feeder temperature on bed bug feeding. All statistics were implemented in SAS 9.4 (SAS Institute, Cary, NC, USA) and PAST (Hammer et al., 2001).

Results

Activation in Response to Heat

The responses of HH bed bugs 40 mm from a cooled or heated target were significantly affected by the temperature of the target, with two distinct treatment effects. First, the number of bugs responding increased with target temperature ($\chi^2_{1,72} = 33.09$, $p < 0.0001$; Fig. 1), and second, activation latency (time to first movement) was negatively related with target temperature ($F_{1,4} = 14.91$, $p = 0.0181$, $r^2 = 0.7884$; Fig. 1, dashed line).

Orientation in Response to Heat

Orientation to the target over a distance of 40 mm was significantly affected by target temperature. The number of HH bugs that reached the target coil increased with temperature, with all bugs reaching the coil at target temperatures >38°C ($\chi^2_{1,87} = 24.19$, $p < 0.0001$; Fig. 2). Higher temperatures resulted in significantly shorter response times for those bugs that reached the coil, although the relationship was non-linear ($F_{2,3} = 12.56$, $p = 0.0348$, $r^2 = 0.8933$; Fig. 2, dashed line).

Walking velocity did not show a significant relationship with target temperature (Fig. 3a; $F_{2,3} = 1.59$, $p = 0.3374$, $r^2 = 0.5153$). Average angular speed increased with temperature in a curvilinear fashion (Fig. 3b; $F_{2,3} = 14.32$, $p = 0.0292$, $r^2 = 0.9052$). The average distance a bug was located relative to the wall throughout the experiment showed a curvilinear relationship with temperature (Fig. 3c; $F_{2,3} = 74.79$, $p = 0.0028$, $r^2 = 0.9803$). The total distance traveled until reaching the coil related to temperature in a curvilinear fashion (Fig. 3d; $F_{2,3} = 12.38$, $p = 0.0355$, $r^2 = 0.8920$).

In addition, coil temperature significantly affected the orientation angle of each HH bed bug relative to the center of the arena (where the coil was located). At lower temperatures, 23°C (r = 0.5513, n = 5, p = 0.2282), 28°C (r = 0.1896, n = 12, p = 0.6590), and 33°C (r = 0.3155, n = 13, p = 0.2792), bed bugs did not exhibit a significant directional orientation (Fig. 4). At higher temperatures however, 38°C (r = 0.5016, n = 18, p = 0.0090), 43°C (r = 0.5418, n = 14, p = 0.0137), and 48°C (r = 0.4937, n = 13, p = 0.0389), bed bugs oriented significantly toward the respective targets (Fig. 4).

Orientation Distance in Response to a Heated Target

HH bed bugs had no significant side-bias, orienting equally to the two arms of the T-maze when the coil was 10 mm away from the T-junction with water at ambient temperature (25°C) circulating through the coil ($\chi^2_{1,30}$ = 0.5333, p = 0.4652; Fig. 5). Bed bugs showed a significant preference for the side with the heated coil (38°C) when it was located 10 mm away from the T-junction ($\chi^2_{1,30}$ = 19.20, $p < 0.0001$), but no significant preferences at 30 mm ($\chi^2_{1,40}$ = 2.47, p = 0.1161) or 50 mm ($\chi^2_{1,30}$ = 0.0000, p = 1.0000) (Fig. 5).

Feeding in Response to Feeder-Blood Temperature

Blood (feeder) temperature significantly and similarly affected feeding in two bed bug strains (Fig. 6). As the feeder temperature increased, the proportion of HH bed bugs that fed increased, with the greatest percentage feeding at 38-43°C; bed bugs did not feed when the blood was at 48°C (Fig. 6). Excluding 48°C from the analysis, the proportion of HH bed bugs that fed significantly related to temperature (°C) (logistic regression, $\chi^2_{1,180}$ = 85.19, $p <$

0.0001; Fig. 6a). The proportion of WS bed bugs that fed similarly increased with feeder temperature (logistic regression, $\chi^2_{1,179} = 64.35$, $p < 0.0001$; Fig. 6b).

Because a small proportion of both HH and WS bed bugs fed at a feeder temperature of 23°C, we performed an additional set of assays being particularly careful to avoid contamination by human odors. At 23°C, 3 out of 28 (11%) individually housed bugs fed on 1 mmol l^{-1} ATP in PBS from a surface-sterilized feeder, while at 38°C, 26 out of 29 (90%) bugs fed. These results confirm that some, albeit few, bed bugs engorge on blood cooled below ambient temperature.

Ambient temperature also significantly affected bed bug feeding (Fig. 7). At ambient temperatures of 20°C and 25°C, the proportion of HH bugs that blood-fed increased monotonically as feeder temperature increased from 23°C to 33°C, with the interactive term (ambient temperature * feeder temperature) not significant ($\chi^2_{2,214} = 0.82$, $p = 0.6625$). At any given feeder temperature, more bugs fed at an ambient temperature of 20°C than at 25°C ($\chi^2_{1,214} = 10.83$, $p = 0.0010$). Feeding responses at a high ambient temperature of 30°C were substantially different, with no clear relationship with feeder temperature, and a high percentage of bugs (>62%) feeding at all feeder temperatures. Feeding increased as feeder temperature increased from 28°C to 33°C. However, at a feeder temperature of 23°C more than twice as many bugs fed at an ambient temperature of 30°C than at either 20°C or 25°C ($\chi^2_{2,108} = 37.59$, $p < 0.0001$).

Discussion

Many hematophagous insects respond to body heat as part of their orientation responses to warm-blooded hosts (Lazzari and Núñez, 1989b; Lees, 1948; Peterson and Brown, 1951). However, few studies have investigated the activation of quiescent insects in response to heated targets alone. This is understandable, as most hematophagous arthropods detect their hosts at some distance and initiate their orientation toward the host using both chemosensory and visual cues. Some arthropods, however, including bed bugs and other cimicids, shelter in close proximity to their host, often within bat roosts or bird nests (Usinger, 1966), and it is plausible that under these ecological conditions, heat emanating from the host may play an important function in early steps of the orientation process. Indeed, heated targets, representing a host, significantly activated quiescent bed bugs at an ambient temperature of 25°C. When the target was cooled to 23°C, only one out of 12 bed bugs moved. These results show that targets above ambient temperature, including temperatures that cause thermal stress and death upon prolonged exposure (Benoit et al., 2009; Kells and Goblirsch, 2011; Pereira et al., 2009) can quickly activate bed bugs from an arrested state.

Orientation toward a target was significantly and characteristically affected by the temperature of the target. At target temperatures ≥28°C, bed bugs oriented toward the heated coil, with >85% of the bugs contacting the coil within 5 min. The time taken to reach the coil decreased as temperature increased. Even with the exclusion of bugs that did not reach the coil, there was still a precipitous decline in response time between 23°C (2°C below ambient) and 28°C (3°C above ambient). These results show that bed bugs are well adapted to detect even small changes in host temperature relative to ambient temperature. Our results

also confirm Rivnay's (1932) findings, which were based on very few replicates, showing that bed bugs can detect temperature differences of 2°C. When the cat flea (*Ctenocephalides felis*) was tested over a similar range of temperatures (27-50°C), response rates and preference for a heated target also increased (Osbrink and Rust, 1985).

Several orientation parameters varied with the temperature of a target positioned in the center of the arena. Average distance to the wall of the circular arena was negatively related to target temperature, increasing with temperature up to 38°C and then leveling off thereafter. The angular speed and total distance traveled by bed bugs that reached the target was positively related with target temperature, with the highest values observed at the extremes of the measured temperatures (23 and 48°C). As expected, at low target temperatures, bed bugs remained closer to the arena wall and travelled farther before reaching the target than at higher target temperatures. As the coil temperature approached 48°C, angular speed increased, despite bugs reaching the coil at a similar time as all intermediate temperatures (28-43°C). Angular speed quantifies the observed "circling" behavior that is typical of bed bugs in response to this unusually high temperature. Bed bugs approached the coil heated to 48°C, circled it, and were slow to make contact with it. Rivnay (1932) reported that target temperatures ≥45°C repelled bed bugs, and it is possible that he considered this circling behavior a manifestation of repellency, although the small size of our heat coil probably resulted in less heat emission and ultimately a more approachable stimulus. Despite this circling behavior, response time to a 48°C target remained low. In addition, orientation angle relative to the center of the arena was only significantly affected by temperatures ≥38°C, showing that even though bed bugs can locate a target heated above

ambient temperature, higher target temperatures result in a more direct orientation toward the target.

There are three important caveats for interpreting these results: the size of the heat source, the size of the arena, and their spatial alignment. Although similar assays with larger arenas and larger heated targets might produce quantitatively different results, we expect the overall patterns to reflect our results. The spatial alignment of the arena and heated target might be more important. All our experiments were conducted on a horizontal plane with minimal air movement, so it is unlikely that convection factored into bed bug orientation behavior. Yet, convection currents play a major role in heat detection in mosquitoes (Peterson and Brown, 1951), and their preference for vertically oriented flight has inspired the design of vertical olfactometers (Feinsod and Spielman, 1979). Moreover, in all our experiments the heat coil remained in contact with the substrate, so it is unclear whether bed bugs responded to conductive or radiant heat, with radiant heat inducing responses in the closely related hematophagous hemipteran, *Triatoma infestans* (Lazzari, 2009; Lazzari and Núñez, 1989b).

Although bed bugs detected heat over a distance of at least 4 cm, as indicated by faster activation at higher target temperatures, directed movement towards a heated target was limited to shorter distances (< 3 cm). In the T-maze assay bed bugs walked up to the junction of the T, and then chose a side with the 38°C heated target positioned at various distances from this junction. Bed bugs showed a clear preference for the target when the heated coil was 10 mm from the T-junction, a marginal (although not significant) preference at 30 mm (61%, $p = 0.1161$), but no preference from 50 mm away from the coil. These

results are not surprising in the context of bed bug ecology. Because *C. lectularius* (along with many other cimicids) shelter in relatively close proximity to their host, typically in locations where the host rests at night (Usinger, 1966), long range host orientation may not be as important as it is for other hematophagous insects. Therefore, host temperature may serve as an important short range cue under these conditions, although long range attraction to larger bodies of heat should certainly be evaluated. Other host-associated cues known to attract bed bugs, such as CO_2 (Wang et al., 2009) and body odors (Aak et al., 2014; Liu and Liu, 2015; Rivnay, 1932) are more likely to serve as long range attractants.

Most bed bugs fully engorged at a broad range of feeder-blood temperatures from 28-43°C, with > 88% feeding at 38 and 43°C, and few bugs feeding at the extreme temperatures (23 and 48°C). In all feeding assays we used rabbit blood which is highly phagostimulatory to bed bugs (Romero and Schal, 2014). Therefore, it is reasonable to conclude that the propensity to feed in our assays was related to feeder temperature and not the quality of the blood. Most interesting was the observation that some bed bugs fed on blood set to 23°C, 2°C below ambient temperature. Although we were careful to eliminate human and blood odorants and tastants from the membrane surface, it was possible that blood constituents permeated the feeder membrane. To control for this, we repeated the assays at 23°C and 38°C with a sterilized membrane and replaced blood with PBS fortified with ATP. Romero and Schal (2014) reported low levels of engorgement on PBS solutions equilibrated at 37°C, but the addition of ATP, a constituent of human blood, induced nearly 100% of bugs to feed. As with blood, some bed bugs (11%) fed on the PBS-ATP solution set to 23°C in our assays. Based on these results and behavioral observations, we suggest that bed bugs naturally probe

all surfaces and if conditions are permissive (e.g., membranous surface) some bugs will penetrate the surface and begin feeding if they taste phagostimulants such as ATP. While these observations also predict that heat alone might increase the rate of probing, it is also possible that the cooler temperature of the glass feeder resulted in a localized increase in relative humidity, a cue that guides other hematophagous insects to hosts (van Breugel et al., 2015). In triatomine bugs, heat is a primary stimulus that triggers biting (Lazzari and Núñez, 1989a), but triatomines are not known to bite targets set below ambient temperature, and neither do tsetse flies, *Glossina pallidipes* (Chappuis et al., 2013). Specifically in triatomines, feeding initiation (biting) appears to be a function of only surface temperature, while the number of bugs that fully engorge and intake rate are controlled by blood temperature (Lazzari and Núñez, 1989a). This does not appear to be the case in bed bugs, with temperature not required to induce feeding, although feeding initiation does increase with increasing surface/blood temperature. In both triatomines and tsetse flies, other sensory cues modulate the probing response. The triatomine *Rhodnius prolixus* bites more when a heated metal surface is covered with latex, suggesting that the decision to probe/bite integrates thermal and mechanical cues (Ferreira et al., 2011). In *G. pallidipes*, skin temperature and humidity synergistically increase the biting response (Chappuis et al., 2013). Mosquito feeding is also affected by temperature, although not shown independently of host odors (Grossman and Pappas, 1991; Willis, 1958).

The feeding response of bed bugs is complicated by the interaction of feeder (host) temperature with ambient temperature. Significantly more bed bugs fed at an ambient temperature of 20°C than at 25°C at each feeder temperature (23, 28, 33°C). These results

suggest that at ambient temperatures < 30°C, feeding responses in bed bugs depend on the relative difference between ambient and feeder temperatures. However, at an ambient temperature of 30°C, 83% of bugs fed at a feeder temperature of 23°C (7°C below ambient). At the same feeder temperature, only 33% and 14% of bugs fed at ambient temperatures of 20°C and 25°C, respectively. This differential feeding response is likely the result of increased movement and thus increased probing of the artificial membrane at high temperatures, although this idea has not been tested. We hypothesize that feeding at low temperatures may relate to the evolutionary history of feeding on bats and even reptiles, whose body temperatures are often close to or only slightly above the ambient temperature (Hock, 1951).

Understanding the activational and appetitive responses of bed bugs to heat should facilitate the development and deployment of traps, other monitoring devices, and direct control approaches. Our results suggest that traps and baits that only use heat to attract bed bugs will need to be deployed at very high densities to overcome their limited active space of < 3 cm. Nevertheless, thermally attractive devices may be highly effective if placed near bed bug sheltering sites or along their foraging paths. While traps may or may not integrate heat with chemoattractants to attract bed bugs (Anderson et al., 2009; Singh et al., 2012; Wang et al., 2009), heat appears to be important in stimulating probing and feeding in bed bugs, an obligatory step in the development of an artificial bait.

To our knowledge, this is the first comprehensive report showing heat to be responsible for activating bed bugs from an arrested state, orienting them toward a heat source, and modulating feeding responses based on both feeder and ambient temperatures.

These results should facilitate the design of monitoring devices and ultimately the development of an artificial liquid bait. Future studies should investigate the sensory structures that bed bugs use to perceive heat (and cold), their distribution on the body of the bed bug, and the genes that encode thermal receptors. The recently sequenced *C. lectularius* genome (Benoit et al., 2016; Rosenfeld et al., 2016) should expedite these investigations. In addition, future work should elucidate the interactions of host-produced heat with other host cues, including CO_2 and body odors, and the orientation patterns of bed bugs at different spatial alignments with the host.

Acknowledgements

We thank Richard G. Santangelo for assistance with experimental animals during this study and Ayako Wada-Katsumata for assistance in preparing figures for the manuscript.

Funding

This research was supported in part by the Blanton J. Whitmire endowment, the Pest Management Foundation of the National Pest Management Association, the U.S. Department of Housing and Urban Development Healthy Homes program (NCHHU0017-13), the Alfred P. Sloan Foundation (2013-5-35 MBE), the National Science Foundation (IOS-1456973), and an award from the National Institute of Environmental Health Sciences (P30ES025128) to the Center for Human Health and the Environment. ZD was supported by the David R. Nimocks, Jr. Fellowship and the North Carolina Pest Management Association Fellowship, and scholarships from the Entomological Society of America.

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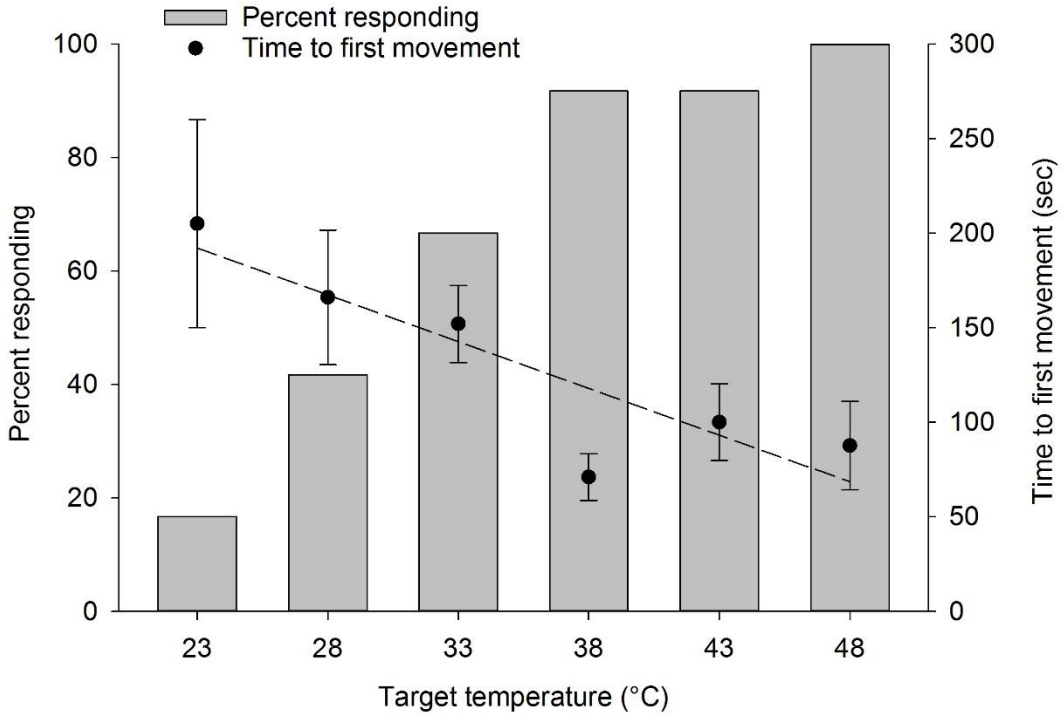

Figure 1 – **Behavioral activation of bed bugs by heated or cooled targets.** The effect of the target coil temperature on the percentage of Harold Harlan strain bed bugs that initiated movement from an arrested state (gray bars) and the latency of the response (sec) of bugs that initiated movement within 5 min after the coil was introduced. Means±s.e.m. are plotted and equations describing the relationships are as follows: Percentage responding = $(e^{-6.947(\pm1.750) + [0.234(\pm0.055) * (°C)]}) / (1 + e^{-6.947(\pm1.750) + [0.234(\pm0.055) * (°C)]})$; Time to first movement (sec) = $305.9(\pm46.8) – 4.95(\pm1.28) * (°C_{target})$. A minimum sample size of n = 12 was used for each temperature.

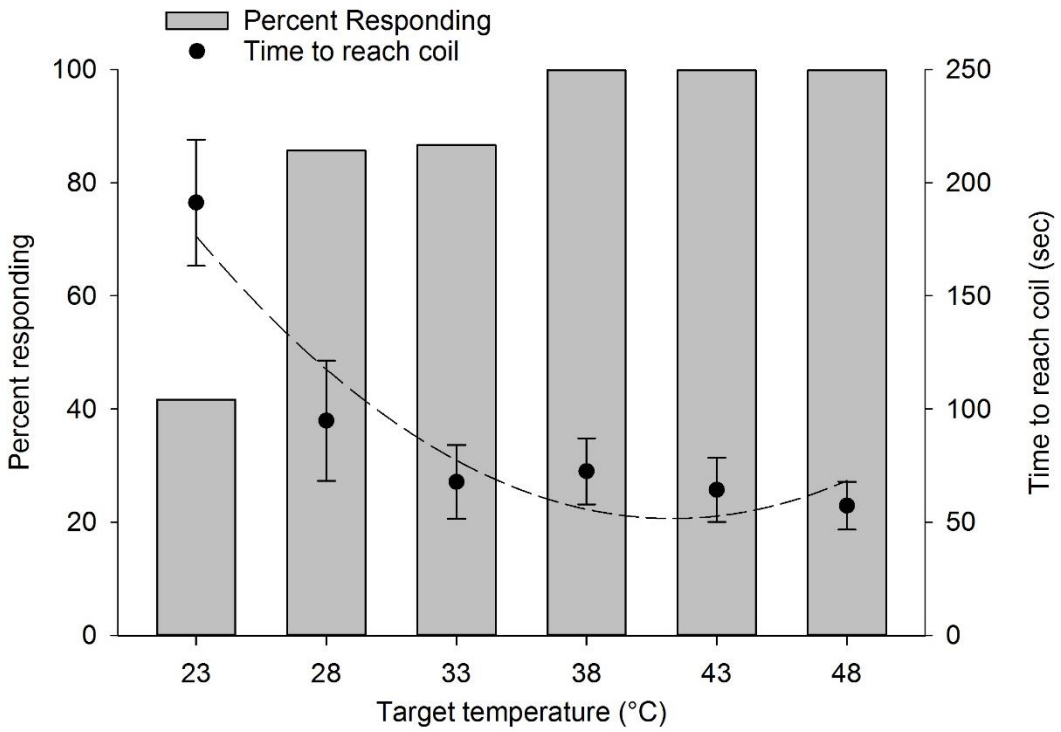

Figure 2 – **Orientation of bed bugs to heated or cooled targets.** The effect of target coil temperature on the percentage of Harold Harlan strain bed bugs that contacted the coil within 5 min (gray bars) and the time (sec) taken to reach the coil (black dots). Means±s.e.m. are plotted and equations describing the relationships are as follows: Percentage to reach coil = $(e^{-6.981(\pm2.395) + [0.294(\pm0.088) * (°C)]}) / (1 + e^{-6.981(\pm2.395) + [0.294(\pm0.088) * (°C)]})$; Time to reach target (sec) = $687.6(\pm169.9) – 30.80(\pm9.96) * (°C_{target}) + 0.373(\pm0.140) * (°C_{target})^2$. A minimum sample size of n = 14 was used for each temperature.

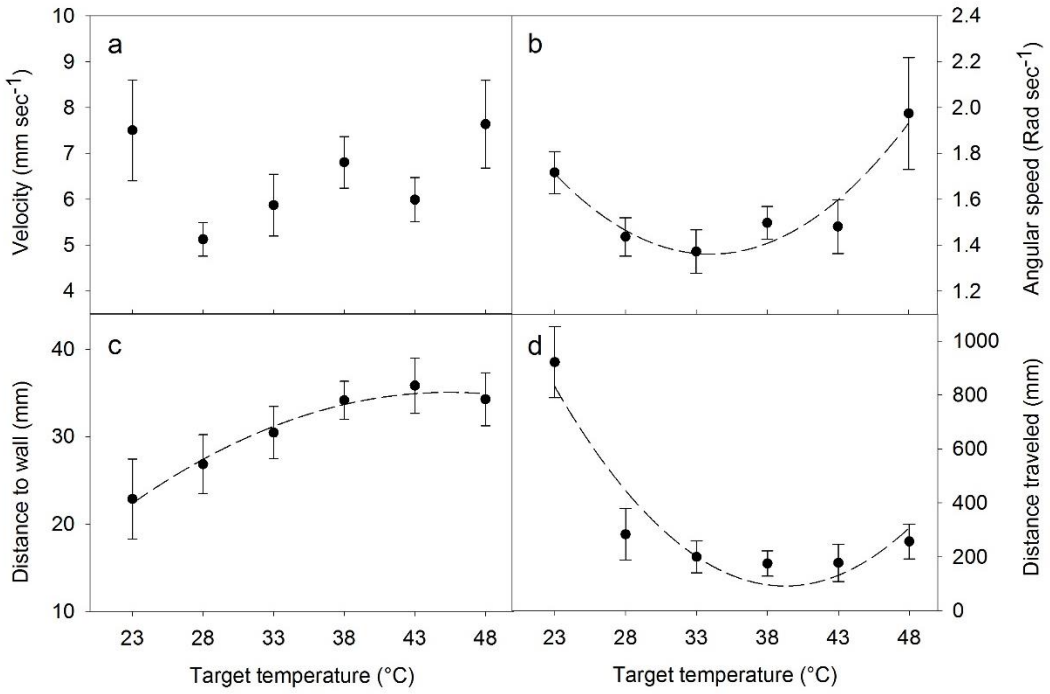

Figure 3 – **Metrics of bed bug orientation to heated or cooled targets.** The effect of target coil temperature on a) velocity (mm sec^{-1}), b) angular speed (Rad sec^{-1}), c) average distance to the wall (mm), and d) total distance traveled (mm) by Harold Harlan strain bed bugs. Only bugs that contacted the coil during the 5 min experiment were included in the analysis. Means±s.e.m. are plotted and equations describing these relationships are as follows: Angular speed (rad sec^{-1}) = 4.711(±0.715) – 0.197(±0.042) * ($°C_{target}$) + 0.0029(±0.0006) * ($°C_{target}$)^2; Distance to wall (mm) = -17.09(±7.28) + 2.296(±0.427) * ($°C_{target}$) – 0.0253(±0.0060) * ($°C_{target}$)^2; Distance traveled (mm) = 4412.6(±981.2) – 220.3(±57.6) * ($°C_{target}$) + 2.81(±0.81) * ($°C_{target}$)^2. A minimum sample size of n = 14 was used for each temperature.

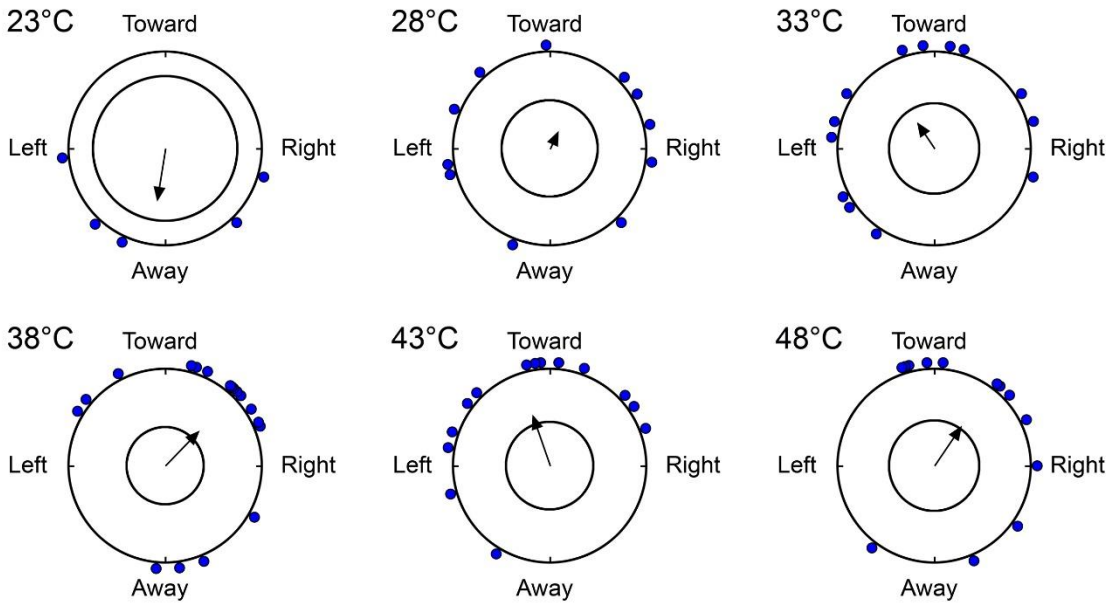

Figure 4 – **Bed bug orientation angles to heated or cooled targets.** Mean orientation angle for each Harold Harlan strain bed bug that responded within 5 min is displayed on the circumference of the circle representing the arena, with the grand mean of all bed bugs that responded indicated by the arrow. The interior circle at each temperature represents the respective $\alpha < 0.05$ (Rayleigh test), and significant deviation from random orientation is indicated by arrows that cross the interior circle ($p < 0.05$).

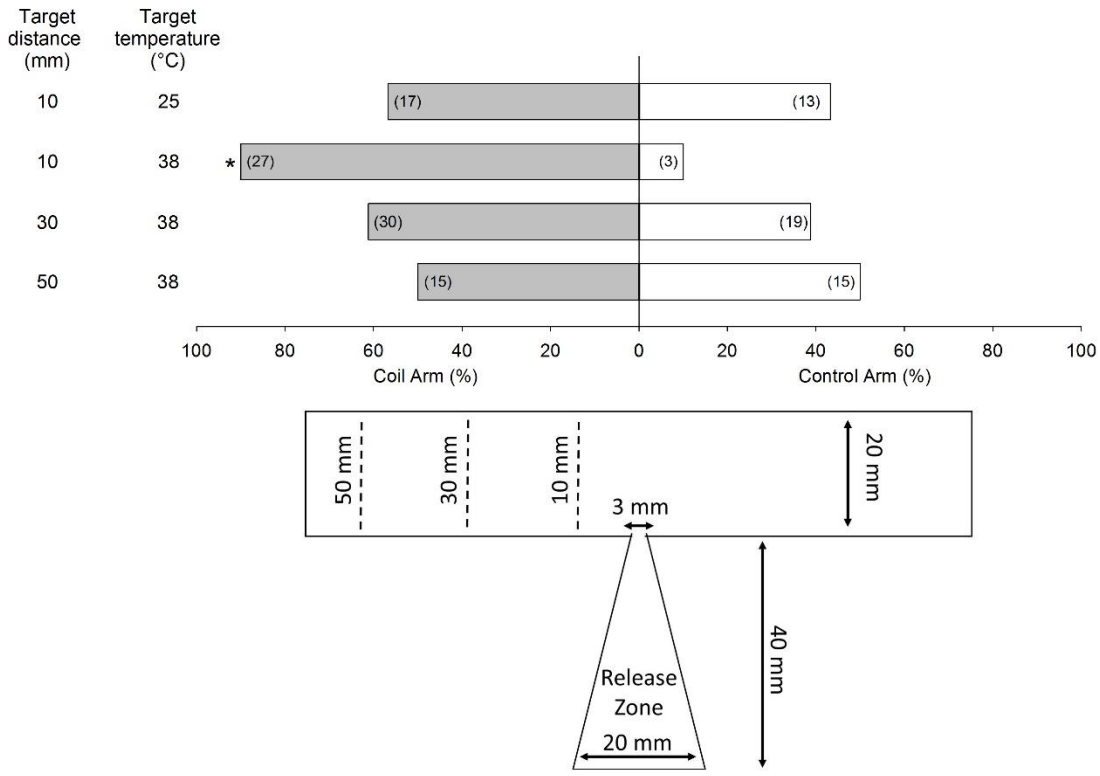

Figure 5 – **Bed bug orientation over distance in a T-arena.** Effects of distance on the ability of Harold Harlan strain bed bugs to correctly orient toward a heated coil (38°C) based on a 2-choice T-maze assay, as depicted below the results. An asterisk (*) indicates significant choice of one arm of the "T" arena (coil or control) based on the Chi-square test ($p < 0.05$). Sample sizes are indicated in parentheses in the bars.

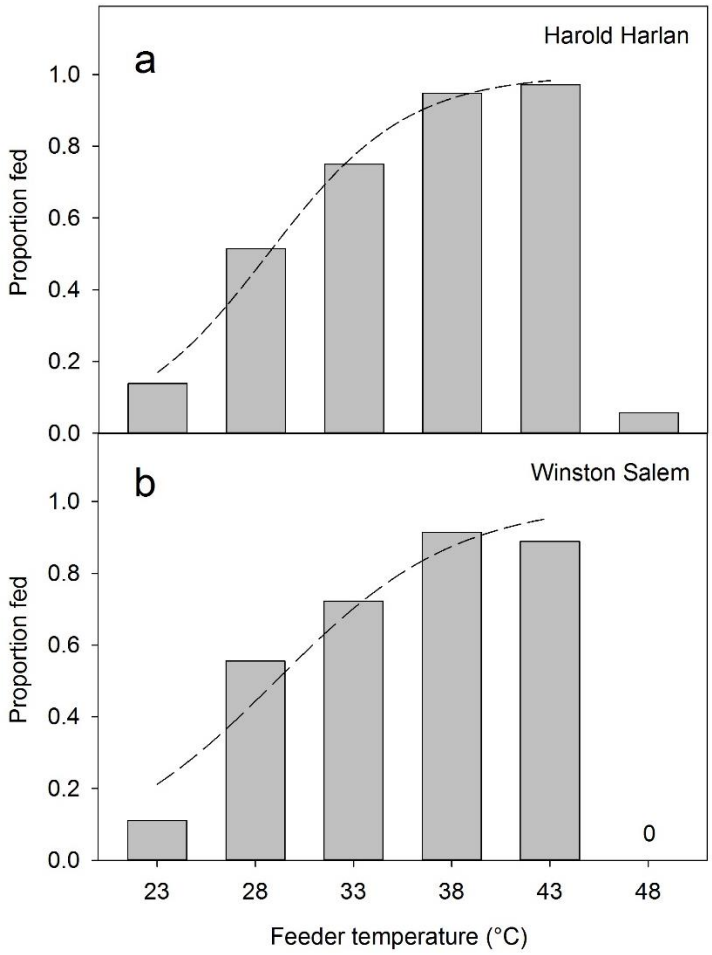

Figure 6 – **Bed bug feeding in relation to feeder temperature.** Effects of feeder (blood) temperature on bed bug feeding for two strains of bed bugs: a) Harold Harlan, and b) Winston Salem. A sample size of n = 36 bugs was used for each feeder temperature and the best fit logistic regression line is displayed for each strain, with the following equations: Proportion Harold Harlan Fed = $(e^{-8.070(\pm1.243) + [0.282(\pm0.041) * (°C)]}) / (1 + e^{-8.070(\pm1.243) + [0.282(\pm0.041) * (°C)]})$; Proportion Winston Salem Fed = $(e^{-6.303(\pm1.032) + (0.217(\pm0.033) * (°C))}) / (1 + e^{-6.303(\pm1.032) + [0.217(\pm0.033) * (°C)]})$. No feeding was observed at 48°C in Winston Salem bed bugs, as indicated by "0" on the graph.

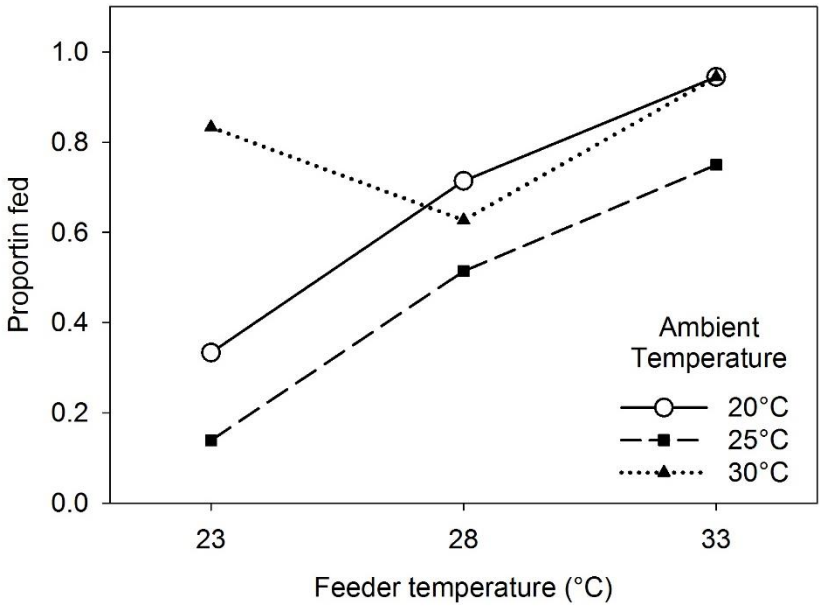

Figure 7 – **Bed bug feeding in relation to feeder and ambient temperature.** Effects of feeder (blood) temperature (23, 28, 33 °C) on the proportion of Harold Harlan strain bed bugs feeding at three different ambient temperatures: 20, 25, 30°C. A minimum sample size of n = 36 bugs was used for each ambient temperature-feeder temperature tested.

CHAPTER 5

Histamine accumulation and persistence in bed bug infested homes

Abstract

Histamine is used in bronchial and dermal provocation, but it is rarely considered an environmental risk factor in allergic disease. Because bed bugs defecate large amounts of histamine as a component of their aggregation pheromone, it is possible that histamine accumulates in infested homes and poses a health risk to residents. We sought to determine if histamine accumulates in household dust from bed bug infested homes, and the effects of bed bug eradication with spatial heat on histamine levels in dust. Dust in homes was collected and analyzed for histamine by GC-MS before and up to three months following bed bug eradication. Histamine levels in bed bug infested homes were remarkably high (> 54 µg/100 mg of sieved household dust) and significantly higher than in control homes not infested with bed bugs (< 2 µg/100 mg of sieved household dust). Heat treatments that eradicated infestations failed to reduce histamine levels, even three months following treatment. We report a clear association between histamine in household dust and bed bug infestations. The high concentrations, persistence, and proximity to humans during sleep suggest that bed bug-produced histamine may pose a serious health risk in the indoor environment.

Introduction

Indoor environmental contaminants pose serious health risks to humans. Well-investigated examples include human-generated contaminants, such as lead paint (*1*), asbestos (*2*) and various pesticides (*3*, *4*), and household pests that produce potent aeroallergens with notable examples being house dust mites (*5*), cockroaches (*6*), and rodents (*7*). These pests share several common features: 1) they are often obligatorily associated with humans and present in large numbers; 2) some of the allergens they produce are excreted in feces and urine and persist in household dust; 3) environmental conditions (sanitation, temperature, humidity) influence both pest populations and allergen persistence; and 4) allergen-containing household dust can become airborne and inhaled when disturbed (*8-11*). Bed bugs (*Cimex lectularius*) share these features, but they are not known to produce allergens beyond those delivered with their bites.

Bed bugs had all but disappeared in the 1960s, but have resurged globally since the early 2000s (*12*) (Fig. 1). Although bed bugs are capable of transmitting *Trypanosoma cruzi* (the causative agent of Chagas disease) (*13*) and *Bartonella quintana* (trench fever) (*14*) in laboratory assays, there is no evidence of natural pathogen transmission by bed bugs. Their bites however can cause severe dermatitis (*15*), as humans become sensitized to nitrophorin proteins in bed bug saliva (*16*). Bed bugs also have strong psychological impacts on their victims, with effects often lasting well after their eradication (*17*). Bed bug eradication is particularly challenging because of high levels of resistance to insecticides, difficulty applying insecticides onto and close to the bed, and costs associated with pest abatement

(*18*). High temperatures ("heat treatments") can eliminate bed bugs, although this approach (on its own) does not offer any residual protection.

Recently, histamine was found to be a component of bed bug feces, serving as a close-range aggregation pheromone component (*19*). Histamine is a known food contaminant, mainly of fish and alcoholic beverages such as beer and wine (*20*). Histamine ingestion is associated with significant adverse effects in sensitive individuals, including hypotension, uticaria, shock, heart palpitations, diarrhea, vomiting, pain, itching, and respiratory distress (*20*). Yet, little information exists on health risks from environmental exposure to histamine. Cutaneous exposure is known to result in dermatitis, primarily in atopic patients (*21, 22*). Respiratory exposure to histamine can reduce forced expiratory volume (FEV) (*23*), and increase nasal mucosa reactivity (*24*), with these effects mostly seen in atopic individuals (*25*). Provocation with histamine is generally administered to assess dermal, nasal or respiratory responses (e.g., bronchial reactivity) (*26, 27*), and these clinical tests suggest that exposure to histamine in the environment would constitute a significant health risk. Indeed, histamine has been found in dust from agricultural hay and thought to be associated with occupational asthma, rhinitis, bronchitis, and related respiratory syndromes (*28, 29*). However, the health effects from chronic respiratory exposure to low levels of histamine are unknown.

We hypothesized that bed bugs elevate indoor histamine levels and designed a study to quantify histamine levels in homes with and without bed bugs. We further hypothesized that bed bug intervention with heat treatments might degrade environmental histamine and thus also constitute an effective histamine mitigation strategy. Results from this study

suggest that bed bug infestations may pose significant and persistent medical challenges to allergic individuals.

Materials and Methods

***Ethics Statement*:** The North Carolina State University Institutional Review Board (IRB) approved this study (#3840). Before participation, adult participants (>21 yrs old) provided informed consent.

***Study Design and Sampling*:** Apartments were located within the same nine-story multi-unit (140 apartments) building in Raleigh, NC. This building has been chronically infested with bed bugs for several years despite recurrent pest control interventions. Therefore, some "un-infested" apartments in this building could have been infested with bed bugs prior to our sampling, or they might represent infested units where we failed to detect low level bed bug populations. To obtain truly un-infested homes, an additional subset of five homes in Raleigh, NC, not associated with the apartment building, were also sampled as external negative controls. Unlike the un-infested units in the apartment building, these homes had no evidence of bed bugs in >3 years, and were located >8 km away from the apartment building.

Residences were surveyed and divided into groups based on their infestation status: infested (14 apartments), un-infested within the same complex (10 apartments), and un-infested >8 km away (5 apartments). The bed bug infested homes were further divided into two treatment groups: infested-controls (no intervention; 9 apartments) and infested-treated (intervention; 6 apartments). House dust was sampled in all homes at baseline, and infested-

treated homes were also sampled 2, 4, and 12 weeks after the intervention. Due to ethical considerations, infested-control homes were sampled only 4 weeks after baseline, with some apartments subsequently switched to the infested-treated arm.

Homes were visually inspected for bed bugs during the initial home visit. This was followed by sampling bed bugs with traps (Climbup Interceptor, Susan McKnight Inc., Memphis, TN) for two weeks. Although quantitative in nature, the trap counts were only intended to indicate the presence or absence of bed bugs, direct our dust sampling efforts and assess treatment efficacy. Dust samples were collected from an area with the highest concentration of bed bugs in either the bedroom or living room. An area of the floor (3 m x 15 cm) near a wall and behind a bed or couch was sampled for 2 min using a Eureka Mighty-Mite 9.0-ampere vacuum cleaner (Eureka Company, Bloomington, Ill) fitted with a Dustream® collector and filter (40 µm, Indoor Biotechnologies Inc., Charlottesville, VA). Samples were placed into glass vials and stored at -80°C.

***Bed Bug Interventions*:** A professional pest control company was contracted by the building management to handle all bed bug abatement efforts. This was entirely independent of our research; our only contact with the pest control company was to coordinate our sampling efforts with their interventions. Bed bug interventions included heat treatments, where the ambient temperature was raised to ~50°C and maintained for >4 hr while fans circulated air throughout the apartment (*18*). Following heat treatments, the pest control technician applied residual insecticide sprays and dusts to bed bug sheltering areas. Although we did not participate in the intervention efforts, we actively monitored bed bugs throughout the study

using visual inspections and traps. Homes where bed bugs were detected at any time post-intervention were discontinued in the infested-treated arm of the study, and only used for their baseline histamine values.

***Histamine Analysis*:** Dust samples were weighed (total mass), sieved (450 µm) and weighed again (sieved mass). Approximately 5–50 mg sieved dust was extracted in plastic centrifuge tubes (Sarstedt Inc., Nümbrecht, Germany) in 1 ml of HPLC grade water. To this mixture, 10 µg of histamine-α,α,β,β-d_4 (Sigma-Aldrich Co. LLC, St. Louis, MO) was added (in 0.1 M HCl) as an internal standard. Samples were shaken on a rocker for 10 min, centrifuged at 2000 rpm, and the supernatants were transferred to glass vials. To each supernatant the following were added: 1 ml toluene, 2 ml alkaline buffer solution (pH = 12; Fisher Scientific, Hampton, NH), and 100 µl of the derivatization agent isobutyl chloroformate (IBCF; Sigma-Alrich Co. LLC). Samples were then capped and shaken on a rocker for 45 min. Derivatized samples were centrifuged briefly, and the top (organic) layer was transferred to a new glass vial. Samples were blown to dryness under a gentle stream of high-purity nitrogen and heat (~30°C), resuspended in 1 ml of toluene, and stored at 4°C.

Samples were analyzed using an Agilent Technologies (Santa Clara, CA) 6890N GC coupled to an Agilent Technologies 5975 mass spectrometer (GC-MS) and operated in EI mode. The GC was fitted with a 30 m x 0.25 mm x 0.25 µm (5%-phenyl)-methylpolysiloxane Agilent J&W HP-5ms column preceded by a 3 m deactivated guard column. The temperature program was: 100°C to 320°C at a rate of 10°C/min, and held at 320°C for 10 min. The IS method of quantification was used with a 9-point calibration curve

ranging from 0.1 µg/ml to 50 µg/ml, and no samples exceeded the upper level of the calibration curve. Quantification ions were selected for both the IS (197) and histamine (194).

***Statistical Analysis*:** Baseline histamine values (before intervention) were cube-root transformed and compared among the infested and un-infested treatment groups from the same apartment building using a one-way ANOVA. Changes in histamine over time in either the infested-control homes or infested-treated (intervention) homes were evaluated using repeated measures ANOVA with means compared using the Tukey-Kramer multiple comparison test. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

Results

***Baseline Histamine Levels in Homes*:** Household dust from homes with active bed bug infestations had significantly higher histamine levels than dust from un-infested-control homes either from the same apartment building or from >8 km away ($F_{2,26}$=14.84, P<0.0001, Fig. 2). Bed bug-infested homes averaged >54 µg histamine/100 mg dust, while control un-infested homes averaged <2.5 µg/100 mg of dust (same apartment building) or <0.3 µg/100 mg of dust (different buildings, >8 km away).

***Effects of Spatial Heat Treatments on Histamine Levels*:** Histamine levels remained unchanged over one month in untreated infested apartments ($F_{1,8}$=0.08, P=0.7782, Fig. 3).

Heat treatments (and apparent bed bug eradication) did not significantly affect histamine levels in infested homes over time ($F_{3,13}$=0.60, P=0.6289, Fig. 3). This indicates that a) histamine in household dust was not degraded by the heat treatment, and b) histamine is highly stable in household dust and persists for months following bed bug eradication.

Discussion

Histamine was detected only at trace levels in homes known to be free of bed bugs, indicating that the issue of environmental histamine is of no concern in residences that are truly un-infested. On the other hand, we found a clear association between high levels of histamine in house dust and the presence of bed bugs, indicating that bed bugs are the major contributor to indoor histamine residues. Low, but detectable, levels of histamine in un-infested apartments within the same building suggest either that bed bugs had been present in the apartment at some prior time, or that some bed bugs were present but we failed to detect them.

The impacts of these finding are substantial, because exogenous histamine can provoke allergic responses and asthma. Histamine receptors occur in many cell types, including epithelial and endothelial cells, dendritic cells, and neutrophils, and in various tissues, including lungs, skin, gut, the nervous system, and the immune system. The histamine concentrations we measured in sieved household dust (>54 µg/100 mg dust) are 10-fold higher than the US-FDA upper limit in edible fish (5 mg/100 g fish) (*30*). Arguably, comparisons of ingested and inhaled histamine are challenging because airborne limits have not been defined. In clinical diagnostic tests of airway hyper-responsiveness in patients with

airway disease, however, patients who breathed saline aerosols containing as little as 24.5 µg of histamine experienced a 20% reduction in forced expiratory volume in one second (FEV_1) (*31*). Additionally, histamine is a potent inducer of pruritus, and cutaneous applications of histamine induce atopic dermatitis (*21*). It is used as a positive control in skin-prick allergy tests at a dose of ~0.5 mg (1 drop of a 1% solution) (*27*). Unfortunately, there is no information on the health effects of chronic low-level exposure to histamine because prior to our study there was no compelling need for such an assessment.

Quantifications of environmental histamine are scarce. Histamine was detected in dairy farm hay dust at up to 0.7 µg/100 mg of airborne dust and up to 0.05 µg/100 mg of bulk hay (*32*). While these levels were far lower than those used in clinical bronchial provocation, they were nevertheless considered a significant health concern. The histamine concentrations in dust collected in bed bug-infested homes were 50-times greater than in agricultural hay.

Importantly, the high concentrations of histamine we recovered were from sieved dust particles which readily become airborne and represent the major route of entry of allergens into the airway, as documented in studies correlating cockroach allergens in settled and airborne dust (*33*). The average histamine concentration we recovered from bedroom dust in bed bug infested apartments was >54 µg/100 mg of sieved dust, while cockroach allergens in kitchen dust average ~1-3 µg/100 mg of dust (*34, 35*), and dust mite allergens average ~0.1-4.8 µg/100 mg of dust (*36, 37*). Although the clinically relevant sensitization and exacerbation thresholds differ for various allergens, exposure to these allergens can stimulate histamine release, resulting in vasodilation, bronchoconstriction, immunomodulation and other allergy and rhinitis symptoms. Thus, exposure to dust-associated airborne histamine

would likely trigger more rapid adverse effects than exposure to aeroallergens. Furthermore, histamine produced by bed bugs could pose a greater risk than some arthropod-produced allergens due to its deposition location and stability. Cockroaches deposit allergens mainly in the kitchen (*34, 35*). In contrast, bed bugs, like dust mites (*38*), aggregate and defecate near their human host (Fig. 1), and histamine is deposited on or near where humans sleep and spend arguably the longest amount of time during the day. Therefore, like potent dust mite allergens, bed bug-derived histamine may pose health risks due to its proximity and persistence in our breathing space. But in addition, cutaneous exposure to histamine that has accumulated on bedding materials is of concern. Histamine appears to induce developmental effects, including suppression of epidermal differentiation and thinning of the epidermis, which impairs the barrier function of the skin (*39, 40*). Persistent contact with high levels of histamine may therefore pose significant dermal challenges.

All bed bug interventions, including chemical and heat treatments (*18*), singularly focus on bed bug abatement because the objectives are to reduce or eliminate bites. Our finding of significant histamine deposits in house dust that persist at least three months past bed bug eradication, underscores the need to develop and validate intervention strategies that mitigate the harmful effects of histamine, in addition to eradicating bed bugs. Prolonged exposure of homes to temperatures of 50°C did not reduce histamine levels in house dust. Therefore, a combination of deep cleaning and pest elimination will likely be needed, similar to the strategies used to reduce German cockroach allergens (*34*). We advocate that a similar protocol should be developed for bed bugs, and evaluated for its efficacy in reducing histamine levels in homes. Additionally, when developing abatement strategies, we must

also consider their effects on the movement and distribution of histamine in homes. It is plausible, for instance, that the high temperatures and air circulation during heat treatments could re-circulate histamine-containing dust particles and deposit them in new locations with higher chances to become contacted, inhaled, or consumed.

Bed bugs have become a major social, economic and health problem since their global resurgence in the early 2000s. Infestations can reach exceedingly high levels, especially among the elderly and in disadvantaged communities, where interventions may be unaffordable. While bed bug bites have been recognized as a dermatological concern that can be exacerbated and lead to secondary infections, bed bugs have not been implicated as disease vectors or allergen producers. The results of this study demonstrate that bed bugs are major contributors to environmental histamine in homes, generating deposits predicted to adversely affect the health of residents. Furthermore, bed bug eradication with heat and insecticides does not appear to reduce histamine levels in homes, suggesting high thermal and chemical stability of histamine. Future investigations should expand the sample size of the present work, to ensure that our findings are not confounded by any other undetected variables. Furthermore, future studies should evaluate the mechanisms of histamine production and excretion, temporal and spatial dynamics of histamine deposition in bed bug-infested homes, health impacts of dermal and respiratory exposure to environmental histamine, and the efficacy of various mitigation strategies to reduce histamine in homes.

Acknowledgement: We thank residents of several housing communities in Raleigh NC who participated in this study. This study would not have been possible without their support and

partnership. **Funding:** Funding for this study was provided by the Blanton J. Whitmire Endowment at North Carolina State University, the US Department of Housing and Urban Development Healthy Homes program (NCHHU0017-13), Alfred P. Sloan Foundation (2013-5-35 MBE) and a National Institute of Environmental Health Sciences grant to the Center for Human Health and the Environment (CHHE, P30ES025128). ZD received the David R. Nimocks Jr. Fellowship and scholarship awards from the Foundation for Agromedicine and Toxicology, Pi Chi Omega, and the Entomological Society of America (Monsanto Research Grant Award, MUVE Scholarship). **Author Contributions:** ZD, RS, and AB conducted field sampling. ZD analyzed samples for histamine. ZD and CS designed the experiment, analyzed the data, and wrote the manuscript. **Competing Interests:** The authors declare they have no competing interests. **Data and Materials Availability**: All data materials are available from the corresponding author, upon reasonable request.

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Figure 1. Bed bugs and signs of an infestation. Photos depiciting (**A**) a typical bed bug aggregation showing blood-fed and unfed bed bugs and fecal spots that contain histamine (photo credit: Matt Bertone), and (**B**) a matress heavily stained by bed bug feces, which contains histamine (photo credit: Mike Waldvogel and Jung W. Kim).

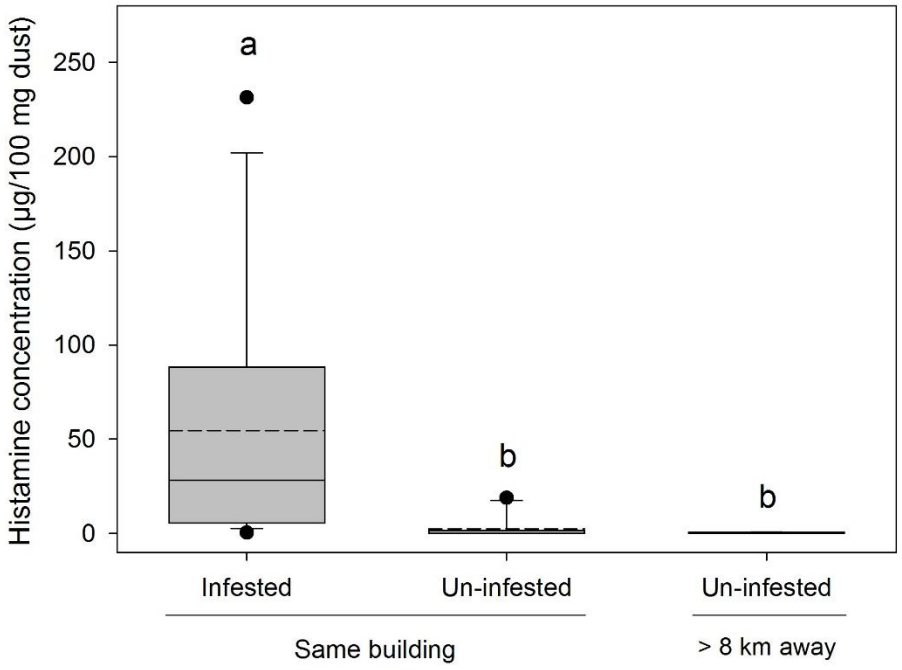

Figure 2. Histamine in bed bug infested homes. Histamine concentrations (mean ± SEM) in house dust collected from bed bug-infested homes (n = 14) and un-infested homes (n = 10) in the same apartment building. Un-infested-control homes (n = 5) are separate apartments >8 km from the apartment building and are not known to have had bed bugs in the past 3 yrs. Significant differences according to the Tukey-Kramer multiple comparison test are indicated by different letters.

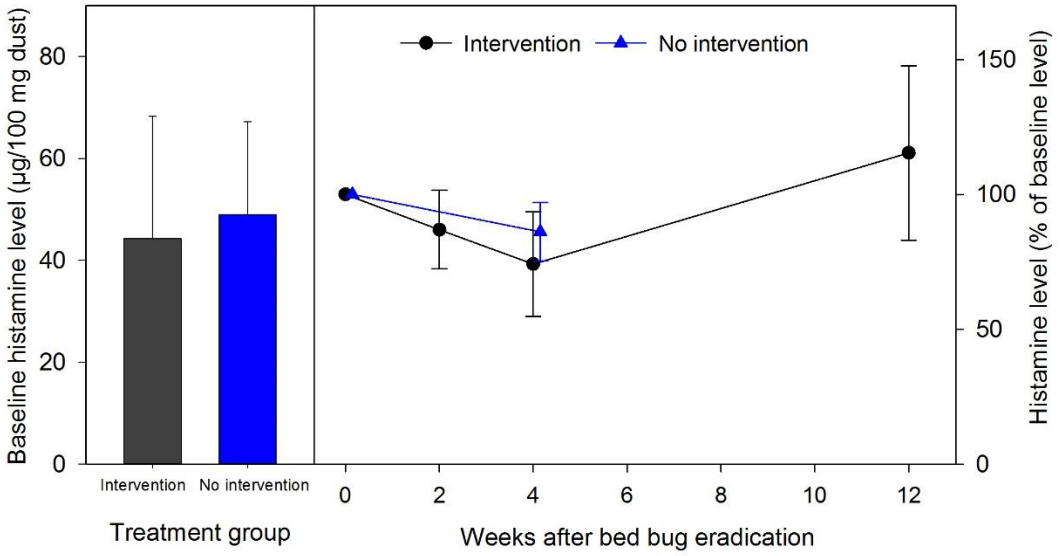

Figure 3. Change in indoor environmental histamine following bed bug eradication.

Percent changes in histamine concentrations (percentage mean ± SEM) in house dust collected from bed bug infested control homes (no intervention, n = 9), and bed bug infested treated homes (intervention at time 0, n = 6). No significant differences were observed for either treatment over time.

CHAPTER 6

Cost-benefit analysis of do-it-yourself indoor residential pesticide applications: A case of social injustice?

Abstract

Differences in pest prevalence in homes result in disparities in pesticide use patterns, the efficacy of pest control products, and health risks associated with exposure to pests and pesticides. Total-release foggers (TRFs) are popular pest control products, commonly used against cockroaches. However, little is known about the efficacy and health risks associated with these products. We investigated the comparative efficacy of four TRF products and two baits against German cockroach (*Blattella germanica*) infestations. In addition, we evaluated the resistance levels of apartment-collected cockroaches, quantified pesticide residues in homes, and conducted a cost-benefit analysis of TRF and bait use. Households with cockroach infestations were enrolled, TRFs and baits were deployed, and their efficacy assessed. Resistance was evaluated molecularly and by topical insecticide. Surface swabs were collected and used to quantify TRF pesticides by GC-MS. TRFs failed to reduce cockroach population, whereas gel-baits did. Resistance of household-collected cockroaches to pyrethroids was extensive and knock-down resistance (*kdr*), a mechanism of target site insensitivity that confers resistance to pyrethroids, was widespread. Swabs of kitchen surfaces revealed large quantities of pesticide residues throughout the kitchen following TRF discharge. The ineffectiveness of TRFs at reducing pest populations, their similar monetary cost compared to highly effective bait products, and the high human pesticide exposure risks associated with TRFs, call into question their utility in the marketplace. Our findings, combined with the disproportionate use of TRFs by people of lower socio-economic status, suggests that TRFs contribute substantially to indoor environmental social injustice.

Introduction

In the United States, 82 million households used insecticides in 2012, and $2.65 billion were spent in the "home and garden sector", representing 50% of all expenditures on insecticides (USEPA, 2017). Indoors, several perennial pests are targeted with various insecticides, most prominently various ants, the common bed bug, *Cimex lectularius*, and the German cockroach, *Blattella germanica*. There are many reasons for eliminating German cockroach infestations, but primary among them is the central role that cockroaches play as etiological agents in allergic disease and asthma (Gore and Schal, 2007; Schal, 2011). Allergens produced by German cockroaches can trigger allergies and asthma in sensitized individuals, and the National Cooperative Inner-City Asthma Study found that asthma morbidity was highest in children with both a positive skin-test response and high exposure to cockroach allergens (Rosenstreich et al., 1997). The National Survey of Lead and Allergens in Housing, a nationwide survey of housing conducted by the NIEHS and HUD, found detectable levels of the cockroach allergen Bla g 1 in 63% of homes (Cohn et al., 2006), and higher concentrations were found in high-rise apartments, urban settings, older homes, and homes of low-income households (Cohn et al., 2006; Rosenfeld et al., 2011). Moreover, because cockroaches move freely between waste and food, they can acquire, carry, and transfer pathogenic bacteria, helminths, fungi, protozoa, and viruses either mechanically or in their digestive system (Ahmad et al., 2011; Brenner, 1995). Thus, the persistence of cockroaches in homes not only lowers the quality of life but also poses significant health risks.

German cockroach infestations are often controlled with residual liquid or aerosol sprays that contain broad-spectrum insecticides, most commonly pyrethroids (Schal, 2011). However, high levels of pyrethroid resistance (Atkinson et al., 1991; Chai and Lee, 2010; Fardisi et al., in press; Hemingway et al., 1993; Wei et al., 2001; Wu and Appel, in press) and their repellency to cockroaches (Hostetler and Brenner, 1994; Ross, 1992; Rust and Reierson, 1978) severely compromise the efficacy of most residual sprays. Moreover, these products can deposit considerable insecticide residues throughout the home (Keenan et al., 2010). Environmental data collected by EPA and HUD on a stratified, nationally representative sample of 1,131 residences found extensive pesticide residues in homes (Stout II et al., 2009). Insecticides formulated as baits offer more effective and safer alternatives in cockroach control (Miller and Meek, 2004; Nalyanya et al., 2009; Williams et al., 2005). Baits are often most effective as part of an integrated pest management (IPM) program that includes sanitation, pest exclusion, and resident education (Arbes et al., 2003; Kass et al., 2009; Wang and Bennett, 2006), but baits have also been shown to be highly effective in stand-alone interventions (Rabito et al., in press; Sever et al., 2007).

Because professional pest management services can be prohibitively expensive, consumer-based pesticide products are commonly used in do-it-yourself pest control. Total-release foggers (TRFs) are often deployed as spatial insecticides, designed to fill a room with fine particles of insecticide, and considered by consumers to be highly effective against all pests (as the common name “bug bomb” implies). TRFs generally contain toxicity category III (based on acute toxicity) active ingredients (pyrethrins and pyrethroids), various synergists meant to inhibit microsomal detoxification by insects, and aerosol propellants that

are often flammable. These products are responsible for substantial acute and chronic health effects, explosions and fires, and persistent environmental contamination. A 2008 "first report in the scientific literature to describe the range of exposure circumstances and acute health problems associated with TRF usage" summarized 466 fogger exposures in eight states over a five-year period, documenting respiratory, gastrointestinal, neurological, ocular, dermatologic, and cardiovascular adverse symptoms (C.D.C, 2008). A similar summary from Texas documented 2,855 fogger exposures over an 8-year period (Forrester and Diebolt-Brown, 2011). Despite these reports, the magnitude of health, economic and environmental damage is poorly documented, and likely underestimated. Indeed, a follow-up report from the New York City Department of Health and Mental Hygiene (2009) stated that the 2008 CDC report understated reported exposures – in a passive surveillance system 344 of 443 calls regarding the use of foggers involved known exposures and >75% of all exposures resulted in acute symptoms. This report also showed that health effects are much more likely to occur from exposures to TRFs than from other pesticide formulations, and moderate or major health effects were more than twice as likely to occur from TRF exposures as from all pesticides, and seven times as likely as from rodenticides. While many of the fogger-associated illnesses and injuries result from inadvertent exposures during their deployment (leaving too late, re-entry too soon, too many foggers discharged, failure to notify others), studies suggest that TRFs deposit large amounts of pesticides in areas easily accessible to humans, especially small children (Keenan et al., 2010; Keenan et al., 2009). The residual pyrethroids on household surfaces can exacerbate a number of chronic health

conditions (Saillenfait et al., 2015), although the health effects from chronic exposure are still under debate.

TRF products appear to contribute significantly to the disproportional pesticide exposure already documented for those living in affordable housing (Adamkiewicz et al., 2011; Evans and Kantrowitz, 2002; Landrigan et al., 1999; Lu et al., 2013). The report from New York City's Department of Health and Mental Hygiene (2009) contends that "the health risks associated with the use of foggers are not justified given their likely poor efficacy". Recently, Jones et al. (2012) showed that over-the-counter TRFs were indeed ineffective at controlling populations of the bed bug. Surprisingly however, there are no reports on the relative efficacy of modern TRFs against the German cockroach.

We designed a study to assess the efficacy of TRFs at controlling German cockroaches, and quantify pesticide residues in the indoor environment following TRF use. As measures of efficacy we exposed caged sentinel cockroaches during TRF discharge and monitored cockroach population levels for a month after TRF use. We used topical applications of insecticides and molecular characterization of knockdown (*kdr*) mutations in apartment-collected cockroaches as measures of insecticide resistance. Pesticide residues on various surfaces in the kitchen were quantified over time. The combined findings on efficacy, resistance, residues, and economics provide a strong foundation for our contention that TRFs have no benefit to the public and are prominent contributors to indoor environmental social injustice.

Materials and Methods

Ethics Statement

The North Carolina State University Institutional Review Board (IRB) approved this study (#1459). Before participation, adult participants (>21 yrs old) provided written informed consent. Demographic data on participants were not gathered in this study, as we were interested in a cockroach intervention and environmental outcomes. Homes were generally in the same communities as reported in Arbes et al. (2003) and Sever et al. (2007).

Recruitment of Participants

Homes in five low-income communities within the city of Raleigh, North Carolina, were visited and surveyed for German cockroach infestations. Homes included multi-unit low-rise apartments, duplexes, and row homes. Residents were first informed of the purpose of the study, provided informed consent, then asked if (1) they had any problems with cockroaches, and (2) if they were interested in participating in the research study. If the resident reported both problems with cockroaches and agreed to participate in the study, the home was visually inspected for the presence of cockroaches. If the visual inspection indicated sufficient numbers of German cockroaches were present, the home was enrolled into the study. A total of 30 homes were recruited into this study.

Interventions

Four different TRFs were used, representing different active ingredients and manufacturers: Hot Shot No-Mess Fogger$_2$ with Odor Neutralizer (Hot Shot 2; 85 g, 0.333%

tetramethrin, 0.834% cypermethrin, 1.667% piperonyl butoxide; Spectrum Group-United Industries, St. Louis, MO), Hot Shot No-Mess Fogger$_3$ with Odor Neutralizer (Hot Shot 3; 170 g, 0.200% tetramethrin, 0.860% cypermethrin, 0.500% piperonyl butoxide; Spectrum Group-United Industries), Raid Max Concentrated Deep Reach Fogger (Raid Deep; 60 g, 1.716% cypermethrin; SC Johnson, Racine, WI), and Raid Fumigator (10 g, 12.600% permethrin; SC Johnson). Five replicate homes were treated with each TRF product, one home in each of five apartment complexes (20 TRF-treated homes).

Each TRF was discharged in the kitchen following the product label instructions and EPA precautions (https://www.epa.gov/safepestcontrol/safety-precautions-total-release-foggers; last accessed April 15 2017). Briefly, all residents vacated the premises for 4-6 hours, windows and doors were closed, air conditioning and gas stove pilot lights turned off, cabinet doors were opened and contents as well as immovable kitchen appliances were covered with newspapers, and aquaria were moved out of the kitchen. Four to six hours later, the home was ventilated, newspapers discarded, dishes rinsed, and residents allowed to re-enter.

Running in parallel, 10 additional homes (not treated with TRFs) were treated with gel baits. Five homes, one in each complex, were treated with a consumer bait, Combat gel bait (0.010% fipronil; Combat Insect Control Systems-The Dial Corporation, Scottsdale, AZ), and another set of 5 homes received a professional bait, Maxforce gel bait (0.010% fipronil; Bayer Environmental Science, Robinson Township, PA). Bait was dispensed as needed at each of three visits up to one month. At the conclusion of the study, all TRF-treated homes were provided a thorough gel bait treatment.

The purchase prices of all products used during the study were recorded. In addition, the total amount of bait used was recorded. We also recorded intervention duration estimates for both TRF and gel bait treatments.

Intervention Efficacy – Cockroach Population Reductions

At baseline, and subsequently two and four weeks after treatment, six glue-board sticky-traps (Victor Roach Pheromone Trap, Woodstream Corporation, Lititz, PA) were placed in kitchen locations where cockroaches likely aggregate. The traps were collected the following day and enumerated in the lab. Changes in each cockroach population (apartment) were assessed relative to the baseline trap catch.

TRF Efficacy – Phenotyping Caged Sentinel Cockroaches

After enrollment, German cockroaches were collected using a modified Eureka Mighty-Mite 7.0-ampere vacuum cleaner (Eureka Company, Charlotte, NC). Live cockroaches were collected into a mesh lined plastic tube attached to the distal end of the vacuum's extension tube.

Apartment collected female cockroaches were reared in the lab for one generation and used for insecticide resistance profiling. Apartment collected male cockroaches were used as caged sentinels for determining product efficacy in the same home where they were collected. Prior to discharging the TRF, 40 laboratory raised, insecticide-susceptible adult male cockroaches and 40 home-specific apartment-collected males were placed into the home as sentinels to determine product efficacy. Twenty cockroaches from both the

laboratory population and the home-specific population were placed in an uncovered cage on the floor 1.0 m away from the TRF (referred to as "floor"), and the other 20 cockroaches from each population were placed in an uncovered cage in an upper cabinet (lowest shelf, referred to as "upper cabinet"). The inside walls of the cages were coated with petroleum jelly to prevent cockroaches from escaping. Four to six hours after the TRF was discharged, and it was safe to re-enter the home, the sentinel cockroaches were collected, returned to the lab, and assessed for mortality 24 hours later.

Insecticide Resistance Bioassays and kdr Genotyping

The caged sentinel cockroaches provided a measure of resistance to the pyrethroid active ingredients in TRFs. Formal testing of resistance was conducted on cockroaches collected from homes prior to TRF deployment. The lethal dose that kills 50% of each population (LD_{50}) was determined by topical application for two insecticides: cypermethrin (a pyrethroid representative of TRF products; Sigma-Aldrich, St. Louis, MO) and fipronil (representative of the gel baits; Sigma-Aldrich). Briefly, adult male cockroaches were anesthetized (CO_2), and separated into groups of 10 in round plastic Petri dishes (90 mm x 15 mm) and 1µl of acetone containing technical grade insecticide was applied to the ventral side between the coxae using a 50 µl syringe in a repeating dispenser (Hamilton Company, Reno, NV). Dilutions ranged from 0 ng (acetone only control) to 50 µg (cypermethrin) or 300 ng (fipronil), based on preliminary toxicity assays. Mortality was assessed 24 hr post-application, with moribund cockroaches (those unable to right themselves or exhibiting uncoordinated movement) considered dead.

Additionally, the pyrethroid synergist piperonyl butoxide (PBO) was tested for its effects on cypermethrin toxicity using two discriminating doses. We used the same protocol as above, except that 1 hr before topically applying either 0.5 or 5 µg of cypermethrin, each insect was topically treated with 100 µg of PBO in 1 µl acetone. Mortality was assessed 24 hr later.

Expressed *kdr* mutations were evaluated in apartment-collected cockroaches representing the five communities. Total RNA was extracted (Qiagen RNeasy kit, Valencia, CA) from 10 cockroaches from each of 10 homes and converted to cDNA (Bioline Tetro cDNA synthesis kit, Taunton, MA). From the cDNA, four different regions of the para-sodium channel that confer *kdr* resistance (Liu et al., 2000) were amplified via PCR (primers listed in Table S1). Amplified DNA was purified (Qiagen Qiaquick PCR purification kit) and sequenced (Sanger) at the Genomic Sciences Laboratory (North Carolina State University, Raleigh, NC).

Pesticide Residue Analysis

Kitchens were sampled for pesticide residues at three time points during the study: before TRF use (baseline), immediately (4-6 hr) after TRF discharge, and one month later. Areas sampled included the floor at 0.5 m and 1.0 m from the site of the TRF, the nearest countertop to the TRF (~0.9 m high), the inside of an upper level cabinet (generally ~1.4 m), and the nearest wall to the TRF at a height of 0.9 m, representing the height of a child. The same areas, but not the same spots, were sampled at each subsequent visit. Samples were collected by wiping an area of 100 cm^2 with a cotton swab wetted with isopropyl alcohol for

1 min. Each swab sample was placed into a 20 ml glass scintillation vial and immediately returned to the laboratory and stored at -30°C until extraction.

Swab samples were analyzed specifically for the active ingredients used in the TRF products tested, which included permethrin (sum of *cis*- and *trans*- isomers), cypermethrin (sum of all isomers), tetramethrin (sum of all isomers), and PBO (pyrethroid synergist). Additionally, all bait-treated homes were analyzed for fipronil residues. Each sample was fortified with 500 ng of the surrogate recovery standard (SRS) $^{13}C_6$-*trans*-permethrin (Cambridge Isotope Laboratories Inc., Tewksbury, MA). Samples were extracted twice using 10 ml of ethyl acetate. After each addition of ethyl acetate, samples were sonicated for 5 min and all solvent was transferred to a new 20 ml glass scintillation vial. Solvent volume was reduced to near dryness using a modified SpeedVac vacuum concentrator, then blown to complete dryness under nitrogen and re-suspended in 1 ml of hexane. Samples were then cleaned using a 3 ml prefabricated solid phase extraction (SPE) column containing 500 mg of silica gel (Supelclean LC-Si SPE Tube, Sigma Aldrich). The SPE column was conditioned with 5 ml of hexane, then the sample was loaded onto the column and eluted with 5 ml of 50% ether (in hexane). The eluant was blown to near dryness under nitrogen and resuspended in 1 ml of hexane. Following the SPE cleanup step, each sample was spiked with 500 ng of the internal standard (IS) 4,4'-dibromobiphenyl (DBBP, AccuStandard Inc., New Haven, CT). Samples were stored at -30°C until analysis.

Samples were analyzed using an Agilent Technologies 6890 GC coupled to an Agilent 5975 mass spectrometer (GC-MS). The GC was equipped with a 30 m x 0.25 mm x 0.25 µm (5%-phenyl)-methylpolysiloxane Agilent J&W HP-5ms column preceded by a 3 m

deactivated guard column. The temperature program was: 100°C for 1 min, then 5°C/min to 225°C, then 2°C/min to 256°C, then 10°C/min to 320°C where it was held for 10 min. Mass spectrometry conditions were: transfer line at 280°C, ionization source at 230°C, and quadrupole at 150°C. One quantification ion was used for each pesticide (Table S2). Ten calibration curve solutions ranging from 0.1 µg/ml to 100 µg/ml for all TRF pesticides (Sigma-Aldrich) were used to generate calibration curves via log-transformed linear regression. Extracted samples were corrected for both the SRS and IS and quantified using the calibration curve. Each calibration curve solution was run a minimum of three times, interspersed evenly among field-collected samples. If any compound exceeded the upper calibration curve point by more than 15%, the sample was diluted and re-analyzed.

Data Analyses

The effects of each intervention on reducing cockroach populations in apartments were evaluated using repeated measures ANOVA (within each treatment), with means compared using the Tukey-Kramer multiple comparison test. At each sampling interval, cockroach trap catches were compared using analysis of variance (ANOVA) with Tukey's post-hoc mean comparison test. Initial population counts were not transformed and compared as raw data (cockroaches trapped). Data for cockroach trap catches at two weeks and one month after intervention were converted to proportions of their respective baseline level and square root transformed to ensure homoscedasticity prior to ANOVA.

Three-way ANOVA was used to compare sentinel cockroach percent mortality (arcsine square root transformed) among TRF products, population (laboratory or apartment-

collected), and placement (floor or upper cabinet). Probit analysis was used to determine the LD_{50} for the insecticides cypermethrin and fipronil for the laboratory and apartment-collected cockroach populations. The effect of the pyrethroid synergist PBO on cypermethrin toxicity (two doses) was evaluated using two-way ANOVA (PBO applied, cypermethrin dose) on arc-sine square root transformed percent mortality.

The effects of each TRF treatment on pesticide residues were evaluated using repeated measures ANOVA (within each treatment) on log transformed values, with means compared using the Tukey-Kramer multiple comparison test. Comparisons were also made among locations for each TRF treatment. Pesticide residues were only evaluated for the Combat gel bait group, and not the Maxforce bait group, at baseline and one month. Total insecticide load in the kitchen was estimated using the average concentration on the four horizontal surfaces scaled up to an average kitchen size of 30 m^2. Dermal exposure (DE) was estimated using the USEPA residential exposure assessment algorithm (USEPA, 2012) for the 1 m floor samples, to best represent potential exposure of a child on the floor. The algorithm was as follows:

$$DE = SR * CF1 * TC * ET$$

where, SR = measured pesticide residues (µg cm^{-2}), CF1 = conversion factor (0.001 mg/ug), TC = transfer coefficient (cm^2/hr, estimated as 1800 cm^2/hr for children), and ET = exposure time (hr/d, estimated at 2 hr/d for children).

All statistical analyses were performed using SAS 9.4 (SAS Institute, 1985).

Results

Effects of Interventions on Cockroach Populations

There were no significant differences in baseline cockroach trap catches, a gauge of population level, among the treatments ($F_{5,22} = 0.97$, $p = 0.4553$, Fig. 1A). Cockroach trap catch was significantly affected by both time after intervention and treatment (interactive effect); therefore, the effect of each product was analyzed separately using repeated measures ANOVA. Only the two baiting interventions resulted in significant declines in trap counts (Combat: $F_{2,8} = 12.40$, $p = 0.0035$; Maxforce: $F_{2,8} = 21.37$, $p = 0.0006$), with trap counts in all TRF treatments not changing significantly from baseline counts ($p > 0.25$, Fig. 1B). For both bait treatments, the Tukey-Kramer multiple comparison test revealed that cockroach populations were significantly lower two- and four-weeks after treatment than at baseline. The percent reduction in trap counts was significantly different among treatments at both two weeks ($F_{5,22} = 11.29$, $p < 0.0001$) and four weeks ($F_{5,22} = 26.21$, $p < 0.0001$, Fig. 1B), but only bait treatments resulted in significant reductions in cockroach trap catches after treatment.

Responses of Sentinel Cockroaches to TRFs

Both the origin of cockroach populations (laboratory or apartments) and TRF product significantly affected sentinel cockroach mortality, while placement (floor or upper cabinet) had no effect ($F_{15,68} = 48.95$, $p < 0.0001$, Table 1). Mean percent mortality was significantly higher in the laboratory cockroaches than in apartment-collected cockroaches. Despite TRF

products having a significant effect on the model, no TRF product provided >38% mortality in the apartment-collected sentinel cockroaches, with some providing <11% mortality (Fig. 2). The differential mortality of the insecticide-susceptible lab cockroaches and the adjacent wild cockroaches in open-topped cages suggested that the latter might be resistant to pyrethroid insecticides.

Pyrethroid Resistance in Home-Collected Cockroaches

All apartment-collected cockroaches were highly resistant to cypermethrin (Table 2). The average resistance ratio relative to the laboratory susceptible population at the LD_{50} dose was 202 ± 33 (SEM)-fold. The average slope for all probit lines for the apartment-collected populations was 2.60 ± 0.32, shallower in comparison to a slope of 5.90 ± 0.48 for the laboratory population, indicating greater tolerance over a broad range of cypermethrin doses. Piperonyl butoxide (100 µg) significantly enhanced the toxicity of topically applied cypermethrin at both 0.5 µg and 5 µg (Fig. 3, $F_{3,28} = 129.31$, $p < 0.0001$). However, even when synergized with PBO, 0.5 µg cypermethrin (~10 times the LD_{50} of the laboratory population) resulted in minimal mortality (7.5 ± 2.4%). These results indicate extensive metabolic resistance to pyrethroids across all apartment-collected German cockroaches.

The L993F *kdr* mutation, known to confer pyrethroid resistance, was predominant in all cockroach populations, with 96% of apartment-collected cockroaches possessing at least one copy of this mutation and 77% being homozygous for this mutation (Table 3). In addition, mutations adaptive for pyrethroid resistance were also found for some cockroaches at both the E434K and C764R sites (Table S3).

Surprisingly, apartment-collected cockroaches were also moderately resistant to fipronil (Table 4). Their average resistance ratio compared to the laboratory susceptible population was 14 ± 2, and the average slope of their probit models was 3.95 ±0.24, compared to 15.32 ± 2.40 for the laboratory population.

Pesticide Residues in Kitchens

Total release foggers significantly contributed to indoor pesticide residues ($p <$ 0.0001 for all TRF-specific pesticides, Figs. 4-7). Pesticide residues varied significantly across sampling locations within the kitchen ($p < 0.0053$ for all TRF-specific pesticides, Fig. 4-7), with all horizontal surfaces collecting significantly more insecticide than the wall. Estimates of the total recoverable insecticide load in the kitchen ranged from 39.3 mg (tetramethrin, Hot Shot 3) to > 500 mg (PBO, Hot Shot 2; Table 5). Corresponding dermal exposure estimates for a child ranged from 0.46 mg/d (tetramethrin, Hot Shot 3), to 4.35 mg/d (PBO, Hot Shot 2; Table 6).

Conversely, there were no significant changes in pesticide residues for the bait-only intervention ($p > 0.3668$ for all pesticides studied). Additionally, fipronil was not detected in any of the bait-treated kitchens at any time point.

Economic Analysis

The applied material cost of TRFs ranged from $2.60 to $4.16, while gel baits were higher ($11.88 to $16.04, Table 7). However, consumers typically cannot purchase TRFs individually, thus a more realistic comparison would be the realized cost (see Table 7 for

equation). The realized cost of TRFs ranged from $7.79 to $12.49, while gel baits ranged from $13.98 to $23.43. Time estimates to complete the interventions were 6 hr for TRFs and 2 hr for gel baits. TRFs require extensive preparation time (estimated at 1 hr), a minimum of 4 hr during deployment when residents must vacate the home, and extensive cleanup following deployment (estimated at 1 hr). Bait placement is much less time consuming, with maximum time required for application estimated at 1 hr, followed by 0.5 hr commitments at two-and four-weeks after the initial application.

Discussion

Lack of Efficacy of Total Release Foggers

Total release foggers are clearly an ineffective cockroach control strategy. All TRFs failed to reduce cockroach populations, providing the first conclusive field-based evidence that these products are inappropriate to use for German cockroach control. While TRFs failed to control German cockroaches, the groups involving gel baits, both consumer-based do-it-yourself and professional, were successful at reducing cockroach populations, as expected based on previous uses of this strategy to mitigate German cockroaches and allergens (Arbes et al., 2003; Arbes et al., 2004; Rabito et al., in press; Sever et al., 2007). In the current study, baits were used on their own, independent of all other IPM tactics.

Although data obtained from population monitoring such as trap catches, provide insight into management efficacy, they do not allow for direct assessment of the effects of TRFs on cockroaches. Therefore, sentinel cockroaches, both laboratory reared and directly collected from each home, were reintroduced in open-top cages during TRF discharge as

direct assays of TRF efficacy. All the TRF products killed the insecticide-susceptible cockroaches, but these assays clearly demonstrated the ineffectiveness of the four TRF products against the apartment-collected cockroaches, with < 38% sentinel mortality for all products tested.

The overall ineffectiveness of TRFs may be caused by any one or combination of the following factors. First, pyrethroid insecticides are known to be repellent (Ebeling et al., 1967; Ebeling et al., 1968; Ebeling et al., 1966). While sentinel cockroaches could not escape the insecticide deposits from TRFs, the wild cockroaches likely displayed a behavioral avoidance of the pesticide residues. Second, aerosolized particles from TRFs likely failed to reach places where cockroaches shelter. We found relatively little pesticide on a vertical wall near the TRF discharge site, compared to horizontal surfaces. Since cockroaches are often found under horizontal surfaces (e.g., under the kitchen sink, under countertops, under shelves), they likely avoid the large insecticide deposits on the tops of horizontal surfaces. Finally, and most significantly, extensive and pervasive pyrethroid resistance has evolved in German cockroach populations over the last 3 decades (Chai and Lee, 2010; Cochran, 1989; Wei et al., 2001; Wu and Appel, in press), rendering even residual spray formulations, which deliver pyrethroids directly to aggregation and foraging sites, ineffective in German cockroach control (Miller and Meek, 2004). We recorded resistance ratios to cypermethrin ranging from 59- to 347-fold relative to the susceptible lab population. Pyrethroids in do-it-yourself TRF and aerosol products continue to impose strong selection pressure on German cockroach populations, especially in affordable housing, Although the levels of resistance observed in the present study are relatively high, they are not unique

(Atkinson et al., 1991; Wei et al., 2001). Resistance to pyrethroids has been documented in multiple populations collected from around the world, indicating that the pattern we observed is not unique to Raleigh, NC, and thus would be generalizable to other geographic locations.

Intense selection for pyrethroid resistance was also evident from the two major resistance mechanisms we observed. Pyrethroids are metabolized by a detoxification system that includes cytochrome-P450s and carboxylesterases (Hemingway and Ranson, 2000). PBO serves as a pyrethroid synergist in various insecticidal products by inhibiting P450s. Pre-treatment with PBO significantly elevated cypermethrin toxicity in our apartment-collected cockroaches, as shown in previous studies (Scott et al., 1990). Nevertheless, even under forced exposure with topical application, high doses of > 5 µg per insect were needed to overcome resistance, showing that German cockroach populations have greatly upregulated their pyrethroid detoxification system. In addition, under persistent selection, first with DDT and then with pyrethroids, German cockroaches have evolved mutations (*kdr*) in the site of pyrethroid action, the voltage-gated sodium channel gene, resulting in target-site insensitivity (Dong, 1997; Dong et al., 1998; Liu et al., 2000). In apartment-collected cockroaches, the leucine to phenylalanine (L993F) mutation was present at high frequency, and two populations appeared to be fixed for this mutation, while most others appeared headed in that direction. Finally, it is likely that these cockroaches have other resistance mechanisms (e.g., reduced cuticular penetration, greater sequestration) that contribute to TRF inefficacy.

TRFs Deposit Large Amounts of Pesticide Residues

TRFs also significantly elevated pesticide residues in homes. Our results for cypermethrin residues on horizontal surfaces (15-80 µg/100 cm^2, depending on the TRF and substrate), were comparable to those of Keenan et al. (2009) who found 7-38 µg/100 cm^2 after TRF deployment. TRF deposits settled relatively evenly on all horizontal surfaces (floor: 0.5 m and 1 m from the TRF; countertop; upper cabinet) 4 hr after discharge, and much less on walls, as also shown by (Keenan et al., 2010). The high concentration of pesticide residues in areas where cockroaches seldom forage, and low deposits on vertical surfaces where cockroaches often aggregate inside cabinets (Schal, 2011), likely contributed to the inefficacy of TRFs. In contrast to TRFs, traditional "crack and crevice" and baseboard applications of residual broad-spectrum insecticides accumulate more pesticide residues close to the wall, with lower concentrations towards the center of the room (Keenan et al., 2010). Thus, the combined use of two popular do-it-yourself products – TRFs and aerosol sprays – would broadly disseminate pesticide residues in all areas of the kitchen.

It is startling how much pesticide was deposited on a kitchen-wide basis, with estimated recoverable amounts ranging from 40 mg to > 500 mg per kitchen, depending on the TRF-insecticide combination. These high deposits would translate to alarmingly high estimates of dermal exposure, where upon re-entry into TRF treated apartments children playing on the floor may be exposed to as much as 4.35 mg/d of TRF active ingredients. These values, however, must be scrutinized with caution because it is unclear what the transfer rate is from the floor to human skin. Moreover, the transfer rate from pesticide deposits on the kitchen countertop to food is unknown, and require further investigation.

Considering the high deposits of pesticides from the discharge of a single TRF, it is not surprising that many studies have found quantifiable "baseline" amounts of a variety of pesticides in homes (including pyrethroids and PBO), without implementing any pest control intervention (Landrigan et al., 1999; Morgan, 2012; Stout II et al., 2009; Trunnelle et al., 2014). The current study is no exception, with quantifiable amounts of several of the pesticides evaluated present at baseline (pre-TRF discharge) sampling.

Integrated Pest Management and Baits

In contrast to the TRFs, both consumer-based gel bait (Combat) and professional gel bait (Maxforce) were highly effective at reducing the cockroach infestations. Moreover, fipronil (the active ingredient in both bait products) was not detected in any of the bait-treated homes, consistent with the findings of Williams et al. (2005), that IPM programs involving baits eliminated most pesticide residues when compared to traditional crack-and-crevice spray treatments.

In addition to the popularity of TRFs, several other obstacles stand in the way of broad adoption of do-it-yourself baits. Consumer education is primary among these, as most residents do not dispense baits in proper amounts and in appropriate locations. Although more classes of insecticides are available in bait formulations than in TRFs and sprays, both behavioral and physiological resistance to bait formulations are escalating (Ko et al., 2016; Silverman and Bieman, 1993; Wada-Katsumata et al., 2013; Wang et al., 2004). We found resistance to fipronil in all apartment-collected cockroach populations (6-23-fold relative to the susceptible lab population) and resistance to it and other bait insecticides (e.g.,

indoxacarb) has been broadly documented (Holbrook et al., 2003; Kristensen et al., 2005; Scott and Wen, 1997; Valles et al., 1997). Two key differences between most bait and spray (or TRF) formulations are that (a) ingestion of an insecticide requires a considerably smaller dose to cause mortality than contact with the insecticide (Sierras and Schal, 2017), and (b) much higher doses are formulated into baits than sprays relative to the LD_{50} dose (Ko et al., 2016). These differences, in combination with bait rotations and careful attention to bait acceptance/rejection by cockroaches, can effectively extend the range of resistance to baits that can be tolerated.

Our effective use of gel baits as a single monitoring-guided intervention, independent of other IPM tactics (e.g., caulking, sanitation, cleaning, trapping), is consistent with an emergent body of literature showing that proper use of baits constitutes the most cost-effective intervention to mitigate the harmful effects of cockroaches and their allergens (reviews: Gore and Schal 2007, Schal 2011). While more comprehensive IPM approaches (e.g. Dingha et al., 2016; Kass et al., 2009; Wang and Bennett, 2006) may promise faster and more effective cockroach control, the additional costs associated with materials, labor and administrative matters also make them less practical in many real-world situations.

Conclusions: TRFs and Social Injustice

Environmental social injustice is prevalent throughout the industrialized world, as people at the lower socio-economic scale are disproportionately exposed to industrial and agricultural pollution (Evans and Kantrowitz, 2002). Likewise, social disparities in exposure to indoor contaminants that impact human health are well-documented for air pollutants,

lead, allergens, and various organic compounds (Adamkiewicz et al., 2011). Pesticides are no exception, and are consistently found at disproportionately higher levels in lower socio-economic households (Carlton et al., 2004; Harnly et al., 2009; Julien et al., 2008; Landrigan et al., 1999; Quirós-Alcalá et al., 2011). To fully understand and ultimately correct social disparities in indoor environmental health requires that each potential contributing factor to these disparities be identified, its associated costs, benefits, and risks comprehensively assessed, and policy changes and regulatory action be considered to mitigate its undesirable effects.

Our findings indicate that TRFs represent a clear example of social injustice in the indoor environment. While TRFs are extensively used in indoor pest control, especially in affordable housing in disadvantaged communities, they provide no control of German cockroach (present study) or bed bug (Jones et al. (2012) infestations. Yet, TRFs contribute substantially to the extensive pesticide load in the indoor environment. The continued use of TRFs ought to be a fundamental societal concern, perhaps on par with other indoor environmental issues such as lead contamination. Foremost, the lack of efficacy allows pest infestations to persist, and in the case of German cockroaches exposes residents potent allergens that can trigger asthma episodes (Gore and Schal, 2007), and increasing the potential for disease transmission (Devi and Murray, 1991; Fotedar et al., 1991; Roth and Willis, 1957). Second, TRFs broadcast large quantities of bioavailable pesticides throughout a room, resulting in persistent environmental contamination, a reservoir for translocation throughout the home, and chronic health effects. Third, substantial (and likely underreported) acute adverse symptoms have been associated with TRF use, as well as

explosions and fires (C.D.C, 2008; Forrester and Diebolt-Brown, 2011). Fourth, the monetary and time costs associated with the deployment of inefficacious TRFs and highly efficacious gel baits are comparable. Finally, TRFs represent a major impediment to adoption of IPM principles and baits in low-income residential settings. The contention that "TRFs can reduce pest populations and often are used by consumers as a low cost alternative to professional pest control services" (C.D.C, 2008) needs to be challenged through public education and training of housing managers.

It has been over 25 years since Fenske (1990) suggested several strategies to mitigate risks associated with indoor pesticide applications, including that "product registrations could be modified or withdrawn for specific applications if an acceptable level of risk cannot be demonstrated." Although his work focused on active ingredients that in fact have been withdrawn from indoor use, the narrative remains the same – TRFs are depositing large amounts of insecticides throughout the home. We now show that TRFs provide no benefits for mitigation of German cockroach infestations. Their associated societal costs, lack of observable benefits TRFs, and the availability of highly efficacious, affordable and environmentally sound alternatives, such as baits, challenge the continued registration and persistence of TRFs in the consumer market.

Acknowledgements

We thank residents of several affordable housing communities in Raleigh NC who participated in this study. This study would not have been possible without their support and partnership. We also acknowledge Michelle McCombs (RTI, NC) for guidance in the initial

steps of GC-MS analysis. Funding for this study was provided by the Blanton J. Whitmire Endowment at North Carolina State University, the US Department of Housing and Urban Development Healthy Homes program (NCHHU0017-11), the US Environmental Protection Agency Pesticide Environmental Stewardship Program (PESP, PE-95450709), and a National Institute of Environmental Health Sciences grant to the Center for Human Health and the Environment (CHHE, P30ES025128). ZD received the David R. Nimocks Jr. Fellowship and scholarship awards from the Foundation for Agromedicine and Toxicology, Pi Chi Omega, and the Entomological Society of America (Monsanto Research Grant Award, MUVE Scholarship).

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Table 1. Results from three-way ANOVA on mortality of caged sentinel cockroaches with two- and three-way interaction terms. Significance indicated with the presence of an *.

Source	Type III SS	*d.f.*	*F*	*p*
TRF product	0.61	3	5.37	0.0022*
Cockroach population (laboratory or apartment-collected)	26.8	1	710.76	< 0.0001*
Sentinel cage placement (floor or upper cabinet)	0.01	1	0.33	0.5698
TRF product x Cockroach population	0.52	3	4.61	0.0054*
Cockroach population x Sentinel cage placement	0.00	1	0.00	0.9978
TRF product x Sentinel cage placement	0.04	3	0.33	0.8071
TRF product x Cockroach population x Sentinel cage placement	0.12	3	1.13	0.3438
Error	2.56	68		

Table 2. Resistance of apartment-collected German cockroach populations to topical application of the pyrethroid insecticide cypermethrin. Cockroach populations are represented from each of five apartment complexes in Raleigh, NC.

Population[a]	*n*	LD_{50} (µg bug^{-1})	LD_{50} 95% CI	Slope ± SE	X^2 (*d.f.*)	RR[b]
Laboratory	600	0.049	0.046 - 0.051	5.90 ± 0.48	11.11 (11)	-
C45	180	17.025	12.90 - 24.50	2.08 ± 0.36	0.02 (3)	347
C50	180	11.568	9.76 - 15.11	3.89 ± 0.83	0.49 (3)	236
F2215	87	4.657	2.67 - 5.72	4.52 ± 1.41	< 0.01 (1)	95
F2225	90	2.874	1.79 - 4.26	1.80 ± 0.32	2.50 (1)	59
P511	120	13.081	9.34 - 17.87	2.23 ± 0.47	0.03 (1)	267
R2919	180	6.905	5.02 - 8.80	2.44 ± 0.48	0.87 (3)	141
R3011	150	15.361	11.50 - 22.10	1.95 ± 0.35	0.09 (2)	313
S1224	120	10.653	6.96 - 15.18	1.81 ± 0.36	0.48 (1)	217
S1320	150	7.213	5.37 - 9.04	2.67 ± 0.54	2.46 (2)	147
Average[c]	9	9.926	-	2.60	-	202

[a] Letters designate unique apartment complexes, and number designates apartment (population)
[b] RR: Resistance Ratio = LD_{50} of apartment population / LD_{50} of laboratory population
[c] Averages for the nine apartment-collected populations

Table 3. Expressed frequencies of the L993F *kdr* mutation. Cockroach populations are represented from each of five apartment complexes in Raleigh, NC.

Population[a]	*n*	S/S[b]	S/R[c]	R/R[d]	S[e]	R[f]
Laboratory	20	0.95	0.05	0.00	0.98	0.02
C45	8	0.00	0.13	0.87	0.06	0.94
C50	9	0.00	0.00	1.00	0.00	1.00
F2225	10	0.10	0.10	0.80	0.15	0.85
F2289	9	0.11	0.22	0.67	0.22	0.78
P511	8	0.00	0.00	1.00	0.00	1.00
P619	10	0.20	0.30	0.50	0.35	0.65
R2919	10	0.00	0.50	0.50	0.25	0.75
R3011	8	0.00	0.38	0.62	0.19	0.81
S1224	10	0.00	0.20	0.80	0.10	0.90
S1320	10	0.00	0.10	0.90	0.05	0.95
Average[g]	10	0.04	0.19	0.77	0.14	0.86

[a] Letters designate unique apartment complexes, and number designates apartment (population)
[b] S/S = proportion homozygous wild-type (considered pyrethroid-susceptible)
[c] S/R = proportion heterozygous
[d] R/R = proportion homozygous for the *kdr* mutation (considered to confer resistance to pyrethroids)
[e] S = proportion "susceptible" allele
[f] R = proportion "resistance" allele
[g] Average of all 10 apartment-collected populations

Table 4. Resistance of apartment-collected German cockroach populations to topical application of fipronil. Cockroach populations are represented from each of five apartment complexes in Raleigh, NC.

Population[a]	*n*	LD_{50} (ng bug^{-1})	LD_{50} 95% CI	Slope (±SE)	X^2 (*d.f.*)	RR[b]
Laboratory	180	2.99	2.87 - 3.11	15.32 ± 2.40	3.93 (4)	-
C45	150	47.48	37.30 - 61.03	3.54 ± 0.55	3.43 (3)	16
C50	90	33.20	23.68 - 42.37	3.83 ± 0.94	<0.01 (1)	11
F2215	89	69.00	54.93 - 85.09	5.07 ± 0.98	<0.01 (1)	23
P511	120	38.74	30.68 - 49.13	3.90 ± 0.68	0.29 (2)	13
R2919	89	16.62	11.84 - 27.85	2.98 ± 0.60	1.57 (1)	6
S1224	90	42.70	33.19 - 54.12	4.06 ± 0.82	<0.01 (1)	14
S1320	90	50.36	39.96 - 63.41	4.30 ± 0.79	<0.01 (1)	17
Average[c]	7	42.59	-	3.95	-	14

[a] Letters designate unique apartment complexes, and number designates apartment (population)
[b] RR: Resistance Ratio = LD_{50} of apartment-collected population / LD_{50} of laboratory population
[c] Averages for the seven apartment-collected populations

Table 5. Estimated pesticide load in the kitchen for each TRF product (±SEM). Values are estimated based on the average pesticide concentration extracted from all horizontal surfaces and an approximate kitchen size of 30 m^2.

TRF	n	PBO (mg)	Tetramethrin (mg)	Permethrin (mg)	Cypermethrin (mg)
Hot Shot 2	4	505.7(±113.7)	116.0(±25.6)	287.9(±70.4)	-
Hot Shot 3	4	82.4(±14.9)	39.4(±6.2)	-	42.8(±3.9)
Raid Deep	5	-	-	-	157.3(±30.7)
Raid Fumigator	5	-	-	126.7(±44.3)	-

Table 6. Estimated dermal exposure (mg/d) for all TRF-contained pesticides (±SEM; see text for equation).

TRF	n	PBO (mg/d)	Tetramethrin (mg/d)	Permethrin (mg/d)	Cypermethrin (mg/d)
Hot Shot 2	4	4.35(±1.05)	1.08(±0.31)	2.66(±0.77)	-
Hot Shot 3	4	0.88(±0.11)	0.46(±0.05)	-	0.52(±0.04)
Raid Deep	5	-	-	-	1.67(±0.19)
Raid Fumigator	5	-	-	1.97(±0.50)	-

Table 7. Monetary costs of TRF-products and gel bait products.

Product	Product cost ($US)	Units per package	Unit cost ($US)	Avg. units used	Applied material cost ($US)	Realized material cost[a] ($US)
Hot Shot 2	$10.17	3	$3.39	1.0	$3.39	$10.17
Hot Shot 3	$10.17	3	$3.39	1.0	$3.39	$10.17
Raid Deep	$7.79	3	$2.60	1.0	$2.60	$7.79
Raid Fumigator	$12.49	3	$4.16	1.0	$4.16	$12.49
Combat	$6.99	1	$6.99	1.7	$11.88	$13.98
Max Force	$23.43	4	$5.86	3.3	$16.04	$23.43

[a] Realized cost = Avg. units used / Units per package (rounded up to the nearest whole number), multiplied by the product cost.

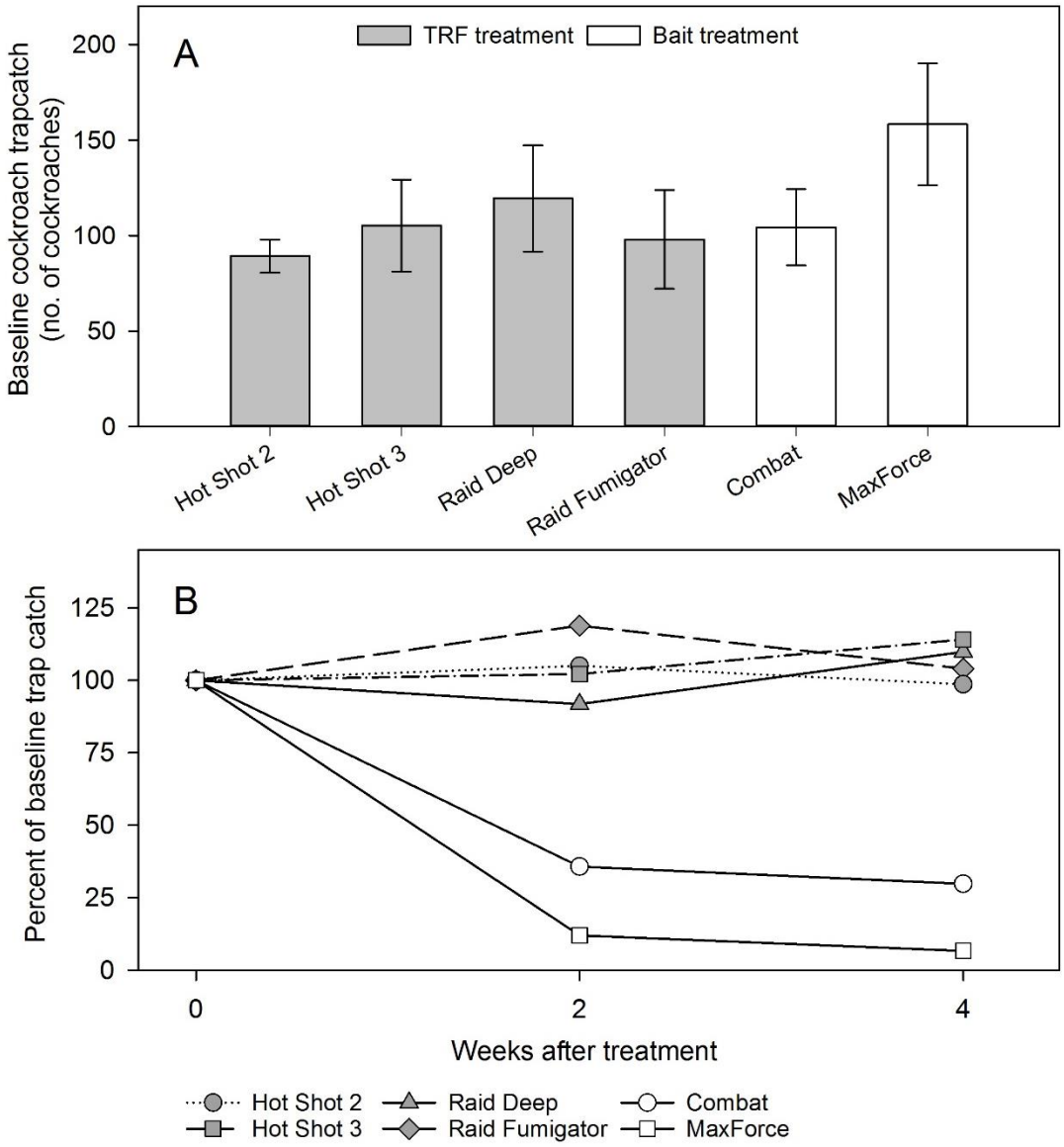

Figure 1 – Effects of six interventions (4 TRFs, 2 gel baits) on cockroach populations. Total cockroaches trapped are displayed for each intervention at baseline (A), and the percentage reduction in trap catches is displayed at 2 and 4 weeks post-treatment (B).

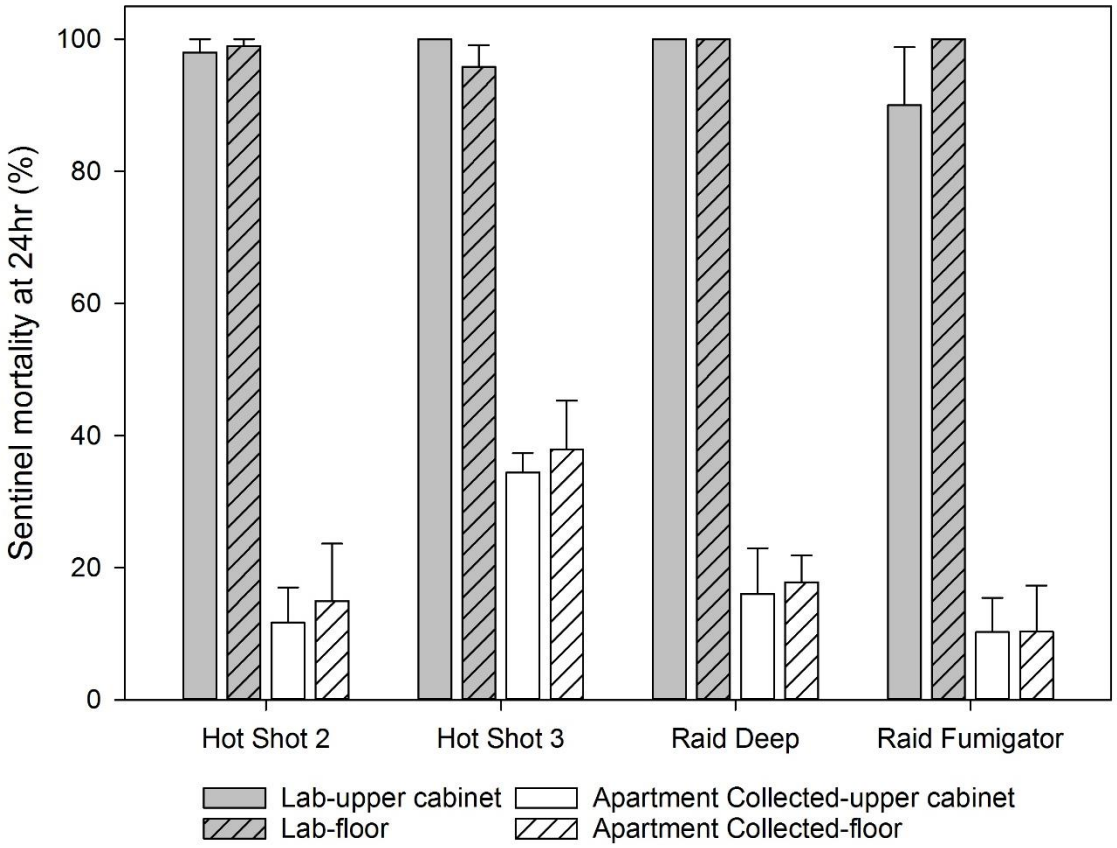

Figure 2 – Effect of TRFs, population origin (Lab, Apartments), and sentinel location (upper cabinet, floor) on mortality of caged sentinel adult male cockroaches. n = 5 homes per TRF type; 20 cockroaches per cage.

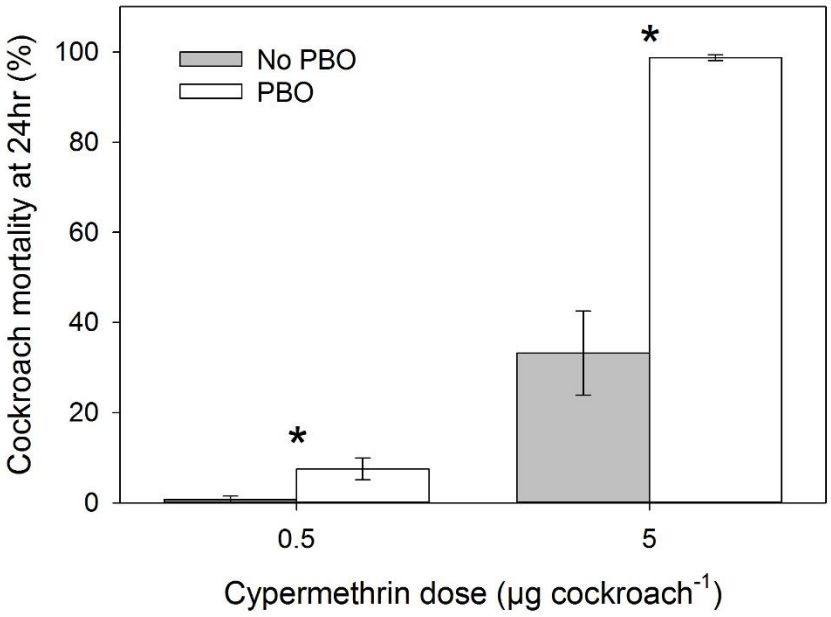

Figure 3 – Effect of the insecticide synergist PBO on cockroach mortality 24 h after topical application of cypermethrin. PBO was applied 1 hr prior to cypermethrin application. Percent mortality was determined for 7-9 populations (n=30 cockroaches per population) and averaged among populations for each of the four treatments. Significance is indicated by an *.

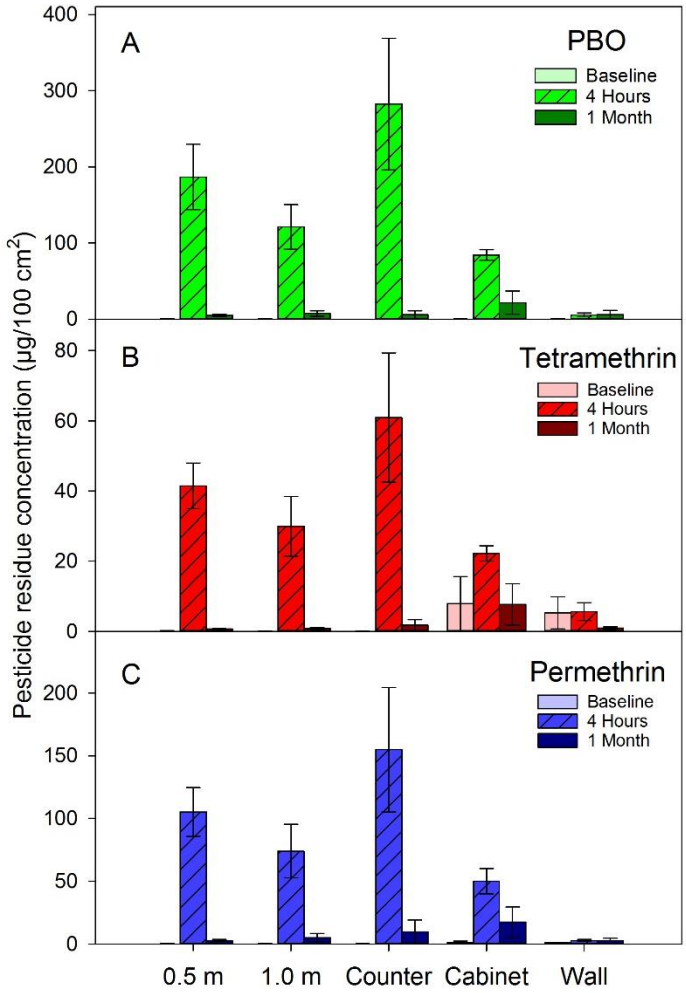

Figure 4 – Pesticide residue concentrations in samples from homes treated with a Hot Shot No-Mess Fogger$_2$ with Odor Neutralizer. Pesticide residues were measured at baseline, 4 hours post-deployment, and 1 month post-deployment. Sites swabbed for pesticide residues included the floor at 0.5 m and 1 m from the TRF deployment site, the nearest countertop to the TRF deployment site, the inside of an upper level cabinet, and the nearest wall to the TRF deployment site at a height of 90 cm from the floor. Pesticides evaluated included PBO (A), tetramethrin (B), and permethrin (C).

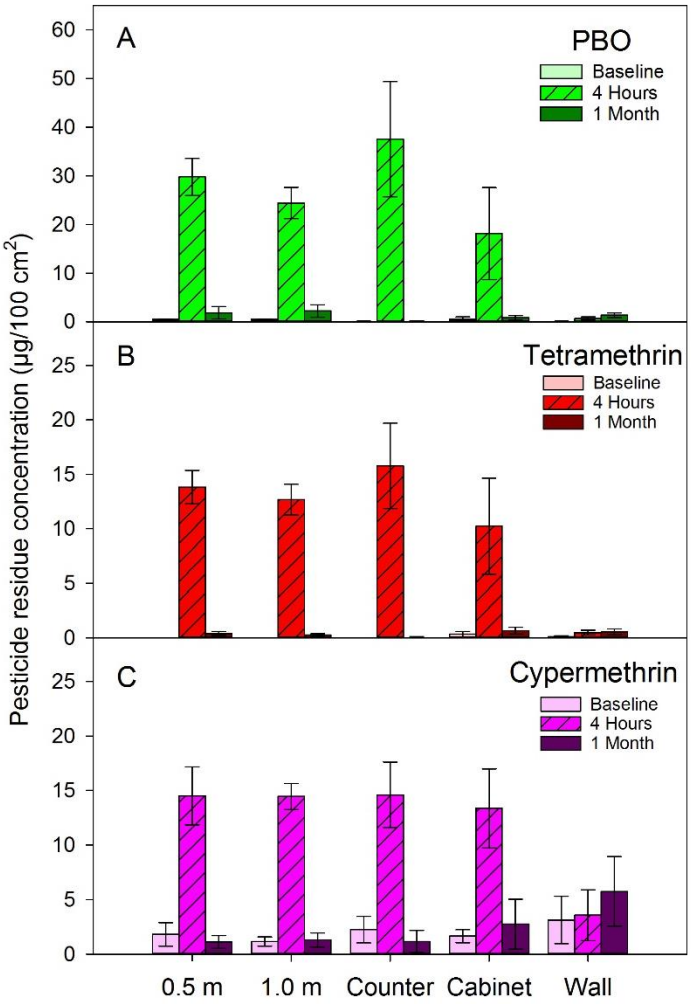

Figure 5 – Pesticide residue concentrations for homes treated with the Hot Shot No-Mess Fogger$_3$ with Odor Neutralizer. Pesticide residues were measured at baseline, 4 hours post-deployment, and 1 month post-deployment. Sites swabbed for pesticide residues included the floor at 0.5m and 1.0m from the TRF deployment site, the nearest countertop to the TRF deployment site, the inside of an upper level cabinet, and the nearest wall to the TRF deployment site at a height of 90cm from the floor. Pesticides evaluated included PBO (A), tetramethrin (B), and cypermethrin (C).

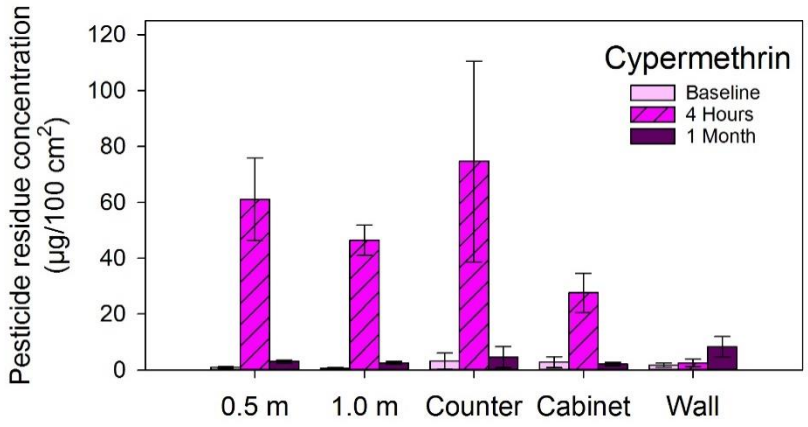

Figure 6 – Cypermethrin residue concentration for homes treated with Raid Max Concentrated Deep Reach Fogger. Cypermethrin residues were measured at baseline, 4 hours post-deployment, and 1 month post-deployment. Sites swabbed for cypermethrin residues included the floor at 0.5m and 1.0m from the TRF deployment site, the nearest countertop to the TRF deployment site, the inside of an upper level cabinet, and the nearest wall to the TRF deployment site at a height of 90cm from the floor.

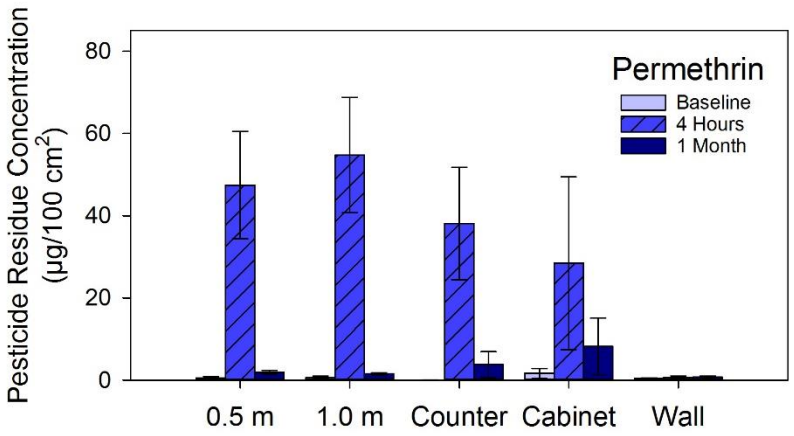

Figure 7 – Permethrin residue concentration for homes treated with Raid Fumigator. Permethrin residues were measured at baseline, 4 hours post-deployment, and 1 month post-deployment. Sites swabbed for permethrin residues included the floor at 0.5m and 1.0m from the TRF deployment site, the nearest countertop to the TRF deployment site, the inside of an upper level cabinet, and the nearest wall to the TRF deployment site at a height of 90cm from the floor.