THE RESPONSES OF THE BED BUG (*CIMEX LECTULARIUS* L.) TO HEAT EXPOSURE AT THE POPULATION, BEHAVIORAL, AND PHYSIOLOGICAL LEVELS

by

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ABSTRACT

Elimination of bed bug infestations remains a challenge for the pest management industry as these insects continue to resurge on a global scale. However, the use of integrated pest management (IPM) to control bed bugs has been effective. A key part of an IPM program is the combined use of chemical and non-chemical techniques, and one effective non-chemical technique is the use of lethal heat. When properly done, heat treatments can eliminate all bed bug life stages. However, household items with variable thermal conductivities can lead to uneven temperatures within the structures being treated. Uneven heating of bed bug harborage areas may allow bed bugs to behaviorally respond to heat exposure by escaping to cooler zones or be exposed to sub-lethal heat when within hiding places. Survival of bed bugs immediately following heat treatments could mainly occur due to two reasons: (1) the ability of bed bugs to develop physiological heat resistance; and (2) their ability to chemically (through release of alarm pheromones) and behaviorally respond to lethal heat exposure by escaping to cooler spots or areas that have been sub-lethally heated, which has not been investigated. Therefore, the main objectives of this study are: 1) Evaluate ability of bed bugs to develop heat resistance or thermo-tolerance at the population level in relation to their history of previous heat exposure, insecticide resistance status, and geographic origin using two different exposure techniques. 2) Determine the temperatures that will stimulate bed bugs to move in search for cooler areas. 3) Identify which *HSP* genes are expressed by bed bugs after heat exposure. 4) Determine if alarm pheromones (APs) are released by bed bugs that are heat exposed and how conspecifics that are not exposed to heat will react to these APs. For the first objective, selection experiments found an initial increase in bed bug survivorship over the first 2–3 generations; however, survivorship did not increase past the fourth generation. The step-function exposure technique revealed non-significant variation in heat tolerance between populations and the ramp-function exposure technique provided similar results. Based on this objective 1 findings, the ability of bed bugs to develop heat resistance within a few generations appears to be limited. Behavioral experiments (objective 2) found that at specific temperatures, different bed bug populations had similar probabilities to flee and their ability to locate unheated areas or survive heat exposure in the arena was not different. Also, if bed bugs are exposed to temperatures of 47–48°C in their harborage while exterior temperatures approach 53–54°C, they will be forced into the open. The RT-qPCR experiments (objective 3) revealed that the expression

of the *HSP70.1*, *HSP70.3*, and *Putative Small HSP* genes were significantly upregulated after heat exposure in all tested populations and are likely critical in their recovery process after heat exposure. For the fourth objective, analysis of head space volatiles, using a SIFT-MS (soft ion flow tube mass spectrometer), revealed that bed bugs responded to lethal and sub-lethal heat exposure by emitting their APs. In separate behavioral experiments, it was shown that conspecifics from both tested populations reacted to APs emitted by heated bed bugs and synthetic APs by frantically moving. The finding that bed bugs emit APs after heat exposure has important implications for pest management professionals that conduct thermal treatments for bed bug elimination. Overall, the results of these thermal biology experiments provide insights into how bed bugs respond to heat exposure at the population, behavioral, and physiological levels. The findings of this dissertation suggest that the behavioral and physiological responses of bed bugs when exposed to heat are critical to their survival particularly when they have an opportunity to flee from a lethally heated environment to a cooler thermally protected area.

CHAPTER 1. BACKGROUND AND OBJECTIVES

1.1 Introduction

Of the ~100 different parasitic pest species in the family Cimicidae, only *Cimex lectularius* (L.) (bed bug) and *Cimex hemipterus* (F.) (tropical bed bug) are associated with global resurgence (Usinger 1966, Hwang et al. 2005, Reinhardt and Siva-Jothy 2007, Doggett et al. 2012). Both, *C. lectularius* and *C. hemipterus* feed on humans, bats, and chickens, live in close association with their hosts and have overlapping distributions in certain areas (Usinger 1966, Newberry 1985, Steelman et al. 2008). The ability of an organism to tolerate environmental conditions influences their geographic distribution (Spicer and Gaston 1999) and these two Cimicids differ in their temperature and humidity tolerance (Omori 1941, Usinger 1966, Newberry 1985). This allows for a widespread distribution of *C. lectularius* in temperate regions, whereas *C. hemipterus* is limited to regions within the 30° north and south latitudes. Despite this, both species are usually sheltered from the outdoor environment and prefer stable conditions (Balvin et al. 2015, Devries et al. 2016a).

Studying the thermal biology of the bed bug, *C. lectularius* is important from the perspective of heat being used for its remediation. Bed bug infestations can be challenging to eliminate because of several reasons (i) chemicals that possess a broad spectrum of insecticidal activity are not applied, (ii) diverse insecticide resistance mechanisms can exist within bed bug populations, and (iii) the inability to apply insecticides in certain areas within a household (Potter et al. 2007, Adelman et al. 2011, Boase and Naylor 2014, Ashbrook et al. 2017). In order to overcome abovementioned bed bug control challenges, whole domicile heating is being used for bed bug remediation (Pereira et al. 2009, Kells and Goblirsch 2011, Raab et al. 2015, Bennett et al. 2015, Devries, 2016a, Ashbrook et al. 2019).

The use of large-scale heat treatments can be effective for eliminating all bed bugs within an infestation. Entire home heating is achieved by directly circulating heated air for several hours (Benoit et al. 2009a, Kells and Goblirsch 2011). There are, however, some drawbacks to bed bug heat treatments. For example, bed bugs may flee and take shelter in cooler wall voids, deep within furniture, or neighboring unheated areas (Doggett et al. 2006, Pereira et al. 2009, Raab et al. 2016, Loudon 2017). Since heat treatments provide no residual protection, fleeing or surviving bed bugs can re-infest previously treated homes. Similarly, bed bugs stunned by heat exposure can fall into

protected areas and then recover (Schrader et al. 2011). Additionally, large scale heat treatments are time intensive, costly, can damage heat sensitive items, and have high energy requirements (Kells and Goblirsch 2011). Relative to other insects, bed bugs are tolerant to heat exposure and are able to withstand heat exposures to 43 °C (CTmax of 45.19 °C for starved insects) (Kells 2006, Benoit et al. 2009a, Pereira et al. 2009, Kells and Goblirsch 2011, Devries et al. 2016a).

Prior heat-exposure studies cited above are valuable since they focused on determining optimal temperature, CTmax, and exposure time/temperature combinations for achieving various levels of mortality for both bed bug species. However, it is difficult to make comparisons between these studies as well as make assumptions about bed bug thermo-tolerance at the population level. No study to date has attempted to screen the bed bug or tropical bed bug populations for variation in their thermo-tolerance or heat resistance, which is indicative of a significant knowledge gap in the scientific literature. Heat exposure experiments using *Drosophila melanogaster* and *Culex pipiens* have reported rapid development of heat resistance within only three or four generations of laboratory selection (Huey et al. 1991, Huey et al. 1992, Gray 2013). Studies on the green house pest, *Tetranychus cinnabarinus*, has revealed that arthropods can develop cross-resistance to insecticides and heat when both are used for control (Liu et al. 2007, Feng et al. 2010). If bed bugs developed resistance to heat in addition to insecticide resistance, it would be exceptionally problematic for bed bug elimination.

Determining how heat resistance could develop within a population is challenging. One potential mechanism for the evolution of heat tolerance or resistance could be mediated by elevated expression of stress-induced heat shock proteins (HSPs) 70 and 90, and levels of certain sugars (trehalose) and amino acids (proline) (Benoit et al. 2009a, Feder and Hofmann 1999, Feder et al. 1999). However, how arthropods express HSPs and other stress-induced genes and metabolites in the field in response to thermal challenges is not well understood. Instead of increasing expression of HSPs and stress induced genes in response to heat exposure, it is possible that insects could flee to cooler areas to avoid heat stress (Feder and Hofmann 1999). Recently, it has been found that bed bugs move from heat-treated apartments to adjacent unheated units to avoid lethal heat exposure (Raab et al. 2015). Loudon (2017) found that some bed bugs move from externally heated luggage to the cooler interior to escape heat exposure. Bed bugs are able to detect and orient towards heated objects (23°C to 48°C) that are 10-30mm away (Devries et al. 2016b). Also, when *C. lectularius* is exposed to a thermal gradient with a range of temperatures, they will preferentially

rest at 27–28°C (Omori 1941). These studies demonstrate that bed bugs are good at detecting and responding to heat at short ranges.

Since bed bugs prefer to harbor within the cracks and crevices of walls or furniture, it is important that the temperatures in these microhabitats reach or exceed lethal limits (>50°C, Kells and Goblirsch 2011) for a sufficient amount of time (6−8h) during a heat treatment. Achieving lethal temperatures in bed bug harborages within a dwelling is a logistically challenging task. Such constraints often lead to bed bugs being exposed to sub-lethal temperatures, which could lead to the development of heat resistance, especially in consideration of bed bugs being able to detect and flee from sub-lethal heat. Additionally, the climatic conditions in the geographic region the bed bug population originated from may influence their thermo-tolerance. As there may be a variety of factors that influence insect thermo-tolerance, this study will simultaneously consider how bed bugs respond to heat exposure at the population, behavioral, and physiological levels in order to understand the long-term consequences of thermal remediation of infestations. These factors give rise to the following hypotheses.

Hypothesis 1: Bed bug strains that have survived heat treatments in the field will require longer exposure times to achieve complete or equivalent mortality relative to bed bugs that have no history of heat exposure.

Hypothesis 2: Bed bug populations that have been previously exposed to heat will be able to detect and escape from lethally or sub-lethally heated areas at higher proportion than bed bug strains without any exposure history.

Hypothesis 3: Sublethal heat exposure will cause significant changes in *HSP* gene expression during the stress recovery process.

Hypothesis 4: Bed bug alarm pheromones (APs) will play a role in the escape response from heated areas.

1.2 Research objectives and rationale

The proposed research will investigate how bed bugs respond to heat exposure at multiple levels by integrating studies on insect thermo-physiology and behavior. The specific objectives for this study are:

Objective 1: Evaluate the ability of bed bugs to develop greated thermo-tolerance at the population level in relation to their history of previous heat exposure, insecticide resistance status, and geographic origin using two different exposure techniques (Ashbrook et al. 2019).

Rationale: In certain instances, bed bugs are known to survive lethal heat exposure. It is not known if the bed bugs are capable of surviving heat exposure due to (1) logistic factors associated with inability to achieve lethal temperatures in bed bug harborage areas for a sufficient time period, (2) re-infestation from bed bugs that escaped to cooler areas within the domicile during heat treatment or (3) development of physiological heat resistance. This objective will determine if bed bug populations with various heat exposure histories, insecticide resistance profiles, and geographic origin show physiological heat resistance. Early detection of heat resistance will have important implications for modifying management protocols that rely on heat treatments alone to eradicate bed bug infestations and thus help ensure long-term effective control.

Objective 2: Determine the temperatures that will stimulate bed bugs to move in search for cooler areas.

Rationale: Reports of bed bugs escaping heated zones during thermal remediation (Raab et al. 2016), moving from the exterior of heated luggage to the cooler interior (Loudon 2017), and incomplete elimination of bed bugs during a heat treatment warrants further investigation into how bed bugs behaviorally respond to heat exposure.

Objective 3: Identify which *HSP* genes are expressed by bed bugs after heat exposure.

Rationale: Determining if strains with greater tolerance to heat exposure also have higher levels of inducible *HSP* gene expression will confirm HSP importance in the bed bug stress recovery process. Screening bed bugs for inducible *HSP* gene expression will identify candidate strains for further heat exposure experiments while also providing additional verification of the heat exposure results. Therefore, it is important to determine how expression of *HSP*s changes over time in response to heat exposure, to shed light on the mechanisms involved in heat stress recovery.

Objective 4: Determine if alarm pheromones are released by bed bugs that are heat exposed and how conspecifics that are not exposed to heat will react.

Rationale: Given the limited ability of bed bugs to develop heat resistance (Ashbrook et al. 2019), their behavior is likely a contributing factor to the insects surviving or escaping heat

exposure during a thermal intervention. Identification of APs emitted by heat-exposed bed bugs in response to thermal stress will serve as a first step to establish the link between "AP release and bed bug escape from heated areas." Results from these experiments will provide insight into how bed bugs respond behaviorally to heat exposure. If the alarm pheromone release causes heat-exposed and non-heated bed bugs to move frantically, it will provide first evidence for interactions between pheromones, heat exposure, and the bed bug escape response. The presence of this interaction will also demonstrate the need for additional control techniques to be combined with heat treatments. For example, when the temperature that causes APs to be released is approached, the rate of temperature increase can be accelerated to trap escaping bed bugs and cause greater mortality. Heat treatments could be modified to incorporate strategies that prevent bed bugs from escaping a heat treatment, such as the deployment of interceptor traps and the application of insecticide barriers. If there are residences adjacent to a domicile that is being heat-treated, they can be inspected once the treatment is completed to be sure no bed bugs have entered from the heated areas or else it will contribute to re-infestation of the treated domicile. Alternatively, APs could be deployed as a flushing agent to remove bed bugs from harborages during a heat treatment for greater heat exposure/contact as was done with desiccant dusts in Benoit et al. (2009c).

CHAPTER 2. BED BUGS (*CIMEX LECTULARIUS* L.) EXHIBIT LIMITED ABILITY TO DEVELOP HEAT RESISTANCE

2.1 Abstract

The global population growth of the bed bug, *Cimex lectularius* (L.) is attributed to their cryptic behavior, diverse insecticide resistance mechanisms, and lack of public awareness. Bed bug control can be challenging and typically requires chemical and non-chemical treatments. One common non-chemical method for bed bug management is thermal remediation. However, in certain instances, bed bugs are known to survive heat treatments. Bed bugs may be present after a heat treatment due to (i) abiotic factors associated with the inability to achieve lethal temperatures in harborage areas for a sufficient time period,(ii) re-infestation from insects that escaped to cooler areas during a heat treatment or (iii) development of physiological resistance that allows them to survive heat exposure. Previous research has investigated the optimal temperature and exposure time required for either achieving complete mortality or sublethally affecting their growth and development. However, no research has examined bed bug populations for their ability to develop resistance to heat exposure and variation in thermo-tolerance between different bed bug strains. The goals of this study were: i) to determine if bed bugs could be selected for heat resistance under a laboratory selection regime, and ii) to determine if bed bug populations with various heat exposure histories, insecticide resistance profiles, and geographic origins have differential temperature tolerances using two heat exposure techniques (step-function and ramp-function). Selection experiments found an initial increase in bed bug survivorship; however, survivorship did not increase past the fourth generation. Sublethal exposure to heat significantly reduced bed bug feeding and, in some cases, inhibited development. The step-function exposure technique revealed non-significant variation in heat tolerance between populations and the ramp-function exposure technique produced similar results. Based on these study outcomes, the ability of bed bugs to develop heat resistance appears to be limited.

2.2 Introduction

Of the ~100 species of blood feeding parasitic pests within the family Cimicidae, only the bed bug, *Cimex lectularius* (L.), and the tropical bed bug, *Cimex hemipterus* (F.), are associated

with the recent global population resurgence (Usinger 1966, Reinhart and Siva-Jothy 2007, Doggett et al. 2012). Both, *C. lectularius* and *C. hemipterus* share hosts and their populations overlap in certain areas (Usinger 1966, Newberry 1989, Steelman et al. 2008). Yet, the ability of these organisms to tolerate environmental conditions influences their geographic distribution because they show differential temperature preference (*lectularius* 28–29°C, *hemipterus* 32–33°C) (Usinger 1966, Newberry 1989, Omori 1941). This allows for widespread distribution of *C. lectularius* in temperate regions, whereas *C. hemipterus* infestations are primarily in tropical/subtropical regions. However, both species have been recently found outside of the previously mentioned areas (Usinger 1966, Omori 1941, Campbell et al. 2016, Gapon 2016, Vinnersten 2017).

Despite differences in their global distribution, both species are usually sheltered from the outdoors as they prefer stable environments (Balvin et al. 2015, Devries et al. 2016a). When indoors, bed bugs negatively impact humans as their bites can leave behind itchy red welts (Usinger 1966). Elimination of bed bugs can be costly as it entails application of chemical insecticides and the use of non-chemical control techniques (Romero et al. 2007, Boase and Naylor 2014, Ashbrook et al. 2017). To avoid the challenges associated with locating all insects in an infestation, pesticide label restrictions on where a product can be applied within a residence and the potential for an insecticide resistant population to be present, whole residence heating is used for bed bug elimination (Pereira et al. 2009, Kells and Goblirsch 2011, Bennett et al. 2015).

Entire home heating is achieved by circulating heated air (55–65°C) indoors for six to eight hours with the ultimate goal of heating bed bug containing objects to >50°C (Kells and Goblirsch 2011). Thermal remediation has many advantages. Not only can it eliminate all bed bug life stages within a residence, but it can also be used in areas or on objects where insecticides cannot be applied (Pereira et al. 2009, Kells and Goblirsch 2011, Bennett et al. 2015, Kells 2018). Additionally, setup of a heat treatment requires less preparation by occupants and it provides more immediate relief to them (Kells and Goblirsch 2011, Kells 2018). However, there are also some drawbacks to the use of heat for bed bug disinfestation. For example, large scale heat treatments are time intensive, costly and do not provide any residual protection against bed bugs (Kells and Goblirsch 2011, Kells 2018). Heat exposure may also damage temperature-sensitive items (Kells and Goblirsch 2011, Kells 2018). Lastly, achieving the necessary lethal temperatures in thermally

insulated areas such as cracks and crevices of walls or furniture where bed bugs prefer to reside is sometimes challenging.

If lethal temperatures are not achieved, bed bugs may detect and respond behaviorally to sublethally heated areas by fleeing to cooler areas such as wall voids, deep within furniture, or in neighboring unheated apartments (Kells 2018, Doggett et al. 2006, Raab et al. 2016). Bed bugs stunned by sublethal heat exposure could fall into protected areas and recover afterwards (Schrader and Schmolz 2011). In one case, it was observed that bed bugs escaped from a heat-treated apartment to an adjacent unheated unit to avoid heat exposure (Raab et al. 2016). Loudon (2017) reported that a single bed bug moved from the heated exterior to the cooler interior of a luggage case in an attempt to escape lethal heat exposure. Furthermore, when bed bugs are placed in an arena at room temperature (25°C), they can detect and orient towards a heated copper coil (28°C to 48°C) that is 10-30 mm away (Devries et al. 2016b), which indicates they are good at responding to heated objects at short ranges. The abovementioned abiotic challenges in achieving lethal temperatures in harborage areas combined with the ability of bed bugs to behaviorally or physiologically respond to sublethal temperature exposure could theoretically select them for increased heat resistance.

There are several examples of arthropods adapting to temperature extremes. Heat exposing *Drosophila melanogaster* in the laboratory resulted in greater temperature resistance within a few generations of selection (Huey et al. 1992, Huey et al. 1992). Gray (2013) showed that plastic temperature tolerance traits can be selected within *Culex pipiens* if they are reared at different temperatures (Gray 2013). *Tetranychus cinnabarinus*, a greenhouse pest, was selected for resistance to abamectin and also showed some cross-resistance to heat exposure due to increased expression of heat shock proteins (HSP) (Liu et al. 2007, Feng et al. 2012). A springtail species, *Orchesella cincta*, was shown to significantly increase expression of the HSP70 family proteins after exposure to non-lethal high temperatures (heat hardening) prior to prolonged heat exposure (Bahrndorff et al. 2009). Although bed bugs do not display heat hardening (Benoit et al. 2009a), repeated sublethal heat exposure could potentially select them for heat resistance, which would be problematic for the use of thermal remediation for their control.

Some of the previous temperature tolerance studies focusing on bed bugs have utilized two different exposure techniques. The first technique, “step-function”, is where the insects are exposed to a rapid increase in temperature (Pereira et al. 2009, Benoit et al. 2009a, Neven 1998).

The second technique is "ramp-function", where the insects are exposed to a slow rate of rising temperatures (Kells and Goblirsch 2011, Benoit et al. 2009a, Clarke 1967, Neven 1998). In another thermal biology study by Rukke et al. (2015, Rukke et al. 2013), the effects of rearing bed bugs at elevated temperatures (34 to 38°C) on survivorship, development and reproduction were reported. However, none of the previous studies have investigated different bed bug populations for variation in thermo-tolerance.

To address the knowledge gaps associated with the potential for bed bugs to develop heat resistance as well as the absence of data on variation in thermo-tolerance of different bed bug populations the goal of this research was two-fold. The first goal was to determine if a laboratory strain of bed bugs could be selected for heat resistance through sublethal heat exposure over multiple generations. The second goal was to utilize the step-function and ramp-function heat exposure techniques to evaluate the temperature tolerance of different bed bug populations. We hypothesized that bed bug strains that have survived heat treatments in the field will require longer exposure times to achieve complete or equivalent mortality relative to bed bugs that have no history of heat exposure.

2.3 Materials and methods

2.3.1 Insects

The insecticide-susceptible Harlan laboratory strain was used for heat selection experiments and as a reference population for thermo-tolerance comparisons. Information on the ten field populations used for heat tolerance screening are outlined in Table 1. Throughout this manuscript, the terms "strain" and "population" are used interchangeably. Field populations of bed bugs were collected from infested dwellings by pest management professionals (PMPs) and university researchers. All bed bug populations were maintained at 25 ±1°C, 50 ±10% RH and a 12:12 h (L:D) cycle in a temperature-controlled environmental chamber (Percival Scientific, Perry, IA). They were fed on defibrinated rabbit blood heated to 37°C using the membrane feeding method (Chin-Heady et al. 2013). Heat selection experiments used large nymphs (4^{th}–5^{th}) that were starved for seven days prior to heat exposure (step-function technique). Similarly, adult bed bugs (1:1 male to female ratio) used for step-function and ramp-function experiments were fed seven

days prior to their use. All field strains were laboratory-adapted and fed readily on defibrinated rabbit blood.

2.3.2 Determination of lethal time estimates for late instar nymphs of the Harlan strain

In order to select the Harlan strain for heat resistance, a LT_{75} (lethal time to kill 75% of the test population at 45°C) was determined for 4^{th}–5^{th} instar nymphs by utilizing the step-function heat exposure method (Pereira et al. 2009, Kells and Goblirsch 2011, Bahrndorff et al. 2009). For the LT_{75} determination, ten Harlan strain nymphs were placed into a 15-mL glass test tube (Fisher Scientific, Pittsburg, PA) with a strip of notecard paper (Roaring Spring Paper Products, Roaring Spring, PA) for harborage (Fig 2.1A). Test tube openings were capped with Parafilm® (Bemis NA, Neenah, WI). These tubes were then placed in a 12x6 plastic rack which was then placed in a water bath (Isotemp 210, Fisher Scientific, Dubuque, IA) heated to 45°C (Fig 2.1B). Rubber bands were used to secure the test tubes and prevent them from floating in the water bath. The exposure periods for nymphs in the 45°C water bath were 10, 12, 13, 14, 16, 17, 18, 20, 21, 22, 23, 24, 25 mins. After the exposure period had elapsed, test tubes were removed from the water bath and bed bugs were placed in a 35x10mm Petri dish (Fisher Scientific, Pittsburg, PA) with a Whatman No. 1 filter paper disc (GE Healthcare, Pittsburg, PA) (Fig 2.1D). Petri dishes were held in an environmental chamber with temperature, humidity, and light conditions identical to those used for rearing. Mortality was scored 24 h after exposure by prodding the insects with a toothpick. Insects were scored as dead if they could not move or right themselves after being prodded.

2.3.3 Selection regimen

The abovementioned step-function heat exposure methods and the probit analysis-determined LT_{75} value (in mins) was used to select the Harlan 4^{th}–5^{th} instar nymphs for heat resistance. An equal subset of nymphs not exposed to heat was maintained as a control colony. Each glass test tube that was used to confine bed bugs during heat exposure contained ten nymphs. Several test tubes were used for heat exposure experiments every generation depending upon the availability of nymphs. After heat exposure at the 45°C LT_{75} time, all nymphs from each individual test tube were transferred to a Petri dish with filter paper and mortality was scored after 24 h. Surviving nymphs from individual Petri dishes were then pooled in a single rearing container with

mesh (Uline, Pleasant Prairie, WI), where they developed into adults and reproduced. Both control and heat-selected colony nymphs were fed one to two times weekly. The selection regime was continued from F0 to F7 generation (except F1) and initially began by selecting 300 nymphs (distributed in 30 test tubes). As the selection process continued (F4 generation and beyond) less insects were used due to lower colony numbers. Therefore, depending on the availability of insects in each generation, anywhere between 50 to 300 nymphs were utilized for selection experiments.

2.3.4 Assessment of blood-feeding and molting ability of heat exposed bed bugs

During the resistance selection procedure, heat-selected bed bugs were also observed for sublethal effects such as the inability to feed to repletion and to successfully molt. Qualitative observations of the sublethal heat impacts on bed bugs led to conducting comparative experiments where the ability of heat exposed insects to feed was assessed. In order to quantitatively evaluate how heat impacted blood feeding, 4^{th}–5^{th} instar Harlan nymphs were exposed to 45°C for 17.45 mins and mortality was scored 24 h later. Survivors of heat exposure were then placed in jars and their ability to feed to repletion on defibrinated rabbit blood was observed on days five, eight, ten and fourteen after heat exposure. Identical numbers of control nymphs were placed in jars and also observed for their ability to feed to repletion at the same time points mentioned above. The number of insects utilized for each replicate was determined by the survival of the bed bugs in response to heat exposure at 45°C for 17.45 mins. Overall, six replicates were performed with an average of 40 bed bugs per replicate.

2.3.5 Thermo-tolerance comparisons among bed bug strains

The procedures used for step-function thermo-tolerance comparison experiments were similar to those used for determining LT_{75} estimates for the Harlan nymphs. For each population, ten mixed sex adult insects (1:1 ratio) were placed into a 15-mL glass test tube with a strip of filter paper as harborage. Test tubes were sealed with Parafilm®, placed in a 12x6 plastic holding rack and then transferred to a water bath heated to 45°C. Insects were exposed to heat (45°C) for 10, 12, 13, 14, 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30 mins to generate exposure time-mortality data. Three to four replicates (10 adults per replicate) were performed for each time point. Additional time points that provided 75–100% mortality were included in step-function heat

exposure experiments to increase the precision of LT_{99} estimations (Rukke et al. 2013). Test tubes were removed from the water bath after the exposure period had elapsed and bed bugs were placed in a 35x10mm Petri dish with a Whatman No. 1 filter paper disc. Petri dishes were held in an environmental chamber with temperature, humidity, and light conditions identical to those used for rearing. Mortality was scored 24 h after exposure using the parameters described under determination of lethal time estimates for late instar nymphs. Control insects were held in test tubes at room temperature and then transferred to Petri dishes.

Procedures for the ramp-function heat exposure bioassay that utilizes a gradual or incremental increase in temperature were similar to those used for the step-function bioassays explained above. Briefly, 15-mL glass test tubes with 10 mixed sex adult bed bugs (1:1 ratio) per tube were placed in a 12x6 plastic holding rack that was transferred to a water bath at room temperature. The water bath was then turned on and the bed bugs were exposed to gradually rising temperatures at the rate of 0.57 °C/min until the water temperature reached 45°C. Once the water bath reached 45°C (~37-min heating time), the insects were held in the water bath for an additional 23 minutes (the Harlan strain LT_{99}; total = 60 mins). After the ramp-up heat exposure period was completed, insects were placed into Petri dishes with filter paper and mortality was scored 24 hours later using previously described criterion under determination of the lethal time estimates for late instar nymphs' section. Three replicates of ten mixed sex bed bugs per replicate were performed for each population, including the Harlan strain, which was used as a positive control for all bioassay tests. Test tubes containing control insects were held at room temperature during the ramp-up heat exposure experiments.

2.3.6 Data analysis

Time-mortality data for 4^{th}–5^{th} late instar nymphs were utilized for PROC probit analysis in SAS 9.4 (SAS 2012, Cary, NC) to determine a LT_{75}. The survivorship data for nymphs from the F0 to F7 generations were analyzed using ANOVA followed by all pairs Tukey's test in SAS 9.4. Comparisons for feeding experiments were made in JMP 13.2 (SAS institute 2016, Cary, NC) using a repeated measures MANOVA with an interaction effect between nymphs and day. Nonparametric Wilcoxon tests were then conducted to determine if feeding response on any particular day was statistically different between heat-exposed and control insects. Exposure time-mortality data from the step-function experiments with adults was analyzed by PROC probit in

SAS 9.4 to determine lethal time (LT_{50} and LT_{99}) estimates and associated parameters for each population. The probit output values (intercept, slope, and covariance) were further used to statistically compare heat-tolerance profiles between different field populations as well as with the Harlan strain (Adelman et al. 2011). Mortality of field populations from the ramp-function heat experiments were analyzed by ANOVA using the PROC GLM function and means were separated using a Tukey's test ($P<0.05$). Logistic regression analysis was performed in JMP 13.2 to determine if there was any correlation between the LT_{50} or LT_{99} values and the latitude for the town/city where bed bug collections were made.

2.4 Results

2.4.1 Response of Harlan strain nymphs to heat selection

Probit analysis conducted on time-mortality data for 4^{th}–5^{th} instar Harlan nymphs indicated a time of 18.15 min at 45°C would kill 75% of test insects (LT_{75}). However, for conducting selection experiments, the lower fiducial limit of the LT_{75} estimate (i.e., 17.45 mins) was used after performing empirical mortality validation tests, which showed that 17.45 min exposure caused ~75% mortality (Appendix A Fig 1). The first round of selection (F0 generation) resulted in an average of 26.3% ± 5.5% of nymphs surviving (Fig 2.2). Survivorship significantly increased in the F2 and F3 generations to 50.5% ± 5.6%. and 55.5% ± 3.6%, respectively (ANOVA results: df =35, 102, F=1.63, P <0.001). However, survivorship in the F4 generation reduced to 31.4% ± 16% survivorship, which was statistically similar to the F0 generation. Exposure of F5 to F7 generation nymphs to the LT_{75} resulted in similar survivorship with an average of 26% ± 9.5%, 20% ± 4.4%, 27.8% ± 9.1% surviving the exposure. Although some of the F7 heat selected nymphs initially survived exposure, the attempt to establish the F8 generation was not successful because bed bugs died out completely likely due to the adverse effects such as reduced feeding and molting issues caused by selection regime.

2.4.2 Impacts of heat exposure on feeding and molting success

As mentioned above, some sublethal effects of heat exposure were observed in surviving insects. Initial qualitative observations suggested that fewer heat-exposed nymphs fed to repletion when offered a blood meal, but all control insects readily fed. Similarly, after feeding, some of the

heat-exposed nymphs failed to escape from their exuvia and died during the molting process (Fig 2.3). The heat-exposed insects that died during molting showed dark pigmentation instead of the opaque and translucent appearance of normal teneral bed bugs. Molting defects were not observed in nymphs of the control strain. To verify the qualitative observations of reduced blood-feeding by heat-exposed nymphs, a separate experiment was conducted where the feeding response of control insects and nymphs that had survived heat exposure was quantitatively compared. Five, 8, 11, and 14 days after heat exposure, a significantly lower proportion of heat selected nymphs fed to repletion in comparison to the control strain nymphs (Fig 2.4, Repeated measures MANOVA results: df =3, 8, F=14.85, P <0.0012, Wilcoxon test results; day 5, Z=-2.80, P=0.005; day 8, Z=-2.80, P=0.005; day 11, Z=-2.74, P=0.006 and day 14, Z=-2.77, P=0.0055.).

2.4.3 Heat tolerance comparison for different bed bug stains: step-function method

The baseline LT_{50} and LT_{99} (and 95% fiducial limits) estimates for the Harlan susceptible strain adults at 45°C were 14.3 (13.7–14.8) and 23.21 (21.7–25.48) mins, respectively. Empirical data showed that 100% mortality of the Harlan adults could be achieved with a 22-min exposure (Appendix A Fig 2). Some differences were observed in the responses of different populations to heat exposure at the LT_{50} level, wherein the KVS strain showed significantly higher heat tolerance or resistance ratios in comparison to the Harlan, Raleigh, Hackensack, Richmond and Poultry House strains ($P<0.05$; S1 Table). However, the LT_{99} values of the KVS strain were not significantly different from the Harlan and all field strains ($P>0.05$; S2 Table). In spite of the lack of statistical support for differences in LT_{99} values for different strains, it was observed that strains with previous heat exposure histories Raleigh and McCall had lower LT_{99} estimates (22.3–26.3 mins) in comparison to some other populations such as Bradenton, Knoxville, KVS and Poultry House (LT_{99} of 27.6 to 29.2 mins). These populations with the highest LT_{99} values also tended to have the highest predicted LT_{50} values, except the Poultry House strain, which had an LT_{50} value close the Harlan strain. No control mortality occurred in any of the bioassay experiments. Lastly, no correlation was observed between the latitude of collection location and the LT_{50} or LT_{99} estimates for different field strains (LT_{50}: $R^2=0.19$, $P > 0.21$, LT_{99}: $R^2=0.23$, $P > 0.16$).

2.4.4 Heat tolerance comparisons for different bed bug strains: Ramp-function method

No variability was found in temperature tolerance of bed bug populations in the ramp-function heat exposure bioassays conducted at temperatures between 25 to 45°C (data not shown). Complete (100%) mortality was achieved for all strains (ANOVA results, df = 9, 20, P > 0.99) including the Harlan population. No mortality was observed in untreated controls.

Table 2.1 Details of bed bug populations used in this study. * Information on the latitude of collection location strains is provided in Appendix A. Table 3** Based on reference Ashbrook et al. (2017) and Adelman et al. (2011)***Based on reference Ashbrook et al. (2017).

Strain name	Strain category	Collection State*	Collection Year	Year Tested
Harlan	Laboratory susceptible strain	New Jersey	1973	2015–2017
Hackensack	Pyrethroid treated before collection	New Jersey	2014	2017
KVS	Unknown	Florida	2006	2017
Bradenton	Unknown	Florida	2013	2017
Raleigh	Pyrethroid and heat treated	North Carolina	2013	2017
Lafayette	Pyrethroid, neonicotinoid, and heat treated	Indiana	2014	2017
McCall	Collected from a heat treated account	Florida	2016	2017
Richmond	Bifenthrin, deltamethrin, and chlorfenapyr resistant**	Virginia	2008	2017
Poultry House	Bifenthrin and chlorfenapyr resistant***	Tennessee	2013	2017
Knoxville	Bifenthrin, deltamethrin, and chlorfenapyr resistant***	Tennessee	2013	2017

Table 2.2 Lethal time (LT) estimates and probit output for bed bug populations exposed to 45°C. *i* Lethal time (LT50 and LT99) values with 95%fiducial limits (FL). All values are expressed in mins. LT values within each column or category (i.e., LT50 or LT99) that are not connected by the same letter are significantly different as their confidence intervals do not overlap with the number "1" (Robertson et al. 2008).

Strain name	N	Slope (±SE)	LT_{50} (95% FL)*i*	LT_{99} (95% FL)*i*	Chi-square (df)
Harlan	540	11.02	14.3 (13.7–14.8)a	23.21 (21.7–25.5)a	11.6 (16)
Hackensack	540	11.39	16.5 (15.9–17.1)a	26.25 (24.7–28.4)a	12.7 (16)
KVS	560	13.46	19.7 (19.1–20.16)b	29.23 (27.8–31.2)a	11.05 (16)
Bradenton	550	11.89	17.6 (17.1–18.15)ab	27.63 (26.1–29.8)a	12.20 (16)
Raleigh	540	17.03	16.3 (15.9–16.6)a	22.30 (21.3–23.6)a	16.21 (16)
Lafayette	550	12.11	16.9 (16.4–17.5)ab	26.42 (24.9–28.4)a	14.81 (16)
McCall	550	12.42	17.1 (16.5–17.6)ab	26.25 (24.8–28.3)a	21.02 (16)
Richmond	560	9.73	15.1 (14.5–15.7)a	26.25 (24.5–28.7)a	8.84 (16)
Poultry House	550	8.28	14.5 (13.8–15.2)a	27.82 (25.7–30.8)a	16.6 (16)
Knoxville	540	11.57	17.4 (16.8–17.97)ab	28.31 (26.6–30.6)a	11.6 (16)

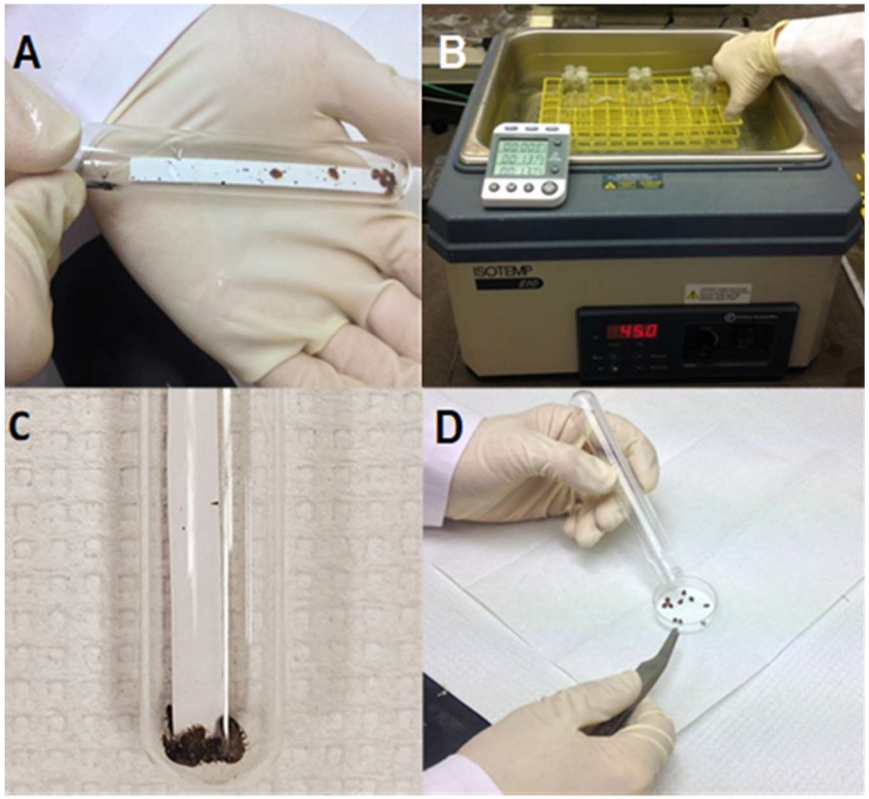

Figure 2.1. A**.** Bed bugs in a glass test tube with a strip of filter paper for harborage prior to heat exposure. B**.** An example of how the bed bugs were heat exposed in the water bath. C. After heat exposure in the water bath, the bed bugs were stunned and have fallen to the bottom of the test tube. D**.** Stunned bed bugs being placed in a Petri dish after heat exposure.

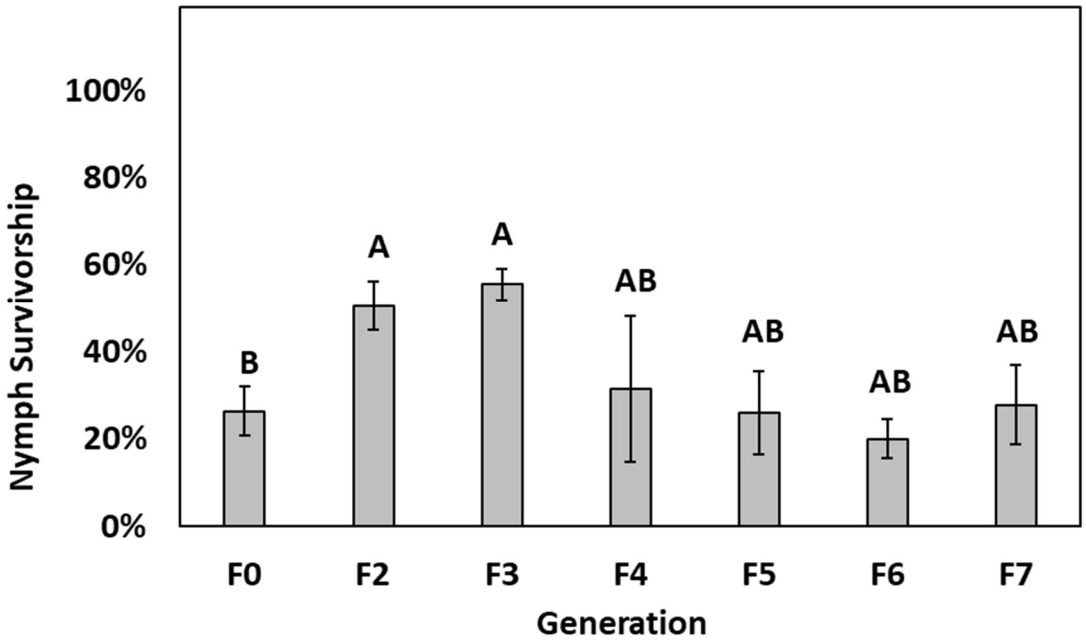

Figure 2.2 Bars depicting average survivorship of late-instar Harlan nymphs (4^{th}–5^{th} instar) after each generation (F0 to F7) of selection or heat exposure at 45°C for 17.45 mins (LT_{75} time). Bars not connected by the same letter show statistically different survivorship rate ($P<0.05$; Tukey's test).

Figure 2.3. A. A large nymph that survived heat exposure, but was unable to complete the molting process. B. A magnified view of a heat exposed bed bug shown in the left image. This insect was attempting to molt, but failed to escape its exoskeleton. The epicranial suture is circled in white appears to have opened, but the bed bug failed to escape through it. C. Depicted in the image from left to right are three heat exposed nymphs that failed to successfully molt to next instar after heat exposure. On the right is an exuvia from a nymph that did successfully molt. Photo credit: John Obermeyer.

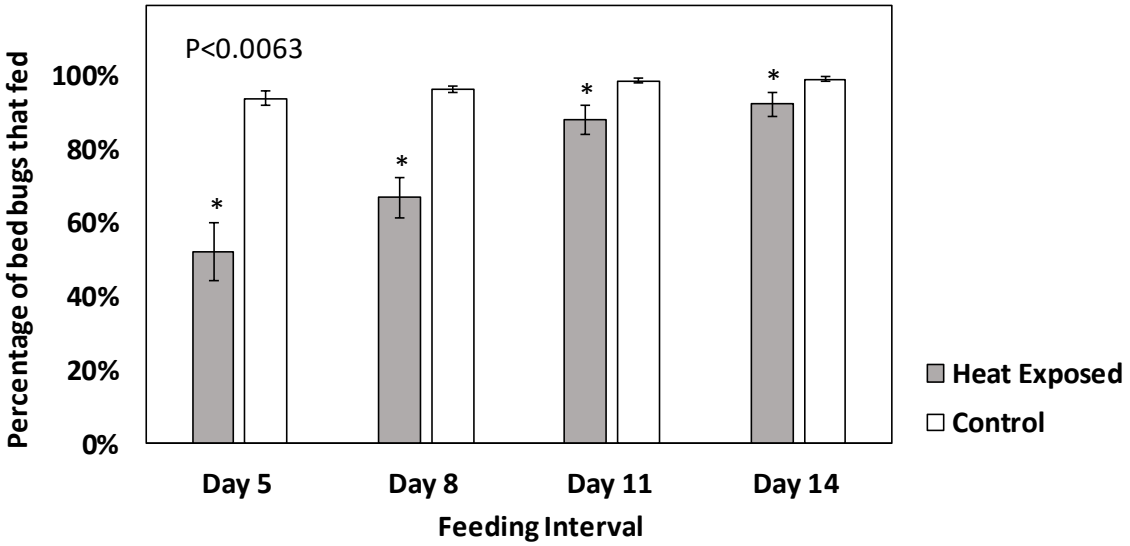

Figure 2.4. Bars representing percentage of Harlan nymphs from heat exposed (dark grey bars) and control (white bars) treatments that fed to repletion. Bed bugs that survived heat exposure at 45°C were offered blood meals at five, eight, eleven, and fourteen days after treatment (n = 40 per replicate). An equal number of control bed bugs that were not exposed to heat were offered a blood meal at the same time intervals. Statistically significant differences were found between the two treatment types and are denoted with an asterisks (*). Nonparametric Wilcoxon tests showed that at all feeding intervals feeding responses of heat-exposed and control nymphs were significantly different ($P<0.05$). Error bars indicate ± standard error (SE) values.

2.5 Discussion

2.5.1 Factors affecting heat resistance development in bed bugs

When inside a human dwelling, bed bugs face a variety of challenges, such as starvation, desiccation, damage by traumatic insemination and local extinction through the implementation of pest management strategies. In comparison to other control strategies such as the use of insecticides, how bed bug populations respond to thermal challenges has been less studied. Late instar (4^{th}–5^{th}) nymphs were utilized to determine if a *C. lectularius* laboratory population could develop heat resistance. This life stage was chosen because the 4^{th}–5^{th} nymphs are close in size to adults, but are still sexually immature. Therefore, these individuals were capable of reproduction only if they survived heat selection and successfully molted to the adult life stage. Additionally, no significant differences in temperature tolerance were observed between late instar nymphs and adults (Appendix A. Fig. 1 and 2).

The Harlan population was selected for heat resistance by exposing them to a pre-determined LT_{75} over the F0 to F7 generations (Fig 2.2). During the selection regime, increased survivorship was initially seen for the F2 and F3 generations. However, when F4 nymphs were heat selected, their survivorship decreased relative to previous generations. In subsequent generations (F5 to F7), survivorship declined further. Although some insects initially survived the heat selection in the F7 generation, none survived long enough to establish the F8 generation and eventually selection could not proceed further. Previous heat selection experiments with other insect species have used a variety of techniques to determine if selection for heat resistance is possible. Laboratory experiments that used ramp-function heat to select *D. melanogaster* found a significant increase survivorship up to the F4 generation; however, survivorship was not reported after this generation (Huey et al. 1992). When two *D. melanogaster* populations were reared at different temperatures for 4 years, the population reared at higher temperature was better at tolerating step-function heat exposure (Huey et al. 1992). However, rearing bed bugs at temperatures greater than 30°C in order to select them for temperature tolerance would likely not select them for heat resistance since research has shown that rearing bed bugs at these temperatures causes mortality, sterility, and developmental issues (Rukke et al. 2013, 2016, Chang 1974).

The initial increase in survivorship followed by a decline in survivorship indicates that bed bugs may have a limited ability to develop greater temperature resistance in a laboratory setting. The limitations on the ability of bed bugs to develop greater heat resistance could be due to many

factors. One of the factors affecting the ability of bed bugs to develop heat resistance, could be the lack of genetic diversity in a laboratory colony (Harlan strain). Adapting insects to laboratory conditions can reduce the genetic diversity of a population compared to wild type populations, which has previously occurred with the sandfly, *Lutzomyia longipalpis* (Mukhopadhyay et al. 1997). Genetic diversity of laboratory colonies such as the Harlan strain can also be reduced when in culture for a long duration. Kim et al. (Kim et al. 2007) found that the Western corn rootworm had decreased genetic diversity when in culture for ~190 generations. Similarly, older laboratory colonies of *D. melanogaster* experience reduced genetic diversity in comparison to recently established colonies (Briscoe et al. 1992). Additionally, genetic studies on bed bugs have found that field populations have low genetic diversity within populations (Booth et al. 2012) and the Harlan population is likely no different. Given the low genetic diversity within different bed bug populations in general, the findings of the laboratory selection study likely also hold true for field populations, i.e., the ability of bed bugs to develop stable and significant levels of heat resistance in a field setting could be very limited.

In addition to the low genetic diversity within bed bug populations, the sublethal effects of heat exposure observed in this study, which were consistent with other studies (Pereira et al. 2009, Rukke et al. 2013, 2016, Chang 1974), may further constrain the ability of bed bugs to develop heat resistance. Similar to previous research (Pereira et al. 2009) when bed bugs were exposed to sublethal heat they were initially stunned (Fig 2.1C) and could not walk, but some recovered and were capable of movement after 24 h. Bed bugs require blood meals in order to molt and reproduce successfully (Usinger 1966). However, heat-exposed bed bugs showed a significantly reduced feeding preference relative to control nymphs for up to 14 days post exposure (Fig 2.4). Similarly, in another study, reduction in bed bug feeding was observed after exposure to sublethal levels of steam (Wang et al. 2018). Bed bugs that did not feed after heat exposure could have been avoiding further stress associated with consuming a hot blood meal. Blood feeding has been shown to increase the body temperature and elicit HSP expression in mosquitoes (Benoit et al. 2011). It has also been reported that bed bugs that feed on overheated blood (39°C) will die, likely due to heat stress (Kells 2018). Although not investigated in this study, the heat selection regime may also have impacted bed bug reproduction by eliminating *Wolbachia* symbionts (Chang 1974). It has been previously shown that rearing bed bugs at 36°C can significantly reduce *Wolbachia* cell counts from their mycetomes, which consequently reduces egg viability for up to 10 weeks after

exposure (Chang 1974). However, if bed bugs are briefly exposed to steam, their reproduction is not impacted (Wang et al. 2018). This indicates that bed bugs must be heat-exposed for a longer duration to eliminate their *Wolbachia* symbionts. In the future, quantitative PCR experiments could be conducted to determine the heat exposure duration required to eliminate the microbial symbionts of bed bugs using the step-function or ramp-function methods.

In some instances, we found that nymphs that survived the step-function heat exposure failed to escape from their exuvia during molting (Fig 2.3). These bed bugs seemed to exhibit characteristics that were similar to hyperecdysonism, where epidermal cells hastily secrete a new cuticle that is incomplete (Williams 1968). Experiments with the flesh fly, *Sarcophaga crassipalpis*, found that some adults were unable to successfully eclose from the puparium after sublethal heat exposure (Yocum et al. 1993). Similar to findings mentioned above, Rukke et al. (2013) reported that *C. lectularius* nymphs reared at temperatures between 34–38°C for two to three weeks failed to molt properly. Studies with other arthropods have shown that physiological adjustments required for overcoming heat stress also have deleterious effects on reproduction and development (Yocum et al. 1993, Krebs et al. 1994, Okada et al. 2014, Zizzari and Ellers 2011).

The deleterious effects of heat exposure on bed bugs, such as reduced blood feeding and molting abnormalities, likely became an important factor regarding survivorship and developmental ability of the heat-selected strain beyond the F4 generation (Fig 2.2). Eventually, the heat-selected colony died out completely after the F7 heat exposure experiment. If the heat-associated sublethal effects of this study are extrapolated to the field, the heat-exposed bed bugs that survive may be less successful in passing their genes to the next generation, which would further reduce the probability of heat resistance evolution

2.5.2 Minimal variation in thermo-tolerance of bed bug strains

The final goal of this study, was to test the ability of field strains to tolerate heat using both the step-function and ramp-function heat exposure techniques (Tables 2.1 and 2.2, Appendix A. Tables 1 and 2). Another objective of these experiments was to determine the influences that geographic origin, insecticide resistance status and previous heat exposure history have on temperature tolerance of bed bug field strains. Adult bed bugs (1:1 ratio of males and females) were used for the thermo-tolerance bioassays because they are one of the most temperature tolerant among the mobile life stages (How and Lee 2014). The temperature tolerance of early instar

nymphs was not determined. However, *C. hempiterus* first instar nymphs have lower temperature tolerance in comparison to adult *C. hempiterus* (How and Lee 2014) and *C. lectularius* may be similar in this regard. Additionally, bed bugs starved for 7 d prior to heat exposure that were used in this study were likely close to an optimal thermo-tolerant state (Devries et al. 2016a). Previous research has shown that bed bugs that were fed 1 d and 21 d prior to heat exposure are less thermo-tolerant than insects fed 9 d fed prior to heat exposure (Devries et al. 2016a). Devries et al. (2014) suggest that there is a metabolic state around this optimal feeding status that maximizes bed bug thermo-tolerance, but what causes this relationship between thermo-tolerance and metabolism is unclear.

Using the step function technique, some variability was observed in LT_{50} times (Table 2.2, Appendix A Table 1), however, none of the LT_{99} estimates were significantly different (Table 2.2, Appendix A Table 2). No clear patterns emerged with respect to the LT estimates and previous history of heat exposure, geographic origin or insecticide resistance status. In comparison to other strains, the bed bug populations that had a history of heat exposure did not show significantly higher LT_{99} values (e.g., Raleigh, NC, LT_{99} 22.3 min, McCall, FL, LT_{99} 26.3 min). This could have been due to the variety of demonstrated impacts of heat exposure found in this study as well the fitness costs documented in other insect species (Pereira et al 2009, Rukke et al. 2013, 2015, Chang 1974 , Wang et al. 2018, Benoit et al. 2011, Yocum et al. 1993, Krebs and Loeschcke et al. 1994, Okada et al. 2014, Zizzari and Ellers 2011). Secondly, the geographic origin (latitude of collection location) of a bed bug population also did not influence their temperature tolerance, likely since indoor environments are relatively stable and based on the preference of the tenant. Bed bugs thus are probably not exposed to sufficiently variable temperatures over many generations to change their thermo-tolerance. In Japan, a study with 30 different *Drosophila* species found that the temperature tolerance did not vary by the geographic latitude of a population, but rather the habitat type (e.g., tree canopy versus open field conditions) (Kimura 2004). Lastly, pyrethroid resistant strains (e.g., Knoxville, Lafayette and Richmond) (Adelman et al. 2011, Ashbrook et al. 2017), did not show significantly different thermo-tolerance based on the LT_{50} and LT_{99} values in comparison to the Harlan strain (Table 2.2, Appendix A. Table 1 and 2) indicating lack of correlation between insecticide resistance status and heat tolerance. However, because of the unknown insecticide resistance status of the KVS strain that shows significant thermo-tolerance at the LT_{50} level, we could not confirm if heat tolerance of this strain is associated with

pesticide resistance. Previously, abamectin (an avermectin class insecticide) resistant mites were also shown to have cross-resistance to heat (Liu et al. 2007, Feng et al. 2012) but since this insecticide is not used for bed bug control, it is likely that they would not develop cross resistance to heat in this way.

The absence of any significant differences in the thermo-tolerance among bed bug populations were further verified using the ramp-function exposure technique. With the ramp-function technique, the temperature is gradually increased, which is similar to how heat is deployed in the field (Kells and Goblirsch 2011, Clarke 1967). This method also allows bugs more time to physiologically respond to thermal stress. However, complete mortality was achieved in all bed bug populations that were tested using the ramp-function technique. It is possible that during the process of establishing colonies of wild type bed bugs in a laboratory setting, the insects may have gone through a significant bottleneck effect that could have further reduced or eliminated any substantial differences in thermo-tolerance that were originally present. Additionally, how arthropods express heat shock proteins, other stress-induced genes, and metabolites such as sugars and amino acids in a field setting in response to thermal challenges is not well understood. Instead of increasing expression of HSPs and stress-induced genes to survive heat exposure, a more optimal response could be to flee to cooler areas to avoid heat stress, and this appears to be the case when bed bugs are exposed to heat (Feder and Hoffmann 1999). Bed bugs express heat shock proteins when heat exposed and it has been shown that they have 13 *HSP* genes (Benoit et al. 2009b, Benoit et al. 2016). However, *HSP* gene expression profiles for the bed bug populations used in this study in response to heat exposure are yet to be determined.

With respect to the role of metabolites in thermal tolerance, *Belgica antartica* is known to increase internal concentrations of trehalose to become more tolerant to both heat and cold (Benoit et al. 2009a). Arthropods can also increase the proportion of saturated lipids and cuticular hydrocarbons (e.g., *n*-alkanes) in their cell membrane and cuticles, respectively, to help reduce water loss and aid in temperature tolerance (Edney 1977, Driifhout et al. 2009). In response to rising environmental temperatures, *Orchesella cincta* can increase the proportion of saturated lipids in their cellular membranes (Van Dooremalen et al. 2011). Similarly, when *Pogonomyrex barbatus* were exposed to higher temperatures and lower humidity for 20 days, they increased the proportion of saturated cuticular hydrocarbons in their exoskeleton (Wagner et al. 2011). Bed bugs are similar to desert-adapted arthropods in their ability to withstand desiccation (Benoit et al. 2007)

and have also shown the ability to evolve modified cuticles to resist insecticides (Koganemaru et al. 2013). However, the roles of metabolites (trehalose) and changes in cuticular hydrocarbon profiles in bed bug heat tolerance are not known and should be further investigated.

It is possible that small differences in LT_{50} and LT_{99} durations of different populations (Table 2.2), although mostly non-significant, could allow some populations such as KVS, Poultry house, and Bradenton to escape insufficiently heated areas in the field more effectively than other bed bug populations. Research indicates that if bed bugs are exposed to sublethal temperatures or if the heat in an area is uneven, they would move to an area with more suitable temperatures (Kells 2018, Doggett et al. 2006). Currently, the bed bug strains tested in this study are being examined for differences in their heat repellency behavior by exposing them to rising environmental temperatures in harborages that are gradually heated (ramp-function method).

2.5.3 Implications for bed bug control

The range of sublethal impacts caused by heat exposure as well as the upper physiological limits of *C. lectularius* heat tolerance has implications for using lethal heat as a control measure for bed bug elimination. First, if bed bugs remain after a heat treatment or are present in a follow-up inspection, the chances that these insects have developed any substantial heat resistance are low. The initial increase followed by a decrease in bed bug survivorship during heat selection experiments in addition to the plethora of sublethal heat impacts, suggest that individuals that are more heat resistant are quickly selected against (negative selection in a few generations). An alternative explanation for insects remaining after a heat treatment is that they were exposed to sublethal temperatures, escaped from high temperature zones, or were re-introduced to the domicile (Kells 2018). If the resident complains of being bitten by bed bugs shortly after a heat treatment, the latter explanation is likely; given that heat-exposed bed bugs will feed at reduced rates for up to two weeks.

In order to ensure that all insects are eliminated within an infestation, temperatures $\geq 50°C$ as well as a sufficient exposure period are required (Kells and Goblirsch 2011), especially if bed bugs are suspected to be harboring deep within objects. Monitoring temperatures throughout heated areas in order to identify heat sinks and/or insulated areas is critical for complete bed bug elimination (Kells and Goblirsch 2011). Since bed bugs have been shown to travel long distances within an infestation (Cooper et al. 2015) and can detect heated objects (Devries et al. 2016b), they

will likely flee to cooler spots or adjacent housing units if sublethal temperatures are used during thermal remediation (Kells 2018, Doggett et al. 2006). Therefore, interception measures should be utilized to trap bed bugs within areas that are heated to 50°C or higher. Placing traps, sealing wall cracks or electrical outlets, and applying silicate dusts or insecticides to create a barrier would prevent bed bugs from escaping. Additionally, if there are areas that are not reaching temperatures ≥50°C, then insecticides can later be applied as spot treatments to those areas and other control strategies can be deployed (Kells 2018). This is a well-known practice that is already utilized by some pest management companies. It is important to note that heat is one of many tools available for bed bug elimination and should be deployed with other IPM strategies and insecticides to maximize control.

CHAPTER 3. DETERMINING THE BEHAVIORAL AND PHYSIOLOGICAL RESPONSE OF THE BED BUG (*CIMEX LECTULARIUS* L.) TO HEAT EXPOSURE

3.1 Abstract

Heat can be effective for bed bug elimination. However, in some cases bed bugs survive heat treatments. The goal of this study was to determine the temperatures that stimulate bed bugs to search for cooler areas and identify which heat shock protein (*HSP*) genes are expressed after heat exposure. To determine the behavioral response of bed bugs to heat, five different populations were exposed to rising temperatures (22–55°C) while in a harborage using a custom-made copper arena. In separate experiments, *HSP* gene expression responses of select bed bug populations were determined using real time quantitative polymerase chain reaction (RT-qPCR). Behavioral experiments found that at specific temperatures, different bed bug populations had similar probabilities to flee and their ability to locate unheated areas was not different. However, the Poultry House population located unheated areas at significantly higher temperatures. The RT-qPCR experiments revealed that the expression of the *HSP70.1*, *HSP70.3*, and *Putative Small HSP* genes were significantly upregulated after heat exposure in tested populations. The expression of these genes was highest at the 15min, 2h, and 4h post-exposure time points and decreased back to baseline levels by 24h. This study shows that when bed bugs in harborage are exposed to temperatures of 47–48°C, while exterior temperatures are at 53–54°C, they are forced into the open. This study also shows the significance of *HSP70.1*, *HSP70.3*, and *Putative Small HSP* in the bed bug recovery process after heat exposure.

3.2 Introduction

Pest insects such as bed bugs (*Cimex lectularius* L.), are susceptible to extreme temperatures and therefore they have been adapted for their control (Oosthuizen M. 1935, Solomon and Adamson 1955). Bed bugs are somewhat cold tolerant and require longer exposure periods to achieve lethality at sub-zero temperatures (-20°C for ~48 hr.) Olson et al. 2013). If bed bug nymphs and adults are exposed to temperatures above 45°C, mortality is achieved faster, but heat tolerance

can vary depending on the exposure technique and feeding status (Pereira et al. 2009, Kells and Goblirsch 2011, Benoit et al. 2009a, Devries et al. 2016a, Ashbrook et al. 2019).

Heat is used for bed bug control because it is lethal to insecticide resistant populations, can be used in sensitive areas, requires minimal preparation by tenants, and locating all bed bug harborage locations in a domicile can be difficult (Kells and Goblirsch 2011, Ashbrook et al. 2019, Bennett et al. 2016, Ashbrook et al. 2017). Heat is used to control bed bugs in three ways. Steam (~100°C) can be applied topically to furniture, items can be heated in compartmentalized units or tents (>50°C), or whole domicile heating (>50°C) can be used (Pereira et al. 2009, Kells and Goblirsch 2011, Doggett et al. 2006, Puckett et al. 2013, Kells 2018). During a heat treatment, heated air (~60°C) is circulated to raise and maintain ambient temperatures above 50°C for several hours (6−8h) to ensure complete bed bug mortality (Kells and Goblirsch 2011). All bed bug life stages can be effectively eliminated when heat treatments are properly done (Kells and Goblirsch 2011).

Heat treatments have some drawbacks. The length of time required to reach and maintain temperatures that are lethal to bed bugs can be disruptive to residents. The cost of thermal remediation is higher than conventional treatments and heat could potentially damage sensitive items (Kells and Goblirsch 2011). Also, bed bugs may flee from lethally heated areas to search for cooler locations or move to neighboring unheated apartments (Doggett et al. 2006, Kells 2018, Raab et al. 2016). Previous experiments that compared thermo-tolerance of different bed bug populations found limited variability in the times required to achieve complete mortality (Ashbrook et al. 2017). These results suggest that bed bugs are not likely surviving heat treatments due to the development of greater thermo-tolerance (Ashbrook et al. 2019).

Other factors that could help explain bed bugs persistence after heat treatments are their physiological and behavioral responses to heat exposure (Benoit et al. 2009a, Rivnay 1932, Devries et al. 2016b). One of the physiological responses to heat exposure is the expression of heat shock proteins (HSPs), which are named based on their molecular weight (kilodaltons or kDa) (Buchner et al. 1997). HSPs act as molecular chaperones and keep cellular constituents such as proteins in the proper conformation or refold them to their original state after exposure to environmental stressors such as heat (Daugaard et al. 2007). HSPs function to reduce deleterious cellular interactions by preventing misfolded proteins from interacting with other proteins and can also degrade damaged cellular constituents for removal or recycling (Feder and Hoffmann 1999).

HSPs can be involved in protecting cells from water loss and modulating the fluidity of cell membranes (Benoit et al. 2011, Prange 1996, Neven 2000, Rensing and Ruoff 2002). Bed bugs physiologically respond to heat exposure at 44°C by expressing two *HSP* genes (*HSP70* & *HSP90*) for up to 24h (Benoit et al. 2009a). The *HSP* gene primers used by Benoit et al. (2009a) for quantifying bed bug *HSP* expression were based on sequences for *Culex pipiens* (*HSP70*), and a combination of sequences from *Trialeurodes vaporariorum*, *Bemisia tabaci*, and *Drosophilia melanogaster* (*HSP90*) (Rinehart et al. 2006). However, the bed bug genome contains 12 unique *HSP* genes (Benoit et al. 2016) and it is unknown which of these candidate genes are upregulated after heat exposure.

Behavioral studies with bed bugs have demonstrated that they are adapted for detecting and responding to heated objects and surfaces. When bed bugs are close to heated objects (<3 cm), they can detect temperature differences of 2°C (Rivnay 1932, Devries et al. 2016b). Bed bug attraction to heated objects increases as their temperature increases up to a maximum of 48°C (Devries et al. 2016b). If bed bugs are placed on a temperature gradient, they preferentially rest in areas that are heated to 28–29°C (Omori 1941). But how bed bug behavior changes in response to rising temperatures when inside their harborage has not been determined. Therefore, the goal of this study was to determine the temperatures that will stimulate bed bugs to move in search of cooler areas and to identify which *HSP* genes are upregulated after heat exposure. We hypothesized that bed bug populations that have been previously exposed to heat will be able to detect and escape from lethally or sub-lethally heated areas faster than bed bug populations without any exposure history. Additionally, we hypothesized that sublethal heat exposure will cause significant changes in *HSP* gene expression during the stress recovery process.

3.3 Methods

3.3.1 Insects

The insecticide susceptible Harlan laboratory population was used as the reference population for heat behavior and *HSP* gene expression comparisons. Details on the field-collected bed bug populations used in this study are in Appendix B Table 1. All bed bugs (Harlan, McCall, Poultry House, Knoxville, KVS, Raleigh) were maintained at 27±1°C, 50±10% RH and a 12:12 h

(L:D) cycle in a temperature-controlled chamber (Percival Scientific, Perry, IA). Bed bugs were fed on defibrinated rabbit blood (37°C) 24h prior to use in all experiments using the membrane feeding method (Hemostat Laboratories, Dixon, CA) (Chin-Heady et al. 2013). Using starved bed bugs revealed that they would probe the heated arena and attempted to feed. Similar observations were reported by Devries et al.(2016b). This highlighted the importance of feeding status when conducting heat repellency experiments with hematophagous insects since heat is an important cue in locating a bloodmeal (Rivnay 1932, Devries et al. 2016b, Rivnay 1930).

3.3.2 Copper arena for heat exposure

A 1mm thick 60.96cmx60.96cm copper sheet (U.S. Brass & copper, IL) was used as the floor of the arena (Figure 3.1). To enable heating of the copper sheet using water, 12.7mm copper tubing (U.S. Brass & copper, IL) was attached underneath the arena surface. First, the coiled copper tubing was cut to fit the copper sheet and straightened. The copper tubes were then welded together using 12.7mm elbow joints (Nibco Inc., IN) and tube connectors. Once enough copper tubing to cover the copper sheet was connected, the tubing was then attached to the underside of the copper sheet by welding 12.7mm tube brackets around the tubing (Nibco Inc., IN) (Appendix B Fig. 1). Thermal paste (Arctic-Silver, Visalia, CA) and thermal grease (Prolimatech, Taiwan, China) was then applied around the brackets and tubing to enhance heat transfer to the copper sheet. To create walls of the arena, four pieces of clear plexiglass (15.24cm, 58.42cm, 8cm thick) were joined at the edges using chloroform (Sigma-Aldrich, St. Louis, MO). The Lauda-Alpha Cooling Circulator (Lauda-Brinkmann, Delran, NJ) was attached to the copper tubing under the arena using 12.7mm vinyl tubing. The copper harborage was made using 12.7mm copper tubing cut to 50.8mm pieces. Then three copper tubes were connected with hot glue (Figure 3.1B, 3.1C). The arena surface, copper harborage, and unheated cups (Fig. 3.1A, 3.1B, 3.1C, 3.1D) were roughened so that the bed bugs could walk and climb on all surfaces. To create unheated harborages for the bed bugs to escape to, plastic cups were painted black and a circular piece of Whatman No 1. filter paper was attached to the bottom. The plastic cups because of bed bugs preference for darker colored objects or areas (Singh et al. 2015).

3.3.3 Determination of bed bug behavioral response to heat exposure

To determine the behavioral response of different bed bug populations when exposed to conductive heat, 15 adult males from individual populations were placed into a copper harborage and allowed to acclimate for 24h. Only males were used because females have a tendency to disperse from aggregations to avoid repeated traumatic insemination (Pfeister et al. 2009). Ten replicates were conducted for each population.

The copper harborage had bed bug fecal spots on the interior to promote aggregation (Gries et al. 2015). After 24h the copper harborage containing bed bugs was placed in the center of the copper arena (Fig. 3.1A). Then conductive heat was used to ramp-up the temperatures of the arena from 22°C–55°C and the harborage (22°C–50°C). Ramp-up heating was achieved by circulating heated deionized water in the copper tubing under the copper arena using a Lauda-Alpha Cooling Circulator for 25min. During the experiment, the temperature was recorded every minute using two thermocouples that were attached to the underside of the copper sheet at different locations (Digi-sense, Vernon Hills, IL, and Fisher-Scientific, Hampton, NJ). To determine the copper harborage temperature, control experiments with no bed bugs in the copper harborage were conducted. This was done by attaching a thermocouple (Fisher-Scientific, Hampton, NJ) to the top of the harborage (Fig. 3.1A) and recording its temperature every minute while also recording the temperature of the copper sheet with separate thermocouples (Digi-sense, Vernon Hills, IL). A thermocouple could not be attached to the harborage with bed bugs in the arena because they would climb on it to avoid heat exposure. Harborage temperature measurement experiments were replicated 10 times.

Both qualitative and quantitative visual observations of the bed bug response to ramp up heat were made concurrently with the temperature and time recordings. Qualitative visual observations included behavioral responses of bed bug to rising temperatures such as their movement in/on the harborage and in the arena (Fig. 3.1B). Quantitative observations included recording the temperature and time at which bed bugs fled the heated copper harborage. If insects left the copper harborage, the observation was scored as flight. The bed bugs that fled the harborage either died on the heated arena surface (Fig. 3.1C) or were able to escape to the unheated black plastic cups that were evenly spaced in the arena (Fig. 3.1A, 3.1D). The number bed bugs successfully escaping to the unheated harborages (i.e. survivors) was also recorded.

3.3.4 Exposure of bed bugs in the copper arena for *HSP* gene expression experiments

To determine if bed bug populations showed differential expression of *HSP* mRNA they were exposed to ramp-up heat (22–42°C harborage and 22–47°C arena floor temperatures) in the copper arena. Heating the harborage and arena to 42 and 47°C, respectively, required 15−16mins. This temperature was chosen based on statistical analysis results from the behavioral experiments. Additionally, if bed bugs fled the harborage at this temperature, mortality would quickly occur if they were unable to locate the unheated black plastic cups. The Harlan, KVS, and Poultry House populations were used for *HSP* gene expression experiments because they either displayed lowest or highest levels of thermo-tolerance (at the median lethal time mortality levels) (Ashbrook et al. 2019). To obtain heat exposed bed bugs for RNA extraction, a group of 50 bed bugs (25 females: 25 males) that represented a single biological replicate were exposed to "ramp-up" heat in the copper harborage using the methods mentioned in section 3.3.3. Bed bugs were acclimated in the copper harborage for 24h before heat exposure. Three biological replicates (50 insects per replicate) were conducted for each population. Once the arena reached 47°C, bed bugs were removed from the arena and then separated into groups of 10 (1:1 female to male ratio) in Petri dishes (Greiner Bio-One, Monroe, NC). Control bed bugs (3 biological replicates with 50 bed bugs per replicate) were also placed in the copper arena, but never exposed to heat.

3.3.5 RNA isolation and cDNA synthesis

At the desired time points after heat exposure (15min, 2h, 4h, 8h, and 24h), each group of 10 bed bugs were cut into small longitudinal/ latitudinal sections and submerged in RNAlater (175 µL) (Thermo Fisher-Scientific, Wilmington, DE) at 4°C for 24h. Control insects that were never exposed to heat were similarly sampled at 15min, 2h, 4h, 8h, and 24h and preserved in RNAlater. After 24h, RNAlater was pipetted off and samples were stored at -80°C until RNA extraction using the Promega® SV Total RNA isolation kit (Madison, WI) following manufacturer instructions. RNA concentration and the purity of extracted samples were determined using a NanoDrop2000 (ThermoFisher Scientific, Wilmington, DE). The Bio-Rad iScript™ cDNA Synthesis kit (Hercules, CA) was used for reverse transcribing RNA to cDNA (complimentary DNA) on a Bio-Rad MyCycler Thermal Cycler (Hercules, CA). The cDNA synthesis reactions (20 µL) volume included 4µL of 5x iScript™ reaction mix, 1µL iScript™ reverse transcriptase, equal

concentrations of RNA (500 ng/reaction) and nuclease free water to make up the remaining volume. cDNA synthesis reactions were processed on the thermal cycler using the following manufacturer recommended time program: priming for 5min at 25°C, reverse transcription for 20min at 46°C, inactivation for 1min at 95°C.

3.3.6 Quantification of bed bug *HSP* gene expression by RT-qPCR

The bed bug genome was used to obtain twelve *HSP* gene sequences for expression quantification (Benoit et al. 2016). Unique forward and reverse primers for specific bed bug *HSP* genes were designed using the NCBI Primer-Blast tool (www.ncbi.nlm.nih.gov) (Appendix B Table 2) and were purchased in desalted form from Sigma-Aldrich (St. Louis, MO). To determine critical threshold (CT) values for each candidate *HSP* gene, RT-qPCR reactions were conducted in a 96-well plate format (Bio-Rad, Hercules, CA) on a Bio-Rad C1000 Thermal Cycler (Hercules, CA). By following SensiFAST™ SYBR® Master Mix No-ROX kit instructions (Bioline, Boston, MA), individual 20µL RT-qPCR reactions were created with 10µL SensiFAST™SYBR® No-ROX mix, 1µL cDNA, 1µL each of unique forward and reverse primers (0.5µM final concentration) and 7µL nuclease free water. cDNA samples from three bed bug populations (Harlan, KVS and Poultry House) and treatment groups (i.e., heat exposed and control) were used. Three biological replicates were conducted for each timepoint and population. A three-step protocol for cDNA amplification was used. In the first step, reactions were heated to 95°C for 3min. In the second step, 40 cycles of denaturation, annealing and elongation were repeated as follows: 15s at 95°C, 15s at 55°C, and 30s at 72°C. In the final step, melt curves were determined starting at 65°C with a 0.5°C increase every 5s to a maximum of 95°C in order to help verify product specificity. Agarose gel electrophoresis of RT-qPCR products was also conducted to verify product amplification and primer specificity (Appendix B Fig. 2).

3.3.7 Data analysis

To compare the flight response of different bed bug populations to ramp-up conductive heat, the temperature and response variable (i.e., on/in the harborage vs. one or more bed bugs fleeing the harborage) for each population was analyzed using logistic regression in JMP Pro 14 (SAS institute 2018, Cary, NC). After logistic regression, inverse probability prediction was used

to determine the temperatures associated with probabilities (25%, 50%, 75%) for bed bugs of different populations to flee the copper harborage. To compare temperatures associated with the predicted probabilities for different bed bug populations to flee from the heated harborage, contingency analysis was conducted. The temperatures at which bed bug populations fled and found the unheated black cups as well as the number of successful escapes (survivorship) for each population was compared using a one-way ANOVA and post hoc t-test.

Relative expression values for each *HSP* gene at the tested time points for heated and control bed bugs was calculated by comparing the average of three biological replicates (CT data) that were first normalized to two reference genes *RiPL18* and *B-Tubulin* (Mamidala et al. 2011). The resulting delta CT (ΔCT) data for heat exposed bed bugs was then normalized to corresponding ΔCT data for temporally created control replicates to obtain delta-delta CT (ΔΔCT) values (Livak and Schmittgen 2001). After normalization of data with two reference genes to obtain ΔCT values, one of the treatment groups was used for normalization of ΔCT values of the other group, which yielded ΔΔCT values that represented baseline expression levels for individual *HSP* genes of each population. For statistical analysis, ΔΔCT data (dependent variable) for different *HSPs* genes, populations and time points (i.e., independent variables) were analyzed using ANOVA and post hoc t-test in JMP Pro 14. Afterwards, ΔΔCT data at individual timepoints were analyzed for differences in expression at the gene and population levels using an ANOVA and post hoc t-test. For visualization of relative expression or fold change data, Z-scores were calculated from $2^{-\Delta\Delta CT}$ data and used to create heatmaps in JMP.

To compare the amino acid residues of HSP proteins, a multiple sequence alignment was conducted in Clustal Omega using the ClustalW parameters (Sievers et al. 2011). After HSP multiple sequence alignment, a principle components analysis (PCA) was conducted in Jalview 2.11.0 (Waterhouse et al. 2009) using BLOSUM62 substitution scores. Afterwards, a phylogram was constructed in Jalview 2.11.0 using BLOSUM62 substitution scores and average distance similarity. The amino acid residues of the three differentially expressed *HSP* genes were aligned separately in Jalview 2.11.0 for target comparison and visualization. The amino acid sequences were then entered into SignalPv.5.0 (Almagro et al. 2019) which was used to determine the possible protein function of the tested *HSP* genes.

3.4 Results

3.4.1 Behavioral responses of different populations to heat exposure

Qualitative observations were made to describe bed bug behavior during heat exposure (Table 3.1). The tested bed bugs became more mobile as temperature increased from 22°C−55°C. At temperatures around 38°C, some bed bugs began to flee the copper harborage. The average arena temperature at which bed bugs from all populations fled the copper harborage was 48.5±0.1°C.

Logistic regression on the binary response data for when bed bugs fled the copper harborage vs. remained on the harborage found that temperature significantly influences the probability of bed bugs to flee (Intercept= -13.6, STD error = 0.58, chi-square= 535.5, P<0.0001, Temperature: slope= 0.28, STD error= 0.01, chi-square= 508.94, P<0.0001). As temperature of the harborage increased, so did the probability that a bed bug would flee. Results from a post hoc inverse probably prediction analysis determined temperatures and 95% fiducial limits associated with the percent chance that a bed bug would flee the copper harborage (Fig. 3.2, Table 3.2). Calculated probabilities to flee and associated arena temperatures are: 25% at 44.2±0.4°C, 50% at 48.1±0.4 C, and 75% at 52.1±0.6°C. Higher probabilities were not calculated because bed bugs were not able to tolerate arena temperatures >54°C (maximal temperature). Contingency analysis revealed no significant differences in probabilities for each population to flee from their harborage at different temperatures (n=2800, df=5, chi-square= 5.35, P=0.37, R^2=0.0017).

A one-way ANOVA on the percentage of bed bugs that located the unheated black plastic cups (i.e., surviving bed bugs) found no significant differences between populations (df=5, sum of squares=0.045, F-ratio=1.79, P>0.13, R^2=0.14) (Table 3.3). Survivorship ranged from 4.0±3.4% (McCall) to 12±8.2% (KVS) and indicates that any particular bed bug population is not better adapted than another to locate unheated areas. However, one-way ANOVA analysis of copper arena temperatures at which fleeing bed bugs found unheated black plastic cups revealed significant differences between populations (df=5, 63, sum of squares=118.78, F-ratio=5.32, P=0.004, R^2=0.29) (Table 3.3). The post hoc t-test found that the Poultry House population (48.8±0.5°C) fled at greater temperatures, followed by the Knoxville (46.5±0.52°C) and McCall populations (46.4±1.2°C) (Table 3.3). Arena temperatures at which the Harlan (45.3±0.6°C), Raleigh (45.5±0.6°C), and KVS (45.8±0.5°C) populations located the unheated black cups were statistically similar (P>0.05).

3.4.2 Heat induced *HSP* gene expression

Results from heat repellency experiments were used to determine harborage and arena temperatures (42 and 47°C, respectively) for heat exposing bed bugs prior to *HSP* expression analysis. These temperatures were chosen because bed bugs had a ~50% chance to flee at this temperature and they would die quickly if they did not find an unheated harborage. *HSP* gene expression of bed bugs was then quantified and some genes were found to be significantly upregulated in comparison to gene expression of control bed bugs at certain tested times (i.e., 15min, 2h, and 4h) following ramp-up heat exposure (22−47°C) (Fig. 3.2). Overall expression levels varied significantly based on the gene and time point (ANOVA: gene, df=11, F=16.1, P<0.0001, time, df=4, F=3.6, P=0.0076). The post hoc t-test found that *Putative Small HSP* and *HSP70.1* were the most significantly upregulated followed by the *HSP70.3*. A post hoc t-test on time points revealed that significantly upregulated genes showed the highest levels of expression at 15min, 2h, and 4h post-exposure (P<0.05). *HSP* gene expression levels at 8h post-exposure were similar to the 4h and 24h times. Gene expression returned to basal levels 24h post-exposure. The time required for expression levels of different *HSP* genes to decrease to baseline levels varied (Fig. 3.3).

Bed bug population also had a significant effect on *HSP* gene expression in the overall model (ANOVA: df=2, F=6.03, P=0.003). T-tests revealed the Poultry House population had higher *HSP* expression and the KVS and Harlan populations had similar expression. One-way ANOVAs were conducted with expression data at separate time points (15min, 2, 4, 8, and 24h) and when statistical significance was observed, post hoc t-tests were performed (P<0.05).

Fifteen minutes post-exposure, the *HSP70.1* gene was significantly upregulated (average upregulation for different populations: Harlan: 73.5±53.2 fold, KVS: 32.8±8.3-fold, Poultry House: 80.1±51.2 fold) (ANOVA: df=11, F=8.87, P<0.0001). *HSP70.3* and *Putative Small HSP* were also significantly upregulated but at lower levels. No differences were found for other genes. Overall, at 15min the Harlan and Poultry house strains had higher expression levels of significantly upregulated genes than the KVS population (ANOVA: df=2, F=4.7, P=0.02).

At the 2h time point, the *Putative Small HSP* was the most upregulated, followed by the *HSP70.1* and *HSP70.3* genes (ANOVA: df=11, F=22.65, P<0.0001). At 2h the Harlan population had lower expression levels than the KVS and Poultry House populations (ANOVA: df=2, F=26.81, P<0.0001). The Poultry House population had the highest expression of the *Putative*

Small HSP (105.03±43.21 fold), followed by the KVS *HSP70.3* (48.6±17.6 fold). The KVS *Putative Small HSP* (34.8±7.09 fold) and *HSP70.1* (31.0 ±7.3 fold) had similar expression levels to the Poultry House *HSP70.1* (39.3±22.0 fold) and *HSP70.3* (29.15±7.5) expression. The *Putative Small HSP* (15.89±7.24 fold), *HSP70.1* (19.3±7.4 fold) and *HSP70.3* (7.21±3.27 fold) genes of the Harlan population were also significantly upregulated.

At 4h post-exposure, the gene and population effects were significant (ANOVA: Population, df=2, F=23.7, P<0.0001, gene, df=11, F=14.9, P<0.0001). The post hoc t-test revealed the *Putative small HSP* was significantly upregulated followed by *HSP70.1* and *HSP70.3*. The t-test on bed bug populations revealed the Poultry House and Harlan populations had greater expression levels than the KVS population. The Poultry House population expression of the *Putative Small HSP* (77.99±20.35 fold) was the highest, followed by the Harlan *Putative small HSP* (45.84±10.62 fold) and *HSP70.1* (31.66±18.52 fold). The *HSP70.1* (KVS: 8.5±5.13 fold, Poultry House: 5.53±2.6 fold), *HSP70.3* (Harlan: 11.79±3.32 fold, Poultry House: 15.0±4.9 fold), and *Putative small HSP* (KVS: 15.52±9.45 fold) expression were also upregulated.

At 8h after exposure, the gene and population characters were significant (ANOVA: gene, df=11, F=18.95, P<0.0001, population, df=2, F=5.32, P=0.01). T-tests separated the KVS and Poultry House populations which had higher expression levels than the Harlan population. A t-test revealed that *Putative Small HSP* (Poultry House: 39.65±10.9, KVS: 24.10±6.23, Harlan: 23.6±4.9) had the greatest expression levels followed by the *HSP70.3* (Poultry House: 2.9±0.8, KVS: 3.2±0.9, Harlan: 2.06±0.7) and then *HSP70.1* (Poultry House: 2.94±1.3, KVS: 6.73±3.12, Harlan: 1.59±0.6). Other *HSP* genes had similar expression levels to the *HSP70.1* because the fold change of the *HSPs* began to decrease at this timepoint.

Fold change in gene expression was not significant at the 24h time point (P>0.05). However, the population effect was significant at the 24h time point (ANOVA results, df=2, F=7.7, P=0.003) because the Poultry House population had overall slightly higher mean expression values *HSP* genes compared to the Harlan and KVS populations.

3.4.3 Comparison of HSP amino acid sequences and functionality predictions

A phylogram and multiple protein sequence alignments showed that HSP70.1 and HSP70.3 were the most similar of all upregulated proteins with a sequence identity match of 88%. The differences between HSP70.1 and HSP70.3 were most common at the C-terminal end of the

protein. When the Putative Small HSP protein sequence was aligned with the HSP70.1 and HSP70.3, 22% and 20% similarity, was found. The Putative Small HSP aligned at the C-terminus of HSP70.1 and HSP70.3. SignalP analysis suggested that the HSP70.2 and Hypoxia Up-Regulated Protein had a likelihood of 99% and 54% of being signal peptides.

Table 3.1. Temperatures of the arena, associated temperature of the top of the copper harborage and descriptions of the behaviors exhibited by the heat exposed bed bugs.

Time (min)	Arena Temp. (°C)	Harborage Temp. (°C)	Behavioral Descriptions
1–4	**24–28**	**24–26**	Initially, bed bugs were at rest in their harborage and a few moved minimally within harborage tubes. Sometimes bed bugs moved from one harborage tube to another.
4–8	**28–34**	**26–31**	More bed bugs began to move within harborage tubes, some bed bugs would move to the inside top of the tubes and then on the outside top of the harborage.
8–13	**34–42**	**31–37**	More than half the bugs had moved to the top of the harborage. Sometimes they climbed on top of each other. Some bugs would flee the harborage at the end of this period. Bed bugs that left the harborage would either return to the harborage or wandered the arena.
13–25	**42–54**	**37–48**	All insects were on top of the harborage tubes and if they returned to the harborage interior, they returned quickly. Any remaining bed bugs would be forced to flee from the copper harborage at the maximum arena temperatures.

Table 3.2. The predicted probability and associated arena temperatures that will stimulate a bed bug to flee.

Prob. To Flee	Arena Temp. (°C) (95% FL)
75%	52.1 (51.5–52.7)
50%	48.1 (47.7–48.5)
25%	44.2 (43.7–44.6)

Table 3.3. The arena temperature at which bed bugs fled and successfully escaped as well as survival percentages for tested populations. † The average arena temperature(°C) at which bed bugs were able to locate the unheated black cups with standard deviation (STDEV). Values not connected by the same letter are significantly different.

Population	Arena Temp. (°C) (STDEV) †	Escape/Survival (%) (STDEV) ‡
Harlan	45.3±0.6[a]	7.3±5.0[a]
Raleigh	45.5±0.6[a]	10.0±9.5[a]
KVS	45.8±0.5[a]	12.0±8.2[a]
Knoxville	46.5±0.52[ab]	11.0±7.4[a]
McCall	46.4±1.2[ab]	4.0±3.4[a]
Poultry H.	48.8±0.5[b]	10.0±7.2[a]

‡ The average percentage of bed bugs that escaped from the heated harborage and arena to the unheated black cups with standard deviation (STDEV). Survival of populations were not significantly different as indicated by similar letters that connect them.

Figure 3.1. Images of the custom-made copper arena used for heat exposing bed bugs. A) Top view of the heat arena. Two thermocouples were attached underneath the arena at separate locations for temperature recordings. Another thermocouple was attached to the copper harborage to determine the temperature of the harborage top during control experiments (i.e., no bed bugs). B) Bed bugs reacting to heat exposure in the copper harborage. To prevent their escape the thermocouple was not attached to the harborage while conducting experiments with bed bugs. C) Bed bug mortality caused by the heated arena. D) The unheated black plastic cup harborages. Encircled in white is a bed bug that fled the copper harborage and found the unheated cup.

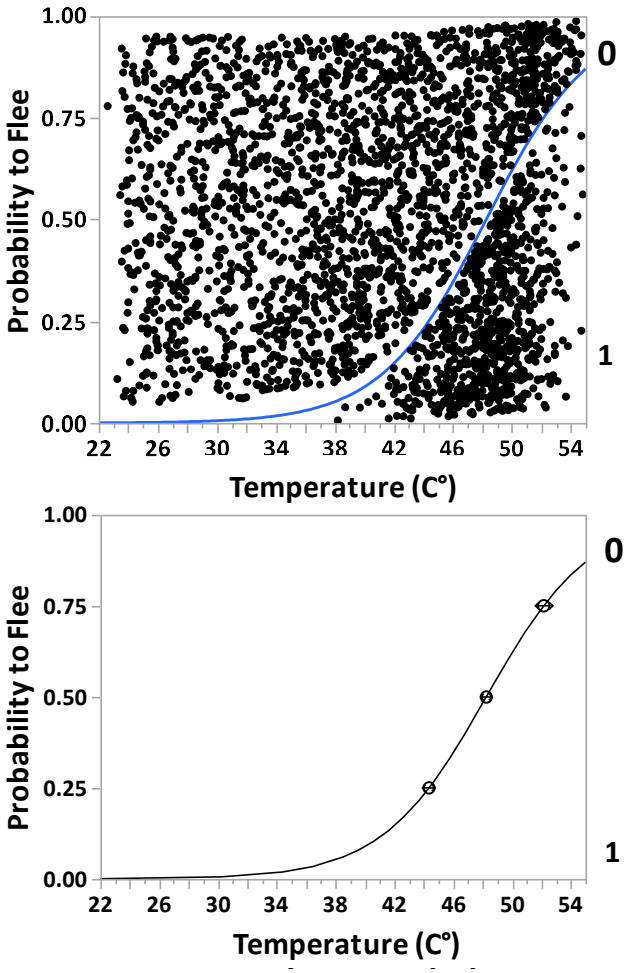

Figure 3.2. Probability curves for bed bugs chance to flee based on temperature (°C). The top graph is raw data of the logistic regression. 0 indicates that the bed bugs were on the harborage and at 1 is where they had fled the heated harborage. The bottom graph depicts the inverse probability prediction analysis results, which was performed for determining temperatures associated with 25, 50 and 75% chance for bed bugs to flee from harborage (detailed in Table 3.3).

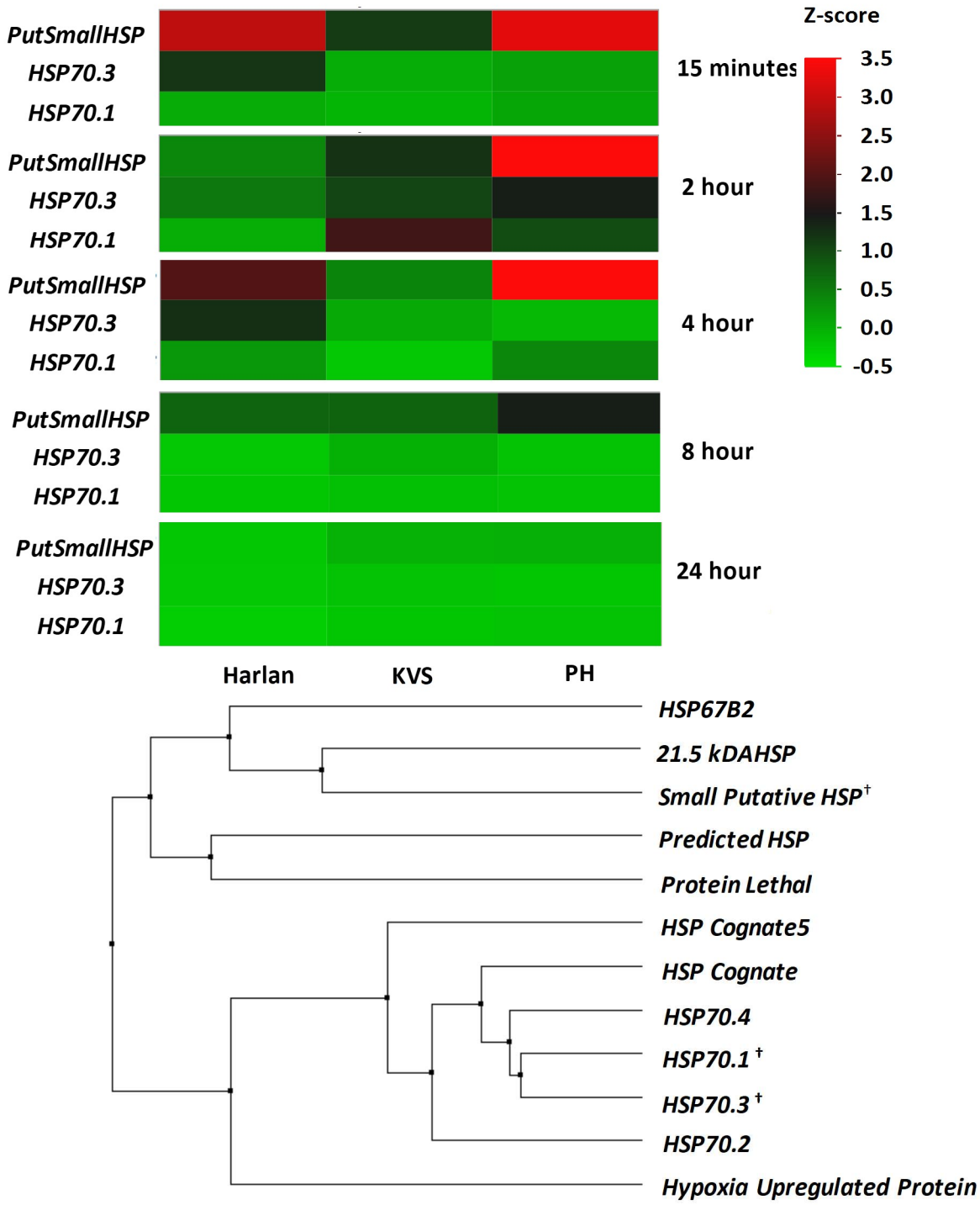

Figure 3.3. Heat maps of *HSP* gene expression showing Z-scores for each population at the tested timepoints as well as a phylogram constructed for the bed bug *HSP* genes. Color intensity indicates mean fold change in gene expression for the upregulated genes. The phylogram was constructed using BLOSUM62 substitution scores and average distance similarity. Dagger sign (†) next to genes on the phylogram indicates the genes that were significantly upregulated after heat exposure.

3.5 Discussion

Exposure of bed bugs to ramp-up heat while inside copper harborages simulates how they would experience conductive heat in cracks and crevices during thermal treatments. However, most household surfaces are not as conductive as copper. Also, whole domicile thermal treatments use convective heat, but this approach was not used in the copper arena tested here. Despite these differences, results from these experiments provide important new insights on behavioral responses of bed bugs to conductive heat while in their harborages. How bed bugs responded to heat exposure in the harborage and arena demonstrated their thermal sensitivity as they displayed a range of locomotive and repellency behaviors (Table 3.1). Their ability to detect differences in temperature was clear at 28−29°C, which is slightly above their preferred resting temperature (Omori 1941). This temperature (28−29°C) caused 6−33% bed bugs to slightly move within the copper harborage. Other studies have confirmed bed bug sensitivity to incremental changes in the temperature of objects and thermal gradients (Rivnay 1932, Devries et al. 2016b, Omori 1941).

Mobility of bed bugs increased as temperatures rose beyond 30°C, but this was not quantitatively measured. Between the temperature range of 30–36°C, some bed bugs temporarily moved to the harborage top, into the lighted area, and then returned to the dark harborage interior (Table 3.1, Fig. 3.1B). When the copper arena floor reached 38°C, some bed bugs began to leave the copper harborage. At these temperatures (~38°C) fleeing bed bugs were repelled by heat and would go back to the copper harborage or would wander the arena. Around arena floor temperatures of 42°C, bed bugs remaining in the harborage would move to the harborage top and if they returned to the interior, they would move to the top immediately. Given the preference of bed bugs for harboring in dark areas it is interesting that heat repellency can overcome their negative phototactic behavior (Singh et al. 2015, Abdoul-Nasr and Erakey 1968). Bed bugs moving from dark areas and into lighted areas is similar to the repellency displayed by German cockroaches in Ebeling choice box assays (Ebling et al. 1966). In Ebeling assays, German cockroaches that are behaviorally resistant to pesticides avoid insecticide treated dark areas and are repelled to untreated light areas of the box. If bed bugs fled the harborage at any point after the copper sheet exceeded 46°C they would wander the arena until mortality occurred or they found an unheated plastic cup.

An important temperature range revealed by using the arena was 53−54°C, which was the maximal copper sheet temperature that bed bugs could tolerate. At this temperature range, bed

bugs on the harborage top (47–48°C) were forced onto the heated arena and mortality would occur. Therefore, if harborage temperatures reach 47–48°C while exterior temperatures are 53−54°C, bed bugs should be forced out of their hiding places and into the open. The maximal arena temperature (53–54°C) that caused all bed bugs to flee from the copper harborage (47–48°C) suggests that if these temperatures are not achieved and maintained during thermal remediation, some insects could survive.

Temperatures and determined probabilities (25, 50 or 75%) for a bed bug to flee the copper harborage found no population-dependent differences (Fig. 3.2, Table 3.2), therefore, conclusions from this study should be generally applicable to other bed bug populations. These results show that bed bugs of certain populations are not escaping lethally or sublethally heated areas because they are more behaviorally responsive to heat than other populations. One difference observed between the populations were the temperatures at which bed bugs located the unheated plastic cups (Table 3.3). Bed bugs from the Poultry House population found the unheated cups at higher temperatures. This result for the Poultry House population indicates that some populations can tolerate lethal heat until they can locate unheated areas. However, locating unheated cups at slightly higher temperatures did not result in greater survivorship of Poultry House bed bugs (Table 3.3). Similar survivorship of tested bed bug populations is a logical result considering how they moved within the heated arena. When bed bugs fled the harborage, they tended to change direction often until mortality occurred or until they located the unheated plastic cups. The behavioral observations and survivorship data suggest that bed bugs could survive a heat treatment if they locate cooler areas within close proximity of their harborages while temperatures are 45−48°C within the domicile. Because behavioral responses of bed bug to heat exposure are driven by differences in temperature, it is possible that items or structures with variable thermal conductivity could mediate bed bug survival during heat treatments.

Temperatures that stimulated bed bugs to respond behaviorally by fleeing also caused physiological changes as evident by upregulation of certain candidate *HSP* genes. Of the twelve investigated bed bug *HSP* genes the *HSP70.1*, *HSP70.3*, and *Putative Small HSP* were upregulated after ramp-up sublethal heat exposure (Fig. 3.3). The highest levels of gene expression were observed at the 15min, 2h, and 4h post-exposure times. At 24h, the *HSP* gene expression levels were at minimal levels for all tested genes (Fig. 3.3, Appendix B Fig. 5).

The expression of significantly upregulated genes was affected by time. Significant upregulation of *HSP70.1* occurred at 15min post-exposure, indicating its likely importance in immediate physiological response to sublethal heat exposure. The *HSP70.1*, *HSP70.3*, and *Putative Small HSP* were upregulated 2h post-exposure with the *Putative Small HSP* showing the highest expression levels. These findings are comparable to those reported by Benoit et al. (2009), where *HSP* mRNA transcripts separated by Northern Blot had the darkest bands (i.e., highest expression) at the 2h timepoint. Peak *HSP* expression by bed bugs at 2h post-exposure follows a similar trend for other arthropods such as Western Flower Thrips (*Frankliniella occidentalis* P.) (Li et al. 2016).

The HSPs from family 70 require energy in the form of ATP for their functionality (Hartl 1996, Bukau et al. 2006). Immediate upregulation of some HSP 70 family genes followed by a decrease in expression at 8h and 24h highlights potential energetic costs associated with their upregulation and functionality (Feder and Hoffmann 1999). Bed bugs are more susceptible to heat exposure when starved (Devries et al. 2016b) and this could be due to having lower ATP levels. Additionally, minimal expression of *HSPs* at 24h may be due to their possible interference with the processes associated with replacing heat damaged or denatured cellular components (Ryan et al. 1992). The functionality of the *Putative Small HSP* is independent of ATP, but likely works with the *HSP70.1* and *HSP70.*3 (Narberhaus 2002, Garrido et al. 2012). *Putative Small HSPs* prevent denatured proteins from interacting with other proteins, until *HSP70.1* and *HSP70.3* can fold proteins back to their proper conformation (Narberhaus 2002, Garrido et al. 2012). This could explain why *Putative Small HSP* was highly expressed in conjunction with *HSP70.1* and *HSP70.3*. The expression of *Putative Small HSP* stayed higher for a longer duration than the *HSP70.1* and *HSP70.3,* suggesting that they could perform additional functions or are still preventing denatured proteins from binding to cellular components until they can be degraded. While recovering from heat exposure, it is possible that bed bugs are more susceptible to insecticides. If bed bugs were treated with a pyrrole insecticide prior to thermal remediation, it could enhance the susceptibility of the insects to heat. Pyrrole intoxication inhibits the ability of insects to create new ATP (Black et al. 1992), which could reduce the functionality of *HSP70.1* and *HSP70.3*.

The protein sequences of HSP70.1 and HSP70.3 were highly similar, which reinforces their co-importance in bed bug thermal biology (Appendix B Fig. 6, 7, 8). The bed bug HSP70.1 and HSP70.3 C-terminus is likely capable of interacting with other cochaperones due to the terminal

EEVD amino acid motif (Bukau et al. 2006, Ryan et al. 1992, Tavaria et al. 1996). This motif at the C-terminus of the HSP70.1 and HSP70.3 also indicates their localization within cellular cytoplasm (Guy and Li 1998). NCBI database searches for genes that are similar to *C. lectularius HSP70.1* and *HSP70.3* found that other Hemipterans (*Tytthus chinensis*, *Halyomorpha halys*, *Orius sauteri,* and *Cyrtorhinus lividipennis*) *HSP70* family genes were >85% similar (E-value= 0.0). The *Putative Small HSP* had high similarity (80−97%) to the *C. lectularius Alpha Crystalline A chain* and *Protein-Lethal2* genes, which was not upregulated after heat exposure (Appendix B. Fig. 3, 4, 5). *Putative Small HSP* gene showed 61% similarity to *Alpha Crystalline A chain* gene from *Halyomorpha halys*. Alpha crystalline chains evolved from HSPs and therefore are similar in structure (Ingolia and Craig 1982, de Jong 1988), but are involved in other stress responses, such as eye lens protection in vertebrates (de Jong 1988). The similarity of *C. lectularius HSP* genes to other Hemipterans highlights the conserved nature of the *HSP* gene family (Feder and Hoffmann 1999). The investigated *HSPs* that were not upregulated could be involved in other responses such as their physiological response to cold, which was not considered in this study.

Based on the findings presented here, bed bugs can survive heat treatments if they escape to unheated or protected areas when the indoor environment is between 45− 48°C, or if temperatures outside their harborages do not reach 53−54°C (harborage temperature 47°C). The bed bug populations tested here had similar behavioral and physiological reactions to heat exposure which indicates that they are not surviving thermal treatments because of underlying differences in behavior or ability to locate cold spots. The results of this study reinforce previous findings that different bed bugs populations have similar heat susceptibility (Ashbrook et al. 2017). If heat exposed bed bugs locate unheated areas, they physiologically recover by expressing *HSP70.1*, *HSP70.3*, and *Putative Small HSP* genes. The *HSP70.1* gene is likely important for immediate thermal protection followed by *HSP70.3.* Based on what is known about the *Putative Small HSP*, it likely aggregates damaged proteins for *HSP70.1* and *HSP70.3* to refold them to their proper conformation.

Finally, the findings presented here on the behavioral responses of bed bugs to rising temperatures can be used for making recommendations to limit bed bug dispersal and prevent them from locating cooler areas during heat treatments. These recommendations are: 1) cracks and openings should be sealed to ensure that bed bugs cannot take shelter in them, 2) traps can be deployed to catch bed bugs moving off of furniture, 3) PMPs can inspect the treated domicile when

temperatures are approaching 45−48°C to prevent bed bugs from locating cooler locations, and 4) when interior temperatures are around 53–54°C (harborage temp. 48°C), covered areas and dense bed bug aggregations can be uncovered for exposure to lethal heat.

CHAPTER 4. DETERMINING IF HEAT EXPOSURE CAUSES BED BUGS (*CIMEX LECTULARIUS* L.) TO EMIT ALARM PHEROMONES AND IF IT TRIGGERS AN ESCAPE RESPONSE IN CONSPECIFICS

4.1 Abstract

Insects use chemical messengers known as pheromones to communicate messages. The chemical ecology of the bed bug, *Cimex lectularius* L., has been recently studied to enhance control efforts. Bed bugs are repelled by high temperatures, which are used for their control. However, the role of bed bug alarm pheromone (AP), a 70/30 blend of (E)-2-Hexenal and (E)-2-Octenal, in heat repellency is unknown. Therefore, the goal of this research was to: 1) determine if bed bugs emit APs in response to heat exposure and 2) quantify the behavioral response of conspecifics to APs emitted by heat-exposed bed bugs. Using a SIFT-MS (soft ion flow tube mass spectrometer), it was found that bed bugs responded to lethal and sublethal heat exposure by emitting APs. The Harlan laboratory population emitted higher pheromone quantities than the field collected McCall, FL population. Females emitted similar amounts of pheromones compared to males for both populations. In separate behavioral experiments, it was shown that conspecifics from both tested populations reacted to APs released by heat-exposed bed bugs (i.e. emitters) by frantically moving within <50mm areas. APs released by one heat exposed emitter also caused multiple conspecifics to respond. At 100mm, the field population was less reactive to APs than the laboratory population. Synthetic APs were also tested in behavioral experiments and caused similar effects to the natural AP blend. The finding that bed bugs emit APs after heat exposure has important implications for pest management professionals that conduct thermal treatments for bed bug elimination.

4.2 Introduction

Insects use semiochemicals as a means for communicating intra- or interspecific messages (Whittaker and Feeny 1971, Howse 1998). Allelochemicals are messengers that elicit a response in other species, whereas pheromones are used to communicate with conspecifics[1]. In some cases, pheromonal communication is more important than using visual, tactile, or auditory modes of communication (Shorey 1973). Volatile pheromones can travel long distances and can be specific blends of chemical compounds that are active at low concentrations (Shorey 1973). Sex

pheromones are usually produced by females and are used to attract pest insects to traps for control or population monitoring (Karlson and Butenandt 1959). Pheromones can also signal danger to conspecifics, which are typically called alarm pheromones (AP or APs) (Wilson 1965).

APs are produced in much greater quantities and can serve a variety of functions such as defense, activity inhibition, aggregation, repellency, or agitation (Blum 1985, Matthews and Matthews 2010, Lofquist 1976). Social insects utilize APs when threatened, which causes a rapid increase in activity and recruitment of nestmates in the area of release (Howse 1998). Several ant species, such as *Formica lugubris* Z., use undecane as an alarm pheromone (Lofquist 1976, Blum 1980). The mandibular glands of *Apis mellifera* L. workers contain 2-heptanone which causes an aggressive response in nestmates (Shearer and Boch 1965). The concentration of APs can influence the response of conspecifics or receiver insects. At low concentrations, *n*-undecane and formic acid can cause aggregation in *Camponotus pennsylvanicus* D., but at high concentrations of only formic acid acts as an AP (Ayre and Blum 1971). Similarly, termite *Trigona subterranea* F. workers either aggregate or attack in the area of AP release depending on the pheromone concentration (Matthews and Matthews 2010).

Hemipteran insects also emit APs when threatened. For example, Hemipterans from the families Pentatomidae, Alydidae, Serinethinae, and Coreidae emit different combinations of either (E)-2-hexenal, (E)-2-octenal, 4-oxo-(E)-2-hexenal, or 4-oxo-(E)-2-octenal in response to threats such as predation (Aldrich and Yonke 1975, Aldrich et al. 1979, Blum 1981, Ishiwatari 1974, Aldrich 1988). The commonality and similar function of these compounds within Hemiptera indicates their evolutionary conservation in certain families (Ishiwatari 1974, Aldrich 1988). In response to the threat of predation, at least 10 different species of aphids emit (E)-β-farnescene, which causes nearby conspecifics to stop feeding and leave the area (Bowers et al. 1972). Emission of APs by aphids to cause dispersal of conspecifics could also be favored by kin selection (Matthews and Matthews 2010, Bowers et al. 1972).

Similar to aphids, bed bugs (Hemiptera: Cimicidae: *Cimex lectularius* L.) are highly related to each other within infestations (Fountain et al. 2014) and adults will emit APs in a 70/30 blend of (E)-2-hexenal and (E)-2-octenal (referred to as hexenal and octenal) as a defensive strategy in response to a threat (Schildknecht 1964, Levinson et al. 1974, Benoit et al. 2009c). Bed bug nymphs emit a slightly different blend of APs, with (E)-2-hexenal and (E)-2-octenal being the main components and 4-oxo-(E)-2-hexenal and 4-oxo-(E)-2-octenal being minor components in

an approximate 20/60/15/5 ratio (Feldlaufer et al. 2010). Bed bugs respond to their AP blend by being repelled from the area of emission and quickly moving away in search for safe locations (Levinson et al. 1974, Benoit et al. 2009c). Alternatively, at low concentrations, the bed bug aggregation pheromone blend ((E)-2-hexenal, (E)-2-octenal, histamine, and other compounds) (Gries et al. 2015) has an arrestant effect on the movement of conspecifics within short distances.

Bed bugs have been shown to emit APs in a variety of situations. Stimuli that will cause AP emission in bed bugs are when they are suddenly uncovered, physically crushed, chewed on by a bat, if sand is poured on them, or if exposed to high concentrations of CO_2 (Schildknecht 1964, Levinson et al. 1974, Usinger 1966, Overal and Wingate 1976). Adult male bed bugs and large nymphs emit APs in response to being mounted by an adult male in order to avoid damage from traumatic insemination, however, females do not (Levinson and Bar 1974, Ryne 2009). Bed bugs are repelled by other stimuli, such as high temperatures (Rivnay 1933, Aboul-Nasr and Erakey 1968, Devries et al. 2016, Chapter 3). The repellent response of bed bugs when exposed to high temperatures has implications for pest management professionals (PMPs) that use heat treatments for bed bug control (Puckett et al. 2013, Loudon 2017, Wang et al. 2018, Kells and Goblirsch 2011, Kells 2018, Raab et al. 2016, Ashbrook et al. 2019). If bed bugs emit APs after exposure to sufficiently high temperatures, it could cause conspecific receivers to flee in search of an area that is at a more favorable temperature. In this regard, it has been found that bed bugs survive heat treatments in some cases (Kells 2018, Raab et al. 2016) and it is likely that heat-induced emission of APs and subsequent dispersal of conspecifics could be one explanation for why they survive heat treatments. However, if bed bugs emit APs in response to heat exposure is unknown. Therefore, the goals of this research are to determine if APs are released by bed bugs at elevated temperatures and how conspecific receivers react to APs emitted by heat exposed bed bugs. The effective radius of bed bug APs and their response to a synthetic AP blend was also investigated. We hypothesized that APs released from heat exposed bed bugs will play a role in the escape response of conspecifics. Similarly, the synthetic AP blend would similarly cause frantic movement in bed bugs.

4.3 Methods

4.3.1 Insects

The insecticide susceptible Harlan laboratory population was used as the reference population for the AP emission and behavioral response experiments. The McCall, FL, field population of bed bugs was used for determining how wild caught bed bugs that are adapted to laboratory conditions would react to heat and APs. Another reason the McCall population was used is that it was collected from a domicile after a heat treatment and, thus, it had presumably survived thermal exposure in the field (Ashbrook et al. 2019). Both bed bug populations were maintained at 26 ±1°C, 50 ±10% RH and a 12:12 h (L:D) cycle in a temperature-controlled environmental chamber (Percival Scientific, Perry, IA). Bed bugs were fed on defibrinated rabbit blood (Hemostat Labs, Dixon, CA) heated to 37°C using the membrane feeding method (Chin-Heady et al. 2013) and were fed 24h prior to use. Recently fed bed bugs were used in all experiments.

4.3.2 Detection of volatile APs emitted from bed bugs exposed to lethal and sublethal heat

To determine when bed bugs emit APs in response to heat exposure, headspace volatiles were continuously sampled from a single bed bug isolated on a digital hotplate (ThermoFisher Scientific, Wilmington, DE) using an inverted 50mm glass Pyrex® funnel (Corning Life Sciences, Oneonta, NY) (Figure 4.1). These volatile collection experiments were conducted from June to August in 2018, November 2019, and February 2020. When experiments were conducted six to ten replicates were run each day. Air samples were taken through the stem of the funnel every 5s using a SIFT-MS (soft ion flow tube mass spectrometry). Using a Defender 510 (Bios International Corporation, Butler, NJ) the airflow rate through the setup was 27.91mL of air/min. The SIFT-MS allowed for real time detection and concentration determinations of (E)-2-hexenal and (E)-2-octenal present in head space volatiles (Syft Technologies, Christchurch, NZ). The reagent ions H3O+, NO+ and O2+ (SIFT-MS calibrated standard gas) were generated in the ion source by a microwave discharge operating in low pressure moist air. The reagent ions formed in the microwave discharge were mass selected by the upstream quadrupole and injected into the flow tube where they were carried along the tube in a stream of helium. The measured amount of (E)-2-hexenal and (E)-2-octenal were automatically calculated using software (LabSyft, Pittsburgh,

PA) in the device from the SIFT-MS calibrated standard gas (Airgas, Indianapolis, IN) connected to the device. Analysis was performed using selected ion mode (SIM) scan for only hexenal and octenal. More information on the SIFT-MS methods is detailed in other reports (Davis et al. 2005, Olivares et al. 2010, Spanel and Smith 2001, Smith and Spanel 2011).

For the first 15min of the head space volatile sampling experiment, one adult bed bug (male or female, from either Harlan or McCall population) placed under the 50mm funnel on a digitally controlled hotplate was exposed to a temperature of 24°C (i.e., near ambient or room temperature) for 15min to determine if placing the bed bug on the hot plate caused APs to be released. Two thermocouples (Digi-sense, Vernon Hills, IL) were attached to the left and right of the hot plate to enable temperature recordings. For the next 15min, the temperature was ramped up from 24−55°C at a rate of 1.86°C/min. Ramp-up heat was used because it is similar to how heat is used to control bed bugs in domiciles (Kells and Goblirsch 2011). The temperature was recorded every 30s while the AP concentration was recorded every 5s by SIFT-MS. However, for statistical analysis, only the AP concentration data that coincided with the temperature recordings (i.e. every 30s) was used. Negative control replicates consisted of empty glass funnels that were heated using the parameters noted above. Additional control replicates used bed bugs that were placed in a freezer 24h prior to use that were also heated and tested for AP emission. Similar heat exposure and SIFT-MS analysis methods to those mentioned above were used for positive control experiments. However, only deceased McCall females were used and were exposed to heat from 24−55°C over a 20min ramp phase. After finding that bed bugs of different sexes 1) emitted similar amounts of APs; 2) and populations tested at similar temperatures react to heat exposure by emitting APs, only one treatment level was investigated for heat exposing deceased insects. Each treatment was replicated six times and a new insect was used each time.

To determine if bed bugs emitted APs in response to sublethal heat exposure, similar methods to those described above were used. However, once the temperature of the hotplate was ramped-up from 24−43°C over 15mins (0.8°C/min), the temperature of 43°C was held constant until the bed bug began to emit APs. The sublethal exposure experiments were terminated after the AP concentrations returned to baseline levels (~8min of exposure at 43°C). Afterwards, the bed bug was removed from the hotplate and placed in a 35x10mm plastic petri dish (Greiner Bio-One, Monroe, NC). 24h later the mortality of the bed bugs was scored. If the bed bug moved or walked in response to prodding with a toothpick, then it was scored as alive. The sublethal heat

exposure experiments were replicated six times. The heated empty blank funnel and heated deceased McCall female data (six reps each), with a similar temperature range as the sublethal heat exposure of live McCall females, were used as controls for statistical comparison. Only McCall females were used for these experiments for the same reasons as mentioned above for the deceased bed bug experiments.

4.3.3 Determining the response of individual receiver conspecifics to APs emitted by bed bugs in 50mm funnels

To determine if a conspecific receiver reacted to pheromones emitted by heat-exposed emitter bed bugs, an inverted funnel design similar to the head space volatile pheromone collection experiments was utilized (Figure 4.2). However, for these behavioral response experiments, the 50mm Pyrex® funnel was placed on a glass Petri dish lid (60mm; Grainger, Indianapolis, IN) lined with a Whatman No 1. filter paper (GE Healthcare, Chicago, IL) on the bottom (Figure 4.2). These behavioral response experiments were conducted using two different ratios of emitter (heat exposed) to conspecific receivers (live bed bug(s) in still air bioassay). The emitter: receiver ratios were either 1:1 or 1:5. In all experiments, emitters were from the same sex and population as receivers.

In 1:1 emitter to receiver ratio experiments, one blood-fed bed bug (male or female, of Harlan or McCall, FL, population) was placed in a Petri dish lid and covered with a 50mm inverted funnel. The receiver bed bug was allowed to acclimate in the setup for 24h. The emitter bed bugs were individually placed into 35x10mm plastic petri dishes with Whatman No. 1 filter paper for 24h prior to use. Separating emitter bed bugs into individual containers ensured that they did not interact with other bed bugs, which could have led to AP emission (e.g., male emission of APs in response to being mounted by another male in an attempt to mate). Control emitter bed bugs were placed in a freezer for 24h prior to use. Before experiments, they were removed and allowed return to room temperature (15mins). To heat expose a live emitter, an individual bed bug was grasped with feather-tip forceps by the head region and quickly passed through a flame 2−3 times, and then dropped into the still air bioassay (Figure 4.2) through the stem of the funnel. Briefly passing the emitter bed bug through flame caused mortality and AP release. The control bed bug was a deceased frozen bed bug that had returned to room temperature and was dropped into the funnel through the stem. For the next 5min, behavioral observations were made on the receiver

conspecific in both the heated and control (unheated) emitter bed bug groups. Behavioral response data was collected on when the receiver conspecific started to move, when they stopped, and if the movement was frantic as described in Levinson & Bar (1974). However, for statistical analysis, the binary data on receiver bed bug movement during the experimental period, which was scored as "yes" or "no" was utilized. Each treatment was replicated 20−22 times. In separate experiments it was verified that temperature of the flame/ heat exposed bed bug had returned to near room temperature (26.6°C) in 15 seconds after exposure. This was done by placing a thermocouple (Fisher-Scientific, Hampton, NJ) directly on the heated emitter bed bug.

4.3.4 Determining the response of multiple receiver conspecifics to APs emitted by bed bugs in 50mm funnels

Procedures used for conducting the 1:5 emitter to receiver ratio experiments were identical to those mentioned above for the 1:1 ratio bioassays. However, in the 1:5 ratio experiments the number of receiver bed bugs that moved during the 5min observation period was recorded in addition to the binary (yes/no) data on if the bed bugs moved. The maximal number of bed bugs that responded to the emitter by moving was used for statistical analysis, but if a motion was initiated in a receiver bug by another receiver bumping into it, the movement was not scored for the numerical response. Logistic regression analysis was also done with the binary receiver bed bug movement data (Appendix C Figure 2). For control experiments, one dead frozen bed bug that had returned to room temperature was dropped into a still air bioassay with five bed bug receivers. Treated and control groups were replicated 20−22 times.

4.3.5 Determining the effect of different heat exposure techniques used on emitters for initiating a response in conspecific receivers in 50mm funnels

To ensure that the heat exposure method (flame vs. hotplate) used on emitters was not influencing the behavioral response of conspecific receivers, additional experiments were conducted with a 1:1 emitter to receiver ratio in 50mm glass funnels. Both live and dead emitter bed bugs were used and heated. Behavioral bioassay procedures for the 1:1 emitter to receiver ratio tests were identical to those described above. One group of emitters (alive or previously frozen deceased bed bugs) were both heat-exposed on a hotplate heated to ~300°C for 2−3 seconds and then dropped into separate 50mm glass Pyrex® funnel setups with one conspecific receiver. The

other group of emitters (alive or previously frozen deceased bed bugs) was heat exposed by the flame heating process methods mentioned above and then dropped into separate 50mm glass Pyrex® funnel setups with one conspecific receiver. Only McCall females were used in these experiments because individual males and females from both populations had similar behavioral responses to APs emitted by heat exposed bed bugs. Also, because the McCall population was field collected from a domicile, their response may be more relevant to how bed bugs would react in the field. For statistical analysis, the focus was on if the bug moved during the experimental period, which was scored as "yes" or "no". Hotplate experiments were replicated 21 times and the flame heated experiments were replicated 30 times.

4.3.6 Determining the response of receiver conspecifics to APs emitted by heat exposed bed bugs in 100mm funnels

To determine the effective radius of bed bug APs, a 100mm glass Pyrex® funnel placed upside-down over a 110mm Whatman No 1. filter paper was used (Figure 3.3). Only 1:1 ratio of emitter to receiver was tested in 100mm funnels. Other behavioral bioassay setup and data collection methods used for 100mm funnel assays were identical to those described for 50mm funnel experiments. For statistical analysis, the focus was on if the bug moved during the 5min experimental period, which was scored as "yes" or "no". Control and live heat exposed bed bug treatments were conducted with adult males and females of both Harlan and McCall strains. Each treatment type was replicated 18−20 times.

4.3.7 Determining the response of bed bugs to synthetic alarm pheromones in 50mm funnels

To determine if AP blend compounds (hexenal and octenal) that were detected by the SIFT-MS could cause frantic movement in receiver bed bugs, behavioral inverted funnel (50mm) experiments were conducted with synthetic analogues of APs. A 100% stock solution of (E)-2-hexenal (98% pure) and (E)-2-octenal (94% pure) was created in a 70/30 ratio (Sigma-Aldrich, St. Louis, MO). The stock solution was then serially diluted in acetone to create 105.8, 52.9, 26.4, 13.2, 8.5, 6.6, 4.2, 0.9, and 0.09 mg/mL solutions. One fed Harlan female bed bug was placed in a 50mm glass Pyrex® funnel placed upside-down over a glass petri dish lid (60mm) with a Whatman No 1. Filter paper on the bottom. Only one of the tested treatment types was used for synthetic AP

experiments because both sexes and populations had a similar behavioral response to APs emitted by conspecifics. Male and female bed bugs also have identical olfactory sensilla have similar responses to different volatile compounds[51]. Harlan female bed bugs were allowed to acclimate in the setup for 24h. Next, a 1mm by 0.3mm strip of Whatman No 1. filter paper was treated with 2µL of AP blend solution (mg per assay: 0.2, 0.1, 0.05, 0.025, 0.016, 0.0125, 0.00625, 0.0016, 0.00016) of abovementioned concentrations or acetone for controls. Once the acetone had evaporated (10s), the strip of paper was dropped through the stem of the funnel. For the next 5 minutes, behavioral observations were made for both the treated and control groups. The focus of these observations was on whether or not the bed bugs frantically moved in response to the synthetic AP blend of (E)-2-hexenal and (E)-2-octenal. The amount of time that the bed bugs were in motion was also recorded. Each concentration tested was tested 6−14 times in conjunction with control acetone replicates (total: 83 replicates).

4.3.8 Data Analysis

To compare the pheromone emission data from the Harlan and McCall populations, and positive control (dead bed bugs that were heated), and empty blank (heated funnel, negative control) treatments, the quantities (mg/mL of air) of hexenal and octenal were first square root transformed. Afterwards, an ANOVA and post hoc t-test were used to compare the quantities of hexenal and octenal in JMP Pro 14 (SAS institute 2018, Cary, NC). Interactions between the population and temperature were included in the analysis. To compare the effect of temperature, sex, and the population had on pheromone quantities, an ANOVA and post-hoc t-test were conducted with the blank and deceased bed bug data excluded. A one-way ANOVA was used to determine if there were any differences between the temperature of AP emission for males and females of both populations. To analyze the pheromone emission response of the McCall females upon exposure to sublethal heat, the data was first square root transformed. Then the sublethal heat data was compared to the heated empty blank funnel, and deceased bed bugs using an ANOVA and post-hoc t-test.

To analyze the response of conspecific receiver bed bugs to APs released by heat exposed emitter bed bugs in the 50mm and 100mm glass Pyrex® funnels, a logistic regression was conducted on the binary response data. The same analysis was used to compare the response of conspecifics to alive and dead bed bugs that were heat exposed using a flame and hotplate. To

analyze the data where there were multiple receivers and one emitter bug, two separate statistical methods were used. An ANOVA followed by a post-hoc t-test was used to compare the number of insects that responded by moving (Figure 4.5) and a contingency analysis followed by a Fisher's exact test to compare the binary response data (Appendix C Figure 1). To analyze the behavioral responses of bed bugs when exposed to different concentrations of synthetic APs, an ANOVA and post-hoc Tukey's test was conducted on the percent time the bed bugs spent moving.

4.4 Results

4.4.1 Emission of bed bug APs after lethal heat exposure

During the temperature ramp-up phase, a temperature-dependent effect on the release of APs was found for both populations, where the temperature that caused bed bug mortality resulted in emission of an AP blend containing hexenal and octenal in high quantities (ANOVA: hexenal, df=1, F-ratio=22.5, P<0.0001, octenal: df=1, F-ratio=22.5, P<0.0001). The temperatures that caused AP emission in live bed bugs exposed to ramp-up heat did not cause release or a spike in AP quantities when deceased bed bugs (controls) that were frozen in advance of ramp-up were heat exposure (Figure 4.3). An increase in AP concentration was also not detected in empty blank funnels (negative control).

Headspace volatile collections of individual male bed bugs revealed that the average temperature that caused males from the Harlan laboratory population to release APs was 45.82±0.29°C, whereas the McCall field population males emitted APs at 45.05±0.44°C. Harlan females would release APs on average at 44.65±0.37°C, whereas McCall females released APs at 44.56±0.47°C (Figure 4.3). Volatile collections or release of hexenal and octenal from bed bugs at temperatures between 44−45°C shows that heat exposure is a stimulus that can ultimately result in AP emission. There were no statistical differences in temperatures that caused APs to be emitted by the bed bug according to sex or population (ANOVA: df=3, F-ratio=2.02, P=0.14, additional statistical details for all ANOVA models are in Appendix C table 1).

The post-hoc t-test revealed that the Harlan population released higher quantities of both hexenal and octenal in comparison to the McCall population (Figure 4.3). However, both Harlan and McCall AP release quantities were significantly higher than empty funnel or blank controls and deceased McCall female bed bugs. The average of the lowest and highest pheromone

quantities detected in individual tested insects are as follows: Harlan females, hexenal: $0.12x10^{-7}\pm0.59x10^{-9}$mg/mL of air to 0.035±0.015mg/mL of air, octenal: $0.311x10^{-7}\pm0.263x10^{-8}$mg/mL of air to 0.011±0.0055mg/mL of air, Harlan males, hexenal: $0.28x10^{-7}\pm0.21x10^{-8}$mg/mL of air to 0.017±0.005mg/mL of air, octenal: $0.35x10^{-7}\pm0.85x10^{-8}$mg/mL of air to 0.006±0.002mg/mL of air, McCall females, hexenal: $0.10x10^{-7}\pm0.21x10^{-8}$mg/mL of air to 0.012±0.009mg/mL of air, octenal: $0.18x10^{-7}\pm0.21x10^{-8}$mg/mL of air to 0.0035±0.002mg/mL of air, McCall males, hexenal: $0.84x10^{-8}\pm0.80x10^{-9}$mg/mL of air to 0.006 ±0.004mg/mL of air, octenal: $0.17x10^{-7}\pm0.36x10^{-8}$mg/mL of air to 0.03 ±0.02mg/mL of air.

To compare the sex by population effect, a separate one-way ANOVA (Figure 4.3) with the blank funnel and deceased bed bug data excluded revealed no differences in the quantities of AP emission between males and females, no differences between the females and males of both populations or the interaction between all three characters (temperature, population, sex) (ANOVA, $P>0.05$ for all treatments). Twenty-four hours after the experiment, survivorship results from the lethal temperature exposure experiments found 100% mortality (0% survivorship) occurred in all tested bed bug groups.

4.4.2 Emission of bed bug APs after sublethal heat exposure

After finding that bed bug females and males from both populations react to the same range of lethal temperatures by emitting an AP blend, only one group of bed bugs (McCall female) was used for sublethal heat exposure experiments. In response to sublethal heat exposure, the McCall females emitted APs beginning at 40.16±0.11°C and pheromone concentration returned to baseline levels around 42°C (Figure 4.4). The peak pheromone concentrations emitted at sublethal temperatures were lower than the concentrations detected during lethal exposure. Low to maximum values detected for hexenal and octenal during sub-lethal exposure are as follows, hexenal: $0.12x10^{-6}\pm0.14x10^{-7}$mg/mL of air to 0.08±0.07mg/mL of air, octenal: $0.88x10^{-7}\pm0.40x10^{-7}$mg/mL of air to 0.01 ±0.009 mg/mL of air. When the bed bugs were emitting alarm pheromones, it was observed that they were lying on their dorsal side and frantically moving their legs suggesting that sublethal heat exposure had led to knockdown. There was a significant difference in the quantities of APs detected between the sublethally heated McCall females, the empty blank treatment, and previously frozen deceased bed bugs, and the interaction between population and temperature (ANOVA, $P<0.05$ for all treatments). Post-hoc t-tests separated the hexenal and

octenal quantities emitted from sublethally heated bed bugs from the empty blank replicates (Figure 4.4). But the heated deceased bed bugs were similar to both the blank and sublethally heated bed bugs even though no spike in AP concentration occurred during heat exposure. Survivorship results from the sublethal temperature experiments McCall female bed bugs found that 5/6 or 83.3% were alive 24h after exposure.

4.4.3 Behavioral responses of individual and multiple conspecific receivers to APs emitted by heat exposed bed bugs

In still air behavioral bioassays conducted in the 50mm funnels at the (1: 1 ratio). The receiver bed bugs were observed if they responded to APs released by the heat-exposed emitters by frantically moving. Logistic regression on the binary response data found a significant difference between the control (room temperature bed bugs that were previously frozen) and heated groups (live bed bugs exposed to lethal heat) for both populations (Figure 4.5) (Logistic regression, $P<0.0001$, Appendix C Table 2 contains additional statistical information for all logistic regression and ANOVA analyses). Individual conspecific receivers responded to emitter bed bugs by moving frantically (Harlan females 85% Harlan males 65%, McCall females 60%, McCall males 80%). In response to APs bed bugs would often change directions and sometimes would wedge themselves underneath the funnel. Although different quantities of APs were emitted by bed bugs from laboratory or field populations, their similar reactions to APs indicate that bed bugs likely react to a range of concentrations after a threshold is reached. There were no differences based on population, sex, or interactions between treatment, population, or sex (Logistic regression, $P>0.05$ for all treatment levels).

Still air bioassays conducted in 50mm funnels with the (1:5 ratio) revealed significant differences in the number of responding receiver bed bugs between the control and treated groups, females and males, and the interaction between treatment and sex (Figure 6.) (ANOVA, $P<0.05$). Multiple receiver conspecifics responded to APs released by a single emitter by frantically moving. The male control treatments groups from both experiments tended to be more active than female controls. A post hoc t-test separated males, females, controls and treatment at all levels of the interaction. No differences were found between the populations and interactions with other variables (ANOVA, $P>0.05$ for all treatments). Contingency analysis of the binary response data from these experiments found an identical trend as mentioned above to the number of bed bugs

moving in response to APs emitted by heated bed bugs (Appendix C Figure 1). Emission of APs by one bed bug causing reactions in multiple conspecifics is logical given their preference to live in dense aggregations.

To determine if high temperature exposure from different heat sources (flame vs. hotplate) causes emission of APs in both live and bed bugs that were deceased prior to use, experiments were conducted where bed bugs (live or previously frozen) were heated and dropped into a funnel with a receiver conspecific (1:1 ratio). Logistic regression for the response of conspecifics to heat exposed bed bugs emitting APs in the 50mm funnels found a significant difference (Logistic regression, P=0.0014) between the heated control bed bugs (hotplate control: 19%, flame heated control: 20%) and heated live bed bugs (hotplate heated: 57%, flame heated, 43%) for insects that were either flame heated or heated with a hotplate (Figure 4.7). No differences between heating method (flame vs. hotplate) or the interaction between the two characters was found (Logistic regression, P>0.05).

Overall, the percentage of Harlan and McCall population conspecific receivers that reacted to APs emitted by heated bed bugs was lower in 100mm still air bioassay funnels in comparison to the 50mm funnels (Figure 4.7). When receivers responded to APs from heated emitters, they displayed frantic movement. Logistic regression for the response data of bed bugs (1:1 ratio) in the 100mm funnels revealed a significant difference between treated and control groups for both populations (P=0.0077). There were no differences in the overall model based on population, sex, or interactions between treatment, population, or sex (Logistic regression, P>0.05). However, when only the response of the McCall population (females 22%, males 11%) was compared to the control (females 11%, males 5.5%), there were not any significant differences between treatment (control vs. heated bed bugs), sex, or the interaction between characters (Logistic regression, P>0.05). When the same analysis was conducted with only the Harlan population (females 42%, males 40%, control: females 10.5%, males 11%), the treatment effect was significantly different (Logistic regression, P<0.05) and sex or the interaction with treatment was not (Logistic regression, P>0.05).

4.4.4 Behavioral responses of bed bugs to synthetic APs

The experimental format here used optimal conditions and 50mm funnels as detailed above. In response to the tested concentrations of synthetic APs (i.e., mixture of hexenal and octenal in

70:30 ratio in acetone), the exposed Harlan female bed bugs responded with frantic movements within the 50mm still air bioassay funnels (ANOVA: df=9, sum of squares=1.41, F-ratio=5.95, P<0.0001). A Tukey's test revealed that APs impacted the percent time spent moving in responding bed bugs in a concentration-dependent way. At lower concentrations of 0.08, 0.85, 4.23, and 6.6 mg/mL responding bed bugs spent on average 12.5±0.1% time moving. The total assay time was 5min. At higher concentrations of 8.46, 13.2, 26.43 and 105.75 mg/mL bed bugs spent on average 22±9.5% of the time moving. The higher concentrations mentioned above were statistically different from control replicates (0.8% respondents; P<0.05), but lower concentrations were similar to control and the higher tested concentrations. One outlier high concentration was 52.87 mg/mL, which caused bed bugs to move for a statistically similar duration as the lower tested concentrations. The behavioral response of bed bugs (i.e. frantic movement within the funnel area) when exposed to synthetic APs was identical to the response exhibited by conspecifics when exposed to heated bed bugs emitting APs. During frantic movement the bed bugs changed directions multiple times in an attempt to move away from the AP source. Bed bugs in control experiments spent a lower amount of time moving in response to acetone treated papers (0.8%). When bed bugs began to move in control experiments, the motion seemed more exploratory and frantic movement was not observed.

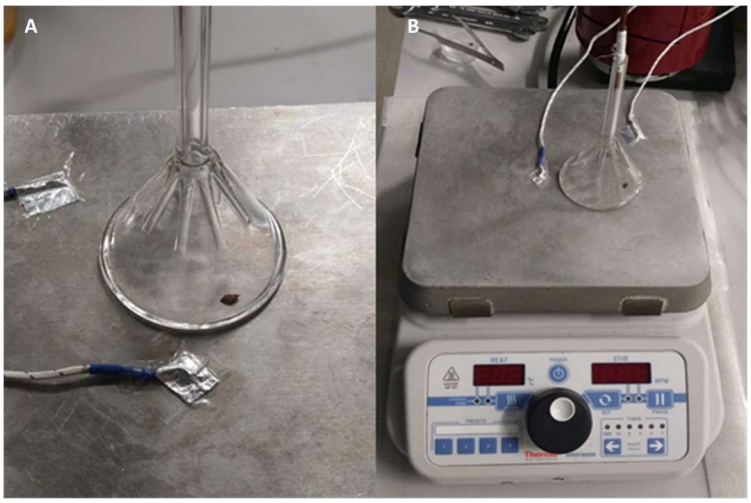

Figure 4.1. Different views of the experimental setup used for bed bug volatile pheromone detection. (A) Two thermocouples are attached to the left and right of the glass funnel allowing for temperature recordings of the hotplate. (B) Air is taken in by the SIFT-MS through the stem of the Pyrex® funnel. One adult male bed bug is placed under an upside-down 50mm Pyrex® funnel for containment and alarm pheromone collection.

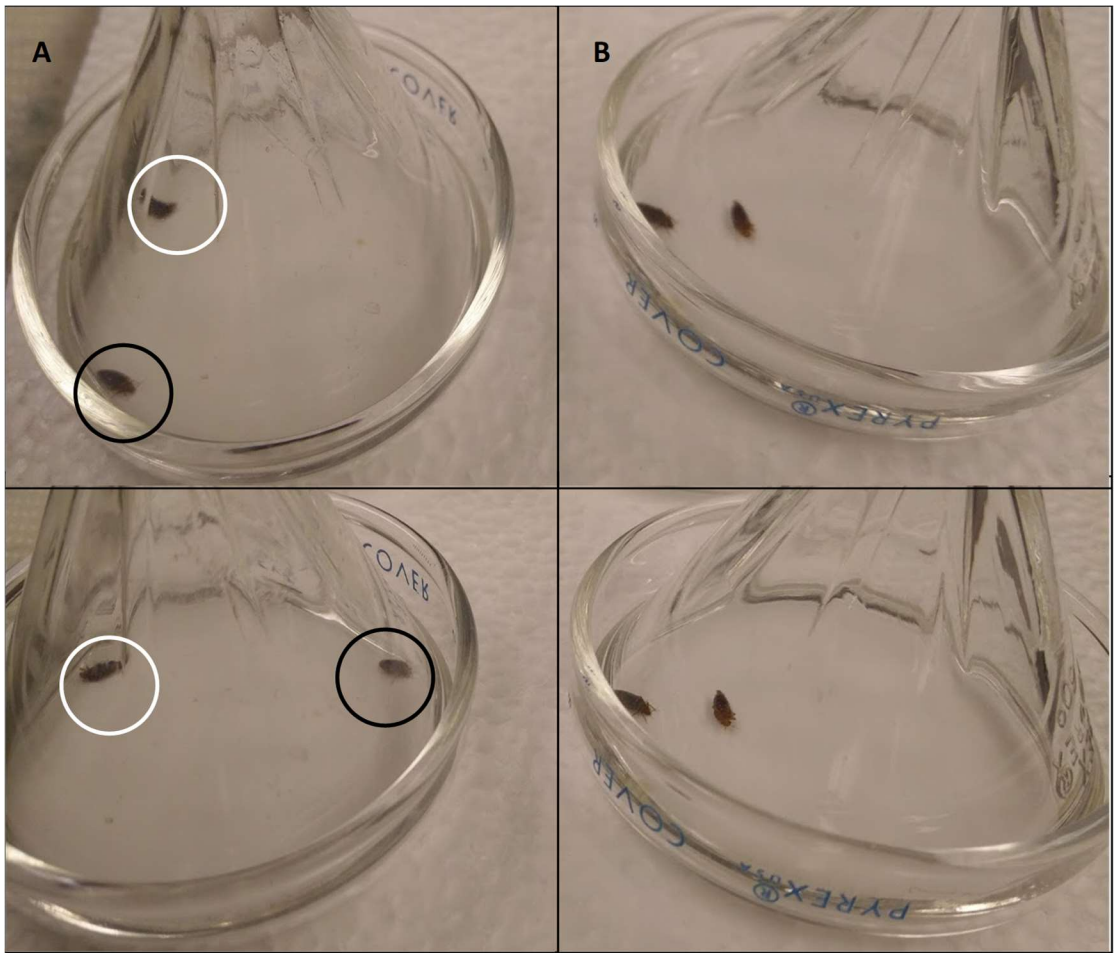

Figure 4.2. The 50mm glass Pyrex® funnel setup for observing the response of receiver bed bugs that are detecting alarm pheromones (APs) released by lethally heat exposed emitter bed bugs. A) The heated emitter bed bug is circled in white in the funnels on the left and the receiver bed bug that showed directional movement in response to APs from the emitter is circled black. (B). The control funnels with a previously frozen deceased bed bug inside. In the control funnels on the right, no motion is observed in response to a bed bug being dropped into the assay setup.

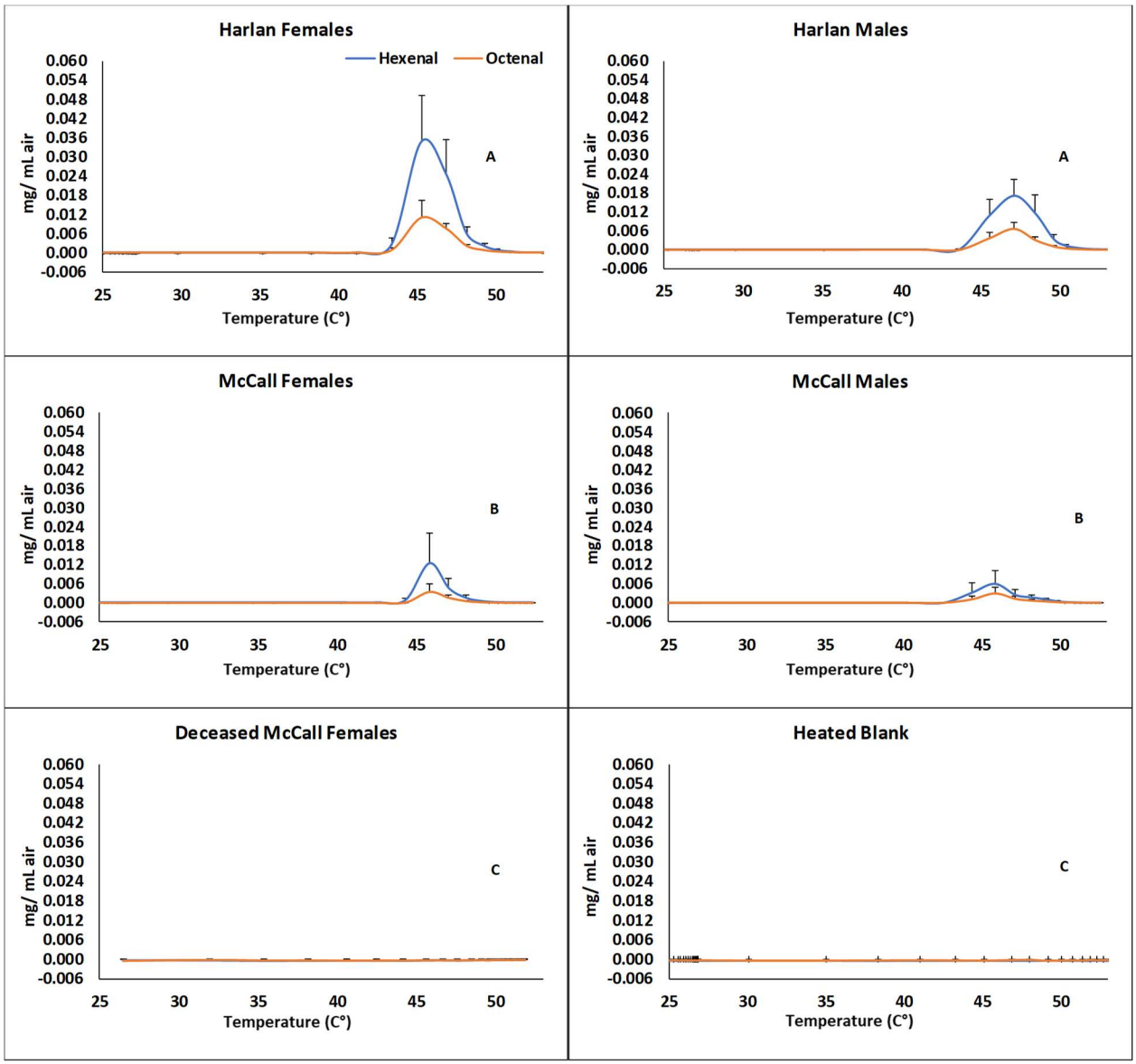

Figure 4.3. Averages of alarm pheromone (AP) emission profiles of adult male and female bed bugs from the Harlan and McCall populations in response to heat exposure as detected by the soft ion flow tube mass spectrometer SIFT-MS. The empty blank funnel and dead McCall female bugs are controls for statistical purposes. The blue lines represent the average detected amount of hexenal, whereas the orange lines represent the average concentration of octenal. Graphs that are not connected by the same letter have statistically different quantities of hexenal and octenal based on population (ANOVA, t-test, $P<0.05$). Error bars represent standard deviation values. Each treatment was replicated six times. Sex related differences in the quantities of hexenal and octenal were not found (ANOVA, t-test, $P>0.05$).

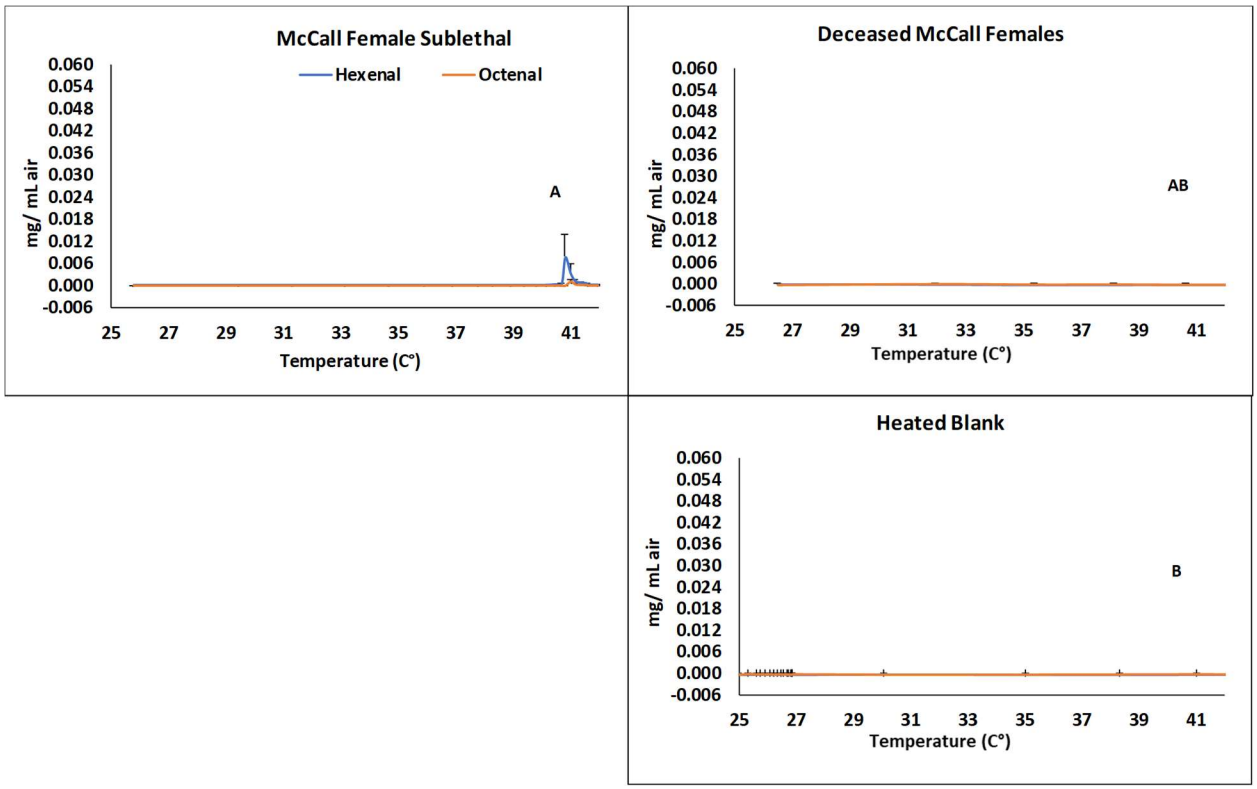

Figure 4.4. The average alarm pheromone (AP) emission profiles of female McCall bed bugs in response to heat exposure as detected by the soft ion flow tube mass spectrometer SIFT-MS. The empty blank funnel and deceased McCall female bugs are controls for statistical purposes. The blue line represents the average detected amount of hexenal, whereas the orange line represents average octenal concentration. Graphs that are not connected by the same letter have statistically different quantities of hexenal and octenal (ANOVA, t-test, $P<0.05$). Error bars represent standard deviation values. Each treatment was replicated 6 times.

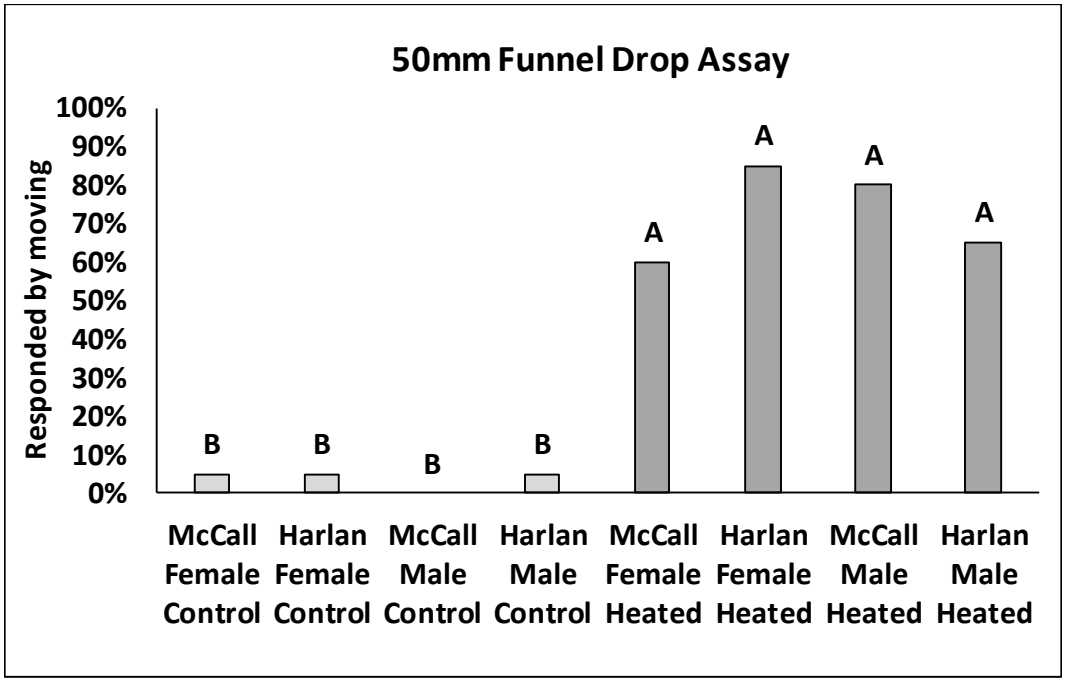

Figure 4.5. The percentage of replicates where receiver conspecifics moved during the 5min period after an AP emitting bed bug was dropped into the 50mm still air bioassay funnel. Each replicate had one receiver conspecific to react to the emitter bed bug. The control bed bugs (light grey bars) were frozen and allowed to return to room temperature before being dropped into the funnel setup. The receivers from the heated group (dark grey bars) were exposed to an emitter bed bug from the same population and sex that was heated with a flame which caused AP emission. Significant differences were found between all the heated groups and the unheated control groups as indicated by different letters above bars (Logistic regression, $P<0.05$).

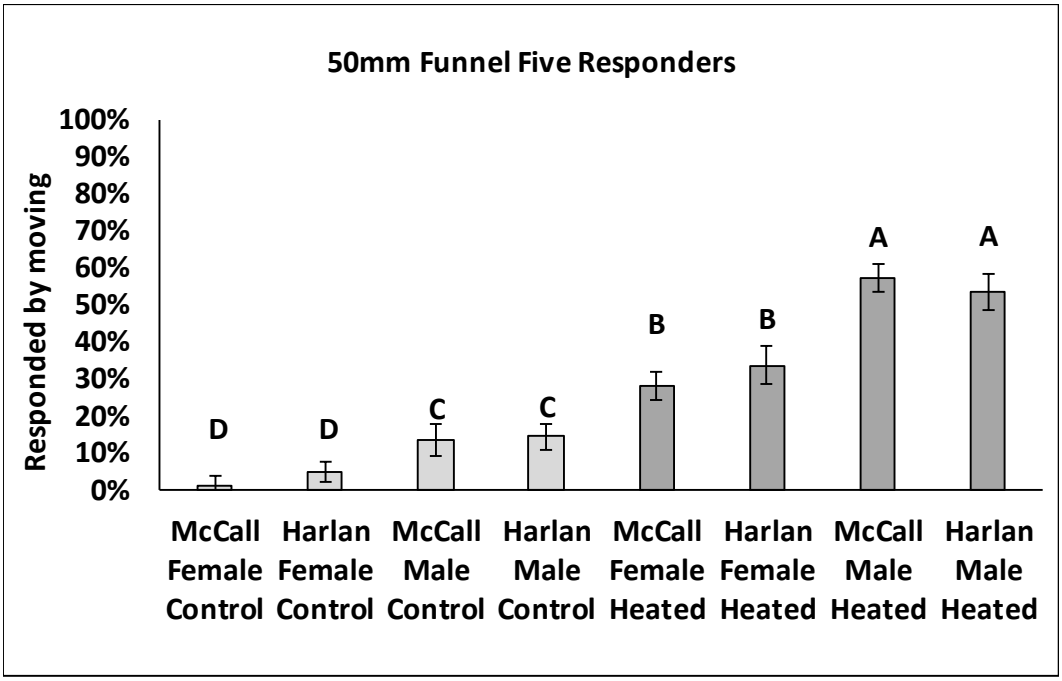

Figure 4.6. Results from the experiments with multiple conspecifics (1:5 emitter to receiver ratio) in the 50mm glass funnel setup. The bars represent the average number of bed bugs that moved during the 5min period after an emitter bed bug was heated and dropped into the setup. The control unheated bed bugs (light grey bars) were frozen and allowed to return to room temperature before being dropped into the funnel setup. The receivers from the heated group (dark grey bars) were exposed to an emitter bed bug from the same population and sex that was heated with a flame which caused AP emission. Significant differences between treatment groups are denoted by the different letters above bars. Statistically significant differences in the number of moving/ responding bed bugs were found between control and heated bed bug treatment groups as well as between the responses of male and female receiver bed bugs (ANOVA T-Test, $P<0.0001$). No significant differences were found between the tested populations or interactions between population and females vs. males (ANOVA, $P>0.05$).

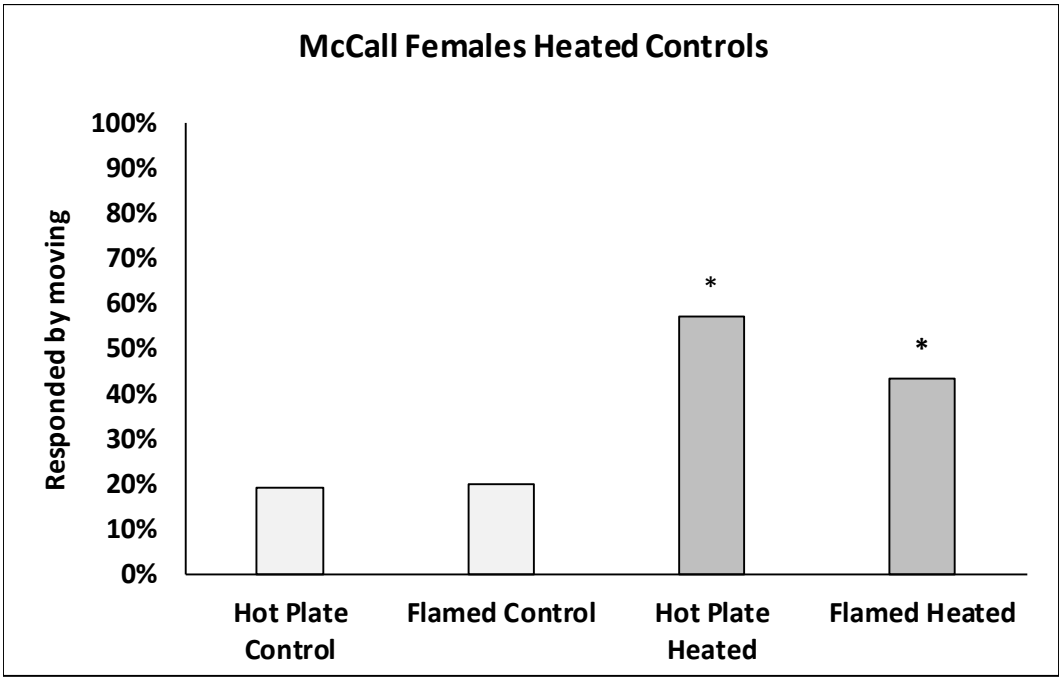

Figure 4.7. The percentage of replicates with receiver conspecifics that responded to emitter (1:1 ratio) bed bugs that were either live (dark grey bars) or dead (control, light grey bars) prior to being heated using either a flame or hotplate. These experiments were conducted to separate the effect of using different heating techniques for initiating a response in conspecific receivers. The asterisks denote statistically significantly differences in the number of replicates that responded to AP exposure between treatment types (Logistic regression, P=0.0014).

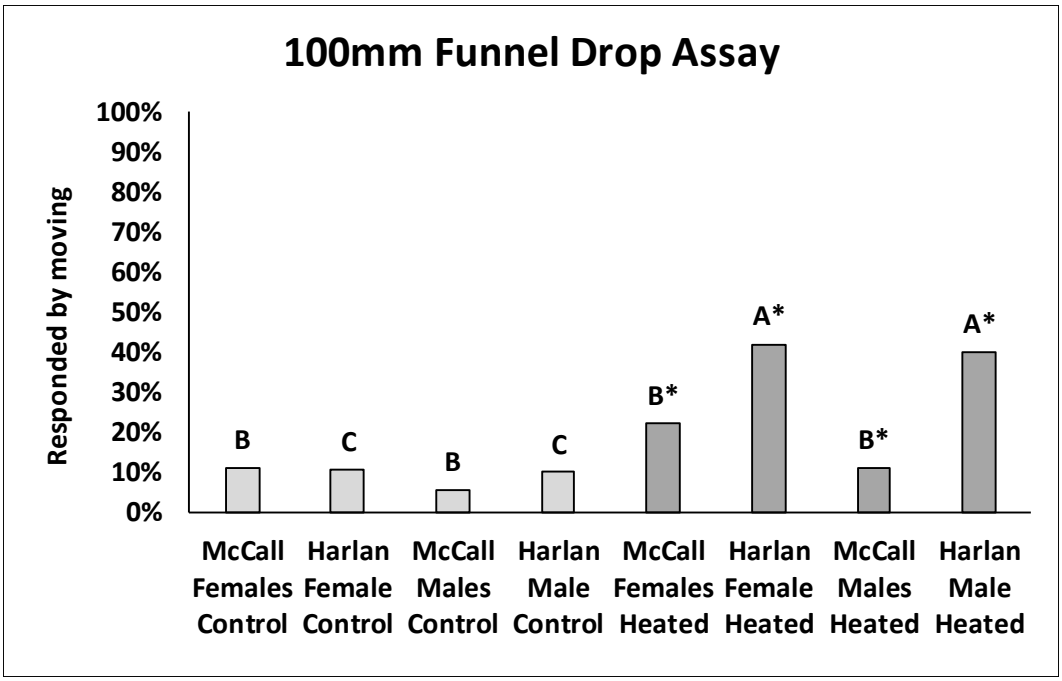

Figure 4.8. The percentage of replicates where receiver conspecifics responded to emitter bed bugs that were alive and then heated (dark grey bars) or deceased (unheated control, light grey bars) prior to being dropped into the 100mm glass funnel. The asterisks denote significantly different number of responders between treatment types in the overall model (Logistic regression, P=0.0098). When the response of the tested populations was analyzed separately, the McCall population was statistically similar to the control groups denoted by the identical letters (Logistic regression, P=0.31), whereas the Harlan population was statistically different than its control group denoted by different letters (Logistic regression, P=0.0036).

4.5 Discussion

Bed bugs can be difficult to eliminate due to insecticide resistance, their cryptic behavior, and inability to treat certain human resting locations (Reinhardt and Silva-Jothy 2007, Romero et al. 2007, Bennett et al. 2016, Ashbrook et al. 2017). Integrated pest management (IPM), which combines population monitoring, chemical, and non-chemical techniques is used to enhance bed bug control (Bennett et al. 2016, Doggett et al. 2012, Cooper et al. 2016). Bed bug pheromones can be utilized within an IPM program. Bed bug aggregation pheromones have been effectively used as lures to increase the efficacy of interceptor traps (Gries et al. 2015, Singh et al. 2015). When bed bug APs are mixed with desiccant dusts, the mixture causes greater mortality (Benoit et al. 2009c).

Another technique used in bed bug IPM is the use of high temperatures. Heat can be used to disinfest items through the application of steam, the use of compartmentalized heaters and/ or

whole domicile heat treatments (Puckett et al. 2013, Loudon 2017, Wang et al. 2018, Kells and Goblirsch 2011, Kells 2018, Raab et al. 2016). Thermal remediation can eliminate all bed bug life stages within an infestation by circulating heated air (at a temperature of ~60°C) in the home for a proper length of time (Kells and Goblirsch 2011, Kells 2018, Raab et al. 2016). In response to heat exposure at sublethal (41°C) and lethal (44−45°C) temperatures, bed bugs of laboratory and field origin emit large enough quantities of APs to be smelled by humans (Figure 4.3, 4.4). This suggests that heat is a stimulus that is perceived as a threat and can cause bed bugs to emit APs to communicate the lethal implications of heat exposure to conspecifics. The concentrations of APs that were emitted by bed bugs in response to sublethal heat were lower than the concentrations emitted by lethally exposed bed bugs. This suggests that pheromone emission was controlled by the bed bugs when exposed to sublethal heat, especially since the majority of them survived heat exposure that resulted in AP emission. Exposure to temperatures that causes mortality, could cause relaxation of muscles controlling the pheromone glands, and therefore emission of APs at higher concentrations.

There were no differences in the quantities of pheromones emitted by males and females, which is likely due to both sexes emitting APs in response to threats (Schildknecht 1964, Levinson et al. 1974, Benoit et al. 2009c). One difference between the Harlan laboratory population and the McCall field population, was that the Harlan bed bugs emitted higher amounts of APs. High variation in the amounts of APs that were emitted by individual bed bugs was observed as well. This is similar to the finding of Liedtke et al. (2015), where the quantities of APs that extracted from *Cimex lectularius* and *Cimex hemipterus* were also highly variable. Liedtke et al. (2015) suggested that the variability could be due to differences in health, size of pheromone glands, or extent of alarm response. Another explanation for the variability in AP quantities was the possibility of AP emission prior to heat exposure. The bed bugs tested in this study were fed 24h prior to exposure and could have emitted APs during feeding or when in their colonies and therefore, had lower quantities of APs to release in response to lethal heat exposure.

The APs that were emitted by heat exposed bed bugs in still air bioassays caused frantic movement in responding conspecifics of both populations and sexes at 50− 100mm distances (Figures 4.5, 4.6, 4.7, 4.8). Sustained frantic movement was seen in almost all the responding receiver bed bugs in all experiments. Additionally, the bed bugs would change directions often when responding to APs, indicating an ability to detect differences in concentrations of the

pheromone blend. Similar frantic movement and directional changes were seen in bed bugs exposed to different concentrations of their synthetic AP blend (Appendix C Figure 1). These results with synthetic APs validate that the heat induced emission of bed bug volatile compounds (hexenal and octenal) detected in the SIFT-MS experiments were very likely the same compounds that elicited frantic movement response in conspecific receivers in behavioral still air bioassays. Not all conspecific receivers reacted to the heat exposed bed bugs emitting APs or exposure to synthetic APs, which could have been caused by variation in pheromone concentration within the still air bioassay funnels. While conspecifics always rested at the edges where the funnel and petri dish met, the heated insects that were introduced to the bioassay would sometimes fall on the opposite end of the funnel. This difference in emitter to receiver location could have resulted in certain conspecifics not detecting the APs because of their longer distance from the emitting source. In this regard, there was generally a lower number of responding insects in the 100mm funnels than in the experiments with 50mm funnels. This indicates a smaller active range for bed bug APs, which is logical given their preference for aggregating together. Therefore, a long-range AP would not be needed to alert nearby conspecifics of a threat. In experiments with 100mm glass funnels, an overall statistical difference was seen in the reaction of the control and treated group bed bugs when the data for both populations was analyzed together (Figure 4.7). However, when only the reaction of the McCall population receivers exposed to APs from heated emitter bed bugs in the 100mm still air bioassays was compared to control groups, they were statistically similar, likely due to emitting lower quantities of APs. However, if the reaction of the Harlan population was compared to their control groups, they were statistically different, likely because they emitted higher quantities of APs and therefore were more likely to detect and respond to APs in the 100mm still air bioassays at a greater frequency.

No differences were found between the reaction of males and females in bioassays with one responder. This absence of differences is likely due to both sexes having identical olfactory neurons (Liedtke et al. 2015) and therefore have similar response to volatile compounds (Harraca et al. 2010). Experiments with multiple receiver bed bugs revealed that one AP emitting bed bug can cause frantic movement in more than one receiving conspecific (Figure 4.5). Females were generally less responsive than males, which was also reported in previous aggregation pheromone bioassays (Harraca et al. 2010). Females may have been less responsive to APs than males in multiple receiver bioassays in order to save energy for egg laying (Olson et al. 2009). Additionally,

control groups of multiple receiver male bed bugs from both populations were more responsive than female control bed bugs and reacted more frequently during the 5min observation period. The higher activity of males in these experiments could have been due to the insects being recently fed, which would cause them to attempt to mate with other male bed bugs (Rivnay 1933). In some control replicates with single or multiple conspecifics it was observed that males would mount the deceased frozen bed bug or other live males. Additionally, there could have been APs released by a conspecific in multiple male receiver experiments because they will emit APs in response to being mounted by a male (Ryne 2009), which could explain their higher activity in these control groups. Nevertheless, despite the greater activity seen in multiple male control replicates, there was still a significantly greater response in treatments where male conspecific receivers were exposed to APs from heated emitter bed bugs (Figure 4.7).

To confirm that using different heat sources causes AP release in emitter bed bugs and to treat the previously frozen deceased bed bugs and live insects in an identical fashion for experiments, they were both heated and dropped into behavioral still air bioassays. Although the effect was statistically significant between live and deceased emitters, some of the control receivers would react as though they were detecting APs by frantically moving after exposure to deceased emitter bed bugs that were heated. In SIFT-MS experiments where previously frozen deceased bed bugs were heated to temperatures that cause AP emission in live bugs, no spike in AP quantities were detected (Figure 4.3). The lack of a spike in AP concentration from the deceased bed bugs was contrary to the lethal and sublethal heat experiments with live bed bugs. Additionally, the quantities of APs that were detected in the previously frozen deceased bed bugs were similar to the blank control experiments (Figure 4.3, 4.4). The difference between the detection of APs from heated bed bugs that were deceased in the SIFT-MS and behavioral experiments could have had to do with the temperatures they were exposed to. To quickly cause morality and ensure that bed bug APs of heated exposed emitters did not completely volatilize before dropping them in the still air bioassay funnels, higher temperatures (>6x) were used in the behavioral experiments. Identical procedures were used to heat expose previously frozen dead bed bugs, which, in some cases, could have destroyed the pheromone gland and released APs that initiated control conspecific receivers to react (~20% of bed bugs). The presence of APs in frozen bed bugs indicates that freezing bed bugs does not cause the emission of high quantities of APs whereas heating bed bugs that are alive leads to AP release. This finding is similar to experiments

showing that live earthworms release APs in response to electrical shock (Halpern et al. 1987), but electrically shocked dead earthworms do not (Colins 1981). Despite the SIFT-MS supported finding that frozen deceased bed bugs do not emit large quantities of APs, the ability of live bed bugs to emit APs in response to lethal and sublethal exposure to different heat sources has implications for how heat is used to eliminate infestations in the field.

In some cases, it has been found that bed bugs survive heat treatments for infestation elimination (Kells 2018) or flee to adjacent unheated apartments (Raab et al. 2016). The finding that thermally stressed bed bugs emit APs in response to sublethal and lethal temperatures and that conspecifics respond to these pheromones by frantically moving could help explain why bed bugs are surviving heat treatments. Bed bugs that detect APs during a heat treatment would respond by fleeing the area that could either lead them to locate thermally protected areas or move into lethally heated areas. Previous research has compared the heat repellency behavior of different bed bug populations when exposed to rising temperatures in their harborages and found that when the exterior environment is heated to 45−48°C, they can locate unheated areas (Chapter 3). Therefore, it is likely that during a heat treatment, bed bugs are stimulated to move by both heat induced repellency and APs emitted by conspecifics, but how bed bugs react to these stimuli in combination is unknown. When *Apis mellifera* workers are exposed to their APs at higher temperatures, they are more active[56] and this could also be the case with bed bug.

The finding that bed bugs are repelled by heat (Rivnay 1933, Devries et al. 2016, Chapter 3) and emit APs at temperatures that can cause knockdown (41°C) and mortality (>45°C) indicates that specific actions should be implemented to prevent bed bugs from escaping lethally heated areas to thermally protected locations or extremities of a domicile during heat treatments. Prior to initiating a heat treatment, interceptor traps could be placed on the legs of furniture or underneath heavily infested items to capture bed bugs as they move away from heat and APs emitted by conspecifics. Cracks and crevices that could create thermal sinks or that would provide thermal protection to bed bugs should be sealed. When the interior temperatures of domiciles reach levels that are lethal or sublethal to bed bugs that would cause them to emit APs, individuals conducting treatments could ensure that conspecifics are not locating thermally protected areas. However, care should be taken to ensure that those working indoors during a heat treatment do not experience to thermal exhaustion. Given that bed bugs are also repelled by synthetic APs, it could be possible to use them to create barriers to prevent bed bugs from escaping lethal heat. Desiccant dusts combined

with bed bug APs could repel insects away from thermally protected locations and also cause mortality in individuals that escape lethal heat exposure if they came into contact with desiccant dust (Benoit et al. 2009c). Bed bug APs could also be applied as a mixture with denatured alcohol containing products that act as flushing agents and also possess contact insecticidal activity. Further research is necessary to determine the practicality of using bed bug APs for control purposes. However, the finding that bed bugs emit APs in response to heat exposure allows pest management professionals to anticipate that pheromone emitting bugs can stimulate conspecifics to move in response to this popular disinfestation method.

CHAPTER 5. SUMMARY

This dissertation research is in part inspired by the question "is it more important for an arthropod to avoid high temperature environments or remain in that environment and develop tolerance to higher temperatures?" with focus on the bed bug, *Cimex lectularius*. The answers to this question have important implications for how heat is used to control bed bugs because in some cases, they are found to survive heat treatments. To answer this question, a series of objectives and experiments were developed to: 1) Evaluate ability of bed bugs to develop heat resistance or thermo-tolerance at the population level in relation to their previous heat exposure history, insecticide resistance status, and geographic origin using two different exposure techniques. 2) Determine the temperatures that will stimulate bed bugs to move in search for cooler areas. 3) Identify which *HSP* genes are expressed by bed bugs after heat exposure. 4) Determine if alarm pheromones (APs) are released by bed bugs that are heat exposed and how conspecifics that are not exposed to heat will react to these APs. The following hypotheses are associated with these objectives: 1) Bed bug strains that have survived heat treatments in the field will require longer exposure times to achieve complete or equivalent mortality relative to bed bugs that have no history of heat exposure. 2) Bed bug populations that have been previously exposed to heat will be able to detect and escape from lethally or sub-lethally heated areas at a higher proportion than bed bug strains without any exposure history. 3) Sublethal heat exposure will cause significant changes in *HSP* gene expression during the stress recovery process. 4) Bed bug alarm pheromones (APs) will play a role in the escape response from heated areas. The results of these thermal biology experiments provide insight into how bed bugs respond to heat exposure at the population, behavioral, and physiological levels.

To determine if bed bugs could develop higher thermal tolerance, a variety of techniques were used to test their upper thermal limits. Heat selection bioassays on bed bugs found an initial survivorship increase (56%), which subsequently decreased over several generations. Heat exposed bed bugs were found to feed at reduced rates, which would impact their development time. In some cases, exposure to sublethal heat impacted the ability of bed bugs to successfully molt. Next, field collected bed bugs had their thermal tolerance determined by using the "step-function" and "ramp up" techniques. No population had significantly greater ability to tolerate heat exposure. Taken together these experiments suggested that the long-term impacts of heat exposure such as

delayed feeding, reproduction, and failure to molt likely prevent bed bug populations with different treatment histories from developing greater thermal tolerance.

To test how bed bug behavior and thermal stress physiology could result in their survival during heat treatments, a custom-made arena was used to expose populations to "ramp up" heat while in a harborage. In behavioral experiments it was found that different bed bug populations had similar behavioral responses to heat exposure. One difference was that the Poultry House population of bed bugs found the unheated harborages at higher temperatures than the other tested populations. This did not result in greater survivorship because no population located unheated areas at greater rates than others. When bugs are exposed to temperatures between 45−48°C, they are most likely to flee and locate unheated areas. If bed bug harborages are heated to 48°C while the exterior environment is heated to 54°C, they should be forced out into heated areas where they immediately succumb to death.

For heat physiology experiments, three populations (Poultry House, KVS, and Harlan) were heat exposed (47°C) in their harborage using the arena. The bed bugs were then collected and analyzed for expression levels of 12 *HSP* genes using RT-qPCR at distinct time points (15min, 2h, 4h, 8h, and 24h). The RT-qPCR results revealed that the *HSP70.1*, *HSP70.3*, and *Putative small HSP* were the most upregulated genes in response to heat exposure. At Fifteen-minute post-exposure the *HSP70.1* was upregulated indicating its importance in immediate thermal protection. At the 2h and 4h timepoint, the *HSP70.1*, *HSP70.3*, and *Putative small HSP* were all upregulated. At the 8h time points, the *HSP* expression levels had decreased, but *Putative small HSP* was still upregulated. At the 24h timepoint, the expression the tested *HSP* genes had returned to baseline levels. From this data it can be concluded that if heat exposed bed bugs are able to locate unheated areas, they will physiologically respond by expressing *HSP70.1*, *HSP70.3*, and *Putative small HSP*.

To determine the role that bed bug APs (a 70/30 blend of (E)-2-hexenal and (E)-2-octenal) plays in their flight behavior in response to heat exposure, headspace volatiles were analyzed in real time from bed bugs exposed to sublethal and lethal heat. In response to lethal heat exposure, field and laboratory origin bed bugs emitted high quantities of APs. When exposed to sublethal heat, the McCall female bed bugs responded by emitting APs. The Harlan laboratory strain emitted greater quantities of pheromones compared to the McCall, FL field population, but no sex related differences were found. These findings show that heat is a threatening stimulus that causes bed bugs to emit APs when sublethal temperatures are reached.

In separate experiments, conspecifics from the Harlan or McCall, FL field population were exposed to heated bed bugs emitting APs and they responded by frantically moving. When bed bugs were exposed to a variety of synthetic AP concentrations, they responded by frantically moving in a similar manner to the experiments with live bed bugs that were emitting APs after lethal heat exposure. The results of the behavioral experiments with synthetic APs validate the behavioral assays findings in which heat-exposed emitter bed bugs cause the same reaction in conspecific receivers.

Finding that bed bugs emit APs in response to heat exposure, which causes conspecifics to flee, that bed bugs flee from heated areas at 45−48°C, and that 3 *HSP* genes are upregulated after heat exposure suggests that these factors are critical to bed bug survival during a heat treatment. The results from sublethal effects heat selection experiments which characterized impacts of thermal stress on bed bug feeding, development and that different populations have similar thermal tolerance levels of suggests that their ability to develop greater heat tolerance is limited. Taken together these results indicate that temperature-dependent behaviors such as heat repellency and emission of APs are more important than developing tolerance by remaining in an unfavorably heated environment.

Determining that bed bugs are repelled to high temperatures and that they emit APs when sublethally or lethally exposed to heat has implications for its use for control. Bed bugs should be prevented from locating thermally insulated areas and the extremities of domiciles from where they could escape to adjacent apartments. Prior to initiating a heat treatment, interceptor traps should be placed under or around heavily infested items to capture bed bugs as they move away from heat and APs. Cracks and crevices that could create thermal sinks or that would provide bed bugs with protection should be sealed. When harborage temperatures of 48°C are reached in harborages while outside temperatures approach 54°C or higher, hidden areas with dense bed bug aggregations can be uncovered to ensure that they are exposed to lethal heat. If any heat exposed bed bugs are able to locate unheated areas, they would physiologically recover by expressing *HSP70.1*, *HSP70.3*, and *Putative Small HSP* for at least 8h. This could be an opportune time to apply insecticides as bed bugs may be more susceptible to them after heat exposure. Bed bugs that survive heat exposure will feed at lower rates compared to unexposed bugs for up to two weeks. Therefore, follow-up inspections for bed bugs could occur approximately a few days after a heat treatment because any survivors that were heat exposed would begin feeding again.

Based on the findings from these experiments there are new research questions to be investigated. The reproductive impacts that heat has on female and male bed bugs could be determined after exposure. The *HSP* gene expression of bed bugs in response to low temperature exposure could be quantified to determine what HSPs play a role in their physiological cold response. Additional research can be conducted to determine the practicality of using bed bug APs for improving the efficacy of heat treatments. Despite these unanswered research questions, the findings on how bed bugs respond to heat exposure at the population, behavioral, and physiological levels have provided insight into what factors contributes to their survival during heat treatments.

APPENDIX A. SUPPLEMENTAL INFORMATION CHAPTER 2

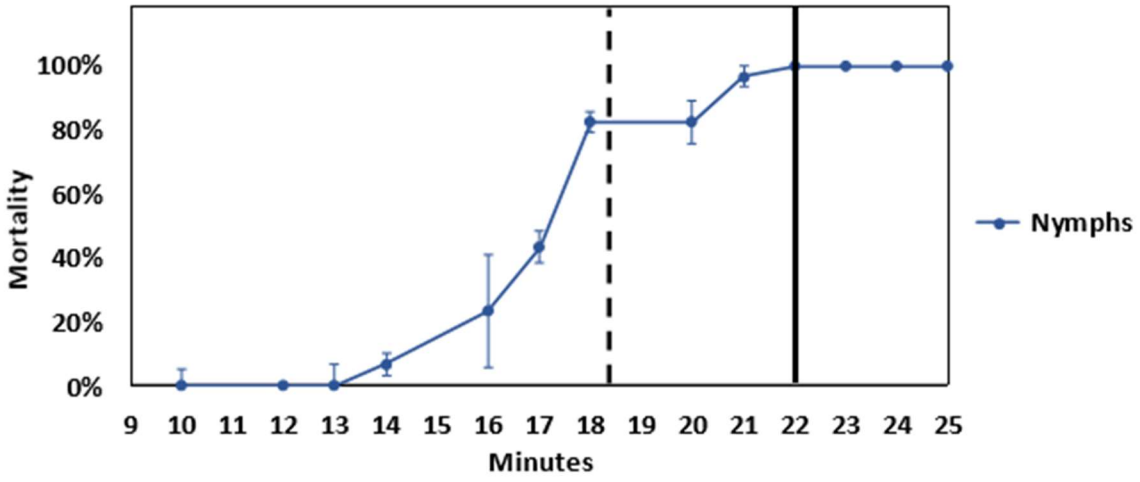

Fig. 1 Harlan nymph time-mortality curve. Time-mortality data for step-function bioassays with Harlan nymphs. All insects were exposed to 45 °C and the exposure times at which they were tested are expressed in minutes. The first dashed vertical line denotes the LT75 exposure time for nymphs, whereas the second black line represents the time at which 100% nymph mortality was achieved

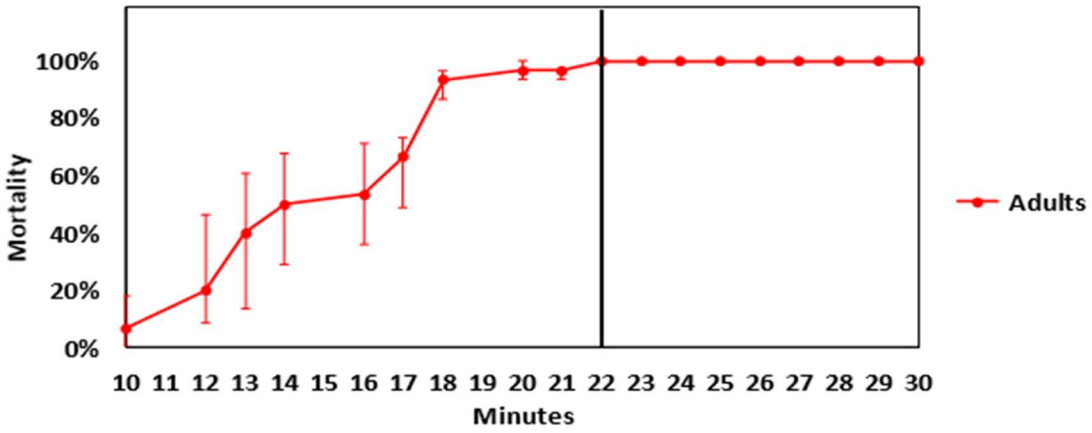

Fig. 2 Harlan adult time-mortality curve Time-mortality data for step-function bioassays with Harlan adults. All insects were exposed to 45°C and the exposure times at which they were tested are expressed in minutes. The solid vertical line indicates the time at which 100% mortality was first observed for adult bed bugs (22 minutes). Results from a statistical analysis test (Robertson et al. 2008) indicated that the life stages (nymphs and adults) were statistically similar to each other. Comparative temperature tolerance ratios between nymphs and adults of the Harlan strain are close to or overlapped with the number one [TR_{50}= 0.84 (0.72–1.00), TR_{99}= 1.09 (0.77–1.53)], indicating that their thermotolerance levels were similar. Error bars indicate (±) standard error values.

Table. 1 Comparison of field strain probit output at the LT50 level. Resistance ratios (RRs) at LT50 and corresponding confidence intervals (in parenthesis) for bed bug strains exposed to 45°C. Significance of RRs was determined using the statistical test described by Robertson et al. (2008). Strains in each column were individually compared to the strains in each row. Within each column RRs that are not connected by the same letter are statistically different. RRs are significantly different if their confidence intervals do not overlap with the number "1". No control mortality occurred during these experiments.

Strain name	Harlan	Raleigh	McCall	Hackensack	Richmond	Lafayette	Bradenton	Poultry House	Knoxville
Harlan	-	-	-	-	-	-	-	-	-
Raleigh	1.14 (1.0–1.33)a	-	-	-	-	-	-	-	-
McCall	1.19 (1.0–1.42)a	1.05 (0.91–1.2)a	-	-	-	-	-	-	-
Hackensack	1.16 (1.0–1.38)a	1.01 (0.88–1.17)a	0.97 (0.82–1.14)a	-	-	-	-	-	-
Richmond	1.06 (0.89–1.27)a	0.93 (0.80–1.08)a	0.89 (0.75–1.05)a	0.92 (0.77–1.08)a	-	-	-	-	-
Lafayette	1.19 (1.0–1.41)a	1.04 (0.91–1.2)a	0.99 (0.85–1.17)a	1.03 (0.87–1.21)a	1.12 (1.0–1.32)a	-	-	-	-
Bradenton	1.23 (1.0–1.46)a	1.08 (0.94–1.68)a	1.03 (0.88–1.21)a	1.07 (0.91–1.25)a	1.0 (0.72–1.40)a	1.04 (0.89–1.21)a	-	-	-
Poultry House	1.17 (1.0–1.37)a	0.9 (0.77–1.0)a	0.85 (0.72–1.0)a	0.88 (0.74–1.0)a	0.96 (0.8–1.15)a	0.86 (0.72–1.0)a	0.83 (0.7–1.0)a	-	-
Knoxville	1.22 (0.86–1.72)a	1.07 (0.93–1.23)a	1.02 (0.87–1.2)a	1.05 (0.90–1.24)a	1.15 (1.0–1.36)a	1.03 (0.87–1.2)a	1.0 (0.84–1.16)a	1.19 (1.0–1.42)a	-
KVS	1.38 (1.16–1.62)b	1.21 (1.10–1.37)b	1.15 (0.99–1.34)a	1.19 (1.10–1.40)b	1.30 (1.11–1.56)b	1.16 (0.99–1.35)a	1.11 (0.99–1.29)a	1.35 (1.14–1.6)b	1.13 (0.98–1.32)a

Table 2. Comparison of field strain probit output at the LT99 level. Resistance ratios (RRs) at LT99 and corresponding confidence intervals (in parenthesis) for bed bug strains exposed to 45°C. Significance of RRs was determined using the statistical test described by Robertson et al. (2008). Strains in each column were individually compared to the strains in each row. Within each column RRs that are not connected by the same letter are statistically different. RRs are significantly different if their confidence intervals do not overlap with the number "1". No control mortality occurred during these experiments.

Strain name	Harlan	Raleigh	McCall	Hackensack	Richmond	Lafayette	Bradenton	Poultry House	Knoxville
Harlan	-	-	-	-	-	-	-	-	-
Raleigh	0.96 (0.69–1.35)a	-	-	-	-	-	-	-	-
McCall	1.16 (0.93–1.45)a	1.17 (0.86–1.60)a		-	-	-	-	-	-
Hackensack	1.13 (0.80–1.61)a	1.17 (0.86–1.60)a	1.0 (0.72–1.38)a	-	-	-	-	-	-
Richmond	1.13 (0.79–1.62)a	1.17 (0.85–1.61)a	1.0 (0.75–1.0)a	1.0 (0.71–1.40)a	-	-	-	-	-
Lafayette	1.14 (0.81–1.61)a	1.18 (0.87–1.60)a	1.0 (0.73–1.38)a	1.0 (0.73–1.39)a	1.0 (0.72–1.40)a	-	-	-	-
Bradenton	1.19 (0.84–1.68)a	1.24 (0.91–1.68)a	1.0 (0.76–1.45)a	1.0 (0.76–1.45)a	1.05 (0.76–1.47)a	1.0 (0.76–1.43)a	-	-	-
Poultry House	1.16 (0.81–1.64)a	1.24 (0.89–1.72)a	1.0 (0.75–1.49)a	1.06 (0.75–1.49)a	1.06 (0.74–1.51)a	1.0 (0.75–1.47)a	1.0 (0.71–1.41)a	-	-
Knoxville	1.22 (0.86–1.72)a	1.27 (0.93–1.72)a	1.08 (0.78–1.48)a	1.08 (0.78–1.49)a	1.08 (0.78–1.5)a	1.07 (0.78–1.47)a	1.0 (0.75–1.41)a	1.0 (0.73–1.44)a	-
KVS	1.26 (0.90–1.76)a	1.31 (1.0–1.75)a	1.11 (0.82–1.52)a	1.12 (0.82–1.52)a	1.12 (0.81–1.54)a	1.11 (0.82–1.5)a	1.0 (0.78–1.44)a	1.0 (0.76–1.47)a	1.0 (0.76–1.40)a

Table 3. Latitude and associated LT_{50} and LT_{99} values for the tested bed bug populations

Population	**LT_{50} at 45°C (mins)**	**LT_{99} at 45°C (mins)**	**Latitude (degrees)**
Harlan	14.3	23.21	40.006
Hackensack	16.5	26.25	40.889
KVS	19.7	29.23	28.3
Bradenton	17.6	27.63	27.48
Raleigh	16.3	22.3	35.76
Lafayette	16.9	26.42	40.41
McCall	17.1	26.25	30.33
Richmond	15.1	26.25	37.533
Poultry House	14.5	27.82	35.97
Knoxville	17.4	28.31	35.97

APPENDIX B. SUPPLEMENTAL INFORMATION CHAPTER 3

Table 1. The background information for the populations that were tested for heat exposure associated behavioral and physiological responses. Insecticide resistance levels in these populations were quantified in Ashbrook et al. (2017). Lethal time (LT_{50}) values and their corresponding 95% fiducial limits (FLs) in minutes required to kill 50% of the tested population at a temperature of 45°C were determined in Ashbrook et al. (2019).

Strain Name and Collection State	Treatment History	LT_{50} (95% FL)
Harlan	Untreated laboratory strain	14.3 (13.7–14.8)[a]
KVS, FL	Field Strain	19.7 (19.1–20.16)[b]
Raleigh, NC	Field Strain, Heat Exposed	16.3 (15.9–16.6)[a]
McCall, FL	Field Strain, Heat Exposed	17.1 (16.5–17.6)[ab]
Poultry House, TN	Insecticide Resistant	14.5 (13.8–15.2)[a]
Knoxville, TN	Insecticide Resistant	17.4 (16.8–17.97)[ab]

Table 2. Primers used for *HSP* target genes and house-keeping or reference genes. (**F**) denotes the forward primer and (**R**) denotes the reverse primer.

Gene Target	Primer Sequence From 5' to 3'
Protein Lethal	**F:** AGACGTTATTGTGGCAGATGTC
	R: ACCACCCTCTCAGCATTTGA
HSP70.1	**F:** GTCTCGGCCAAAGAGAACAG
	R: CTGAGCGTCGTCTTCCTTCT
HSP70.2	**F:** GCCACCAGGTCAGGATAGAA
	R: AACGGAAGAGGTCCATGTTG
HSP70.3	**F:** GTGGCGATCAAAGTTCACAA
	R: GAAGGTCTGGCTCTGTTTGC
HSP70 cognate 5	**F:** ACCATCGAAAGCAGTCAACC
	R: CATCGAGGAGCAATACGTCA
HSP70 cognate	**F:** CCTGTGAAAGGGCAAAGAGA
	R: AAACCTGGCTCTTGTGATCG
Hypoxia Upregulated Protein	**F:** CCGGAAAGCAATAAGACCAA
	R: CAAGCTCGGCACAATGAGTA
21.5kDa HSP	**F:** ACGAATCCCGAGTCAATCAA
	R: TGGGTTATGGGAATGAGCTT
Predicted HSP	**F:** AACGAACCCGACGATTTATG
	R: TGTTTCGCATCCAAAGAGAAG
Putative Small HSP	**F:** ATTGACGGGAAGCATGAAGA
	R: GGAGTGAAAGGACACCATCG
HSP70.4	**F:** TGCACTTTATGACGGTGTCG
	R: ATCGTTGATGGAGCCTTTGT
HSP67B2	**F:** AATTATCGTGTCCTGTCGTTCC
	R: CCAGCCATCCACCATCATA
B-Tubulin (reference gene)	**F:** CGTCGAGAACACAGACGAGA
	R: ATGGTCAGGGAGACGAGATG
RiPL18 (reference gene)	**F:** CAACGACAATTTGGAGGTCA
	R: TCGGATTAACGTAGGCATCA

Figure 1. Underside view of the copper tubing that was welded to the bottom of copper arena floor. A Lauda-Alpha Cooling Circulator was used to move heated deionized water through the copper tubes.

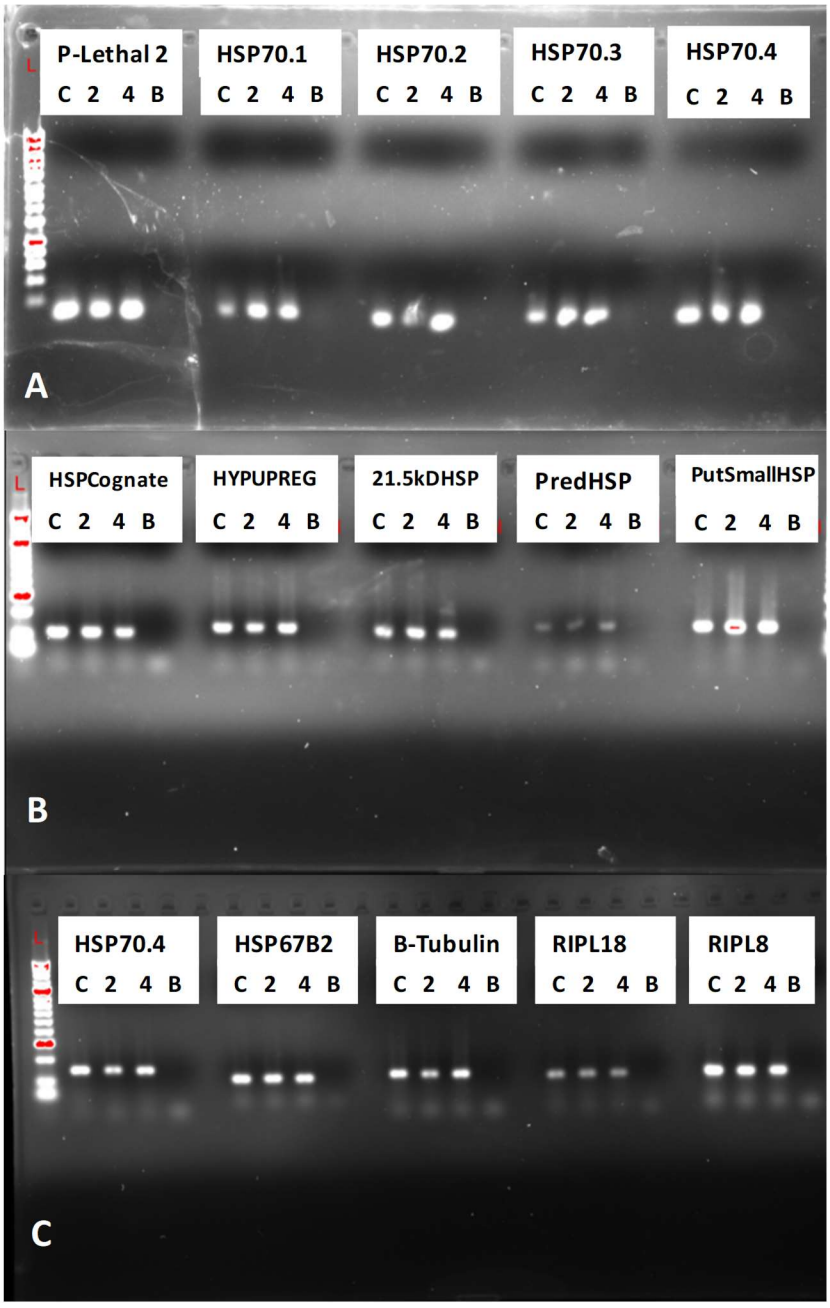

Figure 2. Images of 2% Agarose gels showing amplified PCR products of different candidate *HSP* and house-keeping reference genes used in RT-qPCR experiments. Data are shown for the Harlan strain. The gels were run at 90v for 30min. The C lane is from control samples, 2 is the 2h samples, 4 is the 4h samples, and B are from mRNA extracted from rabbit's blood. Image A) is a 1% TBE/agarose gel (Bioline, Boston, MA) with Hyperladder™ 1kb (Bioline, Boston, MA) on the left side. Images B and C) are 2% TBE/agarose gels with Hyperladder ™50kb run on the left side. Ethidium bromide was used for visualization. RT-qPCR products separated as anticipated in the 100–200bp range. No amplification occurred in the blood samples.

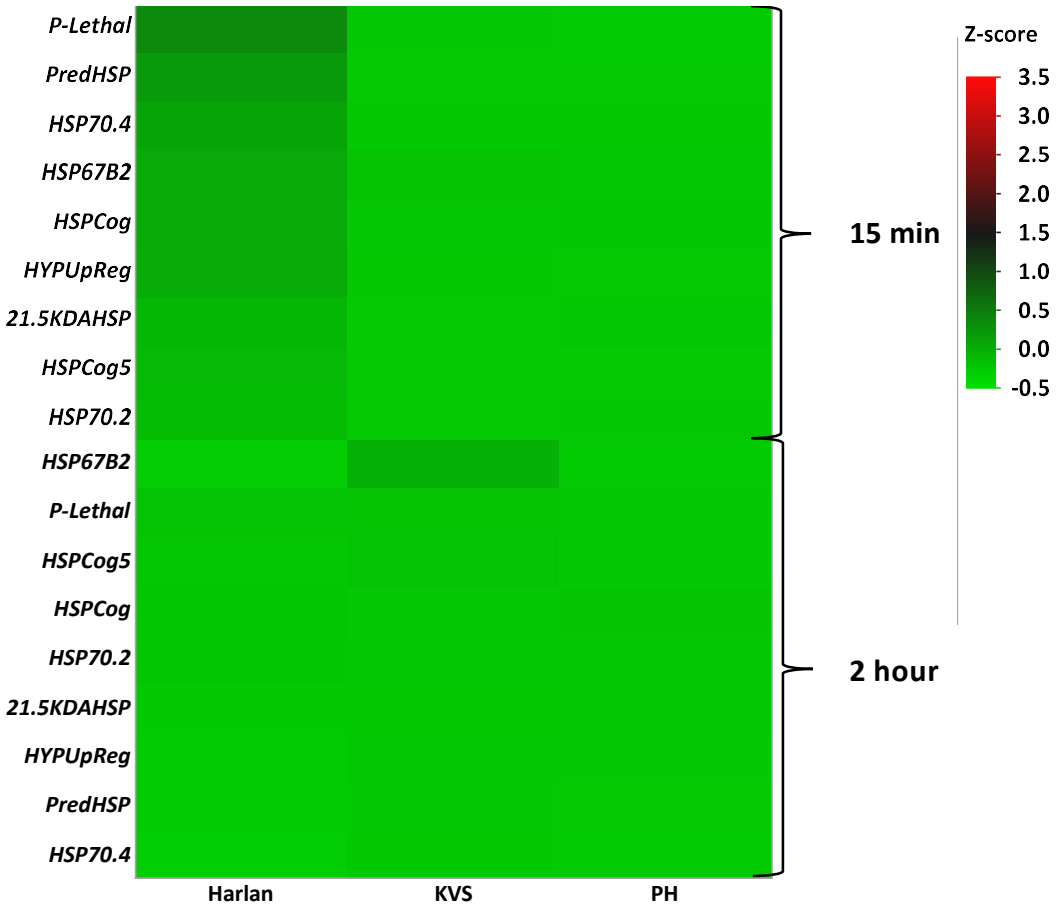

Figure 3. Heat maps of the non-significantly upregulated *HSP* (heat shock protein) genes at 15min and 2h. Color intensity indicates mean fold change values calculated using Z-scores for each population at the different

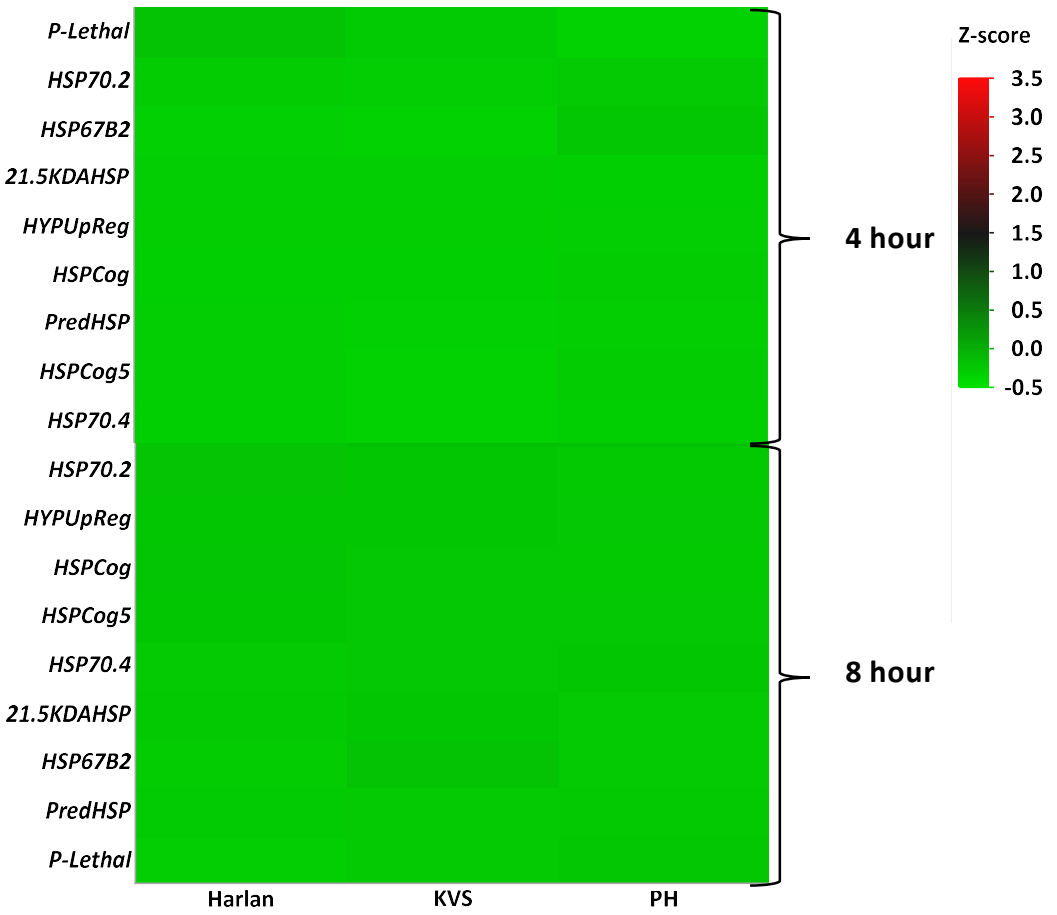

Figure 4. Heat maps of the non-significantly upregulated *HSP* genes at the 4h and 8h. Color intensity indicates mean fold change values calculated using Z-scores for each population at the different time points.

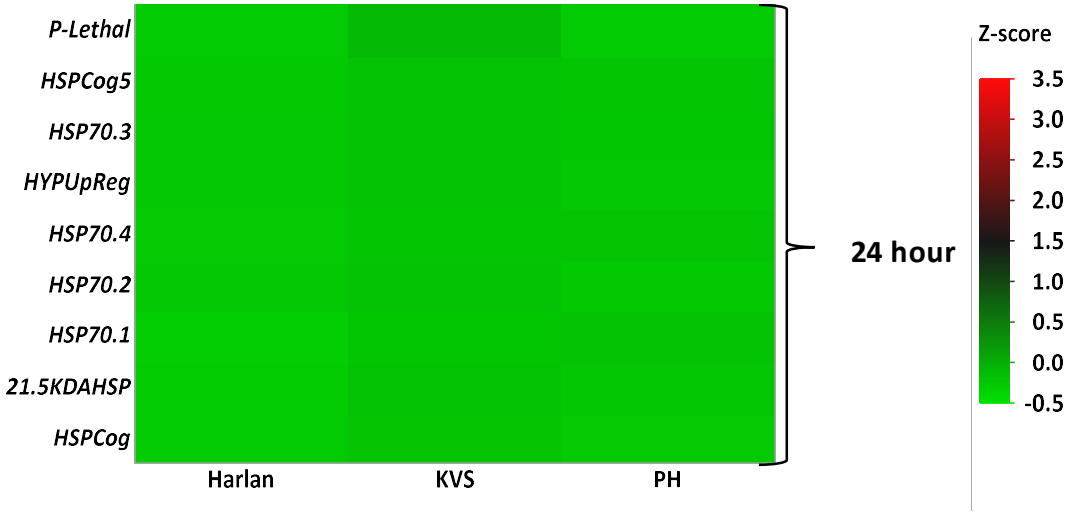

Figure 5. Heat maps of the non-significantly upregulated *HSP* genes at 4h and 8h. Color intensity indicates mean fold change values calculated using Z-scores for each population at the different time points.

Figure 6. Multiple alignment of upregulated HSP protein/ amino acid sequences for *HSP70.1* (XP_014255635.1) and *HSP70.3* (XP_014255633.1) show 88% match.

```
Score = 260.0
Length of alignment = 150
Sequence   XP_014258615.1/1-145 (Sequence length = 190)
Sequence XP_014239635.1/490-636 (Sequence length = 636)

  XP_014258615.1/1-145 MADGGVKRNIPIKLGEFSVLDTEFSNIRERFDAEMRKMEDEMTKFRSEL
                          |  .|| ||  . . |  |  .     |||  | ||.  . |
XP_014239635.1/490-636 ENSTGKSKNIVIK-NDKGRLSKEEIDRMVN-DAEKYKKEDDAQRERIAA

  XP_014258615.1/1-145 MNR-ESNFF--KTTTSTSSNSVSTSSGGLGADKPQN-L-WLESLNSPLI
                        |. |.  |  | .   ... .| |  .    | .. . ||.| ||
XP_014239635.1/490-636 RNQLEAYVFNVKQAVEDAGDKLSQSDKDTIKSKCEDVIRWLDS-NSLAD

  XP_014258615.1/1-145 QEEGDSKMLKLRFDVSQYQPEEIVVKTVDNKLLVHAKHEEKSECKSFYR
                       .|| . |.  |. . . |       .   |      .  .. .  .
XP_014239635.1/490-636 KEEYEHKLKELQSECTPYMTKMHGGQQAPNFTNCGQQAGQRFNTGPTVE

  XP_014258615.1/1-145 EYN
                       | .
XP_014239635.1/490-636 EVD

Percentage ID = 22.00
```

Figure 7. Multiple alignment of upregulated HSP protein/ amino acid sequences for *Putative Small HSP* (XP_014258615.1) and *HSP70.1* (XP_014255635.1) show 22% match.

```
Score = 150.0
Length of alignment = 100
Sequence    XP_014258615.1/1-97 (Sequence length = 190)
Sequence XP_014256533.1/541-639 (Sequence length = 639)

   XP_014258615.1/1-97 MADGGVKRNIPIKLGEFSVLDTEFSNIRERFDAEMRKMEDEMTKFRSEL
                        |     .. .   |.  . ..|   .. | |  .| ....    . |
XP_014256533.1/541-639 EAYVFNAKQAAEDCGD-KLSQSEKDTVKSRCDEAIRWLDNNSLAEKEEY

   XP_014258615.1/1-97 MNRESNFFKTTTS--TSSNSVSTSSGGL-GADKPQNLWLESLNSPLIQE
                        .. |.. |  |   |  .|  .| ||  |.|        . ..| |.|
XP_014256533.1/541-639 EHKLSELQKELTMYMTKLHSGQSSQGGYPGGDFGMPSANKNQSGPTIEE

   XP_014258615.1/1-97 EG
                        .
XP_014256533.1/541-639 VD

Percentage ID = 20.00
```

Figure 8. Multiple alignment of upregulated HSP protein/ amino acid sequences for *Putative Small HSP* (XP_014258615.1) and *HSP70.3* (XP_014255633.1) show 20% match.

APPENDIX C. SUPPLEMENTAL INFORMATION CHAPTER 4

Table 1. Statistical details for various ANOVA analyses. DF stands for degrees of freedom.

Data used for ANOVA analysis	DF	Sum of squares	F-Ratio	P-Value
AP emission temperatures	3	5.95	2.02	0.14
Overall model AP emission by bed bugs, hexenal	7	0.102	24.56	<0.0001
Overall model AP emission by bed bugs, octenal	7	0.41	32.0	<0.0001
AP emission by bed bugs, hexenal*temperature	1	0.01	17.52	<0.0001
AP emission by bed bugs, octenal* temperature	1	0.004	22.5	<0.0001
AP emission by bed bugs, hexenal*population	3	0.03	18.5	<0.0001
AP emission by bed bugs, octenal*population	3	0.015	26.73	<0.0001
AP emission by bed bugs, hexenal*temperature*population	3	0.02	11.64	<0.0001
AP emission by bed bugs, octenal*temperature*population	3	0.007	13.4	<0.0001
Overall model AP concentrations by population, sex, temp, hexenal	7	0.08	13.2	<0.0001
Overall model AP concentrations by population, sex, temp, octenal	7	0.004	16.34	<0.0001
AP concentrations, population, hexenal	1	0.008	9.84	0.0018
AP concentrations, population, octenal	1	0.003	11.7	0.0007
AP concentrations, male vs female, hexenal	1	0.0003	0.35	0.55
AP concentrations, male vs female, octenal	1	0.00003	0.1	0.74
AP concentrations, temperature, hexenal	1	0.06	72.2	<0.0001
AP concentrations, temperature, octenal	1	0.02	91	<0.0001
AP concentrations, temperature* male vs female, hexenal	1	0.00002	0.03	0.86
AP concentrations, temperature* male vs female, octenal	1	0.000001	0.004	0.95
AP concentrations, male vs female*population, hexenal	1	0.00009	0.1	0.74
AP concentrations, male vs female* population, octenal	1	0.0002	0.75	0.38
AP concentrations, male vs female*population*temperature, hexenal	1	0.000007	0.0075	0.93
AP concentrations, male vs female* population*temperature, octenal	1	0.0001	0.49	0.48
Overall model AP concentrations for sublethal heat exposure, hexenal	5	0.11	11.41	<0.0001
AP concentrations for sublethal heat exposure, hexenal*treatment	2	0.013	3.21	0.041
AP concentrations for sublethal heat exposure, hexenal*temperature	1	0.02	10.81	0.0011
AP concentrations for sublethal heat exposure, hexenal*treatment*temperature	2	0.04	10.0	<0.0001
Overall model AP concentrations for sublethal heat exposure, octenal	5	0.007	13.84	<0.0001
AP concentrations for sublethal heat exposure, octenal*treatment	2	0.0003	4.23	0.015
AP concentrations for sublethal heat exposure, octenal*temperature	1	0.0003	13.20	0.0003
AP concentrations for sublethal heat exposure, octenal*treatment*temperature	2	0.0005	11.65	<0.0001
Overall model response of multiple receiver conspecifics in 50mm still air bioassays	7	6.7	31.7	<0.0001
response of multiple receiver conspecifics in 50mm still air bioassays, treatment	1	5.12	167.5	<0.0001
response of multiple receiver conspecifics in 50mm still air bioassays, male vs female	1	1.36	44.13	<0.0001

response of multiple receiver conspecifics in 50mm still air bioassays, treatment*male vs female	1	0.15	5.15	0.025
response of multiple receiver conspecifics in 50mm still air bioassays, population	1	0.004	0.18	0.66
response of multiple receiver conspecifics in 50mm still air bioassays, population*male vs female	1	1.07	0.03	0.30
response of multiple receiver conspecifics in 50mm still air bioassays, population*male vs female	1	0.01	0.62	0.43

Table 2. Statistical details for logistic regression analyses. SE stands for standard error.

Data used for Logistic Regression	Intercept	SE	Chi-square	P-Value
Overall model 50mm funnel individual recipients	-1.16	0.33	12.09	0.0005
50mm funnel indiv. treatment groups	-2.13	0.33	40.52	0.0001
50mm funnel indiv. sex, treatment groups*sex	0.18	0.33	0.29	0.59
50mm funnel indiv. population	0.24	0.33	0.52	0.47
50mm funnel indiv. treatment groups*population	0.11	0.33	0.12	0.72
50mm funnel indiv. treatment groups*population*sex	-0.48	0.35	1.91	0.16
Overall model 50mm funnel heated deceased and live bed bugs, individual recipients	-0.67	0.23	8.44	0.0037
50mm funnel heated deceased and live bed bugs, individual recipients, treatment	-0.74	0.23	10.24	0.0014
50mm funnel heated deceased and live bed bugs, individual recipients, heating method	-0.15	0.23	0.44	0.50
50mm funnel heated deceased and live bed bugs, individual recipients, heating method*control	0.18	0.23	0.63	0.42
Overall model 100mm funnel individual recipients	-1.63	0.24	45.10	<0.0001
100mm funnel indiv. treatment groups	-0.64	0.24	7.10	0.0077
100mm funnel indiv. sex	0.16	0.23	0.46	0.49
100mm funnel indiv. treatment groups*sex	0.18	0.23	0.002	0.95
100mm funnel indiv. population	0.36	0.24	2.30	0.12
100mm funnel indiv. treatment groups*population	-0.25	0.24	1.09	0.29
100mm funnel indiv. treatment groups*population*sex	0.005	0.25	0.09	0.77
Overall model 100mm funnel individual recipients- McCall only	-2.06	0.39	27.13	<0.0001
100mm funnel indiv. treatment groups-McCall only	-0.39	0.39	1.0	0.31
100mm funnel indiv. sex -McCall only	0.39	0.39	1.0	0.31
100mm funnel indiv. treatment groups*sex-McCall only	0.01	0.39	0.002	0.96
Overall model 100mm funnel individual recipients-Harlan only	-1.26	0.31	16.65	<0.0001
100mm funnel indiv. treatment groups-Harlan only	-0.90	0.31	8.49	0.0036
100mm funnel indiv. sex-Harlan only	0.03	0.31	0.01	0.90
100mm funnel indiv. treatment groups*sex-McCall only	-0.007	0.31	0.0005	0.98

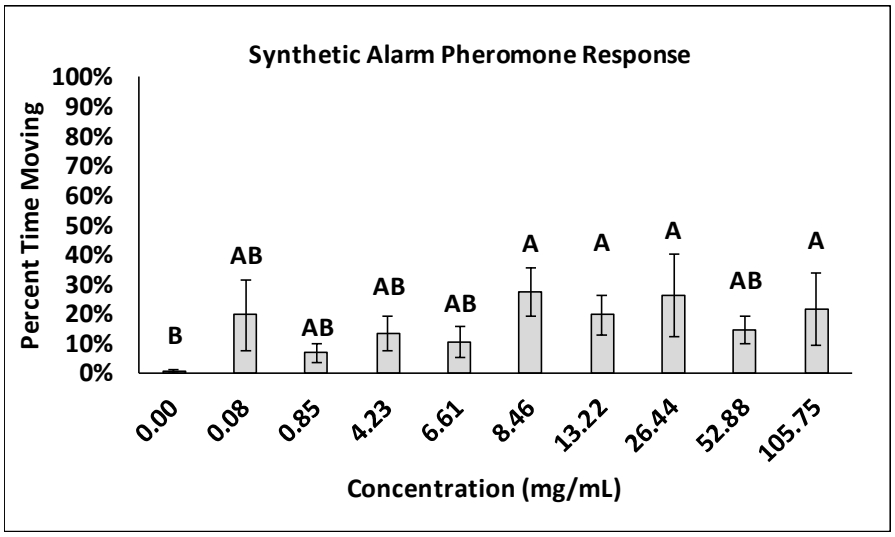

Figure 1. Results from the experiments that tested the response of bed bugs when exposed to various concentrations of synthetic alarm pheromones (APs) in still air bioassay funnels (50mm). The bars represent the average percent time the bed bugs moved during the 5min period after a pheromone or acetone treated paper was dropped into the setup. Each concentration and control (acetone) treatment was replicated 6−14 times. The one-way ANOVA found that pheromone concentration significantly impacted the time spent moving by bed bugs ($P<0.001$). Bars that have no overlap of letters are considered statistically different (Tukey's test, $P<0.05$).

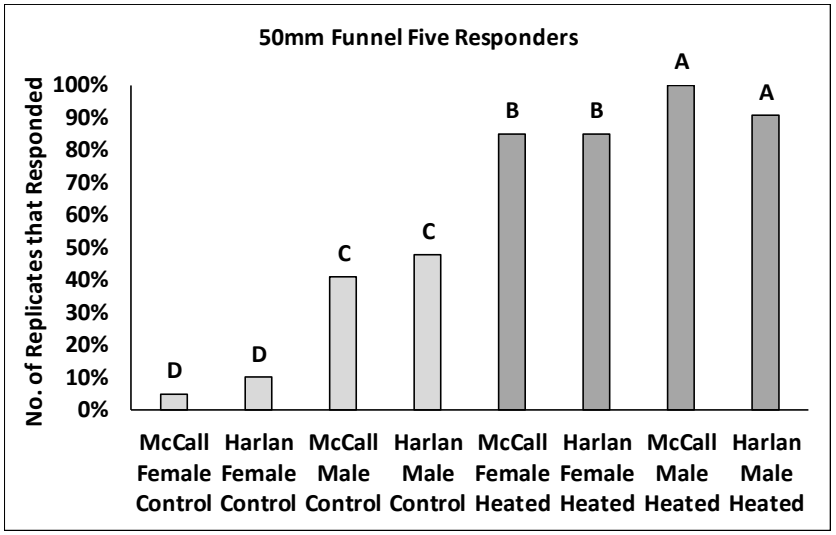

Figure 2. Results from the experiments with multiple conspecific receivers (1:5 emitter to receiver ratio) in the still air glass funnel (50 mm) bioassays. The bars represent the average number of bed bugs that moved during the 5-minute period after a bed bug was dropped into the setup. The control bed bugs (light grey bars) were frozen and allowed to return to room temperature before being dropped into the funnel setup. The receivers from the heated group (dark grey bars) were exposed to a bed bug from the same population that was alive, then heated with a flame which caused pheromone emission before being dropped into the funnel. The contingency analysis and Fisher's exact test used to compare the data found similar results to the ANOVA (Figure 6). There was a significant difference between the control and heated emitter groups (Fisher's exact test: $P<0.0001$) and that males had a different response than females (Fisher's exact test: $P=0.0010$) as denoted by the different letters. No significant differences were found between the tested populations or interactions between the tested characters (Fisher's exact test, $P>0.05$).

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