Reduced Cuticular Penetration as a Contributor to Insecticide Resistance in the Common Bed Bug *Cimex lectularius* L.

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Abstract

The Common bed bug, *Cimex lectularius* L., suddenly reappeared in developed countries in the past 15 years. The factor contributing to the sudden resurgence of the bed bugs is insecticide resistance. In this study, we investigated the mechanisms of reduced cuticular penetration type insecticide resistance in bed bugs. First, we determined and compared the lethal dosage (LD_{50}) of a pyrethroid insecticide using topical and injection application. The resistant strain had demonstrated significantly greater penetration resistance ratios. Second, we determined the levels of gene transcription of their CPR-type cuticle protein genes using real-time quantitative polymerase chain reaction (qRT-PCR). We identified 62 putative bed bug cuticle protein-encoding contigs based on the presence of the Chitin-binding 4 (CB4) domain. Based on the analysis, we found many of the genes were up-regulated in the resistant strain suggesting thickening of the cuticle or increasing the cuticular proteins might be involved in the reduced cuticular penetration. Third, we identified and described the cuticular proteins using the matrix-assisted laser

desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry (MALDI-TOF/TOF). The total of 265 peptides were identified, among which 206 belonged to one of 50 confidently identified proteins. We identified the CPRL, CPF, CPFL, TWDL, and CPAP1 family proteins. The profile of the cuticular proteins between the resistant and the susceptible strains bed bugs were almost identical. Fourth, we determined and compared the cuticular thickness using Scanning Electron Microscopy (SEM). We did not find a correlation between the levels of insecticide resistance and cuticular thickness, though there were strain (population)-level differences. Finally, we identified and described bed bug cuticular hydrocarbon profiles using Gas-Chromatography and Mass-Spectrometry (GC-MS). The total of 87 compounds in addition to *n*-alkanes were extracted and identified. Although *n*-alkanes concentration showed no correlation between the concentration and the levels of insecticide resistance, several additional compounds exhibited the correlation. Overall, we found three lines of evidence to support reduced cuticular penetration as a mechanism of insecticide resistance in some bed bug populations. This study provides additional evidence of the reduced cuticular penetration type insecticide resistance present in bed bugs.

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Attribution

Several scientists contributed to the research design, writing, and statistical analysis parts of this dissertation. A brief description of their contribution is recounted below:

Chapter 1: **The bed bug problem: Past, present, and future control methods**
The majority of work in Chapter 1 was published in the Pesticide Biochemistry and Physiology in July 2013.

Chapter 2. Determination of the lethal dose (LD_{50}) of pyrethroid insecticide necessary to kill pyrethroid-resistant field strain bed bugs using topical and injection application
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Jeffery R. Bloomquist, Ph.D. (Neurotoxicology Laboratory at the Department of Entomology and Nematology, Emerging Pathogens Institute, 2055 Mowry Road, University of Florida, Gainesville, FL 32610), and his student James M. Mutunga, Ph.D. contributed to the designing the experiments.

Karim Fadhloun (Department of Civil and Environmental Engineering (CEE), the Center for Sustainable Mobility, Virginia Tech Transportation Institute, 3500 Transportation Research Plaza, Virginia Tech, Blacksburg, VA 24061) helped me analyze the data.

Chapter 3: **Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes** was published in the Pesticide Biochemistry and Physiology in July 2013.

Michelle A. E. Anderson, the laboratory manager for Dr. Zach Adelman (Entomology Department and Fralin Life Science Institute 360 West Campus Drive, Blacksburg, VA 24061) aided me in the performing the experiments.

Zach N. Adelman, Ph.D. (Fralin Life Science Institute, 360 West Campus Drive,

Blacksburg, VA 24061), an associate professor of the Department of Entomology, contributed to the designing the experiments, analyzing the results, and writing the manuscript.

Chapter 4: Identification and description of the bed bug cuticular proteins using the matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry (MALDI-TOF/TOF).

Richard F. Helm, Ph.D. and William (Keith) Ray, Ph.D. (both at the Department of Biochemistry, 143 and 124 Life Sciences I, Washington St., Blacksburg, VA 24061) guided me in the designing the experiments, provide the bench space, assisted in using the Nano-HPLC, MALDI-TOF/TOF, and associated software programs; they helped to analyze the data, and in the writing of the manuscript.

Zach N. Adelman, Ph.D. (Fralin Life Science Institute, 360 West Campus Drive, Blacksburg, VA 24061), an associate professor of the Department of Entomology, contributed to the designing the experiments and providing his gene annotation data to help identify the cuticular proteins.

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Kathy Lowe, the laboratory manager of the Morphology Service Laboratory (VA-MD College of Veterinary Medicine, 205 Duck Pond Drive, Blacksburg, VA 24061) contributed to experimental design, preparing the samples, and assisting with SEM.

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Geraldine C. Magnin-Bissel, Ph.D., the laboratory manager of the Toxicology Laboratory (VA-MD College of Veterinary Medicine, 205 Duck Pond Drive, Blacksburg, VA 24061) contributed to the experimental design, preparing the samples, and assisted me with using the GC-MS.

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The bed bug problem: Past, present, and future control methods

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ABSTRACT

Bed bugs are wingless hematophagous ectoparasites that have co-existed with humans since they first appeared in the caves of Mediterranean and Middle Eastern regions approximately 65 million years ago. Bed bugs are not known to transmit diseases, most probably due to the lack of sylvatic cycles. Historical control methods include some remedies, but also many useful control methods such as community-wide eradication efforts, insecticidal powders, fumigation, and rigorous cleaning. These intense eradication efforts combined with newly developed synthetic insecticides, such as DDT and malathion, almost eliminated bed bugs during the 1950s. However, there has been a resurgence in bed bug populations during the past 15 years. Recent molecular evidence suggests that bed bugs did not experience a genetic bottle neck, but rather existed in isolated populations. Today, bed bugs are found to have multiple modes of insecticide resistance including reduced cuticular penetration and up-regulation of ABC transporters (ATP-binding cassette protein transporters). Currently available chemical treatments are based on pyrethroid insecticides that are not effective against many insecticide resistant bed bugs, but fumigation and dust formulations have been found to be more effective. Non-chemical control methods are most useful in community-wide integrated pest management. Future bed bug control will most likely to rely on refining the currently available methods and focusing on the research with cooperative efforts.

1. Introduction

1.1. Origin of bed bugs

Bed bugs are obligatory hematophagous ectoparasites that have been one of the most common and annoying pests in human history [1–3]. Adult bed bugs are wingless, reddish brown, approximately 5 mm in length, and dorsoventrally flattened, resembling unfed ticks or cockroach nymphs [2,4]. Bed bug nymphs are translucent, yellowish white in color, and range 1–4 mm in length approximately [2,4]. Bed bugs are believed to have evolved in caves within the Mediterranean [1] and Middle Eastern regions [2], where they were parasites of bats and birds, aggregating in their nests [2]. The exact period of bed bugs emergence is unknown, but ancestral bed bugs are most likely to have evolved approximately 145–65 million years ago (mya), since hematophagous arthropods appeared at six times independently during Jurassic and Cretaceous periods [5], and most of the modern insect fauna was established by 65 mya [6].

Bed bugs are believed to have adopted human hosts when humans started living in the caves of the Middle East and Europe [1,2], sometime during the Pleistocene, Paleolithic, and Neolithic periods [2]. Morphological evidence and the geographical distribution of the Cimex species that feed on bats suggests that bed bugs have probably not originated in Africa [2]. The host–parasite relationship between humans and bed bugs became more established as humans transitioned from their transient lifestyle as hunters gatherers to a more stable community of farmers living in villages [1,2] ca. 8000–5000 B.C. [2]. The earliest records of bed bugs living with humans are those from Greece by 400 B.C. [1,2]. Other bed bug records were discovered from Italy in 77 A.D., China in 600 A.D., Germany in the 11th century, France in 13th century, and England by the late 1500s [1,2]. The first bed bug records in North America date from the 1600s [1,2]. There are at least 57 names used to refer to bed bugs, the majority of them belonging to European, Middle Eastern, and Western Asian languages [2].

Bed bugs, bat bugs, and bird bugs taxonomically belong to the blood feeding family Cimicidae [2,3]. The Cimicidae is one of only two families along with Reduviidae (triatomine) within the order Hemiptera that are blood feeders [7]. All other families of Hemiptera feed on plant tissue fluids [7]. Numerous species of bat bugs and bird bugs have been documented from the nests of bats, swallows, purple martins, swifts, woodpeckers, pigeons, and chickens throughout the world [2,3]; within the New World, parakeets, parrots, and various raptor species such as vultures, eagles, hawks, and owls have also been parasitized with cimicid bugs [8]. Among the 92 species of Cimicidae recognized currently in the world, 16 species were reported in North America [3]. Bat bugs and bird bugs

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primarily feed on their host animals, however they can feed on humans as well, when the nests of the bats and birds are present in domesticated structures and the primary host animals become absent [3].

Only three cimicid species are known to be human ectoparasites [2]: These include Lepto-cimex boueti Brumpt which is present in West Africa [2,5] and primarily feeds on bats but can also feed on humans [5], *Cimex hemipterus* F. found in tropical and subtropical regions [2,3,5] including Florida [3], and *Cimex lectularius* L. which is distributed worldwide but is most prevalent in temperate regions [2,3,5]. All three species of the human bed bugs also feed on bats and chickens, and the common bed bug, *C. lectularius* L., occasionally feeds on domesticated animals [2].

1.2. Medical importance

Bed bugs are known to cause emotional distress, sleeplessness, and anxiety in those people who live with bed bug infestations in their dwellings [9]. Bed bug bites can also potentially cause allergic skin reactions [2–4,10,11]. The bites can be severely irritating [9,12], resulting in a rash with clusters of pruritic, erythematous papules, or wheals as a result of the repeated probing by a single bed bug or multiple bed bugs [4,9,10,13,14] searching for a capillary space from which to feed. An immunoblot analysis using human serum with the common bed bug, *C. lectularius* L., salivary gland extracts and recombinant bed bug saliva proteins found that specific human Immunoglobulin E (IgE) antibodies react with the 32 kDa *C. lectularius* nitrophorin [15]. This study suggests that IgE-mediated hypersensitivity to the bed bug nitrophorin is the cause of skin allergic reaction [15]. The severity of the skin reactions vary significantly among different individuals [16]. Allergic skin reactions to bed bug bites will range from no reaction to a red rash accompanied by intensive itching, that may result in swelling, or even scarring depending on the individual's specific immune response and previous exposure history [16]. The severity of the skin reactions varies significantly among individuals from no reaction to intensive itching, swelling, or scarring for an extended period of time, depending on the previous exposure to bed bugs and various immune responses to protein antigens in the bed bug saliva [15]. Due to this high variability on the reactions, bed bug bites should not be diagnosed solely by skin reactions [16]. The reappearance of bites for a prolonged period of time can be indicative of a bed bug infestation, caused by some other biting insect, or some medical complication. Secondary skin infections from scratching the bites, asthma, anaphylactic reactions [10,15], and sometimes anemia and iron deficiencies in infants and elderly, have been reported in association with high infestations [10,13,17].

To date, there has been no significant evidence of bed bugs transmitting any pathogens that cause human diseases [4–6,11,14,18]. An extensive review of the possible disease transmitted by bed bugs in 1963 reported 32 disease pathogens found from bed bug body parts (gut, head, proboscis, hemocoel, salivary glands, malpighian tubules, hemolymph, midgut and hindgut, intestine, rectum, coelomic fluid, legs, and excreted feces) after being fed infected animals or blood [14]. However, consistent experimental replications showing bed bugs ability to transmit human disease pathogens have been absent [4,5,11,14]. A recent publication showed bed bugs were infected with human Hepatitis B virus (HBV) through feeding HBV-positive blood; and the virus was successfully transmitted through transtadial transmission through different life stages and excreted to bed bug feces (Blow 2001). However, the virus was not transovarially transmitted to the offspring [19]. Another study found human immunodeficiency virus (HIV) was ingested into bed bug guts, but the virus was not replicated within the bed bug bodies, nor detected in bed bug feces, or mechanically transmitted into uninfected blood sources through artificial feedings [20]. Mechanical transmission of the pathogens through contaminated feces or crushing the infected bed bugs while they are still feeding is still possible, and scratching the contaminated surface of the skin might result in accidental infection [14,19]. Any hematophagous insects can potentially transmit diseases mechanically, but pathogens usually cannot survive long outside the host body [5]. The evidence of bed bugs mechanically transmitting human diseases is still lacking [4,5].

The primary reason why bed bugs do not vector nor transmit human diseases might be the lack of sylvatic (forest) cycles, in which obligatory wild-animal hosts are the reservoir of the disease pathogens [5]. All known human disease-transmitting arthropods such as ticks, lice, fleas, kissing bugs, and mosquitoes, have sylvatic cycles; and the pathogens have evolved to live and replicate within the internal physiological environment of the vector arthropod [5]. Theoretically, it is possible that some pathogens may evolve the ability to successfully replicate themselves within the bodies of bed bugs at some point in the future, since some pathogens were found to survive extended periods of time inside the insect body. However, the chance of bed bugs evolving to become a disease vector is probably slim. Bed bugs are most likely to encounter a limited number of the host individuals during the course of their life time, unlike mosquitoes and flies, because they lack wings and heavily rely on passive dispersal by hitchhiking [1–3,5]. Additionally, the common bed bug, *C. lectularius* L. and the tropical bed bug, *C. hemipterus* F., primarily feed on human host during the course of development, hence, exposure to the pathogens and dispersal of the pathogens are limited compared to that of mosquitoes, flies, fleas, ticks, etc. Bed bugs also have a strong instinct to hide in cracks and crevices, and their photophobic, nocturnal, thigmotactic behavior [2,3] may greatly limit the exposure to or the transmission of pathogens and diseases to another host. For these reasons, bed bugs are unlikely to be involved in a human disease outbreak currently or to become a human disease vector in the future.

2. Historical control methods

In the earliest record of bed bug control, found in Greek and Roman literature from 400 B.C., it was believed that bed bugs could be repelled by hanging the feet of a hare, stag, bear skin, or by setting a bowl of water under the bed [1,2]. All of these methods most probably had no effect, but it was around the 1690s–1700s that inspection by professionals was first recommended [1]. Tiffin and Son of London, formed in 1690, was the first exterminator company propagating to treat bed bugs, knowing that the careful inspection where bed bugs could be aggregating and prevention were the key to successful control [1]. In 1730, another exterminator, John Southall, published the first bed bug manual called *A Treatise of Buggs* [2], also emphasizing the importance of examination, and recommending the simplification of beds to make examination and extermination easier [1]. Southall imported Nonpareil liquor from Jamaica to control bed bugs [1,2]. This liquor may have been made with quassia wood, an insecticidal tree from the tropical region, but the formula has been lost [1]. Sassafras wood was believed to repel bed bugs, and it was used to build bed frames in North America around mid 1700s [1]. Setting up traps such as dish pans filled with kerosene or oil and planks of wood with many small holes were also used during that time [1]. Filling the wall cracks with gunpowder and setting a fire was recommended in 1777 from a handbook published as *The Complete Vermin-Killer* [21]. Pyrethrum, the extract of dried chrysanthemum flowers, also known as the Persian Insect Powder, was widely used in Europe and Asia, and in the United States the 1800s [1]. Boiling water

was poured in cracks and crevices of beds and houses [22], and sulfur, arsenic, mercury, grease from salt pork or bacon, were applied [1]. Mercury chloride, bed bug poison, was a corrosive sublimate that was also applied at that time onto infested beddings in households using feathers [1], after beaten together with the egg whites [1]. Although these control efforts gave them some short term reliefs while also poisoning the residents, bed bugs were back often in few weeks or months, since these insecticides gave no residual effects [1].

By the middle of 1700s and 1800s, bed bugs were commonly found in homes, hotels, railroads, and ships; and although bed bugs did not care about the economic status of the human host, bed bugs were often associated with economically poor populations because they often lived in more crowded homes with less rigorous cleaning which was favorable condition to bed bugs to easily multiply and spread the infestation [1]. Economically poor people seemed tolerated bed bugs more than richer [1]. By early 1900s, the central heating system of housings seemed enabled the bed bugs to thrive year around [1], that bed bugs were serious pest in urban environment that one in three houses in cities of Europe had bed bug infestation [1,2]. Bed bugs were common in theaters, restaurants, furniture stores, dressing rooms, laundry services, schools, businesses, and all modes of transportations [1]. The degree if infestation was so high that bed bugs were seen escaping through windows and doors, and walking through walls, pipes, into the neighboring houses [1]. Slam clearance in Europe was done in early 1900s with burning furniture and transferring the tenants to new bed bug free housings, along with rigorous steam disinfecting and fumigation of the beddings and personal belongings with hydrogen cyanide [1,22]. Scotland invented Glasgow System, which reinforced education of the tenants by the Public Health Department informing the importance of cleanliness, bed bug infestations, behavior, and treatments, instructions of rigid prevention protocols, providing inspections of the dwelling at the beginning and at least monthly for the first three months [1]. People were aware of bed bugs, and precautions were taken regularly such as checking the suitcases and clothing during and after traveling, periodical inspections and cleaning of the entire house especially beddings, and replacing heavy wooden beds with simple metal framed beds [1]. Coal oil was used to clean furniture, and beds and furniture were kept away from the walls [1]. Catching traps such as wooden board and a flap of felt, trap made by basket makers, common bean leaves that had trichome hooks to trap bed bugs while crawling, were also used [1]. Despite of the great efforts in 1800s and earlier 1900s, bed bugs were still one of the greatest concerns during the World War I and II as soldiers often suffered with high infestations [1].

Chemical insecticides became the mainstream of the bed bug control methods around the middle of 1900s [1]. Earliest fumigation was burning sulfur [22], a method called fire and brimstone, which produced sulfur dioxide and trioxide that were effective to control all the life stages of bed bugs including eggs [1]. Sulfur was less toxic to the residents than other fumigants, ready-made candles and powders were relatively affordable and easily applied [22], but penetration rate was poor so it had to be applied multiple times in some infestations [1]. Sulfur also easily corroded metals, so had to be coated with lard or Vaseline [1,22]. Fumigation using hydrogen cyanide (hydrocyanic acid as in water formulation) was used during the holocaust in concentration camps as *Zyklon B* pellets and powders, which was also found to be very efficient and 100% effective for bed bug control [1]. It was soon widely used in army facilities and residential settings by exterminators with gas masks, but it was extremely dangerous since the gas could cause unconsciousness in seconds and then death in minutes [1]. Although it was recommended to be used by the professionals, it was available from local pharmacist, consequently killing or injuring many with or without proper training and equipments [1]. However, hydrogen cyanide fumigation was the most effective and efficient [22], it was commonly used till DDT was invented and used in the middle of 1900s [1]. Other chemical compounds used to control bed bugs were rotenone, phenol, cresol, naphthalene, Lethane 384, kerosene, turpentine, benzene, gasoline, and alcohol [1,22]. Those chemicals and fumigation did not have any residual effects, so bed bugs could come back to infest again [1,2].

The biggest success on controlling bed bug infestations happened in early 1940s, when Dichloro-diphenyl trichloroethane (DDT), a chlorinated hydrocarbon (organochlorine), was found to have long residual insecticidal activity against bed bugs [1–3]. Evaluated by USDA Bureau of Entomology and Plant Quarantine, it was reported as the excellent treatment method for the bed bug control [1]. The residual effect was remarkable lasting months [2] or even after 3 year of application [1]. DDT was typically formulated as 5% spray (oil-based, such as kerosene), 10% powder, and DDT-impregnated wall papers and paints, and was available to general public by 1945 in the most stores and food markets by the pesticides companies [1]. DDT was inexpensive and could be applied to almost everything, including beds, clothing, and baby toys, without gas masks, heavy equipments, specialized exterminator, or having to prepare for the application [1]. DDT application by general public in their households was so successful that bed bugs were hard to find within 5 year of application [1,2].

Although DDT was successful in eradicating most of the infestations throughout the world, DDT resistance in bed bug populations were started to be noticed by 1947 [1,2]. The alternative insecticides such as 1% malathion in spray formulation, so as diazinon, lindane, chlordane, and dichlorvos were usually effective in treating DDT resistant bed bug population [1]. Bed bugs were only rarely encountered since then mostly in military facilities but had never been a public concern for the next 50 year in the United States or developed countries, until the sudden resurgence in late 1990s [1,23]. Further discussion about bed bug insecticide resistance and resurgence is discussed in the following sections of this article.

The historical methods to control bed bugs might have helped reducing the bed bug infestations, but many of these residual insecticides are not currently available in the market because of the health risks associated with many of the previously used insecticides. Fast development of the insecticide resistance is also a concern while the chemical insecticides treatments are the common control practice currently used to treat bed bug infestations [24,25]. People, including pest management professionals, in modern societies have never experienced bed bug infestations until last 15 year, and people are accustomed to rely on insecticides. In the past, people took precautious preventions and inspections on a regular basis, and used some alternative treatments such as using steams and rigorous house cleaning in case of bed bug infestations [1]. Perhaps one of the most important factors contributing to the recent bed bug resurgence might be the lack of awareness, knowledge, and experience about bed bugs among the general public [3].

3. Resurgence and insecticide resistance

3.1. Recent bed bug resurgence

Bed bugs were effectively eradicated after the World War II from the United States and other industrialized countries after the widespread use of residual synthetic insecticides [26] such as DDT and malathion [1,3,27], with the greater public awareness to the pest control [26]. In the second half of the 20th century, bed bug infestations only remained mostly in developing countries in middle East, Africa, Central and South America, and some countries

in Europe [11], such as seen in heavy infestations in residential home settings in Tanzania (56% of homes) and India (65% of homes in Hyderabad) [26]. In contrast, worldwide resurgence of the bed bugs has been observed mostly in developed countries within the past 15 year such as from Canada [17], Australia [28], the United Kingdom, Germany, Czech Republic, one of the Arabian Gulf States [26], Italy [29], Denmark [30], Thailand [31], Japan [27,32], etc. In the United States, pest control professionals have been recognizing significant increase in the number of bed bug infestations nationwide since 1998 [33].

Currently, bed bugs have been encountered increasingly in both public and residential settings [33,34], and in poultry facilities [35–37]. The recent surveys conducted nationwide to professional pest control companies in the United States revealed that 95% and 99% of the respondents treated bed bug infestations or asked to treat infestations in year 2009 and 2010 [38]. The sites of bed bug encounters by the pest control professionals were: hotels and motels (80%), college dormitories (54%), nursing homes (46%), office buildings (38%), schools and day care facilities (36%), hospitals (31%), public transportations (18%), movie theaters (17%), clinics (23%), retail stores (21%), laundries (9%), libraries (8%), and restaurants (6%) [38]. Locker rooms, storage facilities, moving vans, prisons, police cruisers, and even in summer camps and funeral homes have been also reported with bed bug encounters [38]. Currently, bed bugs have been reported from all 50 states in the United States [3], and one in five Americans has had bed bug infestations or knows someone who has encountered bed bugs at home or hotels [39].

Several hypothesis to the sudden worldwide bed bug resurgence have been speculated, such as change in our life styles, increased complexity of our systemic modern society [40], increased traveling and immigration [3,23,41], lack of awareness [3], changes in pest management practices and pesticide application methods [3], availability of residually potent insecticides [3,11,23,30,42,43], and the high levels of insecticide resistance among the bed bugs [11,24,44]. Insecticide resistance in bed bugs is discussed later section in this article, and also elsewhere in this special issue publication of this journal articles.

The increase in national and international traveling must have been one of the significant factors contributing to the spread of bed bug infestations [26,29,40]. The genetic analysis of the bed bug populations among 21 infestations collected from eastern parts of the United States revealed high genetic diversity across the populations with no significant correlation to their geographic distributions, strongly suggesting the multiple introductions of the bed bugs into the United States from foreign origins [45]. No correlation of the genetic haplotypes to geographic locations also has been confirmed in some other study suggesting the bed bug populations are highly mixed and traveled [46]. The species of bed bugs that have been reported in the past 15 year increasing the number of infestations in the United States and many other developed countries are mostly the common bed bug, *C. lectularius* L. [3], except in Australia where both *C. lectularius* L. and *C. hemipterus* F. are increasing [28]. However, the bed bug populations that have continuously been infesting developing countries in tropic regions after the World War II is primarily the tropical bed bug, *C. hemipterus* F., not the common bed bug, *C. lectularius* L. [26]. Therefore, the majority of the bed bug populations in the United States and in many other countries that have been concerned the past decades are unlikely originated from the countries in tropical region [26] but from elsewhere in temperate region.

An alternative and novel hypothesis to the sudden resurgence of bed bug populations in the United States is the isolation of bed bug populations from human host to birds and bats during the past few decades, then sudden removal of the barriers allowing these populations of bed bugs to rapidly come back and spread in human environment [11,46]. The analysis of both mitochondrial and nuclear DNA markers among 22 populations of bed bugs collected through various locations in the United States including single family homes, multi dwellings, a hotel, hospital, shelter, laboratories, and poultry facilities, revealed the presence of high heterozygosity in mitochondrial DNA, which supports the bed bug populations in the United States did not go through a genetic bottleneck, as some previously had suspected [46]. The bed bug populations may have been maintained elsewhere perhaps some alternative hosts, such as birds [36,46] and bats [46]. No genetic variations in nuclear rRNA marker was observed suggesting increased gene flow among these populations of bed bugs, including the populations collected from poultry facilities [46]. Bed bug infestations have been reported from poultry facilities often from breeder houses and less frequently from broiler houses [35]. These populations of bed bugs could have been the reservoir of the recently increased bed bug populations in the United States in urban environment [46], because accidental translocations of the bed bugs by the poultry facility workers from severely infested poultry houses to other facilities and other human structures such as homes and the modes of transportations are easily possible [35,36].

3.2. Insecticide resistance as a factor of resurgence

Although the origin of the common bed bug, *C. lectularius* L., in the United States and other developed countries that have been increasing infestations since after the turning of the century is still unknown, the widespread of insecticides resistance among the field populations of bed bugs might be one of the most important factor contributing to the recent bed bug resurgence [11,42]. A field population bed bugs collected in 2006 from Arlington, VA, was more than 300-times less susceptible to 0.06% deltamethrin (Suspend® SC) compared to a laboratory strain, originally collected from Fort Dix, New Jersey, more than 30 years ago that was kept without insecticides exposures, measuring the lethal time to kill 50% of the tested bed bugs (LT50) on insecticide treated panels [47]. Similarly, two field population bed bugs collected in 2008 from a group home in Richmond, VA, and a hotel room in Cincinnati, OH, were both more than 300-times resistant to deltamethrin (0.06%; Suspend® SC) than the laboratory susceptible strain using the similar LT50 tests [48]. The resistance levels of the same Richmond strain using injection bioassay measuring the lethal dosages to kill 50% (LD50) were even greater, reporting resistance levels nearly 5200-fold to deltamethrin and 111-fold to β-cyfluthrin compared to the laboratory susceptible strain [49]. Four field populations of the bed bugs collected from Kentucky and Ohio were not affected by deltamethrin or λ-cyhalothrin when exposed to fresh residues 200–300 times higher than the recommended label rates, whereas two of their laboratory strains collected from New Jersey and Florida, that were kept in laboratory environment without insecticides exposures for more than two decades, reached 100% mortality at concentrations below the label rates [23,24].

Direct spraying of the commercially formulated Suspend® SC (deltamethrin) and Demand CS (λ-cyhalothrin) was not different from the filter paper testing, they killed only a small percentages of the bed bugs in the field [23]. In case of the third-fifth instar nymphs, 14 out of the 16 field populations of bed bugs collected from Kentucky, Ohio, Michigan, New York, Massachusetts, Virginia, Florida, and California, were found to be highly resistant to the technical grade deltamethrin, ten times the labeled rate (0.13 mg/cm^3) reaching 0% mortality, where only the two laboratory strains and one of the field strains collected from California reached 100% mortality within 24 h of exposures [23,24]. A mixed population of bed bugs collected from residential homes and apartments in New York City was 264-fold less susceptible to 1%

deltamethrin compared to a laboratory strain collected from Gainesville, Florida, more than 20 years ago [50]. Neither aerosol formulation of over-the-counter pyrethroids foggers (Hotshot Bedbug and Flea Fogger, Spectracide Bug Stop Indoor Fogger, and Eliminator Indoor Fogger) showed little or no mortalities to the five field populations of bed bugs collected from residences in Columbus, Ohio, between 2010 and 2011, when bed bugs were provided optional harborage [51].

Insecticide resistant bed bug populations were not only reported from the United States or among the common bed bug, *C. lectularius* L. populations, but also from other countries and the tropical bed bug, *C. hemipterus* F. populations [52]. The tropical bed bug, *C. hemipterus* F., populations from three districts of Sri Lanka, were reported to have DDT (organochlorine), malathion (organophosphate), and propoxur (carbamate) resistances, even though DDT had not been used for 25 years [53]. In Thailand, both *C. lectularius* L. and *C. hemipterus* F. populations have been collected from various hotels in wide geographical areas, and all the populations showed resistance to organochlorines (DDT, dieldrin), organophosphates (malathion, fenitrothion), carbamates (bendiocarb, propoxur), and pyrethroids (cyfluthrin, deltamethrin, permethrin, λ-cyhalothrin, etofenprox) when tested with the conventional bioassay based on the World Health Organization insecticide-impregnated paper assays [31]. In several villages in Tanzania, the use of pyrethroid-treated bed nets to control malaria mosquitoes initially eradicated the local tropical bed bug, *C. hemipterus* F., populations that had been infesting the majority of the houses [44]; however, the bed bugs reappeared after 6 years of disappearance in these villages showing resistance to alphacypermethrin and permethrin, while the bed bugs in other villages without bed nets did not have resistance to the pyrethroids insecticides [44].

The insecticide resistance to pyrethroids among bed bugs have only been reported within the past few decades, however the insecticides resistance to DDT and other classes of insecticides among the bed bugs has already been documented widely since 1947, only several years after the DDT application started worldwide [2]. The first DDT resistant population of bed bugs were reported from barracks of the Naval Station at Pearl Harbour, Hawaii [2]. By the 1950s, DDT resistance was reported worldwide from Greece, Poland, Israel, Lebanon, India, Belgian Congo, French Guiana, Gambia, Somaliland, Iran, Taiwan, Hong Kong, Singapore, Bombay State [11], Japan, Korea, Ohio, the United States naval vessels on boards [11,54]. Several bed bug populations collected in Israel were also resistant to gamma-BHC (lindane, a hexachlorocyclohexane organochloride) at low levels [55]. The National Pest Control Association on the United States was suggesting to use malathion, an organophosphate, as an alternative insecticide to replace the DDT in order to control the DDT resistant bed bug populations by 1956 [11]. The bed bug populations resistant to both DDT and gamma-BHC in Israel were successfully controlled by the use of malathion [55]. While DDT was removed from registration in the United States in 1972 and also from many other industrialized countries thereafter, the insecticide resistance to DDT in bed bug populations persisted through the 1960–1980s in some other counties where DDT has been used to control mosquitoes [11]. In recent study, three out of the four field collected bed bug populations that were confined on the surface treated with 5% DDT were resistant, showing only 5%, 10%, and 30% mortalities after five consecutive days of exposure [23].

The most recent studies of the insecticide resistance mechanisms in bed bugs using 24 populations revealed that more than 70% of the studied populations possessed multiple mechanisms of insecticide resistance such as knock-down resistance (kdr), increased metabolic activities of detoxification enzymes cytochrome P450s, ABC transporters (ATP-binding cassette protein transporters), and up-regulation of the cuticular-related protein genes that might be involved in reduced cuticular penetration [56]. A study conducted using next-generation RNA sequencing (RNA-Seq) of three insecticide resistant and three susceptible populations also found involvement of the putative cytochrome P450s, ABC transporters, and cuticle protein genes from the up-regulated ESTs, also suggesting the multiple mechanisms of insecticide resistance in bed bugs [57]. The multiple mechanisms of insecticide resistance exhibited in a single population of bed bugs was also documented from a population collected from Virginia that exhibited (1) L925I amino acid substitutions in a voltage-sensitive sodium channel gene that confer kdr resistance, (2) enhanced activities of the general esterases (>35%) and cytochrome P450 monooxygenases (41%), and (3) over-expression of several genes related to cytochrome P450s, esterases, and one GST [49]. The first molecular evidence of insecticide resistance was reported from the two mutations on the voltage-gated sodium channels genes causing two amino acid substitutions, V419L and L925I, in bed bug populations collected from New York that showed high levels of pyrethroid resistance to deltamethrin that confirmed kdr resistance in bed bugs [50]. The same mutations were investigated in 110 bed bug populations collected within the United States, and 88% of the bed bug populations were found to contain at least one mutation or both mutations in tested populations [58]. The transcriptomic analysis of the pyrethroid resistant bed bug populations collected from Ohio revealed the transcripts of cytochrome P450 genes were elevated, and the quantitative PCR (qPCR) analysis also showed elevated levels of the cytochrome P450 mRNAs in all stages of bed bugs, particularly highest in early instar nymphs [59]. In this population of bed bugs, glutathione S-transferases (GSTs) genes were expressed in the late instar nymphs, but no significant levels of GSTs genes were expressed in adults [59]. A genetic crossing of an insecticide resistant bed bug with a susceptible bed bug resulted an intermediate level of resistance in F1 offspring [23,24]; this suggested the inheritance of the insecticide resistance related genes in this bed bug population was not simply dominant or recessive genes, but rather an incomplete dominance involving one or more genes [23,24].

Insecticide resistance in bed bugs has been contributing the worldwide bed bug resurgence [24], and insecticide resistance is most liable explanation for the bed bug resurgence [11]. The widespread of insecticides resistance in field bed bug populations and the multiple mechanisms of resistance within a population significantly hinders bed bug control efforts, if only traditional chemical control methods using insecticides application was used in the field. Since bed bugs has been showing insecticide resistance to the all classes of insecticides, re-registration of the previously available insecticides is less likely to control current populations of bed bugs. The alternative but effective control methods and insecticides with new modes of action are already being applied in order to prevent fast spreading of the bed bug infestations [60].

4. Current control methods

4.1. Chemical control methods

Chemical treatments using insecticides are still the most used control method to treat bed bug infestations today. Almost all (99%) of the pest management professionals in the United States are applying insecticides, among all other currently used methods such as mattress encasements (86%), laundering (86%), vacuuming (65%), disposal of infested items (62%), and steaming (43%) [38]. Pyrethroids are the most common type of insecticides used by the pest management professionals in Europe and Australia [11]. Although pyrethroid resistance is widespread in field populations of bed bugs [11,23,24,58,60], 77% of the pest management

professionals are still reporting satisfactory levels of bed bug control using chemical control methods [38]. Change in regulatory restrictions have banned other classes of insecticides that had longer residual effects toward bed bugs such as organochlorides (DDT), organophosphates, and carbamates [11,23]. Only pyrethroids, insect growth regulators (IGRs), and pyrroles, are still permitted to use indoors for treating bed bug infestations [23].

The pyrethroids insecticides currently available to treat bed bug infestations in the United States are deltamethrin, permethrin, phenothrin, resmethrin, tetramethrin, bifenthrin, lambda-cyhalothrin, cyphenothrion, esfenvalerate, zeta-cypermethrin, cyfluthrin, beta-cyfluthrin, prallethrin, imiprothrin, bioallethrin, and gamma-cyhalothrin [61]. Pyrethrins are also registered for bed bug insecticides products [61]. According to a recent survey by the pest management professionals, beta-cyfluthrin (TempridTM, formulated with imidacloprid, a neonicotinoid), sumithrin (Bedlam®), bifenthrin (Transport®, formulated with acetamiprid, a neonicotinoid), deltamethrin (Delta Dust® and Suspend® SC), and cyfluthrin (Tempo®), are the most commonly used insecticides to treat bed bug infestations (42%, 28%, 15%, 12–11%, and 11%, respectively) [38]. A field study conducted using beta-cyfluthrin (Tempo® SC Ultra), deltamethrin (Suspend® SC), and hydroprene (Gentrol®), the traditional bed bug treatment, in low income housing apartments significantly reduced the bed bug infestation [43]; however in laboratory studies, pyrethroid products were not significantly effective against a field strain bed bugs [47], and they did not kill 100% of a susceptible strain, either [62]. Interestingly, in another study, type I and type II pyrethroids showed different levels of toxicity (<5% and >80%, respectively) when applied to a field population of bed bugs [63]. To increase the toxicity of pyrethroids insecticides, synergists such as piperonyl butoxide (PBO) and MGK 264 (N-Octyl bicycloheptene dicarboximide) are often formulated with the pyrethroid insecticides [64]. However, the addition of such synergists may not always work to control pyrethroid resistant bed bug populations [11,65]. For example, PBO and MGK 264 are the inhibitors of cytochrome P450 monooxygenases [64], but insecticide resistance in a population of bed bugs may also involve other mechanisms of resistance [60,65]. Many pyrethroid insecticides are currently available to the general public in retail stores as over the counter products, some specifically labeled for bed bug treatments [51]. However, a recently study found that none of the three foggers that are available to the general public consumers over the counter were effective to control field collected pyrethroid resistant bed bugs [51]. The efficacy of pyrethroid insecticides seems to vary greatly among different bed bug populations [24,47,65], formulations [63], and specific pyrethroids and products [47,62,63].

Only a few types of neonicotinoids are currently available in the United States to control bed bug infestations today, such as acetamiprid, imidacloprid, and dinotefuran [61]. As described above, two of the most commonly used insecticides by the pest management professionals are TempridTM and Transport® [38,60,66], that ranked in the second and the sixth of the most commonly used insecticides for bed bug treatments [38]. When TempridTM and Transport® were exposed to bed bugs as fresh dried residues, they effectively killed field collected pyrethroid resistant bed bugs in a constant surface exposure test, reaching 100% mortality within several days [66]. In contrast, bed bugs that were exposed to Suspend® SC (0.06% deltamethrin) never exceeded 72% mortality after four days of exposure using the same test [66]. By combining pyrethroids and neonicotinoids, bed bugs are exposed to two different pesticide classes with different modes of actions, which increases efficacy against pyrethroid resistant populations [66]. Theoretically, it is assumed that developing two mechanisms of resistance in parallel to two classes of different insecticides with different modes of actions might be more difficult [60]. However, since most of the current populations of bed bugs in the field have already developed multiple modes of insecticides resistance mechanisms [56], it is possible that the bed bugs may easily develop insecticide resistance to neonicotinoids as well [60,66].

Insect growth regulators (IGRs) or juvenile hormone (JH) analogs, such as S-hydroprene, ethofenprox, pyriproxyfen, and cold-pressed Neem oil are also available (EPA) and used for the control of bed bug infestations in the United States [38]. Currently, hydroprene (such as Gentrol®) is the fourth most common (16%) insecticide used in the United States by the pest management professionals to control bed bug infestations [38]. However, a laboratory study using hydroprene (Gentrol®) showed no delay on molting among nymphs, and all of the surviving nymphs (72–98%) successfully molted to adults in 4–5 weeks [62]. Some of the adults successfully produced F1 progeny, early instar nymphs, by the end of the study [62]. However, hydroprene also caused significantly higher mortality (66–100%) among the adults and nymphs, rupturing their gut after feeding and filling the haeomocoel and legs completely with blood [62]. This high mortality in early adulthood may have some impact controlling bed bug infestations [62]. Because IGRs are slow acting, currently they are often used or formulated to aid more fast acting, other classes of insecticides [23,25].

Pyrroles are a new class of insecticide available to treat bed bug infestations. Pyrrole is a contact and stomach poison [64] activated by cytochrome P450 enzymes [60]. Once activated, pyrrole becomes toxic by inhibiting the production of ATP in the mitochondria [60]. The only pyrrole insecticide available is chlorfenapyr [64], and is registered for bed bug control in the United States [25,60,61]. Currently chlorfenapyr (Phantom®) is the most popular insecticide used by the 51% of the pest management professionals in the United States to treat bed bug infestations [38]. Chlorfenapyr has longer residual effects compared to pyrethroids [60] remaining effective as a residue four months after being sprayed [25], but chlorfenapyr is significantly slowed acting [23,47,66]. Furthermore, chlorfenapyr did not prevent pyrethroid susceptible bed bugs from mating, laying eggs, and hatching from the eggs during the two week periods of continuous exposure [47]. In the field, it is unlikely that bed bugs remain exposed to chlorfenapyr treated surfaces for a prolonged period of time, so the practical effectiveness of using chlorfenapyr is questionable [47,60]. Therefore, chlorfenapyr is currently used in combination with more fast acting insecticides [66].

Organophosphates and carbamates are not readily available in the United States and other countries for use in the household [11]. One exception is the organophosphate 18.6% dichlorvos (DDVP) strips [3,60,67]. DVVP is applied to the inside of an impermeable plastic bag or containers lightly filled with bed bug infested items, tightly closed and sealed to allow the volatile DDVP vapor to fill the bag and kill both adult bed bugs and eggs [3,67]. However, DDVP can take more than 2 weeks to kill bed bugs, and still may not reach 100% mortality, if the internal air was not circulated sufficiently in the bag [60]. Some materials such as computer keyboards, book-binding, and the toe of a shoe had lower mortality at two weeks, so DDVP treatment may need to be administered for longer time periods and allow proper air circulation for items and materials that are harder to penetrate [60]. Temperature also affects the volatility of DDVP, so that lower ambient temperature should be avoided [60]. Propoxur (1%), a carbamate insecticide, is also available to control bed bug infestations only by prescription bases to apply to commercial and industrial buildings, hospitals, nursing homes, warehouses, motels, food storage areas, food processing facilities, public transportations except for aircraft, as long as children and elderly will not be exposed [68]. In Asia, Mexico, and Central and South America, where organophosphates and carbamates are still available for bed bug treatments, bed bugs are not

considered a difficult pest to control [38]. Organophosphates and carbamates are often effective against controlling bed bugs that are resistant to pyrethroids insecticides [38]. However, as it was discussed above in previous section of this article, bed bugs have shown resistance to organophosphates (malathion, fenitrothion, diazinon) and carbamates (bendiocarb, propoxur, fenobucarb) in a countries where both insecticides have been applied to control bed bugs [31]. Organophosphates and carbamates may not remain effective for long as bed bugs populations continue to develop resistance.

Boric acid, silica gel, and diatomaceous earth (Silicon dioxide) are used as desiccants for bed bug treatments in the United States [61]. The desiccants stick to the outer surface of the bed bugs and absorb the insect epicuticular wax layer [60,64,69]. Exposed bed bugs die within a few days [69]. Desiccants are often used in conjunction with pyrethroids to formulate dusts, but also can be applied without pyrethroids [61,69]. Diatomaceous earth is one of the safest insecticides on its own, when it is not formulated with other insecticides as it is also available as an animal food additive [69], and can be applied to broad range of environments [61,69]. Heat-treated filter grade diatomaceous earth is not registered for bed bug treatments because the structure of the diatomaceous earth is changed and is an inhalation hazard [69]. A study found that dust formulated insecticides did not lose efficacy after 24 weeks, whereas spray formulated products showed reduced residual toxicity after the same time period [63]. In the same study, the survival half-life (S1/2) of bed bugs exposed to silica dusts were not significantly affected with or without 1% pyrethrins or PBO, suggesting the efficacy of the tested products were a result of using the desiccant but not the pyrethrin [63]. Another study found that installing "dust bands", consisting of 3.8 cm wide fabric band on furniture legs and then brushing Tempo® dust (1% cyfluthrin), decreased bed bug infestation by 95% in 12 weeks, compared to 85% reduction observed using quick insecticidal spray application done by the contract pest management professionals [70]. Desiccant dusts and dust formulated insecticides might be a practical alternative to control insecticide resistant bed bug population of bed bugs [11].

Gas fumigation using sulfuryl fluoride, sold as Vikane® [71], Zythor®, and Master Fume® have been used to treat structural damaging insects like termites [64,72–74]. Fumigation is extremely effective in controlling structural pests but has not been applied widely to control general household pests, including bed bugs, because the cost is considerably more expensive [72]. Using fumigation in the United States for bed bug treatment is underutilized [71] as it is only used by 16% of pest management professionals [38]. Fumigation may be infrequently used because many of the previously registered fumigants such as hydrogen cyanide, methyl bromide, dibromochloropropane (DBCP), and chloropicrin (tear gas) [75] caused severe injuries and deaths in the early 1900s [1]. However, high volatility [64,75] and the ability to diffuse easily into large masses [64] make fumigation an ideal control method in wall voids, crack and crevices, various personal belongings, and furniture [72]. Fumigation is also useful treating items that other treatment options are not feasible [71] such as musical instruments and other valuable items. Fumigation is also ideal to treat areas with children and elderly as multiple exposures to residual insecticides can be harmful [72]. Sulfuryl fluoride was 100% effective at treating infestations [71,72], killed all the bed bugs in all states including eggs, and fumigated eggs did not hatch after 2 weeks of the treatment [72]. There was no residues after aeration, and successfully penetrated all types of furniture [71,72]. Fumigation using CO_2 was also found to be effective alternative to control bed bugs [76]. The CO_2 fumigation was conducted using 158 l (42 gallon) heavy duty garbage bags or double-zipper plastic bags filled with fabrics and boxes and various stages of bed bugs then fumigated using dry ice or tanked CO_2; the fumigation successfully killed 100% of all the bed bug life stages when the bags were fumigated with $\geqslant$94% CO_2 for 24–48 h in room temperature [76]. The minimum lethal concentration of CO_2 was approximately 30% in 25 °C with 24 h of exposure, but the eggs were not completely killed if the concentration was lower than 80% [76]. Since bed bug treatments are already quite expensive [9,77,78] generally needing multiple treatment applications, fumigation is also used as an alternative option for treating bed bug infestations in the places where it is available.

Many insecticide formulations are available as liquids, dusts, or aerosols for bed bug chemical treatments. Liquid formulations are generally applied to ceiling wall junctions, baseboards, crack and crevices [79]. Dusts are commonly applied in wall outlets, wall voids, and on the floor under the baseboards [79]. Aerosols are mostly applied to mattresses, box springs, furniture, and crack and crevices, but the labeling instructions may vary by the products [79]. Didecyldimethylammonium chloride (DDAC), benzalkonium chloride (ADBAC; alkyl dimethyl benzyl ammonium chloride), isopropyl alcohol, and 2-phenethyl propionate are also added to insecticide formulations [61]. The most common insecticide formulation used currently in the field by the pest management professionals in the United States to control bed bug infestations are liquids (96%), followed by dusts (91%), aerosols (52%), insecticide impregnated resin strips (21%), fumigants (16%), total release foggers (2%), and miscellaneous others (1%) [38]. Changes in insecticide formulations from alcohol and water-based insecticides to emulsifiable concentrates and pressurized aerosols seem to be more effective, as long as bed bugs were sprayed directly [23]. Dusts formulations containing silica gel or diatomaceous earth kill pyrethroid resistant bed bugs [23,63], but application sites are limited [23] to minimize human exposure [63].

Chemical treatment is still an important part of the bed bug control, and has been reported to be effective in the field [38]. Changes in insecticide formulations have improved the efficacy of the available insecticides [23], and some seem to have higher efficacy and residual effects than the other formulations [23,63]. Chemical treatments are currently used in conjunction with other types of control methods such as mattress encasements, laundering, vacuuming, etc. [38]. Today, people are more concerned about using insecticides [1], as reflected by the development of more selective, environmentally biodegradable (and less residual) compounds with less mammalian toxicity. Insecticides with longer residual activities are not readily available to pest management professionals [1], and bed bugs have developed multiple mechanisms of resistance towards several classes of insecticides currently available. However, heavy reliance on chemical treatment will result in higher selection pressure, amplifying the frequency of resistance genes in a given population of insects [64,80]. Resistance to hydrogen cyanide, a fumigant, was observed in scale insects [64], so it is possible that bed bugs can develop resistance to all classes of chemical insecticides including fumigants, if chemical treatments are used intensively. For the reasons stated above, heavy reliance on chemical treatments should be avoided to discourage further development of the insecticide resistance in bed bug populations. Using alternative control methods, such as non-chemical control methods, is also an essential component to a successful bed bug control.

4.2. Non-chemical control methods and integrated pest management (IPM)

In bed bug management non-chemical treatments are not simply an option or a substitute for chemical treatments [81]. Unlike management for other pests, in bed bug management

non-chemical treatments are mandatory [81]. Non-chemical treatments should be the first in the list of treatment methods, and chemical treatments should be used less intensively in spot treatments [81]. As bed bug populations become increasingly resistant to insecticides [11,23,25], non-chemical treatments are now essential components in current bed bug management practices [3,25]. Bed bug control efforts often fail if chemical treatments are only used [3]. Currently, effective chemical treatments are lacking, and the general public is seeking for more environmentally safe treatment options [77]. Chemical treatments often pose health risks [77], and can bring an economic burden [9,77,78] due to the need of repeated applications. The lack of public awareness [1] is also leading to the late detection of bed bug infestations, the increase in infestation levels, and the rapid spreading of bed bug infestations [3,77].

In the past decades, our society has become more interconnected and complicated [40]. In such society, it is important to identify and treat the reservoirs of bed bug infestations in order to effectively control bed bugs [40]. The increase of personal belongings and clutter in dwellings has also brought a new challenge for the control of bed bug infestations; it provides additional harborages for the bed bugs, making the population more dispersed and difficult to eliminate [69,77,78]. Currently, successful bed bug control does not only rely on the application of insecticides to infested items, but also on an integrated approach using a variety of non-chemical treatment efforts, such as mattress encasements, extreme temperature treatments, physical removal of the bed bugs [3,69], community-wide education, awareness, and cooperation [3,77,78]. The development of non-chemical bed bug control methods has been mostly based on the trials and errors in the field, because little research has been carried to evaluate the efficacy of the treatment methods [81]. Many of the treatments have been evaluated only based on the numbers of re-treatments [81].

A current fundamental non-chemical treatment, part of a reputable bed bug management program for a successful bed bug control, is the installation of mattress encasements [69]. Bed bugs are mostly found where the host sleeps and rests [3]. A study found that 70% of a bed bug population was associated with beds, 23% with upholstered furniture, and only 7% were found in areas not associated with beds [3]. In the early stages of infestation, bed bugs are most likely to be located close proximity to beds and upholstered furniture [3]. Bed bugs start being found outside of these areas more often as the infestation level increases [3]. Typically, bed bugs form aggregations in cracks, crevices, and seams of headboards, bed frames, mattresses, box springs, and upholstered furniture [3,16]. Replacing the infested mattresses and box springs would remove a large portion of the infestation, but it can be expensive, unnecessary, and often not effective [82]. Replacing mattresses and box springs are not effective controlling bed bug infestations because the new mattresses and box springs are frequently re-infested by bed bugs from surrounding harborages [3,82], such as other furniture, personal items, wall voids, behind base boards, and floor boards. Box springs provide excellent harborage for bed bugs [69,82,83] because box springs provide complex environments where bed bugs can be easily hidden and protected from many of the currently available control practices [69,82]. Furthermore, the insecticides that are currently available for mattress and box spring treatments are also limited and often non-residual [61]. Some pest management professionals would prefer not to apply insecticides on mattresses and box springs [82]. It is more important to encase the box springs if one cannot afford to encase both the box spring and the mattress [69]. During the course of the bed bug treatment, it is essential to install mattress and box spring encasements because not only it prevents bed bugs from further infesting the mattresses and box springs [69,77,82,83], but also it physically encloses bed bugs preventing them from feeding and eventually killing them by starvation [69,77,82]. Mattress encasements can also be used as preventative method when getting new mattresses and box springs to protect them from being infested [3,77,82], or in the absence of bed bug infestations [3,82]. Encasements for mattresses and box springs also make bed bug inspection and treatments much easier, efficient, and successful [3,69,77,81,82], because bed bugs are restricted to only the exterior surface of the encasements that can be easily detected and treated with other control methods [3,82]. There are several manufacturers of encasements on the market, however, effective encasements have to be durable [3,82], have a specially designed tight teeth zipper that completely seals the entire box springs, securely close at the end stop in order to prevent bed bugs from entering or escaping [82,83], be smooth, with no folds or pockets that provide area for bed bugs to hide or lay eggs, and prevent bed bugs from feeding from inside the encasement (bite-proof material) [82]. Felt strips can be applied to rough and uneven surfaces of the bed frames to prevent ripping the box spring encasements [3]. Currently, 86% of the pest management professionals in the United States are using mattress encasements as one of the methods to treat bed bug infestations; 81% is treating the infested beds with insecticides, and among them 73% is treating both the mattresses and box springs with insecticides [38].

Exposure of bed bugs to extreme temperatures such as hot temperature laundering and drying [3,60,81,84], steam [3,69,81], portable heat boxes [3,60,84], whole house heat treatments [3,60,69], and application of CO_2 snow, are also currently being used as non-chemical treatments for bed bug control [3,60,69]. These methods are commonly used in the control of the structural, wood, and stored food products pests, and have been adopted for bed bug control [3]. Some studies found that the common bed bug, *C. lectularius* L., became less active at temperatures approximately 13–15 °C (55.4–59 °F) [2], but could tolerate as low as −15 °C (5 °F) for overnight [85]. At −18 °C (−0.4 °F), 100% of adults and nymphs died in one hour [85]. Another study reported that bed bug adults and nymphs tolerated temperatures below freezing (0 °C or 32 °F) at least for several days, and eggs survived up to 30 days [3]. Experiments testing upper threshold temperatures found that the common bed bug development ceased at 36–37 °C (96.8 °F–98.6 °F), whereas tropical bed bugs *C. hemipterus* F. had higher temperature threshold [2]. In Johnson (1941), all bed bug eggs survived 100% after 1 h treatment of 43 °C (109.4 °F), but conversely all died at 45 °C (113.0 °F). In the same study, the thermal death-point of adult bed bugs was 1 °C lower than that of eggs [85]. Other studies reported that bed bugs and eggs died in less than one minute in 50 °C (122 °F) [3,86]. This temperature (50 °C) and duration (1 min) for heat treatment is being currently used as a standard by most pest management professionals [86]. The thermal death was found to be 40 °C (113 °F) for 15 min for bed bug adults and nymphs, and 60 min for eggs [3]; but the adults survived up to 90 min at 45 °C (113 °F) and up to 8 h when the temperature was slowly raised (14.2 °C or 6.5 °F per hour) [3]. Bed bugs were also found to survive starvation for extended periods of time especially at lower temperatures [2]. Usinger [2] stated that the mean survival days in starvation were 142.6 for adult bed bug males and 83.7 days for first and second instars, at 22 °C and 40–45% relative humidity (RH). Based on these information, leaving bed bug infested items outside in the cold is unlikely to kill the bed bugs [3], and leaving a room unoccupied for days will not get rid of bed bug infestations, either [9,34]. Similarly, leaving bed bug infested items in a closed vehicle [60] or black plastic bags [9] under the direct sunlight is also unlikely to be effective in killing bed bugs, because the internal vehicle temperature may not reach a lethal temperature for enough time, except for warmer seasons [60]. Contrarily, hot temperature drying kills bed bugs and their

eggs [69] in as little as 5–10 min, even in medium to high heat cycles [38]. Currently 86% of the pest management professionals are recommending laundering the infested items to their clients [38]. The infested clothing must be contained in plastic bags and sealed until they can be washed [81]. However, the use of hot temperature dryer to eliminate bed bugs from infested items is not infallible, because dryers vary in their upper temperatures and some even have automatic shutoff failing to reach an appropriate temperature for enough time [60].

Commercial portable steam units with head attachment that reach temperatures up to 100 °C (220 °F) are also currently being used by 43% of the pest management professionals (Potter 2011 survey) to kill both live bed bugs and eggs [3]. The steam can reach up to 6 cm deep within cracks and 2 cm below the fabrics [3], so it is often applied on mattresses [3,69], couches [69], cracks and crevices on furniture, and upholstered furniture [3]. Puckett et al. [87] recently found that the application of steam treatment at a rate of 10 s per 30.50 cm was sufficient to kill all bed bug adults, nymphs, and eggs, when the steam head was placed directly on top of them. Heat chambers and portable heating units can be also used to treat furniture and items that cannot be laundered [3]. Heat treatments do not require to be applied by certified pest management professionals because no pesticides are involved and it is not toxic [84]. Heat chambers are now used by the rental furniture industry [3]. An affordable homemade heat chamber can also be built using polystyrene sheathing boards, oil-filled electrical space heaters, desktop fans, tapes, and electric cords, in less than $400 [88]. Within 6 h of the treatment, bed bugs are effectively controlled [88]. Portable heat chambers that can be plugged into a regular wall outlet to treat smaller items that cannot be treated with insecticides or laundered, such as luggage, toys, etc., are also available [3,84]. Heat chambers take longer to kill bed bugs than dryers, but they allow treating almost everything that fit inside of the chamber [84].

Whole structural heat treatments using propane generated heat or electric heaters are also being used as a non-chemical bed bug treatment [3,9,69]. For this treatment, the room temperature is raised to 57.2 °C (135 °F), monitored, and left for several hours [69]. While whole structure heat treatment is proven to be effective in most of the cases [3,69], the use of propane is restricted in some regions due to associated risks of fires, and the availability of enough electric power might be challenging in some cases [3]. The equipment cost for this treatment is also relatively expensive [88], exceeding $60,000 [84]. Additionally, some construction materials such as wood or slab, the condition of the structure such as how well the structure is sealed, and the amount of the furniture and clutter, dramatically affect the length and efficacy of the treatment [3,69]. Using heat treatment in a room with a fire extinguisher such as automatic sprinkler has been also challenging [86]. The fire extinguisher is activated by the high temperatures posing a significant safety risks with the propane heaters or electric heaters in the room, and disabling the fire extinguisher is illegal in many states [86].

Cold temperature treatments for bed bug control require exposure to −5 °C (23 °F) for at least five days, or flash-freeze at −26 °C (−15 °F), which is the supercooling point of eggs [81]. The Cryonite® system, that releases CO_2 dry ice snow at −60 °C (−76 °F), instantly kills bed bugs and eggs in direct contact, and it is currently being used for bed bug treatments [89]. This system requires proper distance and lower releasing pressure [3], but it can be applied to almost any surface without damaging, does not leave any residues, and is environmentally safe allowing the treatment to be applied without stopping production in a factory or removing the items [89]. An apartment complex, a single family home, and a homeless shelter were successfully treated for bed bug infestations in a study with only two treatments using Cryonite® [89].

Extreme temperature treatments are effective, but the treatments are usually a slow process that requires a lot of time and patience, thorough applications, and some of these treatments are quite expensive [69]. Extreme temperature treatments are safe to use in many surfaces because it does not leave any residues, and can be applied without certification [69]. While the lack of residue makes them safe to use, it also allows for bed bug to re-infest the treated surfaces right after finishing the treatments. Insulation may protect the bed bugs from the extreme temperatures during the treatments [3]. In whole room heat treatments some cold spots in certain constructions provide refuge for bed bugs [69]. Due to the limitations in the efficacy of extreme temperature treatments, it is recommended that they are applied along with chemical treatments [69].

The physical removal of bed bugs by vacuuming and disposal of infested items is also being used as part of the non-chemical control methods [3]. Currently, 65% of the pest management professionals use vacuuming, and 62% use disposal of the bed bug infested items as part of the treatment of bed bug infestations in the United States [38]. Physical removal by hand picking the bed bugs is usually impractical and ineffective, only potentially being effective when the population only consists of a few individuals without eggs [77]. Vacuuming is an effective and efficient method to physically removing large numbers of bed bugs when used in targeted areas where bed bugs aggregate [3,77]. Inspections are also facilitated [69] by vacuuming molted skins, hatched egg shells, and debris [69,77]. Although vacuuming reduces bed bug populations quickly, it does not completely eliminate them, failing to remove bed bugs from cracks and crevices [3,77] or cemented eggs [3,69,77]. Due to this limitation, other chemical or non-chemical treatments are necessary in addition to vacuuming [77]. Upon finishing vacuuming, the vacuum bag should be removed immediately, sealed in plastic bag, and discarded outside [3,69,77] to avoid infestation of the vacuum itself or spreading the bed bugs to uninfested rooms [77]. Discarding infested items is also often used by pest management professionals [38] since it is the fastest method to remove large numbers of bed bugs from an infested unit [77]. The decision is made weighing the cost of treatment, cost of replacement of the item, and the emotional value of the item [3,77]. Among the most discarded items are infested mattresses and box springs; however, replacing them or any other items before completing successful bed bug elimination often results in reinfestation of the new items [3,77]. Therefore, discarding infested items should not be used as the only solution to a successful bed bug elimination; instead, it should be used in combination with other methods [77]. Unfortunately, discarded items that are not sealed and marked as bed bug infested are often picked up by unsuspecting individuals [3]. Infested items should be wrapped tightly in plastic or storage bags prior to transferring to avoid the unintentional dispersal of bed bugs and eggs [3], otherwise it will contribute to the passive bed bug dispersals. As of January 2011, the New York City Department of Sanitation established a new law mandating the full encasement of mattresses and box springs prior to disposal in order to prevent bed bug dispersal, with a $100 fine being issued to anyone who violates this law [90].

Creating physical barriers such as pulling the furniture out several inches away from the wall, making sheets and blankets tucked in, and placing interception device under the bed legs, are still used today as part of the non-chemical control methods [3]. Historically, the interception devices were small tuna cans containing water [69], metal cans or glass jars [3,91] containing kerosene, petroleum jelly greasing the legs of the bed, or double-sided sticky tape around the legs of the bed [3]. New interception devices such as the ClimbUp® insect interceptor passive monitoring device have been used not only for monitoring, but also as controlling device [3]. The ClimbUp® interceptor uses human as bait and catches

bed bugs as they climb up the legs of the beds to feed, or climb down the legs to disperse away from the host [3,69]. Placing the ClimbUp® interceptive devices under the legs of the beds after successfully eliminating bed bugs from the beds will create physical barriers, if all other physical bridges to the bed are eliminated by pulling the bed away from the walls and placing the sheets fully tucked in [3,77].

Several monitoring devices were developed and are currently being used in addition to visual inspections to detect bed bug infestations and to monitor the activities of bed bugs [92]. Some of the devices seem to trap large numbers of bed bugs and may also help reduce the bed bug population [93,94]. In Anderson et al. [93], a pitfall trap baited with CO_2 gas (50–400 mL/min), heat (37.2–42.2 °C), with or without a chemical lure gel mixture (33.0 μg proprionic acid, 0.33 μg butyric acid, 0.33 μg valeric acid, 100 μg octenol and 100 μg L-lactic acid) developed to attract kissing bugs, *Triatoma infestans*, caught 5898 bed bugs in 9-trap days in an unoccupied, one-bedroom, high-rise apartment without furniture after the resident was relocated. In the study, traps without CO_2 gas trapped 656 bed bugs in 29-trap days [93]. The number of bed bugs caught in the baited traps declined exponentially as a result of trapping [93]. Anderson et al. [93] found that the trap was effective catching host-seeking unfed bed bugs of all stages with or without furniture or the human host present in the room, and that CO_2 was the most important factor attracting the host-seeking bed bugs. Similarly, Wang et al. [76] also found CO_2 as the most important attractant for bed bugs, capturing bed bugs using a home-made dry ice trap as effectively as two active monitors currently available in the market equipped with CO_2, heat, and synthetic lures (components have not been disclosed). In the same study, the ClimbUp® device trapped a similar number of bed bugs to the dry ice trap, more bed bugs than the two other commercially available monitoring devices, and was more effective than visual inspections detecting low levels of bed bug infestations [94]. The ClimbUp® device effectively reduced the bed bug population during the field experiment conducted in a high-rise low-income apartment, catching the average of 219 ± 135 bed bugs per unit in 10 wk, while the visual inspections only caught 39 ± 22, respectively [94]. Visual inspections and resident reports are found to be unreliable when determining the bed bug infestation [91]; among 40 total apartment units that previously reported bed bug infestations, visual inspections and residents reports only identified 24 and 12 units, respectively, with bed bug infestations [91]. In the same study, many bed bugs were still found trapped in the interceptor device after eliminating all bed bugs that were visually found during visual inspections in each apartment units [91].

However, the feeding status of bed bugs significantly affects the attractiveness of the bait [93], since the attractiveness of a host cues such as heat and sebum diminishes as bed bugs engorge with blood [92]. Bed bugs leave the host as soon as finishing feeding to remove themselves from the vulnerable location [92], and may stay in harborages digesting the blood for days until they need another blood meal. Also, the ClimbUp® device only catches the bed bugs when they are actively moving and passing through the device [92]. Several traps and monitoring devices are currently available equipped with heat or chemical lures or both, but the exact components of the semiochemical attractants in these lures are unknown, and only a few scientific studies have been conducted to study the efficacy of these traps [92]. Several studies have focused on the identification and the location of bed bug olfactory sensilla, and their behavioral and electrophysiological responses to various semiochemicals, including alarm and aggregation pheromones, and host kairomones [92]. However, many of the currently available monitoring devices or traps are not as attractive as a natural human host, and are not economical, practical, or efficient to attract both fed and unfed bed bugs.

An innovative, potentially useful, mechanical trap has been investigated recently based on the historical eastern European method of entrapping the bed bugs on the surface of bean leaves [95]. The method consisting of leaving fresh bean leaves scattered under the beds before going to bed and burning the entrapped bed bugs with the leaves next day was historically used in Baklan countries [95]. A study on the ability of plant leaf trichomes, microscopic hooked hairs, trapping insects was first published in 1943, but the idea was never pursued because soon afterward synthetic insecticides effectively reduced bed bug infestations [95]. Recent research using the scanning electron microscopy (SEM) and videography revealed that the trichome tips of fresh Kidney beans, *Phaseolus vulgaris* L., were mechanically piercing specific locations on the bed bug tarsi within seconds of bed bugs coming in contact with the leave, making the bed bugs permanently entrapped and unable to escape [95]. The piercing locations identified were underneath the pretarsal craws and in the intersegmental membrane between the first and second tarsal subsegments [95]. Microfabrication of the trichomes using polymer molds similar to plant cell walls did not trap bed bugs as effectively as the fresh bean leaves, only snagging the bed bugs temporally, not permanently [95]. This microfabrication of biomimetic surfaces may have a great potential as a non-chemical treatment to control bed bug infestation in the near future [95].

Currently, integrated pest management (IPM) approaches, using both chemical and non-chemical control methods, are recognized as more effective and useful long-term solutions for controlling bed bug infestations, compared to only using insecticides for treating bed bug infestations [9,94]. Chemical control alone is less likely to eliminate the bed bug infestations today, because bed bugs have become highly resistant to most of the currently available insecticides [11,23,25]. Direct application using spraying insecticides or dust formulated insecticides [63,70] may kill bed bugs, but due to the highly cryptic and nocturnal nature of bed bugs [2,96], many bugs are missed during visual inspections [94] and never come in contact with the insecticides. Also, non-chemical treatments such as steam, traps, mattress encasements, may work well on particular items in an infested room, but may not effectively treat bed bugs harboring in the wall voids or deeper inside upholstered furniture. Therefore, an effective IPM program involves careful designing and planning of action plans [3]. The action plans are mostly dependent on dynamics of the bed bug infested environment, so there are differences in protocols between hotels, apartment buildings, single family homes, shelters, schools, etc., in each unique case [3]. Although there are differences in treatment methods and durations based on the severity of infestation, the components of the integrated pest management must contain (1) prevention and early detection of infestations, (2) identification of the bed bug species and confirmation of the infestation, (3) education of all involved in eradication efforts, (4) inspection and application of the treatments (combining several non-chemical and chemical treatments), and (5) follow-up visits for efficacy evaluation and application of additional treatments, as required [3]. Each step is crucial component of the integrated pest management in order to successfully control a bed bug infestation [3].

Some of the preventive methods used in the field are mattress and box spring encasements, interception devices, mechanical barriers, routine inspections for signs of infestations, periodical detailed inspections, professional inspections, and active monitoring devices [3]. Routine inspections and periodical detailed inspections can be performed by staff and residents by checking for obvious signs of infestations [3], such as itchy bite marks on skin [2,3,9,16,33], or the presence of live bed bugs [2,3,16,33]. Periodical detailed inspections include checking mattresses and box springs along the seams and holes, along the wood or metal frame of the box springs, behind head boards and foot boards, along the seams

of upholstered furniture, behind the bed frames, behind pictures on the wall, ceiling and wall junctions, along the base boards, and at electrical outlets, looking for other signs of infestations, such as the presence of black fecal deposits, molted caste skins (exuvia), eggs, live and dead bed bugs [3,16,33]. Correct identification of the bed bugs to the species level is important because bed bug caste skins often resemble that of dermestid (carpet) beetle larvae, and live bed bugs can be confused with immature cockroaches [3]. Fecal spots from bed bugs can appear very similar to that of German cockroach feces, however bed bug fecal spots are smooth when touched because they only consume blood, compared to the fecal spots of German cockroaches, which have a more granular feel because they contain solid wastes [16]. In addition, bed bugs, bat bugs, and bird bugs can all appear similar, but require different treatment depending on the species and the location of the infestations [3]. Professional inspections can be done by pest management professionals or using special diagnostic tools such as canine scent detection [97] on a yearly basis or several times a year [3]. Properly trained bed bug detection dogs are very efficient inspecting large areas such as hotel rooms, college dorm rooms, and apartments, in a short period of time compared to a typical visual inspection by a pest management professional [3]. Detection dogs can distinguish between live bed bugs. Viable eggs, dead bugs, cast skins, and feces with 95% confidence when trained properly [97]. Using canine detection is becoming increasingly more popular, up from 16% in 2010 to 43% in 2011 [38], but the success rate seems to vary depending on the dogs and handlers, and training [9]. Active monitoring devices (such as those described earlier) equipped with baits such as CO_2, heat, and chemical lures are currently used for the inspections in unoccupied hotel room, apartment units, etc. [3].

The cost effectiveness of IPM programs have not been scientifically evaluated, but one study revealed that the mean treatment costs of using diatomaceous earth dust-based IPM was $463 versus $482 for chlorfenapyr spray-based IPM for a 10 week period [94]. Both treatments included a seminar and educational brochure for residents and staff, installation of mattresses and box springs encasements, steam treatments, hand-removal of the bed bugs, and placing the bed bug intercepting devices for diatomaceous earth dust-based IPM treatment units [94]. The infestations were monitored every 2 weeks for 10 weeks, re-treated as needed; at the end of the program, bed bugs were eliminated from 50% of the apartment units in each IPM group [94].

An IPM approach minimizes the risk of new infestations, finds infestations in earlier stage, effectively eradicates the bed bug infestations, and maintains sustainable bed bug control [77]. A successful IPM program requires pest management professionals to be knowledgeable, detail-oriented, and work in a methodical manner [3]. A study found that adult female bed bugs actively disperse away from aggregations, potentially leading to control failures [98] because one female can easily be undetected. Another study found adult bed bugs were nine times more likely to disperse than nymphs, and the adult bed bugs were using hallways to disperse [91]. The total number of adults caught in interceptive devices placed behind entry doors in apartment units were 138 individuals [91]. In the same study, 53% of the apartment units adjacent to bed bug infested units also had infestations, but 50% of the residents were not aware of their bed bug infestation [91]. For IPM approaches to be successful, pest management professionals must understand bed bug ecology and life cycle, thoroughly examine the area, and must have the ability to work closely with the clients [9].

The application of non-chemical treatments and an IPM program has been found to be effective to control bed bug infestations, but education is an essential part of successful bed bug treatments [3,77]. Education and cooperation of the residents directly affects the treatment efficacy because uneducated residents may unknowingly allow bed bugs to survive in untreated items and contribute to the new spread of infestations [77]. Bringing the infested items back to the apartment unit also contributed to the failure of the treatment [94]. Lack of public awareness is also an important factor contributing to unintentional spread of the bed bugs [1,3,77]. Failure to understand whether there is an ongoing infestation, the dynamics of the infestation, including the route of dispersal, and the degree of infestation correctly can also contribute to unsuccessful bed bug control [91]. Therefore, current bed bug management needs a community-based IPM program that takes into consideration the demographics of the residents and using affordable control methods to prevent this challenging pest from spreading further in the society [77].

5. Future of the bed bug control

Because there is currently no single effective, low cost treatment methods available for controlling bed bugs, the number of bed bug infestations will continue to increase worldwide. In the United States, the restrictions on insecticides permitted for indoor use will likely remain strict due to heightened public concerns with the use of synthetic insecticides. The development and registration of new insecticides with new modes of actions is remarkably expensive, so effective novel insecticides will most likely not be available [9]. Improving the formulation of currently existing insecticides to target effective penetration of the bed bug cuticle is an area that should be pursued [9]. Also, the economic impact of bed bug infestations is still not fully understood or reported [9]. Implementing community-wide awareness, continuous education, and improving IPM protocols are also necessary to better control bed bug infestations [3,77].

Currently, pest management industries, researchers, public health departments, and federal agencies are increasing efforts to address the bed bug resurgence. Continuous efforts to increase the cooperation among them is essential not only to develop a successful community-based bed bug elimination programs, but also to better understand bed bug biology, ecology, biochemistry, physiology, and toxicology, all aimed at developing better bed bug control methods. Investigation of the current bed bug populations, behavior, chemistry, genetic, and insecticide resistance mechanisms are necessary to gain a better understanding of this fast spreading pest.

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6 Reduced cuticular penetration

Recent bed bug studies have documented two mechanisms of physiological resistance; enhanced detoxification enzyme activity (metabolic resistance) and *kdr*, specifically a mutation at the alpha-subunit of the sodium channel. These studies are summarized in Chapter 3, Section 2. However, direct evidence of the reduced cuticular penetration type resistance in bed bugs has been lacking. To date, there have been two indirect indicators suggesting reduced cuticular penetration type resistance in bed bugs. The first indicator was presented in a recent study describing the gene expression in 24 bed bug populations (Zhu et al. 2013). In this study, the authors observed the up-regulation of some cuticular-related protein genes in addition to cytochrome P450s, esterases, *kdr*, and ABC transporters genes (Zhu et al. 2013). Another study (Koganemaru et al. 2013) published a detailed investigation of the bed bug cuticle protein genes present in both a pyrethroid resistant field-strain and a susceptible laboratory strain (see Chapter 3). Koganemaru et al. (2013) observed elevated transcripts of multiple putative cuticle genes across all types of cuticular-related protein genes (RR-1, 2, and 3 of the CPR families) in the pyrethroid-resistant strain (see Figure 4 of Chapter 3). While both of these studies

demonstrating upregulated cuticular genes certainly suggest reduced cuticular penetration type resistance in bed bug field populations, these studies provide only indirect, one-dimensional (transcriptional) evidence to support this conclusion.

More direct evidence for reduced cuticular penetration type resistance has been documented in other orders and species of urban and agricultural insect pests. Studies to investigate the possibility of reduced cuticular penetration type resistance have been conducted by measuring the amount of topically applied radioactively labeled insecticide that penetrate through the insect cuticle into the body cavity of the insect. Examples include the DeVeries (1981) study where a strain of house fly *Musca domestica* L. was topically treated with radioactively labeled (1R,S)-trans-[14C]permethrin. Suspected resistant fly strains were found to have significantly less penetration of the insecticide into the body than a known susceptible strain. Another study investigating a resistant strain German cockroaches *Blattella germanica* L. were found to have a reduced accumulation of [14C] fenvalerate compared to a susceptible strain when they were treated with a topical application (Wu et al. 1998). Furthermore, a resistant strain cotton bollworm *Helicoverpa armigera* Hübner exhibited the delayed penetration of [14C] deltamethrin compared to a

susceptible strain; they were treated with a topical application in series of different time intervals in hours, and the percentage of the radioactive insecticide penetrated through the larval cuticle was calculated by subtracting the amount of radioactivity in the cuticle rinsate from the applied dosages (Ahmad et al. 2006).

Although the above studies provide evidence to support that reduced cuticular penetration resistance exists in multiple insect species, the specific mechanisms of reduced cuticular penetration type resistance are still unknown. In some laboratory strains of house flies, reduced cuticular penetration is believed to be controlled by the penetration delaying (*pen*) gene located on chromosome III, followed by the increased detoxification enzyme activity type resistance controlled by desethylation (gene *a*) on chromosome II (Sawicki 1970). In addition, Wood et al. (2010) observed a positive correlation between the thickness of the cuticle and the levels of pyrethroid resistance in a malaria vector mosquito *Anopheles funestus*. Therefore, thickening of the cuticle is thought to be involved in reduced cuticular penetration. Similar observation of thickening of the cuticle was made by Pedrini et al. (2009) in a study involving different populations of the Chagas disease vector, *Triatoma infestans*.

The relative differences in cuticular thickness between resistant and susceptible strain insects might be a result of increased levels of cuticular protein gene transcription. In the case of mosquitos, significant up-regulation of certain cuticular protein genes were observed in pyrethroid resistant *An. stephensi* (Vontas et al. 2007) and *An. gambiae* populations (Awolola et al. 2009). A significant up-regulation of some cuticle protein genes might also change the composition and cross-linking structures of the insect cuticle (Cornman and Willis 2009). These changes in cuticular protein composition and cross-linkage ability of the cuticle proteins could potentially alter the density, metal-binding properties, and hardiness of the insect cuticle (Cohen 2010, Rubin et al. 2010). It is not hard to imagine that any modifications of these cuticle properties might have a significant effect on the penetration ability of different insecticide formulations (oil or water-based).

The epicuticular wax layer covering and protecting the insect cuticle may also play a significant role in reduced cuticular penetration type resistance. Both the composition and the concentration of cuticular hydrocarbons that make up the wax layer may create a substantial barrier to the penetration of insecticide through the insect cuticle. Pedrini et al. (2009) observed that populations of pyrethroid resistant individuals in *Triatoma infestans*

had significantly larger quantities of the surface cuticular hydrocarbons than the susceptible individuals. When investigating bed bugs, Feldlaufer and Blomquist (2011) extracted cuticular hydrocarbons from a laboratory susceptible strain, and compared the hydrocarbon composition from different sexes of live bugs, and those of cast skins. The hydrocarbon profiles for the laboratory (susceptible) bed bugs were characterized by a peak area of the alkanes. However, no studies have yet been conducted to characterize or quantify the hydrocarbons of field collected bed bug strains.

Regardless of which mechanisms might be involved in reduced cuticular penetration type insecticide resistance, all mechanisms are most likely the result of human selection of specific advantageous phenotypes. Because the bed bugs share their habitat with humans, it is highly likely that our domestic pesticide use has selected for those phenotypes that allow bed bugs to survive repeated insecticide application that would be deadly to less resistant populations (Koganemaru 2013).

Unlike the increased detoxification enzyme activity or *kdr*, that have been observed in other studies, reduced cuticular penetration type resistance in bed bugs is still largely uninvestigated. Changes in the levels of gene expression, cuticular thicknesses, cuticular

proteins, and hydrocarbons, can be all measured and analyzed to test the hypothesis that the bed bugs have the reduced cuticular penetration type physiological resistance. The purpose of this study is to first measure and quantify the levels of insecticide resistance among different bed bug populations (susceptible and resistant). Next, a detailed investigation into the genetics and proteomics of the bed bug cuticle will provide insight into potential physiological processes that might be involved in bed bug reduced cuticular penetration. Finally, measurements of cuticular thickness, hydrocarbon profiling, and hydrocarbon quantification studies will be completed to determine if there are actual physiological cuticular differences between resistant and susceptible strain bed bugs.

7 Research objectives

Based on the above literature review, the following five research objectives were selected for this dissertation:

1. To determine the lethal dose (LD_{50}) of pyrethroid insecticide necessary to kill pyrethroid-resistant field strain bed bugs using topical and injection application methods.
2. To determine the levels of gene transcription specific to the development of the insect

cuticle in a pyrethroid-resistant field strain bed bugs collected from Richmond, VA, using real-time quantitative polymerase chain reaction (Published).

3. To identify and describe bed bug cuticular protein profiles using the matrix-assisted laser desorption/ionization time-of-flight/time-of-flight high-resolution tandem mass spectrometry (MALDI-TOF/TOF).
4. To determine and compare the cuticular thickness of resistant and susceptible bed bug strains using Scanning Electron Microscopy (SEM).
5. To identify and describe bed bug cuticular hydrocarbon profiles using Gas-Chromatography and Mass-Spectrometry (GC-MS).

This study provides additional evidence of the reduced cuticular penetration type resistance in bed bugs. Not only this study reveals some mechanisms behind the reduced cuticular penetration type resistance, but also offers some useful knowledge to the industry to help develop better formulation of currently available insecticides.

Chapter 2: Determination of the lethal dosage (LD_{50}) of pyrethroid insecticide necessary to kill pyrethroid-resistant field strain bed bugs using topical and injection application

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Also, some other part of this chapter was published in Pesticide Biochemistry and Physiology 106 (2013): 190-197. I requested and was granted permission from Elsevier B.V. publisher to include the journal article, in full or in part, in this dissertation.

2.1 Introduction

Sudden resurgence of the common bed bugs, *Cimex lectularius* L., in the U.S. and other developed countries since the late 1990s has raised a substantial public health concern (Potter 2006). The cause of this sudden worldwide bed bug resurgence is still unknown, but widespread of insecticide resistance across the field population of bed bugs has been identified a contributing factor (Romero et al. 2007, Davies et al. 2012). For example, two field-collected populations of bed bugs from Arlington and Richmond, VA, and one bed bug population from Cincinnati, OH, were determined to be more than 300-fold resistant to 0.06% deltamethrin (Suspend® SC) and 0.05% permethrin (Dragnet)(Moore and Miller 2006, Polanco et al. 2011). Fourteen out of sixteen populations of field-collected bed

bugs across the U.S. exhibited no mortality when they were exposed to a fresh residues of deltamethrin and λ-cyhalothrin formulated at 200-300 times higher than the label concentration (Romero et al. 2007). Several total release aerosol products were also evaluated for efficacy in controlling five different bed bug populations (Jones and Bryant 2012). In each of these evaluations, no bed bug mortality was recorded (Jones and Bryant 2012). Because high levels of insecticide resistance is undoubtedly inhibiting successful bed bug control in the field, insecticide resistance has been suggested to be one of the most significant factors contributing to the recent bed bug resurgence (Davies et al. 2012).

There are three types of insecticide resistance that have been reported in bed bugs; target-site insensitivity, enhanced detoxification enzyme activities, and reduced cuticular penetration. The first type of insecticide resistance, target-site insensitivity (knockdown resistance, *kdr*) was first reported in bed bugs by Yoon et al. (2008). The target-site insensitivity type of insecticide resistance in bed bugs is caused by the two-point mutations, the Valine to Leucine mutation (V419L) and the Leucine to Isoleucine mutation (L925I), of the voltage-gated sodium channel α-subunit genes (Yoon et al. 2008). These mutations were reported in the bed bug population collected from New York City, and were found to be responsible for the *kdr* to deltamethrin in the bed bug population (Yoon et al. 2008).

Zhu et al. (2010) reported 88% of the 110 bed bug populations collected in the U.S. had either one or both of the sodium-channel mutations. The target-site insensitivity type of insecticide resistance seems to be common in many modern bed bug populations (Zhu et al. 2010).

The second type of insecticide resistance, enhanced detoxification enzyme activity, has been reported in several bed bug studies. Enhanced detoxification enzyme activity is caused by the insect producing either excessive amounts of detoxifying enzymes such as cytochrome P450 monooxygenases, carboxylesterases (CE), and glutathione-S-transferases (GST) (Yu 2008) or the enzymes themselves have increased detoxifying activity. Romero et al. (2009) found cytochrome P450 monooxygenases detoxification activity was involved in two deltamethrin resistant population of bed bugs collected from Cincinnati, OH, and Worcester, MA. Romero et al. (2009) also observed that the levels of deltamethrin resistance in these field strain bed bugs were reduced when piperonyl butoxide (PBO), a cytochrome P450 monooxygenases inhibitor, was applied two hours prior to deltamethrin treatment. However, the levels of deltamethrin resistance remained high when the two population of bed bugs were not pre-treated with PBO (Romero et al. 2009).

A transcriptomic analysis of an insecticide resistant field population of bed bugs

collected from Columbus, OH, performed by Bai et al. (2011) revealed increased level of a cytochrome P450 (CYP9) transcripts compared to that of a susceptible laboratory Harlan strain bed bugs. Another transcriptomic analysis conducted by Adelman et al. (2011) of a pyrethroid-resistant field-collected Richmond strain bed bugs discovered an up-regulation of multiple detoxifying enzyme genes across P450s, CEs, and GSTs, in addition to the L925I substitution on the sodium channel gene.

The third type of insecticide resistance that have been suspected to exist in bed bugs is reduced cuticular penetration. Two reports have recently documented the up-regulation of putative cuticular protein genes in field-collected bed bug populations (Zhu et al. 2013; Koganemaru et al. 2013). Zhu et al. (2013) reported multiple putative cuticle genes were up-regulated across many field-collected bed bug populations that had up-regulated P450 genes, other detoxifying enzyme genes, and ABC transporter genes. Koganemaru et al. (2013) also identified many of the putative cuticle protein genes, and found that many of these genes were up-regulated in the pyrethroid-resistant (Richmond) strain.

While the up-regulation of cuticular protein genes has been documented, we have no hard evidence that reduced cuticular penetration type resistance is actually present in

field population of bed bugs. Therefore, we decided to compare the LD_{50s} necessary to kill pyrethroid-resistant and susceptible populations of bed bugs using two different application methods – topical and injection. Our hypothesis is that the ratio of the topical LD_{50} value over the injection LD_{50} value (penetration ratio) determined for the resistant bed bug strain will be greater than the penetration ratio for the susceptible laboratory strain bed bugs.

2.2 Materials and Methods

2.2.1 Bed bug colonies

Collection and rearing of the common bed bug, *C. lectularius* L., was as described previously (Adelman et al. 2011), except defibrinated rabbit blood was used as the bed bug food source. The total of four bed bug populations (strains) were used in this study: the Harlan (H), a pyrethroid susceptible laboratory strain; the Richmond (R), a pyrethroid resistant strain (both described in Adelman et al. 2011); the British (B), a pyrethroid susceptible strain which was originally collected from an apartment complex in London, United Kingdom, and sent to our laboratory in 2010; and the Epic Center (E), a pyrethroid resistant strain collected from a hotel room in Cincinnati, OH, in 2009. The B strain was

determined to be slightly resistant to pyrethroid insecticides based on the calculated LT_{50} values (resistance ratio [RR] = 2.6), but not as resistant as the R (RR = 390.5) or the E strains (>340) when exposed to deltamethrin (0.06%) on hardboard panels (Polanco et al. 2011).

2.2.2 Topical and injection bioassay methods

Both topical and injection application bioassays were performed using technical grade β–cyfluthrin to determine the level of pyrethroid insecticide susceptibility across the four strains. The topical application bioassay was conducted as described by Koganemaru et al. (2013). The injection application bioassay was conducted as described by Adelman et al. (2011). Ten adult male bed bugs (7-days after final molt) were placed in a glass Petri-dish that was chilled on ice for few minutes. Technical grade β–cyfluthrin was applied dissolved in acetone for the topical application, and 10% DMSO with distilled water for the injection application. Figure 2.1 shows the topical and injection application sites of the insecticide on the bed bug. After the topical or injection application of insecticide, bed bugs were placed in a plastic Petri-dish (10 individuals per Petri-dish) that contained a filter paper. Mortality was recorded at 24, 48, and 72 hrs post-exposure.

The LD_{50} data for both topical and injection bioassays was analyzed using Polo Plus Probit and Logit Analysis computer software (version 1.0; LeOra Software Company®, Petaluma, CA). The LD_{50} values were calculated as well as the 95% confidence levels (CL; $P < 0.05$), and the slope $\pm$ the standard errors (SE) of the dose-response probit analysis. The results were also confirmed using MATLAB® R2013a (version 8.1.0.430; The MathWorks®, Inc., Natick, MA). Resistance ratios (RRs) were calculated by dividing the LD_{50} of each strain by the LD_{50} of the H strain. Penetration resistance ratios were used to quantify the bed bug cuticle's ability to reduce insecticide penetration into the insect body.

2.3 Results

As expected, both H and B susceptible strains had significantly lower LD_{50} values than that of R and E resistant strain in both the injection and topical bioassays. For the topical application bioassay, the LD_{50} values of H, B, R, and E strains were 3.1, 0.6, >500,000, and >244,000 ng/insect, respectively (Table 2.1). The R and E strain LD_{50} values could not be calculated because the mortality never reached 100% for the highest concentrations applied (>349,500 ng/insect). The highest concentrations of topical

application was almost saturated with the technical grade β-cyfluthrin dissolved in acetone, so the insects had a white, powdery spot (dried pure technical grade β-cyfluthrin) at the application site on their abdomen. Yet, majority of the resistant individuals (>90% for R, and >50% for E) showed no signs of toxic nerve effects. A few of the bed bugs were excited, and attempted to mate with the other males, but they failed because the other males were not receptive. In contrast, the highest concentration of insecticide topical applied to the H and B strains was approximately 35 ng/insect (10,000-fold less from that of R and E) yet all of the individuals from the H and B strains were dead within 24 hrs post-application. The topical bioassay LD_{50} of the H strain was significantly different from that of B strain, as the 95% confidence intervals (CIs) calculated did not overlap (Table 2.1). The 95% CI and Slope $\pm$ SE for the R and E strains for the topical application could not be calculated, because the highest mortality never reached 80%. Based on the 95% CI, the B strain topical application LD_{50} value was significantly lower from that of H, R, and E strains. Interestingly, the B strain topical application LD_{50} value was significantly lower than that of H strain, even though both were pyrethroid-susceptible strains. The R and E strain topical application LD_{50} values were significantly greater than that of H or B strains, as expected. The RRs of the B, R, and E strains for the topical application bioassay were 0.2,

>162,338, and >79,324, respectively.

For the injection application bioassay, a similar pattern of LD_{50} values for resistant strains was observed. The LD_{50} values of H, B, R, and E strains were less than those calculated in topical application bioassays at 0.037, 0.031, 4.430, and 2.780 ng/insect, respectively. Note that there were 100-folds differences between the susceptible (H, B) LD_{50} values and resistant (R, E) LD_{50} values. However, for all the strains the LD_{50} values of the injection application bioassay were less (>20-fold less) than that of topical application bioassays. Interestingly, unlike the topical application bioassay results, H and B strains had almost identical LD_{50} values (0.037 and 0.031 ng/insect) and substantially overlapping the 95% CIs. Similarly, both resistant strains (R, E) were not significantly different although there was an almost 2-fold differences between their injection LD_{50} values.

The RRs of the B, R, and E strains for the injection application bioassay (H strain LD_{50} in denominator) were 0.8, 120, and 75, respectively. The injection RRs (H strain LD_{50} in denominator) of the R and E resistant strains (120 and 75) were considerably lower than those of the RRs of the topical application bioassays (>162,338 and >79,324).

The penetration resistance ratios (the LD_{50} values for topical application were

divided by injection LD_{50} values within each strain) for the H, B, R, and E strains were 83, 20, >112,867, and >87,884, respectively. The penetration resistance ratios of the susceptible strains (H, B) were strikingly lower than those of the resistant strains (R, E). The penetration resistance ratio of the resistant strains (R, E) were more than 1,000-fold greater than that of H strain, and more than 4,000-folds greater than that of B strain. The large differences in penetration resistance ratios between the susceptible (H, B) and resistant (R, E) strains suggest the presence of reduced cuticular penetration type resistance in the two resistant strains.

2.4 Discussion

The results of this experiment provided a significant evidence in support of reduced cuticular penetration type resistance existing in bed bugs. The differences in topical application resistance ratios between the susceptible (H and B) and resistant (R and E) strains were reduced substantially when the insecticide was injected directly into the body. During the injection bioassay, when the cuticle barrier was removed the resistant ratios were reduced by 100-fold to approx. 100,000-fold. The resistant strains were still resistant (compared to susceptible strains) regardless of the insecticide application method;

however, the significantly greater penetration resistance ratio in the resistant strains (R, E) provided strong evidence that the cuticle is a contributing factor in their insecticide resistance level. The penetration resistance ratio of the susceptible and resistant strains would be similar if the *kdr* or enhanced mixed function oxidases (MFOs) activity were the only mechanisms of resistance in these two strains. Because the penetration resistance ratios were so much greater in the resistant strain bed bugs, the ratios clearly indicate that the resistant strain cuticle was less permeable to insecticides than that of the susceptible strains.

The results of this study have a very significant practical application when you consider that historically, bed bug insecticide treatment over the last century have changed as each mode of action has been rendered ineffective by the development of insecticide resistance. Specifically, the most common insecticides applied in domestic environment started with chlorinated hydrocarbons (such as DDT), then shifted to organophosphates, carbamates, and finally to pyrethroids (Potter 2011). The development of reduced cuticular penetration type resistance suggests that any active ingredient (even those with novel modes of action) may not be effective for controlling bed bug populations because the insects will only have limited exposure to the toxicant. In bed bugs, we have only

tested two field-collected pyrethroid-resistant populations. However, the fact that both strains exhibited high levels of penetration resistance ratios compared to those of two pyrethroid-susceptible strains may indicate that reduced cuticular penetration type resistance might also be widespread in other field populations of bed bugs. Molecular evidence reported by Zhu et al. (2013) showing up-regulation of putative cuticle genes across different population of bed bugs might also indicate the widespread of reduced cuticular penetration type resistance in field bed bug populations.

In conclusion, this study provided a solid evidence supporting the reduced cuticular penetration type resistance in field-collected populations of bed bugs. The resistant strain bed bugs had significantly greater RRs compared to that of the susceptible strains in both topical and injection bioassays, confirming they were highly resistant to pyrethroid insecticides. Both the R and E resistant strains exhibited significantly greater penetration resistance ratios compared to that of H and B susceptible strains. This is indisputable evidence that the resistant strain cuticle was much less permeable than those of our susceptible strain bed bugs, reducing and delaying the insecticide penetration. While molecular evidence supporting reduced cuticular penetration as a mechanism of bed bug insecticide resistance is available (Zhu et al. 2013, Koganemaru et al. 2013), the details of

the physiological process is still not well understood. Because reduced cuticular penetration type resistance might be common in field populations, further investigation into the specific mechanisms of reduced cuticular penetration type resistance (thickness, cross-linkage, etc.) in bed bugs is still needed.

2.5 Acknowledgements

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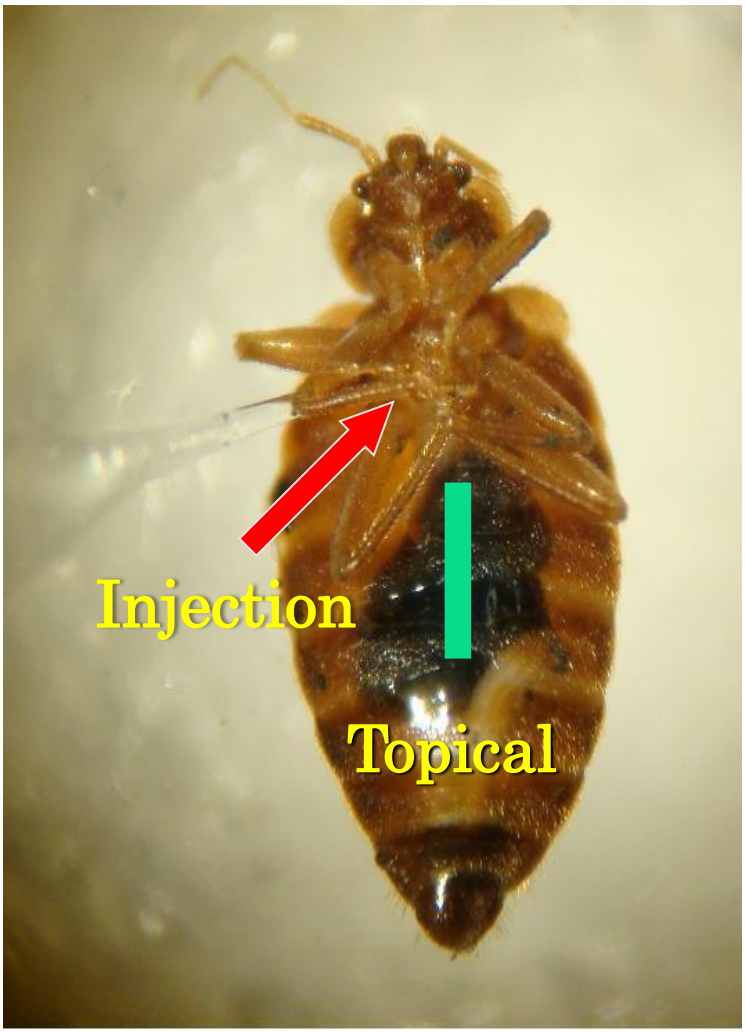
Table 2.1 Comparison of the lethal dosages (LD_{50s}) among four bed bug strains: Harlan (H) susceptible, British (B) susceptible, Richmond (R) resistant, and Epic Center (E) resistant. Technical grade β-cyfluthrin was applied using topical and injection methods. The total number of insects used to calculate the LD_{50}s are listed in column n. Resistance ratios (RRs) were calculated using the H strain LD_{50} values in the denominator. Penetration resistance ratios were determined by dividing the topical by the injection LD_{50} values within each strain. The H and R data were collected in 2011 (from Adelman et al. 2011, Koganemaru et al. 2013), whereas B and E data were collected in 2014. The LD_{50} values, 95% confidence intervals (CI; $P < 0.05$), and the slope $\pm$ the standard errors (SE) were calculated using Polo Plus Probit and Logit Analysis software (ver. 1.0). Significant differences were indicated by the failure of the CIs to overlap, and are followed by different letters.

Application	Strain	n	LD_{50} (ng/insect)	95% CI	Slope $\pm$ SE	RR
Topical	H	150	3.080	1.131 - 8.931[a]	1.173 $\pm$ 0.207	1
	B	250	0.614	0.383 - 0.954[b]	0.993 $\pm$ 0.112	0.2
	R	100	>500,000	n/a[c]	n/a	>162,338
	E	190	>244,318	n/a[c]	n/a	>79,324
Injection	H	90	0.037	0.025 - 0.054[a]	3.184 $\pm$ 0.737	1
	B	190	0.031	0.025 - 0.039[a]	2.731 $\pm$ 0.348	0.8
	R	100	4.430	3.189 - 6.089[b]	3.087 $\pm$ 0.683	120
	E	190	2.780	2.125 - 3.693[b]	2.007 $\pm$ 0.252	75
			Penetration Resistance Ratio (Topical LD_{50} / Injection LD_{50})			
	H		83			
	B		20			
	R		>112,867			
	E		>87,884			

Figure 2.1 Topical and injection application sites on a bed bug. Green line represents the site of topical application on the ventral side of the abdomen using a 25 µl Hamilton glass syringe with a repeating dispenser. The red arrow represents the site of injection using a glass capillary needle, custom-made by pulling a Kwik-Fil Silicate Glass Capillaries with a P-2000 Laser micropipette puller. The image was taken during injection application, using a DiMAGE Xg digital camera equipped with Konica Minolta zoom lens (focal length: 5.7 – 17.1 mm; Maximum aperture: f/2.8 – f/3.6; Konica Minolta Camera, Inc.) placed directly on top of a ocular lens of a dissecting microscope.



Chapter 3. Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes

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Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes

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ABSTRACT

Bed bug (*Cimex lectularius* L.) populations are increasing worldwide, with resistance to insecticides thought to be a major contributor. Several recent reports have documented widespread *kdr*-type mutations at the target site for pyrethroid insecticides, and there is substantial published evidence for metabolic resistance mediated through cytochrome P450-mediated oxidation. Here we report that resistance ratios for a bed bug strain collected in Richmond, VA, increase by three orders of magnitude when the route of insecticide treatment is changed from inoculation to topical. This increase suggests that reduced cuticular penetration plays a powerful role in bed bug resistance to insecticides. We identified 62 putative cuticle protein-encoding contigs from bed bug transcriptome data containing the Rebers and Riddiford consensus sequence. We classified these contigs as to CPR type, and compared the amino acid composition of the different types to that of the entire proteome. Quantitative PCR analysis indicated that many of these transcripts were substantially upregulated in resistant bed bugs, with some more than 20-fold higher than in the susceptible strain. These results suggest the possibility that thickening or remodeling of the bed bug cuticle may contribute to decreased insecticide penetration.

1. Introduction

Insecticide resistance is one of the most important factors contributing to the increase in bed bug (*Cimex lectularius* L.) infestations within the United States over the past 10 years [1]. Almost all modern bed bug populations collected within the US have been found to be highly resistant to insecticides in the pyrethroid class [1]. However, pyrethroid insecticides make up the great majority of insecticides that are currently available for bed bug treatment. Therefore, pyrethroid resistance has allowed the common bed bug (*C. lectularius*) to proliferate throughout the United States [1].

Knockdown resistance (*kdr*) to pyrethroid insecticides is caused by one or two point mutations (V419L and L925I) in the α-subunit of the voltage-sensitive sodium channel. These mutations were first observed in a population of bed bugs collected in New York [2]. After *kdr* resistance was documented in the New York strain, the same mutation(s) was found to be widespread among US bed bug populations [3]. Similarly, a modern population of the tropical bed bug, *C. hemipterus,* from Sri Lanka has also been found to have *kdr* resistance. The Sri Lankan resistant strain (collected in 2007), suggests that *kdr* resistance was not limited to US populations or even populations of *C. lectularius* [4].

Another type of insecticide resistance is characterized by the insects' ability to metabolize insecticides via enhanced detoxification enzyme activity (metabolic resistance). A bed bug strain collected in Richmond, VA was found to have increased cytochrome P450 and esterase activity in crude lysates. This enhanced activity of the detoxification enzymes was correlated with significantly higher levels of transcription among some P450 and carboxylesterase genes [5]. Studies using piperonyl butoxide (PBO) as a synergist for deltamethrin have also documented cytochrome P450 involvement in pyrethroid resistance within bed bug populations collected from Cincinnati OH and Worcester MA [6]. Similarly, silencing of the bed bug NADPH-cytochrome P450 reductase was found to increase bed bug susceptibility to pyrethroid insecticides [7]. In *C. hemipeterus*, elevated activities of esterases and carboxylesterases were detected in many populations, yet some P450 monooxygenase and acetylcholinesterase insensitivity was also detected [4].

Unlike the two mechanisms of physiological resistance described above the role of reduced cuticular penetration in bed bug insecticide resistance has not yet been robustly examined. The insect cuticle is composed of three layers [8]. The outer most layer of the cuticle is referred to as the envelope, and is approximately 10–30 nm thick. The envelope may provide some

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protection to the epidermis from chitinase activity during the molting process [8,9], though recent data indicates that the Knickkopf protein is essential in this regard [10]. Under the envelope layer is the epicuticle, which is approximately 1 μm thick and is stabilized by quinones but does not contain chitin [8,9]. Directly underneath the epicuticle is the procuticle (exo- and endo-cuticle), which is composed of a chitin and cuticular protein matrix arranged in stacks of laminae in specific orientations [8,9]. Differences in cuticular chitins are minor and only vary in chain length and the degree of acetylation; however cuticular proteins are highly diverse [11]. Most cuticular proteins are secreted by the epidermis directly into the cuticle and are covalently cross-linked to form a stable, sclerotized cuticle [8,12]. As the insect grows, cuticular proteins are added to the inner region; some are also added among the older proteins by intussusception. [9]. Thickening of the cuticle changes as the insect grows or ages [13], with different cuticular proteins located in different body regions, and/or appearing during different metamorphic stages [9].

By far the most common insect cuticle-associated proteins belong to the CB4 (chitin-binding 4)-domain containing protein family (CPR; Cuticular Protein with the Rebers and Riddiford Consensus; [14]). The CB4 was first recognized as a 28-residue region by Rebers and Riddiford [15], now known as the R&R consensus. This motif is highly conserved not only among all six orders of insects, but also among arachnids and crustaceans [16]. Currently there are three recognized forms of an approximately 68 amino acid extended R&R consensus, referred to as RR-1, RR-2, and RR-3 [17,18]; reviewed in [14]. RR-1 type cuticular proteins are thought to be associated with soft, flexible parts of the cuticle, while the RR-2 type proteins are associated with hard, rigid cuticle [17–19]. Cuticular proteins with the RR-3 consensus have not been associated with specific cuticular characteristics [14], but they have been found in post-ecdysal cuticle [18].

We recently described a bed bug (*C. lectularius* L.) strain collected in Richmond, VA that when exposed to deltamethrin and β-cyfluthrin had calculated resistance ratios of ~200–5000. This strain was determined to have both target site (*kdr*) and metabolic resistance [5]. However, these resistance ratios were based on LD_{50} data following the injection of insecticide directly into the bed bug hemocoel. This method of insecticide inoculation completely bypassed the cuticle, eliminating from our consideration the bed bug's most significant protective barrier from insecticides in the field. Here we demonstrate that resistance ratios in the Richmond strain exceed 10^5 when pyrethroid insecticides are applied topically to the bed bug cuticle. In fact, resistant bed bugs could not be killed at all using this method, even when the insecticide was applied at concentrations that were no longer soluble. These data suggest that the cuticle plays a significant role in further reducing resistant insects' susceptibility to insecticides in the environment. We also report a revised transcriptome assembly of the adult male bed bug to include some recently published Illumina data [20]. From this dataset, we have identified 62 putative CPR-encoding contigs. Quantitative PCR analysis revealed that approximately 70% of bed bug CPRs examined were significantly overexpressed in the pyrethroid-resistant strain. Thus, these findings suggest that possible thickening of the bed bug cuticle, through increased production of CPRs, may be an important mechanism of pyrethroid resistance in modern bed bug populations.

2. Materials and methods

2.1. Bed bug colonies and topical insecticide assays

Collection and rearing of pyrethroid susceptible (Harlan strain) and resistant (Richmond strain) bed bugs (*C. lectularius L.*) was as described previously [5]. Both strains were maintained by feeding on citrated chicken blood, through artificial membranes. For the topical application bioassay, technical grade pyrethroid insecticides (deltamethrin and β-cyfluthrin; Sigma Aldrich, St. Louis, MO) were dissolved in acetone. Stock solutions of the highest concentrations were made and serially diluted 10-fold. Solutions were applied (0.5 μl) onto the ventral side of the abdomen of 7-day-old adult male bed bugs with a 25 μl Hamilton glass syringe using a repeating dispenser. Each concentration of both pesticides was applied on ten individual bed bugs from each strain. The lethal doses (LD_{50} values) were calculated by counting mortality 24 h after application. The data was analyzed using Polo Plus Probit and Logit Analysis computer software version 1.0.

2.2. RNA extraction/qRT-PCR

Adult male bed bugs were frozen in liquid nitrogen 7 days after the final molt and stored at −80 °C. Total RNA extraction, polyA mRNA purification, and cDNA synthesis were performed as described previously [5]. Primers used to amplify each CPR gene are listed in Table S1.

2.3. Transcriptome assembly, analysis and annotation

Raw Illumina data corresponding to the Harlan strain (PS) from Mamidala et al. [20] were downloaded from the GEO (accession #GSE31823). Reads were filtered using the FASTX toolkit to remove low quality sequences (<80% of bases with quality score of >20) and sequence artifacts, followed by collapsing duplicate sequences to produce a non-redundant set of initial reads. Trinity [21] was used to produce an initial assembly. Output contigs were reduced to remove highly redundant isoforms and were assembled together with the contig set described in Adelman et al. [5] using CAP3 [22] to produce the final assembly (containing both .contigs and .singlets). To identify RR-type cuticle proteins, we queried the final assembly with pfam000379 using NCBI-BLASTX [23]. Contigs matching at an e-value of 1e−5 or lower were extracted and used as queries in a second TBLASTX search to identify any related sequences. Matching sequences were analyzed for the presence of a CB4 domain using Pfam [24]; those sequences without an identifiable domain were discarded. CB4 domains from each sequence were extracted manually and fed into MEGA5 [25]; protein alignments were performed using ClustalX, and were manually adjusted to conform to the known R&R extended consensus sequence [14,18]. Assignments of RR-1 and RR-2 type designations were made using cuticleDB [26] along with placement in the phylogenetic tree. Bed bug contigs were conceptually translated using OrfPredictor at http://proteomics.ysu.edu/tools/OrfPredictor.html [27]. The final updated transcriptome, along with each of the identified CB4-containing sequences, raw amino acid composition data, as well as cuticleDB, Pfam and SignalP predictions, are provided as Supplemental file S2.

3. Results

We demonstrated previously that a bed bug strain collected in Richmond, VA displayed both target site (*kdr*) and metabolic resistance to pyrethroid insecticides [5]. To explore the possibility of reduced insecticide penetration as an additional resistance mechanism, we performed topical LD_{50} bioassays on Richmond strain bed bugs and susceptible controls (Table 1). For both deltamethrin and β-cyfluthrin, we were unable to obtain LD_{50} data for the resistant strain, due to the fact that bed bugs did not die, even at the highest possible concentration of insecticide. This represents a resistance ratio of at least 10^5 between the two populations,

Table 1
Topical resistance to pyrethroid insecticides.

Insecticide	Strain	*N*	LD_{50} (ng/insect)	95% CI	Slope ± SE	Resistance ratio
Deltamethrin	Richmond	60	>80,000	?	?	>200,000
	Harlan	60	0.40	0.16–0.98	2.15 ± 0.7	
β-Cyfluthrin	Richmond	100	>500,000	?	?	>160,000
	Harlan	150	3.08	1.13–8.93	1.17 ± 0.2	

Table 2
Further improvement to the bed bug transcriptome.

	# Contigs	# Bases in contigs	N_{50}	Ortholog hit ratio > 0.8[a] (%)	Ortholog hit ratio > 0.5[a] (%)
Adelman et al. [5]	23,036	17,014,701	1036	22	41
Mamidala et al. [20]	51,492	na	1150	33–35	48–50
Combined assembly	29,751	30,574,174	1885	43	61

[a] Determined by blastx comparison to the *A. pisum* geneset [42].

several orders of magnitude greater than when the same assays were performed using inoculations [5]. Thus, we confirm that there are at least three resistance mechanisms operating in this strain, with the end result being complete resistance to two different pyrethroids, at any dose, when applied externally.

One possible explanation for this extreme phenotype is that the expression of proteins involved in cuticle formation and structure are overproduced in the resistant strain. An initial survey of our previous 454-based transcriptome data [5] revealed 24 distinct potential CPR-encoding genes. Compared to hymenopterans (32–62 CPRs) and dipterans (101–156 CPRs), this was likely only a small subset of the total bed bug CPR repertoire. In order to identify the most complete set of CB4-type proteins, we merged our previous transcriptome assembly [5] with a more recent set of Illumina data derived from the same strain (Harlan) of bed bug [20]. As shown in Table 2, merging these two datasets resulted in a substantial increase in both the N_{50} (number of contigs containing more than 50% of the assembled bases) and the number of *Acyrthosiphon pisum* orthologs covered at >80% compared with either assembly alone.

Using the CB4 domain as a query (Pfam000379), we identified 62 contigs potentially encoding CB4-type cuticle proteins. Of these, 41 appeared to be full length based on the presence of an initiator methionine and a termination codon following the open reading frame. Ten contigs were truncated at the 5′ end (no start codon), seven at the 3′ end (no stop codon), and four contained truncations at both ends. Analysis with the software SignalP 4.0 [28] and Phobius [29] yielded a prediction that all contigs with an intact 5′ end had an identifiable signal peptide (Table S1). Two contigs (5191, 8456) with 5′ truncations were also predicted to encode a signal peptide, indicating in these cases that the amount of missing information was relatively small. No domains other than CB4 were identified in any of the predicted protein sequences, with most contigs containing a single CB4 domain (Table S1). The three exceptions to this were contigs 3553, 4795 and 2034, which encoded for two, two, and five CB4 domains. Putative CPR protein sequences were further classified with cuticleDB [26] into RR-1 and RR-2 subgroups. Alignment of the 68 CB4 domains from all 62 contigs confirmed the strong conservation of the R&R extended consensus sequence [14,18] among bed bug CPRs (Fig. 1). From this, we deduce a bed bug-preferred consensus of YxFxYxxxDx(14–25)GxYx(5)DGx(4)VxYxADx(2–4)GF, where x is any amino acid. We note that four putative CPRs lack the final GF motif, with two of these (Contigs1870 and 1629) containing a pair of cysteines within the R&R consensus region (Fig. 1).

A neighbor-joining tree was produced using this protein alignment to further visualize the relationships among all 68 identified CB4 domains (Fig. 2). All RR-2 and RR-1 type CB4 domains formed two discrete clusters, confirming the overall accuracy of the cuticleDB prediction tool. (Fig. 2). Four of the unclassified CB4 domains clustered with the RR-2 clade. Of these, three (contigs 1800, 5711, and 11109) were predicted by cuticleDB as RR-2, but below our score cutoff (40 for both RR-1 and RR-2), while the last (11057) was a truncated sequence. Thus, we designate 14 contigs as encoding for RR-1 type CPRs, 31 contigs to the RR-2 type, and a single RR-3 type CPR. The remaining unclassified CB4 domains, representing 16 additional putative CPRs, formed five distinct clades (I–V). Each of these clades received strong bootstrap support, though the placement of each clade with respect to the RR-1 and RR-2 clusters as indicated in Fig. 2 was arbitrary.

We next examined the amino acid composition of each of the bed bug CPRs to determine if the unclassified groups were more biochemically similar to the RR-1 or RR-2 types. As expected, the predicted protein sequences for all classes of bed bug CPRs (following removal of the predicted signal peptide) were found to be highly enriched for the amino acids A, G, and P, as well as Y, when compared to the entire predicted bed bug proteome (Fig. 3A, and Supplemental file S2). Several other trends in amino acid composition were observed between the RR-1 and RR-2 groups. Bed bug RR-1 type proteins displayed a stronger preference for P compared with RR-2 proteins, and were highly enriched in Q, whereas RR-2 proteins contained Q at levels similar to the average across the proteome. Conversely, RR-2 proteins were specifically enriched in V, D, and H and showed a stronger preference for Y than RR-1 type proteins. Several amino acid residues were found to be underrepresented in bed bug CPRs than the genomic average, with some interesting trends based on RR type. While all CPRs contained far less I and L than the genomic average, these amino acids were significantly lower in RR-2 type proteins than RR-1. In fact 19 of the 31 RR-2 proteins were completely devoid of I, an interesting observation considering the strong enrichment for V (5–20% of the total protein) in this group. Usage of residues E and K were also lower across all CPRs, with only RR-2 type proteins showing a preference away from N. In general, amino acid usage among the unclassified proteins was similar to that of the RR-1 type, with the only difference being a greater presence of R. More specifically, this trend was likely due to the contributions of groups-II and -III, where all 8 members were found to be P and Q rich but V and H poor. Two of the three group-IV proteins (4474 and 3553), as well as the RR-3 type, were more similar to RR-2 type proteins, being V and H rich and Q poor. The two group-I proteins (2034 and 4795) were G and A rich and H poor, but were also highly enriched for S (14% and 15% of the total protein). Likewise, all three group-V proteins displayed an enrichment for W, an amino acid that is rare or absent

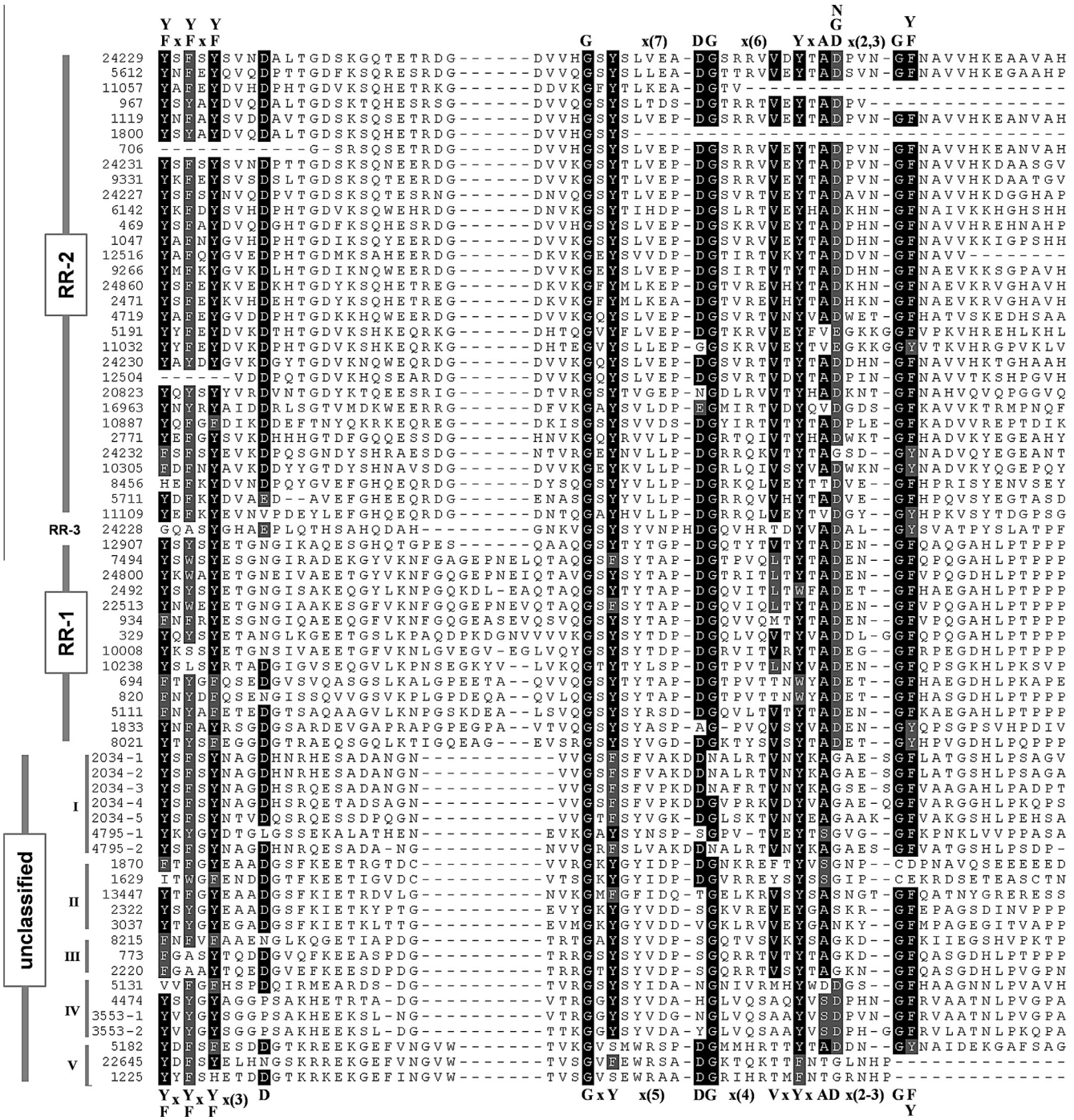

Fig. 1. Sequence alignment of the CB4 domain (Pfam00379) of putative bed bug cuticle proteins. The Rebers and Riddiford extended consensus (above) and bed bug consensus (below) sequences are indicated. Identical (black) and similar (gray) residues present in >70% of sequences are indicated.

in all other CPRs. As has been described for other insects, both C and M were extremely rare in all classes of bed bug CPRs. Complete descriptions of the amino acid composition for each putative cuticle protein are listed in Supplemental file S2.

Resilin is an important component of insect cuticles due to its rubber-like qualities: high elasticity, stretching, and the ability to return to form after deformation (reviewed in [30]). Pro-resilin is used to refer to the initial protein monomer prior to cross-linking. Pro-resilins are characterized by a signal peptide, a conserved N-terminal peptide, a high glycine content (typically >30%), and an RR-2 consensus sequence flanked by upstream and downstream sets of glycine-rich repeats [30]. Based on these criteria, we identify contig24232 as the bed bug pro-resilin gene. At 36.8% G, this is by far the most glycine rich CPR gene, and the N-terminal sequence (DAPVN-SYLPPKS) matches closely to the consensus sequence deduced from dipteran and hymenopteran pro-resilins (EPPVN-SYLPPS) [30]. The RR-2 consensus sequence is preceded by four 9 a.a. repeats [consensus G-F/Y-G-G-X-X-X-X-G] and is followed by seven 8 a.a. repeats [consensus G-X-G-Y/F-P-G-G]. The CB4-domain of bed bug pro-resilin forms a distinct clade within the RR-2 cluster with 5 other CPRs (Fig. 2). We compared the amino acid composition of these 6 pro-resilin-like sequences to the

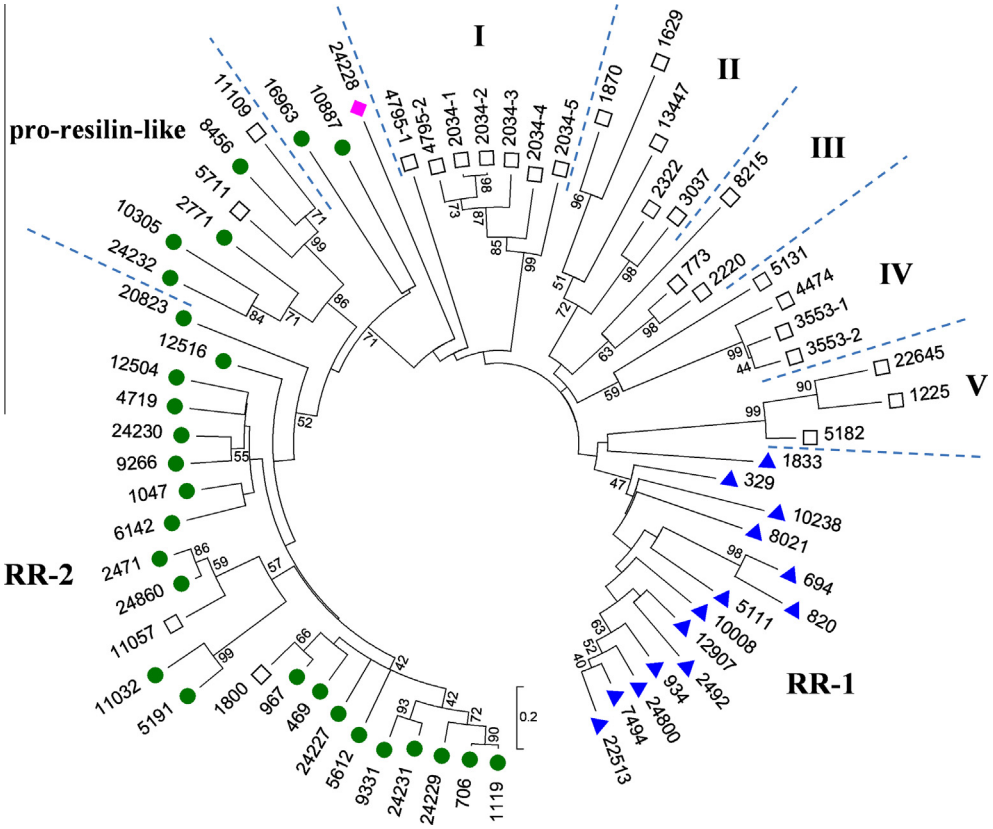

Fig. 2. Alignment of CB4 domains from assembled bed bug contigs. Predicted CB4-domain protein sequences were aligned with ClustalW, with manual adjustments to conform to known critical residues within the R&R consensus. The Neighbor-joining method was used to construct the tree, bootstrap value over 40% (of 2000 replicates) are indicated. Predicted RR-1 (triangles), RR-2 (circles) and RR-3 (diamond) type, as well as unclassified (open squares) adult cuticle protein-like genes are indicated.

rest of the RR-2 cluster (Fig. 3B). Pro-resilin-like sequences differed from the main RR-2 group only in the usage of G and V; pro-resilin-like sequences were G rich and V poor, while the main RR-2 CPRs were V rich, with lower levels of G (Fig. 3B).

To determine if the expression of the any of the bed bug CPR-encoding mRNAs were altered in our pyrethroid-resistant bed bug strain, we performed RT-qPCR analysis on a subset of CPR-encoding transcripts (Fig. 4). Of the 23 CPR mRNAs analyzed, we observed a significant increase in mRNA transcripts in 16 (69%). Increases in steady state transcript levels were substantial, as seven CPRs had more than a 10-fold increase in mRNA transcripts, with two found to increase by more than 20-fold. Upregulation of CPR transcripts was largely independent of type, as RR-1, RR-2, RR-3 and unclassified CPRs all had members displaying strong differential regulation. However, of the 7 RR-2 genes examined, the four displaying significant increases all derived from the main RR-2 cluster, while the two pro-resilin genes showed no change. For the unclassified CPR contigs, we observed differential regulation from members of groups-I, -II and -III, but not -IV or -V. Overall, these data suggest that the overexpression of CPR genes, should be highly relevant to the formation and thickening of the adult cuticle. Future studies based on proteomic analysis of the bed bug cuticle will be required to confirm that the products of the upregulated genes we observed are true structural components of this tissue.

4. Discussion

The properties of insect cuticle including strength, flexibility, and permeability are determined not only by the chitin sclerotization, construction, and hydration, but also by the combinations of different cuticular proteins and their arrangements [11]. Classification of cuticular proteins has largely focused on representative motifs found in the primary protein sequence; the most obvious example being the R&R consensus sequence, the most abundant motif found in cuticle proteins known to be responsible for anchoring the protein to the chitin matrix. RR-1 and RR-2 type CPRs are thought to differ with respect to their association with soft/elastic or hard/rigid cuticle types. This association with specific cuticle types is thought to be primarily regulated by the number of histidine and lysine residues available on the outer surface of the protein for cross-linking [19]. Histidine, lysine, and tyrosine are all expected to be able to serve as cross-link points during cuticular sclerotization [12,31]. Indeed, other studies have shown an enrichment of both histidine and lysine in RR-2 type proteins when compared with RR-1 types [9,19]. We found that bed bug RR-2 type proteins were significantly enriched for histidine and tyrosine, but not lysine as compared with RR-1 types. This difference suggests that these residues may play a more general role in the sclerotization of harder cuticle structures of the bed bug. Several other biochemical characteristics are typical of CPR proteins. For example, almost all structural cuticular proteins are devoid of cysteine and methionine residue in mature proteins, most probably because they interfere with sclerotization [9]. It is interesting then, that of the 62 putative bed bug CPRs we characterized, two contained a pair of cysteines within the R&R consensus region. The role of these highly unusual proteins in bed bug cuticular structure remains to be determined, but we note that one of these (contig1629) was upregulated in the pyrethroid-resistant strain.

Because RR-2 type CPRs are thought to derive primarily from hard cuticle structures, (as determined by structural modeling [19] and histidine content [9]), the presence of the RR-2 motif in

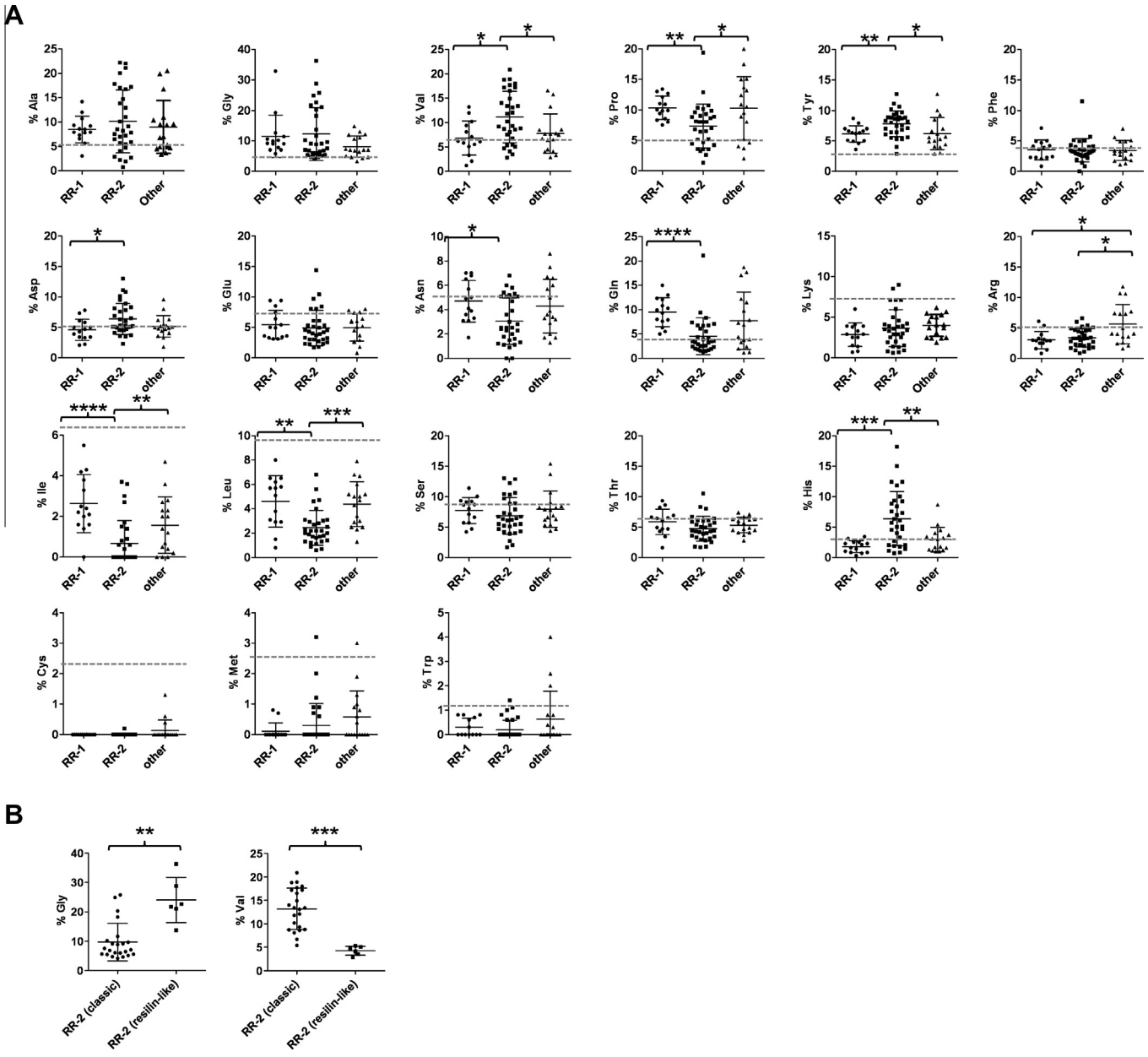

Fig. 3. Amino acid composition of bed bug CPR genes based on *in silico* translation. (A) The percent of each amino acid (AA) found in the presumptive mature protein summarized for all beg bug CPRs as a function of RR-1 or RR-2 designation. RR-3 and unclassified (I–V) are grouped as other. The average amino acid usage across the entire bed bug proteome (as determined by conceptual translation of our assembled contig set) is indicated by the dashed horizontal line for each plot. (B) Amino acid composition of pro-resilin-like RR-2 proteins with the main RR-2 cluster as described in Fig. 2. Statistical significance for all pairwise combinations was determined using the Mann–Whitney test. Differences between groups were not significant unless indicated otherwise ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

pro-resilins is counter-intuitive [30]. Pro-resilins are highly elastic and deformable, rather than rigid, yet all characterized to date contain the RR-2 motif [30]. Our data suggest that pro-resilins form a separate subgroup of RR-2 type proteins. These proteins are phylogenetically distinct from the main cluster, both in the primary sequence of the extended R&R consensus and in amino acid composition, with the glycine-rich pro-resilins expected to be more flexible and elastic than their valine-rich RR-2 counterparts [30].

Recently, Mamidala et al. [20] reported on an RNA-seq study conducted on both Harlan strain bed bugs (the susceptible strain used in our experiments as well), and a resistant bed bug strain collected in Columbus, OH. Among the more than 5000 genes Mamidala et al. found to be differentially regulated between the two strains were a number of potential components of the cuticle. Unfortunately, we were unable to determine the amount or extent of overlap between the CPRs we describe and the cuticle genes identified in the Mamidala et al. study [20] due to the fact that the contig assembly was not included in their publication, and was not available in a public repository as of the time of this submission. Interpretation of the data in Mamidala et al. [20] was further complicated by the fact that no measure of cuticular penetration (change in insecticide resistance ratios between bed bug treated topically versus injection) was obtained for the sequenced strains. Thus, the magnitude of resistance associated with cuticular penetration in their resistant strain, and the correlation of that resistance with any upregulated sequences, remains unknown.

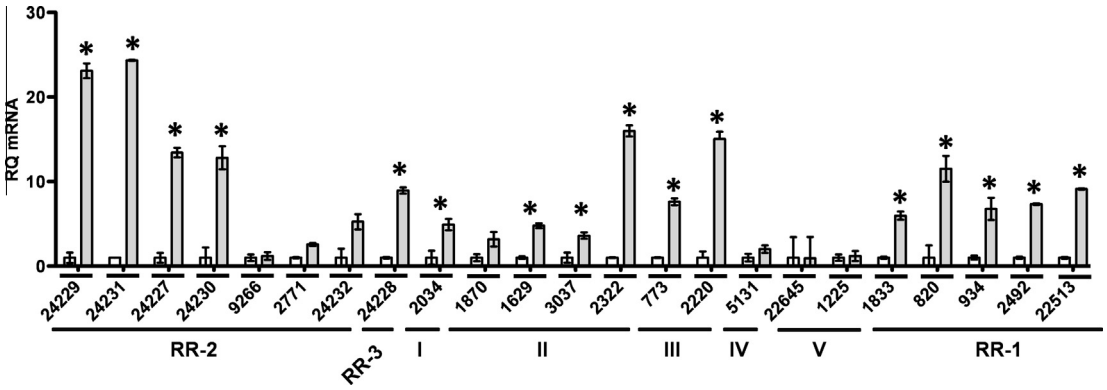

Fig. 4. Increased transcript levels of putative bed bug cuticle genes in a resistant population. Quantitative real-time PCR was performed using cDNA extracted from Harlan (susceptible, white bars) or Richmond (resistant, gray bars) for 23 genes with a potential role in the cuticle. Each bar represents the mean of two biological replicates; each performed in triplicate and normalized using two endogenous control genes (tubulin and myosin). Statistical significance using a modified t-test (see materials and methods) is indicated ($^{*}P < 0.05$).

Reduced cuticular penetration as a form of insecticide resistance has been documented in several insect species. Many of the resistant insect species described below have a long history of insecticide exposure(s) due to their pest status. The cotton bollworm *Helicoverpa armigera* Hübner exposed to [14C] deltamethrin exhibited delayed cuticular penetration (and enhanced detoxification) where the rate of penetration and the internal concentrations of [14C] deltamethrin was inversely correlated with the magnitude of pyrethroid resistance [32]. A fenthion-resistant strain of house fly *Musca domestica* L. selected first with biorethmethrin then later with (1R)-trans-permethrin showed decreased cuticular penetration when exposed to (lR,S)-trans-[14C] permethrin. The flies also displayed decreased nerve sensitivity when exposed to other pyrethroid insecticides [33]. Reduced cuticular penetration has also been documented in a strain of German cockroaches *Blattella germanica* L. exposed to [14C]fenvalerate [34] residues. In the malaria vector *Anopheles funestus*, cuticular thickness was positively correlated with the level of known metabolic resistance to permethrin [13]. Pyrethroid-resistant populations of *Triatoma infestans*, a vector for *Trypanosoma cruzi*, have also been reported to have significantly thicker cuticles than non-resistant populations [35]. Finally, a transcriptional analysis of a pyrethroid resistant population of *Anopheles stephensi* showed a significant upregulation of a member of the low-complexity family of cuticle proteins (CPCL8, now CPLCG3 [36]), thought to be involved in thickening of the cuticle [37]. Likewise, microarray analysis revealed that transcripts for both CPLCG3 and CPLCG4 were upregulated in a pyrethroid-resistant strain of *An. gambiae* [38].

It is not surprising that more than a century of insecticide exposure would select for populations of bed bugs with relatively thick or dense cuticles. However, the physiological processes involved in how and when these additional cuticular proteins are deposited in the cuticle is unknown. In this study, only bed bugs that were 7 days post-eclosion adult were selected for testing. This was to insure that their cuticular sclerotization was complete, and that adult hormone levels were stabilized. However, the greater abundance of CPR transcripts we observed in resistant bed bugs at 7 days post eclosion leave us to hypothesize as to how the mechanistic processes of cuticular protein deposition might be altered. One possibility is that CPR transcripts in the resistant strain are present in greater abundance throughout the deposition of the adult cuticle. Alternatively, CPR transcripts may be present in similar amounts between resistant and susceptible populations initially, but extended synthesis or stability in the resistant strain leads to a thicker or denser cuticle. In the former case resistant adult bed bugs, regardless of their time since eclosion, would produce very consistent resistance ratios when exposed to a particular concentration of insecticide. However, in the latter instance, the resistant strain would become increasingly resistant (would produce greater resistance ratios) over time. Future studies examining topical resistance throughout the bed bug lifespan should resolve these two scenarios.

Our results confirm that reduced cuticular penetration type resistance in bed bugs may be a significant contributor to the total resistance ratio, though direct measurements of the movement of pyrethroids across the bed bug cuticle in various formulations are still needed. Other factors may also contribute to the observed phenotype, such as the increased activity of ABC transporters [39–41]. We observed that topical resistance adds more than three orders of magnitude to the total resistance ratio when combined with target site and metabolic resistance. Similarly, we conclude that at least one (and probably many) US bed bug population has all three forms of physiological resistance (reduced cuticular penetration, enhanced metabolic activity, and target site insensitivity). It will be critical to identify genetic markers associated with this reduced penetration resistance phenotype to allow for population studies based on geographic distribution (similar to *kdr*-based studies) [3].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pestbp.2013.01.001.

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Chapter 4: Cuticular proteins of the Common bed bugs, *Cimex lectularius* L. Identification and description of the bed bug cuticular proteins using the matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry (MALDI-TOF/TOF)

4.1 Introduction

Cuticular proteins (CPs) are one of the two important elements in insect cuticle along with chitin (Rubin et al. 2010, Willis 2012, Iconomidou et al. 2005). Chitin, the main structural component of arthropod exoskeleton, is a homopolysaccharide composed of β-(1-4)-*N*-acetyl-D-glucosamine residues, which in insect cuticles, forms antiparallel crystalline sheets (Cohen 2010). CPs are much more structurally diverse, providing a wide range of insect cuticle properties based on the ratio, type, and cross-linking structures (Rubin et al. 2010). CPs are believed to be distributed in the arthropod cuticle cross-linked to each other forming a matrix that is tightly bound to chitin polymers (Cohen 2010). CPs are synthesized in the epidermal layer of the cuticle and subsequently transferred to the upper procuticle region where they are deposited. Depending on the CPs deposited and degree of sclerotization, cuticles can be extremely elastic or rigid (Iconomidou et al. 2005, Rubin et al. 2010, Willis 2012), leading to soft and hard cuticles, respectively (Gullan and Cranston 2005). Sclerotization is an irreversible chemical reaction that initially involves the hydroxylation of tyrosine (Y) aromatic ring to 3,4-dihydroxylphenylalanine (DOPA). DOPA is subsequently decarboxylated to

dopamine and *N*-acylated to either *N*-acetyldopamine (NADA) or *N*-β-alanyldopamine (NBAD). Both of these molecules are sclerotization precursors, as oxidation to *O*-quinones leads to *p*-quinone methides and catechol derivatives (Andersen 2010). These pool of reactive intermediates can react with themselves or with N-terminal or side chain amino groups of CPs leading to a complex crosslinked polymeric network where the water-filled space between CPs will be filled with polymerization products during the sclerotization event (Andersen 2010).

There are two types of proteins that exist in insect cuticle: structural and non-structural (Willis 2012). The non-structural proteins that can be found in insect cuticle include pigments, various enzymes, defense proteins, and arylphorins (Willis 2012). Structural CPs were first identified and characterized by Andersen et al. (1995) directly sequencing 40 purified CPs (Willis 2012). Thirteen families of CPs have been recognized to date, and while several are found to be extremely conserved across many Orders of insects (Diptera, Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Orthoptera, Dictyoptera, Phthiraptera, and Collembola), some are Order specific (Willis 2012). Currently, there are more than 300 CPs that have been identified and confirmed, and hundreds of sequences putatively identified through genomic efforts (Willis 2012).

The CPR family of cuticle proteins is the most common, containing a characteristic chitin-binding 4-domain (CB4), which was originally identified by Rebers and Riddiford and

hence named R&R consensus domain (Willis 2010). Originally, it was identified as 28-amino acid sequence but now it is extended to 68 (extended R&R Consensus) (Andersen 1998, 2000). The extended R&R Consensus can further be divided RR-1, RR-2, and RR-3 types. RR-1s are associated with soft cuticles, RR-2s with hard cuticles, and RR-3s with post-ecdysal cuticles (Andersen 2000). Our recent investigation of the bed bug transcriptome (Koganemaru et al. 2013, Chapter 3) showed that the putative CPR family CP genes were highly up-regulated in Richmond (R) resistant strain bed bugs relative to the Harlan (H) susceptible strains. Zhu et al. (2013) observed that many of the CP genes they examined in their resistant strain bed bugs were also up-regulated. Wood et al. (2010) reported there was a significant correlation between the resistance levels and cuticular thickness of a malaria-vector mosquito, *Anopheles funestus* Giles. These studies support the hypothesis that resistant strain bed bugs have evolved a strategy to thicken or modify their cuticle leading to reduced insecticide penetration.

To test the above hypothesis at the protein level, we extracted and identified the proteins present in cast skins of resistant and non-resistant bed bugs using mass spectrometry-based proteomics techniques. The results verify the presence of several CPs that were identified in our previous transcriptome analyses and also demonstrate the presence of many additional CPs.

4.2 Materials and methods

4.2.1 Bed bug colonies

Collection and rearing of the pyrethroid susceptible Harlan (H) strain and resistant Richmond (R) strain bed bugs, *C. lectularius* L., was as described previously (Adelman et al. 2011), except defibrinated rabbit blood was used since 2013. The colonies were maintained by feeding only the defibrinated rabbit blood through artificial membranes. A new colony of the H strain was obtained from Dr. Harold Harlan of the National Pest Management Association (Fairfax, VA) again to establish a fresh colony soon after the blood was switched.

4.2.2 Cuticule preparation and washing

Bed bug cast skins from the 5^{th} instars were collected at each molting cycle and stored in separate glass jars at room temperature by strain. The collected shed skins were then ground in a liquid nitrogen using a mortar and a pestle, until the cuticles formed a powder-like pellet from which 20 mg was processed further, with the remaining material stored at -20ºC.

Subsequent processing to remove impurities essentially utilized the procedures described by Okot-Kotber et al. (1996). The powders were mixed with 50 mM DTT-SDS buffer (600 µl) that was made using 2x Laemmli SDS sample buffer (Biorad) and LC-MS grade water, and heated for 15 min at 98ºC on a heating block after securing the cap with an aluminum foil

wrap. The microtubes were allowed to cool down to a room temperature, then centrifuged at 17,000 x g for 5 minutes. The supernatant were collected into a separate tube. The procedure was repeated five times, adding a fresh 50 mM DTT-SDS buffer each time as the washing reagents. The washing reagent was then removed from the pellets and washed with 25 mM ammonium bicarbonate (NH_4HCO_3), repeating five times at 15 minutes each, centrifuged at 13,000 x g for 10 minutes in between the washing processes.

4.2.3 Protein digestion and peptides extraction

To digest and/or extract the cuticular proteins, enzyme Trypsin was used for the peptide extractions. Mass spectrometry grade trypsin (Sigma) was used to digest the cleaned cuticles. Aliquots were prepared by re-suspending 20 μg lyophilized protease powder with vigorous vortexing in 40 μl 50 mM acetic acid to give the 0.5 μg / μl stock solution. Then it was diluted with 300 μl freshly-prepared 100 mM ammonium bicarbonate. For each a total of 3 μg trypsin was used per digest. The samples were incubated overnight for 16 hours at 37ºC. The digested cuticle peptides solutions were brought to 0.25% trifluoroacetic acid (TFA) utilizing a 5% stock solution (prepared by adding a 1 ml ampule of TFA with 19 ml LC-MS grade water). The cuticular peptides solutions were then adjusted to 1% formic acid using high-purity (≥ 98%) formic acid, mixed well using vortexing and then centrifuged at 10,000 x g for 2 minutes to

separate the peptides (digested proteins) from solids (undigested pellets). The aqueous fraction was removed and desalted using 100 μl Omix C18 solid-phase extraction pipette tips (Varian, Inc., Walnut Creek, CA) following the manufacturer's instructions. The peptides were eluted from the tips using 50:50 (v/v) acetonitrile:water containing 0.1% (v/v) trifluoroacetic acid (TFA), and then concentrated in a centrifugal vacuum concentrator to dryness. The peptide samples were then re-suspended in 40 μl 98:2 LC-MS grade water: LC-MS grade acetonitrile containing 0.1% TFA.

4.2.4 Separation of the peptides and sample preparation for the MALDI-TOF/TOF plate

Ten microliters of the re-suspended sample was introduced via a manual injection valve into a 50 x 0.1 mm Magic C18 AQ, 200A, 3 μm (Michrom Bioresources, Inc.) reversed-phase column packed in-house using Integrafrit fused-silica capillaries. The column was connected to the low-flow rate channel of a 2D-HPLC system (NanoLC-2D, Eksigent part of AB Sciex, Redwood City, CA) and maintained at 60ºC using a nanocolumn heater (Phoenix S and T). The effluent from the column was spotted onto a 384 spot MALDI target plate (AB Sciex) utilizing a spotting robot (Ekspot, Eksigent). Solvents were 98:2 LC-MS grade water: LC-MS grade acetonitrile containing 0.1% TFA (solvent A) and 2:98 LC-MS grade water: LC-MS grade acetonitrile containing 0.1% TFA (solvent B). The column was equilibrated for 4 minutes at 95%

A. Once the injection valve was switched to have the sample in-line with the column, solvent was changed to 90% A. This concentration was maintained for 10 minutes. This was followed by a linear gradient from 90% A to 64% A over 100 minutes, then a 1 minute gradient change to 25% A which was maintained for the rest of the run. Flow rate was 700 nl/min and column effluent was spotted from 19 to 179 minutes advancing from spot to spot every 25 seconds. The MALDI plate spots were then overlaid with alpha-cyano-4-hydroxycinnamic acid matrix solution (See Appendix 4.1 for the preparation protocols of the alpha-cyano-4-hydroxycinnamic acid matrix). The spots were air dried and the plate was loaded into the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry.

4.2.5 Peptides analysis and protein identification

The mass-spectra peaks of the peptides separated on the MALDI plate were detected and analyzed using matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF/TOF, ABSciex 4800 MALDI-TOF/TOF). The MALDI-TOF/TOF data were imported and analyzed using the Scaffold Q+ software (ProteomeSoftware, Inc., Portland, OR, USA) to identify the contigs that match the genome assembly sequence (Clec_Bbug02212013.contigs.fa) available from the Bed Bug Genome Project website (Baylor College of Medicine Human Genome Sequencing Center, Houston, TX) at

https://www.hgsc.bcm.edu/arthropods/bed-bug-genome-project. The results were filtered limiting peptides to those with >95% confidence. For the determination of peptide and protein levels, only the peptides that satisfied above conditions were used. The exclusive unique peptide counts were used for the semi-quantitative analysis, comparing the peptide counts among different biological samples (H or R, replication 1, 2, or 3) only within each contig and within the exact peptide sequence match.

To identify the proteins present in the bed bug cast skin extracts, the identified contigs confirmed by the presence of peptides that satisfied above statistical conditions were first examined for the presence of transmembrane structures and/or the signal peptides using the Phobius online server (SBC [Center Stockholm Bioinformatics], Stockholm University, Solna, Sweden) at http://phobius.sbc.su.se/. Then the contigs sequences were queried using NCBI-BLASTP (NCBI-protein blast) to identify predicted or related proteins. The locations of the bed bug contigs on the bed bug genome was manually aligned and annotated by using Web Apollo Jbrowse web-based sequence annotation editor tool (Lee et al. 2013 Web Apollo) through the BCM-HGSC website, comparing mostly with the female RNA sequences, male RNA sequences, embryo RNA sequences, and Koganemaru et al. 2013 data already available in the Web Apollo Jbrowse.

4.3 Results

Cuticles from resistant and non-resistant bed bugs were collected from shed skins and processed at approximately equal protein loading levels to test the hypothesis that pesticide resistance is associated with a modified protein composition of the cuticle. Peptides generated from trypsin digests of shed skins from both the non-resistant Harlan strain and the resistant Richmond strain were analyzed using LC-MALDI. A total of 265 peptides were identified with a 95% confidence level, 206 of which represented amino acid sequences belonging to only one of 50 confidently identified proteins (Appendix A). The other 59 peptides were amino acid sequences found in 2 or more of the identified ORFs (Appendix A). The majority of the peptide sequences identified exhibited low sequence complexity, enriched with A, G, P, Y and V, with short sequence repeats, characteristics observed within sequences of many previously identified cuticular proteins (Willis 2012 and 2014).

Many of the proteins identified were members of previously described families of CPs including CPR (RR-2 and RR-1), CPRL, CPF, CPFL, TWDL, and CPAP1 (Table 4.1). Two proteins, NPLP3 and a peroxidase, were not considered to be cuticle-associated proteins while several contained sequence motifs similar to other CPs but could not be confidently assigned to any specific family. A few had predicted signal sequences suggesting they were secreted

proteins while roughly half of the proteins identified share no homology with previously described families of CPs or other characterized proteins. These proteins therefore must represent a new family or families of CPs.

As can be seen in Table 4.1, no protein was confidently identified in only Harlan or only Richmond cuticle digests. Two proteins appear to be identified more readily in Richmond than Harlan digests: protein number 15 (identified as CLEC004438-PA) that appeared in all 3 R replicates but only 1 H replicate, and 21 (not associated with CLEC) in which all 3 quantifiable proteotypic peptides exhibit an R/H greater than one. Unfortunately, not enough data was collected for these two proteins to provide statistical proof that this truly reflects a relative abundance change of these two proteins in Richmond versus Harlan. Three other proteins, 48 and 49 (both possibly associated with CLEC000732-PA) and 18 (CLEC001964-PA), also exhibited consistently higher R/H peak area ratios for all quantifiable proteotypic peptides but without enough information available for confident statistical analysis.

While protein levels may be the same between strains, single point mutations or modification of particular residues in a protein may account for the differences in resistance observed. Hence we sought to compare changes in the proteome at the peptide level by comparing peptide peak areas for those peptides found only in particular proteins (proteotypic

peptides). Table 4.2 shows the seven peptides that had significantly different ($P \leq 0.05$) average R/H peak area ratios. The Richmond to Harlan ratio for the protein represented by the ORF CLEC007768-PA (CPR84), for example, was found to be 0.69 ± 0.01 (p-value 0.05) based on the measurements of a proteotypic peptide. Similarly, proteins encoded by CLEC004093-PA (CPR32), CLEC002505-PA, CLEC011522-PA and CLEC004437-PA were also significantly less abundant in Richmond versus Harlan digests at the peptide level (Appendix A). The only protein to be statistically greater ($p = 0.04$, 11 measurements of 4 separate proteotypic peptides) in Richmond versus Harlan, encoded by CLEC011386-PA/CLEC011388-PA, was found to be almost 1.3x more abundant in Richmond but with a variation of almost 35%.

When the peak areas of individual peptides were compared between Richmond and Harlan for the proteins above, most exhibited the same trend observed when averaging the peak area ratios for the proteotypic peptides. The average ratio for 3 of the 4 proteotypic peptides assigned to CLEC011386-PA/CLEC011388-PA indicated a higher relative abundance in the Richmond sample than the Harlan sample. Ratios for both proteotypic peptides from CLEC004437-PA indicated a lower abundance in Richmond relative to Harlan as did all proteotypic peptides from CLEC011522-PA and CLEC007768-PA (CPR84), while most, but not all, proteotypic peptides from CLEC004093-PA (CPR32) and CLEC002505-PA exhibited the

same trend. Finally, most peptide ratios that exhibited a fold change greater than 2 or a p-value less than 0.05 were those exclusively associated with one of the proteins described above.

Peptides that did exhibit a significant change for which the corresponding protein did not, such as TYGYSGLGYGR from protein 13 (CLEC004439-PA) which has an R/H ratio of 0.58 versus near 1 for the other two proteotypic peptides from this protein (Table 4.2), may be the result of changes in access to cleavage sites recognized by trypsin, as crosslinking and other post-translational modifications may hinder access of the protease to the cleavage site. Another example is protein 5 (CLEC011346-PA) for which the R/H ratio is greater than one for three quantifiable proteotypic peptides but less than one for the other three (Appendix A).

Identified peptides with sequences found in multiple ORFs allowed the grouping of these ORFs into 6 families of homologs: proteins 1, 4 and 5; 13, 14 and 15; 2, 3 and 11; 6 and 23; 24, 29, 30, 34, 35, 40 and 42 (CPR-like); and, 41, 47, 48 and 49 (CPRL-like) (Table 4.1). Interestingly, many of the genes for these ORFs belonging to the same family appear to be grouped together as evidenced by being present on the same genome sequencing scaffold number. This suggests the possibility that homologs may be due to gene duplication events.

4.4 Discussion

In this study, we confirmed the presence of several previously predicted bed bug cuticle proteins (CPs) within tryptic digests of sclerotized shed cuticles, including members of the RR-2 family of CPs. Members of the RR-2 family are known to be associated with hard cuticular material such as the shed cuticles analyzed in this study (Koganemaru et al. 2013). We also confidently identified many proteins that had not previously been predicted to be associated with bed bug hard cuticle including representatives of other families of known CPs; RR-1, CPRL, CPF, CPFL, TWDL, and CPAP1. These cuticular protein families have previously been identified and annotated in other species of Hemipetra (Gilbert 2012, Willis 2012) but this study is the first to confirm the presence of representatives of these cuticular protein families in bed bug cuticles. In addition, several proteins not belonging to previously described cuticle protein families, unclassified hypothetical secreted proteins that may represent new families of CPs, and some additional proteins known to not typically be associated with the cuticle were also identified. These results have allowed us to expand upon our previously proposed list of 62 putative CPR-type cuticle proteins (Koganemaru et al. 2013) bringing our new list to close to 300 putative bed bug CPs covering a broad range of known CP families as well as possible new families of CPs. Added to the list are those proteins identified in this study as well as homologs of the identified proteins discovered by searching preliminary genomic data (Adelman,

unpublished).

The lists of proteins identified from digests of Harlan strain and Richmond strain shed cuticles are essentially the same. This suggests no major shift in cuticle proteome composition causes the pesticide resistance observed in the Richmond strain. However, these results do not rule out the possibility that the resistance is associated with more protein but with individual proteins still present at the same relative ratios. Though several CPs for which quantifiable information was obtained from two or more exclusive unmodified peptides did exhibit a statistically significant difference between digests of Harlan and Richmond strains, it cannot yet be determined if such subtle changes could in fact be responsible for pesticide resistance. The protein with the largest statistically significant change, CLEC004415-PA, was found to only be 3-fold less in Richmond versus Harlan. It may be possible that such small shifts in proteome composition may have drastic effects on cuticle structure if the proteins with altered levels were those primarily associated with crosslinking or the subtle changes observed here may not necessarily indicate a proteome composition change at all but instead reflect different levels or types of crosslinking between the two strains. Such changes would be expected to alter apparent protein levels determined in this experiment since this would be expected to alter the accessibility of various parts of each protein to the trypsin used to generate peptides for study. The issue of protease accessibility is highlighted by the fact that even after a 16 hour digestion

using trypsin, a majority of the cuticular material remains insoluble.

Another possible reason for peptide level variability that is not observed for every peptide from a protein is the presence of some type of post-translational modification (PTM) on some but not all molecules of a peptide. The search software used recognizes many common forms of PTMs, none of which were observed in confidently identified peptides except often observed artefacts that result from sample processing. Due to crosslinking and other factors involved in sclerotization of cuticular proteins, the exact mass of the PTMs possibly present in proteins identified in this study is not yet known, and unfortunately, peptides containing modifications due to crosslinking or other sclerotization processes or two peptides joined together by crosslinking could not be identified using the data analysis methods employed here.

4.5 Future Work

The three proteins, proteins 14, 25, and 39 (identified as a new protein, hypothetical secreted protein, and CPR 84), should be the focus of the future analysis, because these three proteins were all significant in both the peptide counts and peak area analysis (Table 4.1 and 4.2). Especially the CPR 84, since it is already known to be associated with CP, must be investigated the functionality and location where it is expressed in the bed bug cuticle.

The bed bug cuticle is a very complex matrix of crosslinked proteins and chitin. This would be expected to hinder the accessibility of a cleavage reagent the size of a whole protein. The use of a different cleavage reagent, especially utilizing a small molecule such as acetic acid, may result in more efficient cleavage than that observed using an enzyme such as trypsin. Creation of peptides for analysis from shed cuticles using acid hydrolysis could provide a completely different and possibly complementary set of peptides for analysis. In fact, acid hydrolysis using severe conditions (boiling with hydrochloric acid) is often used to completely degrade proteins to their constituent amino acids for analysis. Such conditions could be used on cuticular material to help characterize the nature of crosslinks present within the bed bug cuticle by identifying molecules in addition to amino acids present in such hydrolysates. This knowledge would aid in the data analysis of not only acid-generated peptides but also possibly peptides generated by any number of cleavage techniques since knowing the mass shifts associated with crosslinking and the most likely targeted amino acid residues would allow adjusting parameters within protein identification software to recognize such mass shifts.

Finally, the type of acquisition method employed by the MALDI Tof/Tof utilized here as well as many other commercially available mass spectrometers does not guarantee a quantitative measure for the same set of peptides successive analyses. This results in incomplete quantitative information as evidenced by the many blanks in the peak area tables of

the peptide summary. A newer instrument recently acquired by the mass spectrometry incubator utilizes a different type of acquisition method that ensures quantitative information for every peptide observed over many sample runs. This would provide a more complete picture of the changes in the proteome than what could be obtained using the MALDI Tof/Tof. In addition, this instrument uses a different ionization technique (electrospray) which often results in observing a complementary set of peptides compared to MALDI ionization.

4.6 Acknowledgements

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Table 4.1 Proteins extracted and identified from Harlan (H) susceptible and Richmond (R) resistant cast skin extracts with the total and unique peptide counts, R/H ratios, and the type of the CPR (RR-2, RR-1, others) are shown. Proteins that showed P $\leq$ 0.05 are in bold.

Protein	Genome Annotation	Unique Peptides	Total Peptides	R/H	R/H std	p-value	n	Quant Pep	Identified Protein	Type
1	CLEC011347-PA, CLEC011348-PA, CLEC011350-PA, CLEC011352-PA	2	6	0.83	0.39	0.73	4	2	New	
2	CLEC011389-PA, CLEC011391-PA	2	18	1.00	0.35	0.83	6	2	New	
3	CLEC011354-PA	10	15	0.91	0.24	0.67	17	6	Unclassified CP	
									Unclassified CPs (clusters)	
4	CLEC011353-PA	4	15	0.95	0.27	0.68	11	4	New	
5	**CLEC011346-PA**	**6**	**16**	**0.71**	**0.69**	**0.03**	**11**	**5**	**New**	
6	N/A	1	16	1.19			1	1		
7	CLEC010982-PA	2	2	1.18	0.54	0.97	4	2	hypothetical secreted protein	
8	CLEC009177-PA, CLEC011344-PA	10	10	1.06	0.29	0.31	9	5	New	
9	CLEC006846-PA	2	3	1.28	0.31	0.18	6	2	new	
10	CLEC006545-PA	3	3	1.23	0.60	0.36	6	3	NPLP3 (?)	
11	**CLEC011386-PA, CLEC011388-PA**	**4**	**19**	**1.28**	**0.43**	**0.04**	**11**	**4**	**Unclassified CP**	
									New	
12	CLEC004440-PA	3	3	1.00	0.30	0.66	4	2	new	
13	CLEC004439-PA	4	6	0.84	0.32	0.09	8	3	new	

14	**CLEC004437-PA**	**2**	**5**	**0.33**	**0.15**	**0.02**	**5**	**2**	**new**	
15	CLEC004438-PA	3	7	1.15			1	1		
16	CLEC004435-PA	2	2	0.99	0.09	0.96	2	2	new	
17	CLEC004384-PA	13	13	1.11	0.66	0.43	27	11	new	
18	CLEC001985-PA	3	3	1.63	0.46	0.14	3	2	unknown	
19	CLEC002329-PA	3	3	1.13	0.51	0.82	2	1	Peroxidase	
20	CLEC000231-PA	3	3	1.22	0.37	0.48	4	3		
21	N/A	4	4	1.57	0.56	0.28	3	3	unknown	
22	**CLEC011522-PA**	**7**	**7**	**0.50**	**0.17**	**0.02**	**7**	**5**	**CPAP1-N, hypothetical secreted protein**	
23	CLEC002629-PA	5	8	0.84	0.18	0.11	8	4	CPF3b, CPFL	
51	CLEC002626-PA	10	13	0.88	0.21	0.15	19	8		
									CPF1, 2, 3a	
24	CLEC000304-PA	2	3	0.45			1	1		
25	**CLEC002505-PA**	**18**	**18**	**0.83**	**0.27**	**0.02**	**43**	**17**	**hypothetical secreted protein**	
26	CLEC000744-PA, CLEC000747-PA	2	2	0.95	0.31	0.26	4	2		
27	CLEC011867-PA	4	4	0.98	0.30	0.49	8	4	New	
28	CLEC000748-PA	3	3	1.17	0.37	0.16	5	2	CPR 15	RR-2
29	**CLEC004093-PA**	**10**	**14**	**0.82**	**0.27**	**0.03**	**18**	**9**	**CPR 31, 32**	**RR-2**
30	CLEC004096-PA	2	6	0.91	0.62	0.86	2	2	CPR33, 34, 35	RR-2
31	CLEC004802-PA	2	2	1.02	0.16	0.82	2	1	CPR 48, 49	

32	CLEC004782-PA	4	4	0.86	0.43	0.14	9	3		
33	CLEC006890-PA	2	2	0.86	0.59	0.42	5	2	CPR 54	RR-1
34	CLEC005785-PA	2	6	0.64			1	1		
35	CLEC005789-PA	3	5	0.43			1	1		
36	CLEC005565-PA	2	2	1.12	0.64	0.89	4	2	CPR 79	CPR other
37	CLEC005566-PA	7	7	0.87	0.29	0.10	8	5	CPR 80	CPR other
38	CLEC005568-PA	3	3	1.21			1	1		
39	**CLEC007768-PA**	**3**	**3**	**0.67**	**0.03**	**0.03**	**4**	**2**	**CPR 84**	
40	CLEC008665-PA	2	11	1.42			1	1	CPR 88, 89	CPR other
41	N/A	1	10	0.79	0.11	0.10	3	1		
42	CLEC011465-PA	5	7	0.85	0.19	0.12	10	5	CPR 94	
43	CLEC011415-PA	6	6	0.91	0.26	0.58	10	6	CPR 99	
44	CLEC011589-PA	2	2	1.05	0.58	0.73	3	1	CPR 104	CPR other
45	CLEC012184-PA	3	3	1.16	0.30	0.68	5	3		
46	CLEC002176-PA	2	2	0.97	0.06	0.54	3	1	TWDL 1-3	
47	CLEC000743-PA	2	10	0.94			1	1	CPRL 4, 5	
48	CLEC000743-PA	2	4	1.75	1.15	0.75	4	2		
49	CLEC000743-PA	2	9	1.95	1.17	0.45	2	1	CPRL7, 8	
									CPRL 9, 10	
50	CLEC000743-PA	2	3	0.91			1	1		

Table 4.2 The seven proteins and the proteotypic peptides ($P \leq 0.05$) that were extracted and identified from Harlan (H) susceptible and Richmond (R) resistant cast skin extracts showing their peak area, average R/H ratios, and the identified proteins.

Protein	Sequence	Theor m/z	Avg R/H	Std	n	p-value	Genome Annotation	Identified Protein
13	TYGYSGLGYGR	1193.56	0.58	0.14	3	0.03	CLEC004439-PA	new
14	AAPAPVAVAHAAPVATSYANTYR	2269.17	0.34	0.19	2	0.03	CLEC004437-PA	
25	TVAPAVSYAAPAVSYAAPAVASVR	2289.22	0.72	0.07	3	0.04	CLEC002505-PA	hypothetical
25	TVGPAVSYATPAVAALR	1643.91	0.72	0.07	3	0.02		secreted protein
26	AAHFAAVAR	913.50	0.92	0.00	3	0.04	CLEC000744-PA, CLEC000747-PA	
39	DQVYQFQPK	1152.57	0.69	0.01	2	0.05	CLEC007768-PA	CPR 84
42	VAAPVAYASPAYGYHH	1673.81	0.78	0.07	3	0.03	CLEC011465-PA	CPR 94

Chapter 5. Determination and comparison of the cuticular thickness of resistant and susceptible bed bug strains using Scanning Electron Microscopy (SEM)

5.1 Introduction

The insect integument, including its many diverse structures, forms, colors, morphology, and biological properties has been widely studied since the early 1900s (Casas and Simpson 2010). Wigglesworth (1933) provided the first detailed investigation of insect cuticle, and determined the primary structure of the insect integument and physiology using a kissing bug, *Rhodnius prolixus* (Triatomidae, Hemiptera) as a model. Wigglesworth (1933) determined that the insect integument was secreted from epithelial cells in the epidermis. Structure of insect integument consists of a basement membrane that separates the exoskeleton from internal organs of the body (Gullan and Cranston 2005). At the base of cuticle is the epidermis which is a single layer of cells that secretes the cuticle. The procuticle, on the top of epidermis, is separated into three layers: the endocuticle, exocuticle, and epicuticle (Gullan and Cranston 2005). Pore canals extend from the endocuticle to the outer surface of the procuticle (Gullan and Cranston 2005). The purpose of the pore canals is to transport various compounds, predominantly hydrocarbons of the integument, to the outside of the body forming a wax layer (Gullan and Cranston 2005). The purpose of the wax layer is to aid in retaining moisture inside the

insect's body (Blomquist and Bagneres 2010). Thickenss of the procuticle ranges from 10 µm to 0.5 mm, and consists largely of chitin and cuticular proteins (Gullan and Cranston 2005, Cohen 2010). Chitin is an amino-sugar polysaccharide that is composed primarily of β-(1-4)-linked units of *N*-acetyl-D-glucosamine (Cohen 2010). The chains of chitin are bundled and embedded tightly in a protein matrix, forming sheets (lamellae) that are layered in slightly changing orientations (Gullan and Cranston 2005, Cohen 2010).

The arthropod integument has been found to range widely in both strength and flexibility (Gullan and Cranston 2005, Cohen 2010). Depending on the orientation of the tertiary structure folds, the linkage structure of the chitin, the components of the chitin and proteins, and additional elements present within the chitin-protein matrix, the integument may have wide variety of properties and strength (Cohen 2010, Rubin et al. 2010). For example, figure 5.1 shows two different structures of cuticle within an individual bed bug showing rubber-like flexible part of the cuticle and less-flexible, harder structure around the anus. Although hydrogen bonding between the adjacent chitin chains gives great strength to the cuticle, sclerotization of exocuticle provides the additional structural strength (Gullan and Cranston 2005).

Sclerotization is the linkage between adjacent protein chains within the cuticle

that is a function of quinone tanning (phenolic bridges) (Suderman et al. 2006), dehydration of the cuticle protein-chitin matrix, or both (Gullan and Cranston 2005, Rubin et al. 2010). During quinone-tanning, proteins are oxidatively conjugated (covalently cross-linked) with catechols (a post-translational process called catecholation), and then crosslinked to form a chain (Rubin et al. 2010, Suderman et al. 2006). Pigment such as melanin is often deposited within the exocuticle as it sclerotizes. However, the presence of melanin (contributing to darkened color) is not always necessary for sclerotization (Gullan and Cranston 2005). Incorporation of metal elements such as Fe, Zn, and Cu, have also been reported to be found in arthropod integuments, most likely be related to strengthening the cuticle (Rubin et al. 2010). Cuticle dehydration also takes place during and immediately after the eclosion process removing the moisture content from the cuticle protein-chitin matrix, hence hardening the insect integument (Gullan and Cranston 2005, Rubin et al. 2010). In insect procuticle, endocuticle does not sclerotize but remains flexible relative to exocuticle (Gullan and Cranston 2005).

The timing and duration of the insect molting is controlled by series of intricate encodocrine processes (Gullan and Cranston 2005). The corpora allata secrets juvenile hormone (JH) which regulates metamorphosis and reproduction events (Gullan and

Cranston 2005). Ecdysteroids (i.e. ecdysone) are released from prothoracic glands into the hemolymph and initiates molting (Gullan and Cranston 2005). Bursicon controls cuticular sclerotization (Gullan and Cranston 2005). Deposition of the cuticle, as described in Riddiford (2012) when observing the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae), seems to be occurring in between ecdysis when the hormone titers were lower (Gullan and Cranston 2005, Riddiford 2012).

The purpose of insect cuticle is not only to help retain moisture inside the body, but also to protect insect from exposure to various xenobiotics including insecticides in urban environment (Gullan and Cranston 2005, Yu 2008). Reduced cuticular penetration type resistance has been reported in multiple species of pest insects since the early 1980s. Studies involving direct application of radioactive insecticides such as (1R,S)-trans-[^{14}C] permethrin, [^{14}C] fenvalerate, or [^{14}C] deltamethrin on the outer surfaces of house flies (*Musca domestica* L.), German cockroaches (*Blattella germanica* L.), or cotton bollworm (*Helicoverpa armigera* Hübner), all demonstrated reduced cuticular penetration type resistance in known resistant strains of these insects (DeVeries 1981, Wu et al. 1998, Ahmad et al. 2006). Although these studies provided evidence of reduced cuticular penetration type resistance, the detailed mechanism of this type of resistance has remained

largely unknown.

Simple thickening of insect cuticle might reduce the penetration of insecticides, but studies comparing cuticular thickness within the same species across different populations in relation to insecticide resistance have rarely been conducted. One exception is a report from Wood et al. (2010) measuring cuticular thickness of an adult malaria vector mosquito *A. funestus* using scanning electron micrograph (SEM). After measuring a cross section of the leg using SEM, Wood et al. (2010) found a positive correlation between cuticular thickness and the levels of pyrethroid resistance. Therefore, Wood et al. (2010) concluded that cuticular thickness contributed to increasing the level of pyrethroid resistance in the mosquito population.

Two studies have already provided has been some genetic evidence to support involvement of reduced cuticular penetration type insecticide resistance in bed bugs (Zhu et al. 2013, Koganemaru et al. 2013). Usinger (1966) provided a summary of bed bug morphological studies describing bed bug cuticle, based on observations made using light microscope and histological sectioning. However, a detailed study of bed bug cuticle morphology supporting the genetic evidencefor reduced cuticular penetration has not yet been conducted.

We hypothesized that thickening of the bed bug cuticle might be a mechanism of reduced cuticular penetration type insecticide resistance. To investigate this hypothesis, we decided to physically measure the cuticular thickness using SEM among different bed bug strains that had different levels of insecticide resistance.

5.2 Materials and methods

5.2.1 Bed bug colonies

Collection and rearing of the common bed bug, *C. lectularius* L., was as described previously (Adelman et al. 2011), with the exception of defibrinated rabbit blood being used as the food source for rearing bed bug populations. The total of seven populations (strains) were used for this study: the Harlan (H), a pyrethroid susceptible laboratory strain (Adelman et al. 2011); the Macaroni (M), a pyrethroid susceptible field strain which was collected from an infested furniture on a city street in Denver, CO, and sent to our laboratory in 2011; the British (B), a susceptible field strain which was originally collected from an apartment complex in London, United Kingdom, and sent to our laboratory in 2010; the Kramer (K), a pyrethroid resistant field strain which was collected from Arlington, VA, in 2006 (Moore and Miller 2006); the Richmond (R), a pyrethroid resistant

field strain which was collected from an elderly group home in Richmond, VA, in 2008 (Adelman et al. 2011); the Epic Center (E), a pyrethroid resistant field strain collected from a hotel room in Cincinnati, OH, in 2009; and the Royal Oaks (RO), a pyrethroid resistant field strain which was collected from a single family home by a pest control professional in Royal Oaks, MI, in 2009.

The B strain was determined to be almost susceptible based on the calculated LT_{50} values (resistance ratio [RR] = 2.6). The K strain has increased susceptibility to pyrethroid insecticides over the past several years since 2006 (RR = 339 to 3.8 based on the LT_{50} values calculated using 0.06% deltamethrin exposure on hardboard panels), as they were maintained in the laboratory environment without exposure to insecticides. The R (RR = 390.5) and the E strains (RR >340) were highly resistant to pyrethroid insecticide when exposed to deltamethrin (0.06%) on hardboard panels (Polanco et al. 2011). The RO strain was believed to be resistant, since the population persisted over the several course of insecticide treatments by pest control professionals in the field.

5.2.2 Sample preparation

Sample preparation procedures for SEM was similar to those described by Echlin

(2009; Table 8.6, Section 7.3.2.1.1). The sample preparation was designed to accommodate an animal tissue epithelia, but we used the same procedure to prepare the hard, sclerotized insect (bed bug) cuticle, extending approximately double or triple the time of each treatment steps. To prepare the bed bug samples for the SEM, first, seven day old adult males (7 days post 5^{th} instar molt) from each strain were submerged in general purpose fixative (approx. 2 ml; 5 % glutaraldehyde, 4.4 % formaldehyde, 2.75 % picric acid in 0.05 % sodium cacodylate buffer) inside a screw-top glass vial, one strain per vial. Individuals were still alive at the time of submergence, but eventually died. Individual bed bugs were added into the vial as they became available until the total of 25 – 45 individuals were in each vial. The total of 7 vials (7 strains) were prepared. Vials were stored in 4 ºC for at least 48 hrs to allow fixative to penetrate each insect. Fixed bed bugs were removed from vials and individually decapitated at head-thorax junction while still submerged in the fixative using a surgical blade. The decapitated bed bugs were submerged again in the fixative and stored in 4 ºC for $\geq$ 3 days to allow the fixative to penetrate the body cavity. After more than 3 days of submergence, the decapitated bed bugs were bilaterally sectioned from the prothorax to the abdomen tip. Sectioning was performed from the ventral (V) surface, so the blade could be positioned between the

appendages (legs) along the center of the body. Legs were not removed from the body sections so that the specimen could be easily handled using soft forceps. Legs were also used to facilitate distinguishing the V from the dorsal (D) surfaces of the body when scanning the images. One of the two bed bug sections was immediately submerged in a glass vial with fresh fixative. The other half was kept in a separate glass vial as a back-up. Each sample (half section bed bug) was stored in 4 ºC for additional 72 hrs to allow complete penetration of the fixative into the sample.

To prepare for dehydration step, the samples were rinsed three times for 15 minutes with 0.1 M sodium cacodylate buffer to remove the general fixative. The samples were then stained and stabilized with a 1% aqueous solution of osmium tetroxide (OsO_4) and held at room temperature for 1 hr. Following the staining, the samples were rinsed twice for 10 min in 0.1 M sodium cacodylate buffer to remove the osmium tetroxide. Samples were dehydrated by submerging the bed bugs in series of ethanol solution at increasing concentrations (15, 30, 50, and 70 %) for 15 min at each concentration until reaching 70 %. When the treatment reached at 70 % ethanol, the sample bed bugs were then submerged overnight at 4 ºC to allow ethanol to penetrate and completely remove the remaining fixative in the bed bug internal body cavities. Approximately 18 hrs later, the

70 % ethanol was replaced with a fresh 70 % ethanol. Finally the samples were submerged for 15 min each in 95 %, 100 %, then in 100 % ethanol for the second time. The 100 % ethanol dehydration step was repeated twice to ensure complete removal of all moisture from the bed bug samples.

A Ladd Critical Point Dryer (CPD; Ladd Research Industries, Inc., Williston, VT) was used to complete the dehydration process by replacing the 100% ethanol with liquid CO_2. The bed bug samples were enclosed in a metal basket and then placed in the oven compartment of the CPD. The CPD temperature was set at 9 ºC, and then lowered to 0 ºC by repeatedly filling and venting the oven compartment with the liquid CO_2 every 5 min for 1 hr. This filling and venting allowed replacement of the 100% ethanol in the samples with liquid CO_2. The CPD oven compartment temperature was then gradually increased to 42 ºC, and kept constant at that temperature while gradually increasing the pressure (up to 250 psi) to evaporate the CO_2. The bed bug samples were dried for approximately 20 min, then the oven was turned-off and allowed to cool to room temperature. After drying, the bed bug samples were kept in a desiccant box until ready to be mounted on a scanning electron microscope (SEM) mounting stub.

The bed bug samples were mounted on a SEM mounting stub so that the cut-

surface of the insect was facing upward to show the cut edges of both the dorsal (D) and ventral (V) side cuticle. To position each sample, a drop of silver conductive paint was placed on a SEM mount, and allowed to dry slightly (1 – 2 min) to increase viscosity. Then the bed bug sample was placed perpendicular to the mounting stub, so that the lateral edge of the sample was held in place by the silver conductive paint. The sample was put in position by holding the legs with soft forceps so that the lateral edge could be placed in the paint without damaging the bed bug sample. The sample was carefully observed during the next 5 – 10 min while the silver conductive paint was drying. If necessary, the angle of the sample could be adjusted slightly by pushing the surface sides of the sample with forceps tips. Up to three bed bugs were mounted on each SEM mounting stub. All individual bed bug samples were numbered on the stub with a permanent marker so that they could be identified under the SEM. Strain and sample preparation dates were also recorded on the back side of the SEM mounting stub. After mounting, all the samples were placed overnight in a desiccant box.

Prior to SEM, the bed bug samples were sputter-coated with gold particles using a SPI Module Sputter Coater (SPI Supplies, division of Structure Probe, Inc., West Chester, PA) inside a vacuum chamber to evenly distribute electron charge in the SEM. Sputter-

coated bed bug samples were then placed in the Carl Zeiss EVO40 SEM (Carl Zeiss AG, Oberkochen, Germany) for scanning. Specific points on the bed bug cuticle were selectively scanned at increasing magnification ranging from 65X to 55,000X. The average magnification range used for measuring particular points on the cuticle was between 30,000X – 45,000X. The digital images were obtained during scanning (Figure 5.2a – d).

5.2.3 Measuring the cuticular thickness

To compare the cuticular thickness between bed bug strains, the 2nd and 3rd abdominal segments (both dorsal and ventral) were selected for measuring and comparison purposes. The 2nd and 3rd abdominal segments were chosen because they appeared somewhat "softer/thinner" in their location at center of the abdomen than other abdominal segments that were located closer to posterior-end of the insect. We hypothesized that bed bugs are more exposed to insecticide treated surfaces if they rest their abdomens on the treated surfaces. A total of 3 point locations (Ps) were selected for measuring on each of the 2nd and 3rd segments (Figure 5.2b). The 3Ps were chosen on each segment because cuticular thickness appeared to vary at different points on the same segment depending on

the distance from the anterior end of the sample. P1 was positioned at anterior (closest to the head) side of the segment where the cuticle surface appeared thinner and smoother. P2 was positioned at the mid-point between the anterior-posterior end of the segment, generally where the cuticle was the thickest, and setae was present on the outer surface. P3 was positioned at posterior of the segment where the cuticle was thinner relative to the mid-point of the segment.

The thickness of the cuticle was measured from the exterior surface of the insect to the transitioning point to the endocuticle (Figure 5.2c) using a two-point distance calculator/converter tool of the SmartSEM computer software program (Carl Zeiss AG, Oberkochen, Germany). The SEM stage was tilted and rotated for each P measurement as necessary to make the cut-surface as perpendicular as possible to the SEM electron beam. Each P was measured three times at the highest magnification allowed for viewing the entire exocuticle on the computer screen. The three measurements were then used to calculate an average measurement of cuticular thickness at each point. Total of 12 Ps were measured per individual bed bug sample, and at least 10 sample replicates were collected at each point. When a specific measurement was not possible (i.e. view was blocked by a dust particle, or sample was not clean-cut), the data was not taken at that

specific P, and other sample was used until all 10 replicates were collected.

5.2.4 Statistical analysis

The type 3 Test of Fixed Effects (a specific type of ANOVA) was used to analyze the cuticular thickness data to determine which variables of the statistical model were affecting the cuticular thickness measurement. The test of fixed effects was selected because the variables (strain, side of the surface, or points where measurements were taken) were deliberately selected and not chosen randomly. The variables were nested in groups (Ps were nested within segments, and segments were nested within D or V surfaces), and the data distribution was non-parametric. Known levels of insecticide resistance among strains were also categorized into high (R, E, RO strains), medium (K, B strains), and susceptible (low) (H, M strains). The B strain was categorized as medium level for resistance because the strain was initially reported to have organophosphate resistance (however the result of Chapter 2 obtained after finishing the SEM analysis revealed the B strain was rather susceptible). Following the Type 3 Test of Fixed Effects one way ANOVA, strain by strain comparisons of each of the 12 positions (P1 – P3), the 4 segments (2V, 3V, 2D, and 3D), and the 2 surface (V and D) surface sides were analyzed using

Kruskal-Wallis test. The Wilcoxon Signed Rank Test non-parametric one way ANOVA was used to compare the X^2 distribution and to calculate the *P*-values. Box-and-wiskers graphs of the median and quartile distributions for each of the position were also created. All data was analyzed using Statistical Analysis Software (SAS)® 9.3 (SAS Institute Inc., Cary, NC, USA). MATLAB® R2013a (version 8.1.0.430; The MathWorks®, Inc., Natick, MA) was used to graph the data distribution.

5.3 Results

A total of 3924 cuticular measurements were taken across all bed bug strains and the total of 2733 measurements were used for statistical analysis (Table 5.1). The test of fixed effects revealed that all of the variables (strain, D or V surface side, 2^{nd} or 3^{rd} segment, and P1 – P3 point locations) were significant factors for affecting the cuticular thickness measurements (Table 5.1). Figure 5.4 summarizes the distribution of the measurement data across the seven strains at each point position. In general, the measurements of bed bug cuticular thickness were greater on the D surface side compared to those of the V surface side (Figure 5.4). The median and quartiles range of the strains for each of the point position was represented in Figure 5.5 and 5.6.

Compared to the H susceptible laboratory strain, the strain by strain comparison of the each point position revealed significant differences across the seven strains ($P < 0.05$) at majority of the 12 measurement points, segments, and surface sides (Table 5.2). Figure 5.3 represents some example of two different individual bed bugs exhibiting a continuous average cuticular thickness (Figure 5.3a) and some greater than the average thickness (Figure 5.3b). However, when the M susceptible field-collected strain was used to compare the cuticular thickness measurement at the each point position instead of H susceptible laboratory strain, many of the seven strains did not have statistically significant differences in cuticular thickness measurements in majority of the each point position (Table 5.2). Since both H and M strains were pyrethroid-susceptible strains, there were no significant correlation found between the thickness of the bed bug exocuticle and the levels of insecticide resistance.

Although there were no statistical correlation found between the thickness of the cuticle and the levels of insecticide resistance, there were some strain differences observed across the cuticular thickness depending on the measurement point positions. The H strain cuticular thickness measurements were almost always significantly different from those of other strains for the majority of the point positions (Table 5.2, Figure 5.5 and 5.6). The

RO strain cuticular thickness were often significantly different from those of the other strains. The B, K, and R strains cuticular thickness were revealed to be statistically different at some of the measurement point positions.

5.4 Discussion

Based on the result of this experiment, we found that the thickness of exocuticle was not related to the levels of insecticide resistance in bed bugs, unlike the mosquito species previously reported (Wood et al. 2010). However, there were some strain (population-level) differences in cuticular thickness measurements across the strains, depending on the point position. The H susceptible laboratory strain demonstrated the cuticular thickness statistically different from any of the other seven strains. The H strain has been maintained in a laboratory environment for decades in relatively higher humidity which may not require the population to maintain the thicker cuticule. After many generations of inbreeding, the genetic variability to produce thick strong cuticle may have been lost in such favorable environment in the H strain, in comparison to the other field strains. The result of this experiment may suggest that the H strain may not be a good strain to represent *C. lectularius* species in future experiments.

The differences in cuticular thickness among different strains (populations) in bed bugs may also indicate that some population potentially be selected in the field to have greater cuticular thickness. Two lines of evidences we observed supporting this hypothesis are 1) the H strain demonstrating statistically thinner cuticular measurements, and 2) the RO strain, in contrast, exhibiting thicker cuticular measurements. Thickening the cuticle may not be the major factor contributing to the insecticide resistance; however, it might be an additional factor supporting the reduced cuticular penetration type insecticide resistance in some of the resistant populations (i.e. the RO strain).

In this experiment, we measured only the thickness of the sclerotized exocuticle based on the assumption that sclerotized cuticle may have greater effect on reducing cuticular penetration compared to unsclerotized endocuticle. The unsclerotized endocuticle may not substantively contribute to the reduced cuticular penetration in bed bugs as much as the sclerotized exocuticle. However, if the thickness of the unsclerotized endocuticle is substantially increased, then it may start to have some effect reducing cuticular penetration of insecticide. Therefore, the thickness of the unsclerotized endocuticle should also be investigated in relation to the levels of insecticide resistance in bed bugs. Another physical property of the bed bug cuticle we must investigate is the

cuticular density. One might easily imagine that denser cuticle may reduce the penetration rate of insecticides. This might also be a mechanism of the reduced cuticular penetration type resistance in bed bugs. Thus, these two experiments are essential to further investigate next into the reduced cuticular penetration type insecticide resistance in bed bugs.

5.5 Acknowledgements

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Table 5.1 Results of the Type 3 Tests of Fixed Effects (ANOVA) showing statistical significance ($P < 0.05$) of each variables (bed bug strains, D or V surface sides, 2nd or 3rd abdominal segments, and P1-3 point locations on each segment) contributing to the model to predict the cuticular thickness measurements of bed bugs.

Variable (Effect)	Number of Variables	*P*-value
Strain	1	**<.0001**
Surface side	1	**<.0001**
Segment	1	**0.0097**
Point location	1	**<.0001**
Total Number of Observations		**3924**
Total Number of Observation Used		**2733**

Table 5.2 Strain by strain comparison of the cuticular thickness measurements across the 7 strains based on the Harlan or Macaroni strain at three different levels (positions) of comparisons: the point locations (V2P1 – V3P3), abdominal segments (V2-3, D2-3), and surface sides (D or V). The *P*-values were calculated using the non-parametric one way ANOVA Wilcoxon Signed Rank Test.

Position	Strain		n	Median (Range)	*P*-value (based on Harlan)	*P*-value (based on Macaroni)
V2P1	Harlan	H	12	2.92 (1.41 – 3.47) [a]		**0.0046**
	Macaroni	M	10	3.73 (2.48 – 4.54) [b]	**0.0046**	
	British	B	13	4.35 (2.69 – 5.16) [b]	**0.0005**	0.0721
	Kramer	K	10	3.63 (2.75 – 5.03) [b]	**0.0122**	0.5967
	Richmond	R	10	3.96 (2.91 – 4.81) [b]	**0.0006**	0.5453
	Epic Center	E	11	3.76 (2.12 – 5.27) [a,b]	0.0848	0.8327
	Royal Oaks	RO	10	4.24 (2.90 – 5.75) [b]	**0.0006**	0.1736
V2P2	Harlan	H	13	2.67 (1.98 – 4.90) [a]		0.0825
	Macaroni	M	10	3.53 (2.22 – 4.33) [a, b]	0.0825	
	British	B	13	3.80 (2.64 – 4.81) [b]	**0.0111**	0.3522
	Kramer	K	10	4.09 (3.26 – 9.64) [c]	**0.0008**	**0.0233**
	Richmond	R	10	3.24 (2.28 – 7.53) [a, b]	0.0721	0.8798
	Epic Center	E	10	3.54 (3.12 – 5.95) [b]	**0.0110**	0.4057
	Royal Oaks	RO	10	4.18 (3.45 – 7.22) [c]	**0.0010**	**0.0191**
V2P3	Harlan	H	13	2.39 (1.57 – 3.72) [a]		0.2387
	Macaroni	M	10	2.73 (1.70 – 3.53) [a, b]	0.2387	
	British	B	13	3.43 (2.47 – 5.77) [c]	**0.0003**	**0.0131**
	Kramer	K	11	3.21 (2.24 – 7.13) [b]	**0.0028**	0.1392
	Richmond	R	10	3.05 (2.21 – 7.20) [b]	**0.0184**	0.1988
	Epic Center	E	10	3.00 (2.35 – 3.93) [b]	**0.0256**	0.3258
	Royal Oaks	RO	10	3.70 (3.24 – 4.34) [c]	**0.0002**	**0.0007**
V3P1	Harlan	H	11	2.81 (2.31 – 3.65) [a]		0.2004

	Macaroni	M	11	3.46 (2.07 – 5.00) [a, b]	0.2004	
	British	B	14	3.84 (2.50 – 5.60) [b]	**0.0375**	0.4115
	Kramer	K	13	3.99 (2.36 – 6.66) [b]	**0.0028**	0.3391
	Richmond	R	15	3.30 (2.07 – 14.65) [a, b]	0.2429	0.9793
	Epic Center	E	10	3.63 (3.42 – 4.67) [b]	**0.0009**	0.5262
	Royal Oaks	RO	10	4.02 (3.09 – 4.90) [b]	**0.0019**	0.3600
V3P2	Harlan	H	12	3.00 (2.13 – 6.77) [a]		0.2184
	Macaroni	M	11	3.38 (2.37 – 4.74) [a, b]	0.2184	
	British	B	12	3.94 (2.27 – 4.98) [b]	**0.0941**	0.5383
	Kramer	K	12	4.12 (3.19 – 5.50) [c]	**0.0067**	**0.0743**
	Richmond	R	10	3.45 (1.71 – 8.12) [a, b]	0.4683	0.8880
	Epic Center	E	10	3.76 (3.06 – 9.60) [b]	**0.0349**	0.4813
	Royal Oaks	RO	10	3.98 (3.64 – 5.43) [b]	**0.0101**	0.1053
V3P3	Harlan	H	11	3.00 (1.87 – 3.74) [a]		**0.0167**
	Macaroni	M	10	3.40 (2.71 – 3.85) [b]	**0.0167**	
	British	B	13	3.39 (1.87 – 4.34) [a, b]	0.2129	0.8041
	Kramer	K	13	3.20 (2.06 – 5.09) [a, b]	0.2350	0.8041
	Richmond	R	10	3.15 (2.16 – 4.06) [a, b]	0.4386	0.7055
	Epic Center	E	10	3.42 (2.48 – 8.08) [a, b]	0.0573	0.8798
	Royal Oaks	RO	10	3.87 (3.50 – 4.89) [c]	**0.0002**	**0.0065**
D2P1	Harlan	H	14	4.42 (3.14 – 6.14) [a]		0.7843
	Macaroni	M	11	4.28 (3.16 – 5.93) [a, b]	0.7843	
	British	B	10	5.47 (4.06 – 6.12) [c]	**0.0084**	**0.0486**
	Kramer	K	12	4.48 (3.53 – 6.16) [a, b]	0.6250	0.6225
	Richmond	R	10	6.01 (4.98 – 6.74) [c]	**0.0002**	**0.0031**
	Epic Center	E	10	5.52 (4.53 – 6.55) [c]	**0.0010**	**0.0075**
	Royal Oaks	RO	10	5.88 (5.31 – 6.78) [c]	**0.0002**	**0.0019**
D2P2	Harlan	H	11	4.67 (3.82 – 6.31) [a]		**0.0167**
	Macaroni	M	10	5.70 (4.41 – 7.44) [b]	**0.0167**	

	British	B	10	6.29 (5.11 – 7.13) [b]	**0.0012**	0.2899
	Kramer	K	12	5.56 (4.20 – 7.74) [a, b]	0.0743	0.5097
	Richmond	R	10	6.05 (4.60 – 8.92) [b]	**0.0060**	0.6501
	Epic Center	E	10	5.38 (4.81 – 6.44) [b]	**0.0137**	0.4057
	Royal Oaks	RO	10	6.23 (5.44 – 7.95) [b]	**0.0015**	0.1306
D2P3	Harlan	H	11	3.48 (2.61 – 4.42) [a]		**0.0031**
	Macaroni	M	10	4.52 (3.54 – 6.21) [b]	**0.0031**	
	British	B	10	5.53 (3.82 – 7.77) [b]	**0.0002**	0.0821
	Kramer	K	12	4.84 (3.22 – 6.75) [b]	**0.0081**	0.5978
	Richmond	R	10	3.43 (2.65 – 5.29) [a, c]	0.5732	**0.0494**
	Epic Center	E	10	4.19 (3.96 – 4.75) [b]	**0.0006**	0.4963
	Royal Oaks	RO	10	5.08 (3.98 – 6.24) [c]	**0.0001**	**0.0588**
D3P1	Harlan	H	11	3.84 (3.23 – 5.13) [a]		**0.0018**
	Macaroni	M	11	5.05 (4.10 – 6.05) [b]	**0.0018**	
	British	B	10	6.37 (4.37 – 9.91) [b]	**0.0007**	0.0573
	Kramer	K	11	4.75 (2.79 – 8.37) [a, b]	0.1228	0.3410
	Richmond	R	11	5.06 (3.69 – 7.34) [b]	**0.0043**	0.8182
	Epic Center	E	10	5.23 (4.19 – 6.57) [b]	**0.0025**	0.8880
	Royal Oaks	RO	10	5.72 (4.53 – 6.38) [b]	**0.0003**	0.0910
D3P2	Harlan	H	11	4.13 (3.04 – 5.70) [a]		**0.0007**
	Macaroni	M	11	6.22 (4.21 – 6.99) [b]	**0.0007**	
	British	B	10	6.65 (5.00 – 10.05) [b]	**0.0003**	0.1925
	Kramer	K	11	5.30 (4.36 – 8.22) [b]	**0.0014**	0.6224
	Richmond	R	11	6.08 (4.30 – 6.96) [b]	**0.0007**	0.8182
	Epic Center	E	10	5.30 (4.62 – 6.15) [b]	**0.0019**	0.0783
	Royal Oaks	RO	10	6.25 (5.56 – 7.25) [b]	**0.0001**	0.2313
D3P3	Harlan	H	10	3.15 (2.52 – 4.28) [a]		**0.0012**
	Macaroni	M	11	4.68 (3.44 – 7.27) [b]	**0.0012**	
	British	B	10	4.42 (3.43 – 6.37) [b]	**0.0012**	0.5732

	Kramer	K	11	4.91 (4.04 – 10.39) [b]	**0.0002**	0.6224
	Richmond	R	11	4.26 (2.97 – 5.68) [b]	**0.0167**	0.1077
	Epic Center	E	10	4.33 (3.58 – 5.23) [b]	**0.0041**	0.2313
	Royal Oaks	RO	10	5.22 (4.35 – 6.17) [b]	**0.0002**	0.4386
V2	Harlan	H	38	2.63 (1.41 – 4.90) [a]		**0.0017**
	Macaroni	M	30	3.32 (1.70 – 4.54) [b]	**0.0017**	
	British	B	39	3.82 (2.47 – 5.77) [c]	**<0.0001**	**0.0110**
	Kramer	K	31	3.60 (2.24 – 9.64) [b]	**<0.0001**	0.0607
	Richmond	R	30	3.66 (2.21 – 7.53) [b]	**<0.0001**	0.2254
	Epic Center	E	31	3.47 (2.12 – 5.95) [b]	**<0.0001**	0.3634
	Royal Oaks	RO	30	4.00 (2.90 – 7.22) [c]	**<0.0001**	**0.0002**
V3	Harlan	H	34	2.92 (1.87 – 6.77) [a]		**0.0056**
	Macaroni	M	32	3.40 (2.07 – 5.00) [b]	**0.0056**	
	British	B	39	3.64 (1.87 – 5.60) [b]	**0.0026**	0.5712
	Kramer	K	38	3.87 (2.06 – 6.66) [b]	**<0.0001**	0.0941
	Richmond	R	35	3.33 (1.71 – 14.65) [a,b]	0.1159	0.5894
	Epic Center	E	30	3.63 (2.48 – 9.60) [b]	**<0.0001**	0.1950
	Royal Oaks	RO	30	3.95 (3.09 – 5.43) [c]	**<0.0001**	**0.0025**
D2	Harlan	H	36	4.10 (2.61 – 6.31) [a]		**0.0080**
	Macaroni	M	31	4.67 (3.16 – 7.44) [b]	**0.0080**	
	British	B	30	5.77 (3.82 – 7.77) [c]	**<0.0001**	**0.0061**
	Kramer	K	36	4.81 (3.22 – 7.74) [b]	**0.0040**	0.8112
	Richmond	R	30	5.23 (2.65 – 8.92) [b]	**0.0006**	0.2256
	Epic Center	E	30	5.13 (3.96 – 6.55) [b]	**<0.0001**	0.3867
	Royal Oaks	RO	30	5.86 (3.98 – 7.95) [c]	**<0.0001**	**0.0005**
D3	Harlan	H	32	3.88 (2.52 – 5.70) [a]		**<0.0001**
	Macaroni	M	33	5.28 (3.44 – 7.27) [b]	**<0.0001**	
	British	B	30	5.71 (3.43 – 10.05) [b]	**<0.0001**	0.2130
	Kramer	K	33	4.91 (2.79 – 10.39) [b]	**<0.0001**	0.5946

	Richmond	R	33	5.06 (2.97 – 7.34) [b]	**<0.0001**	0.3266
	Epic Center	E	30	5.03 (3.58 – 6.57) [b]	**<0.0001**	0.1104
	Royal Oaks	RO	30	5.78 (4.35 – 7.25) [b]	**<0.0001**	0.0959
V	Harlan	H	72	2.82 (1.41 – 6.77) [a]		**<0.0001**
	Macaroni	M	62	3.37 (1.70 – 5.00) [b]	**<0.0001**	
	British	B	78	3.76 (1.87 – 5.77) [c]	**<0.0001**	**0.0240**
	Kramer	K	69	3.81 (2.06 – 9.64) [c]	**<0.0001**	**0.0098**
	Richmond	R	65	3.36 (1.71 – 14.65) [b]	**<0.0001**	0.7393
	Epic Center	E	61	3.55 (2.12 – 9.60) [b]	**<0.0001**	0.1186
	Royal Oaks	RO	60	3.96 (2.90 – 7.22) [c]	**<0.0001**	**<0.0001**
D	Harlan	H	68	3.93 (2.52 – 6.31) [a]		**<0.0001**
	Macaroni	M	64	5.05 (3.16 – 7.44) [b]	**<0.0001**	
	British	B	60	5.74 (3.43 – 10.05) [c]	**<0.0001**	**0.0071**
	Kramer	K	69	4.86 (2.79 – 10.39) [b]	**<0.0001**	0.7732
	Richmond	R	63	5.21 (2.65 – 8.92) [b]	**<0.0001**	0.8320
	Epic Center	E	60	5.11 (3.58 – 6.57) [b]	**<0.0001**	0.5687
	Royal Oaks	RO	60	5.78 (3.98 – 7.95) [c]	**<0.0001**	**0.0002**

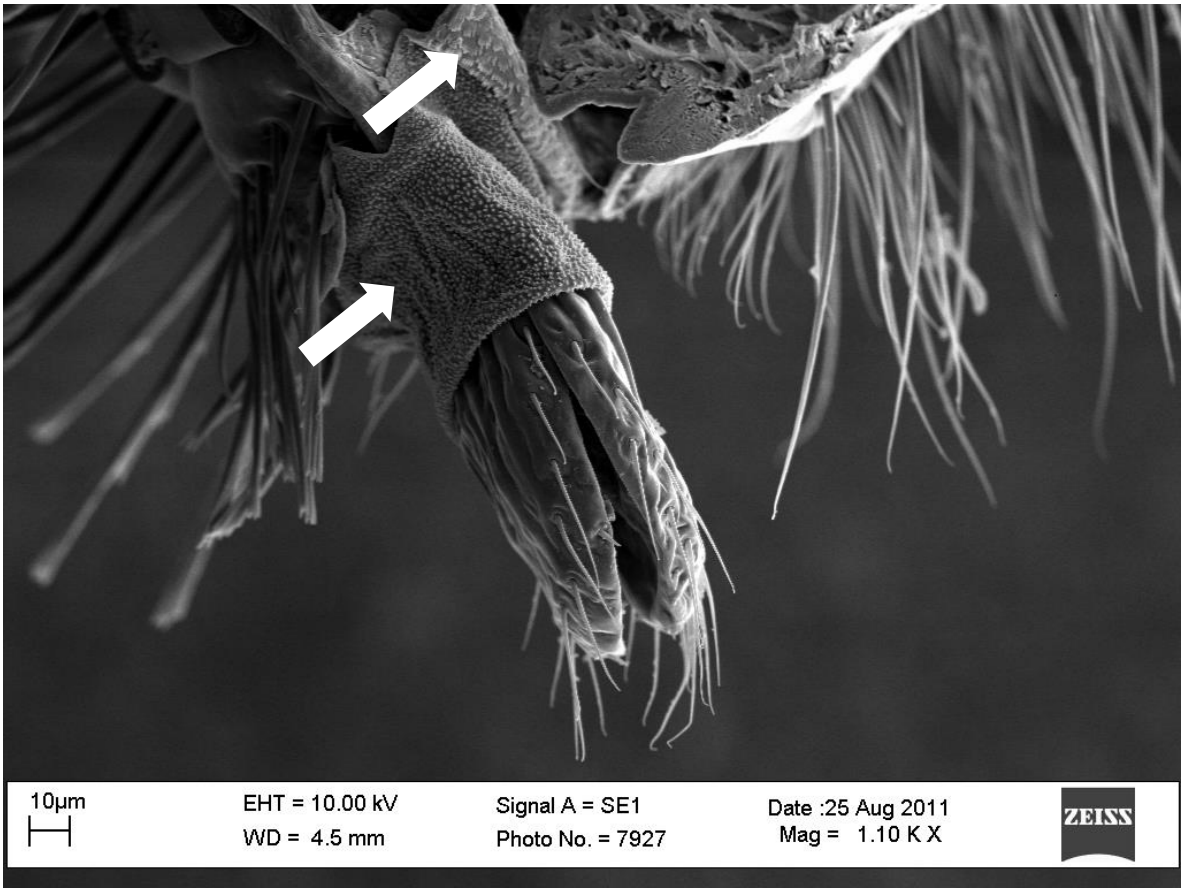

Figure 5.1 Two different appearances of rubber-like soft-cuticles surrounding the anus of a bed bug (white arrows). One shows small dotted projections, and the other one shows finger-like structures. Sample image is represented using Harlan sample No. 3 (H3).

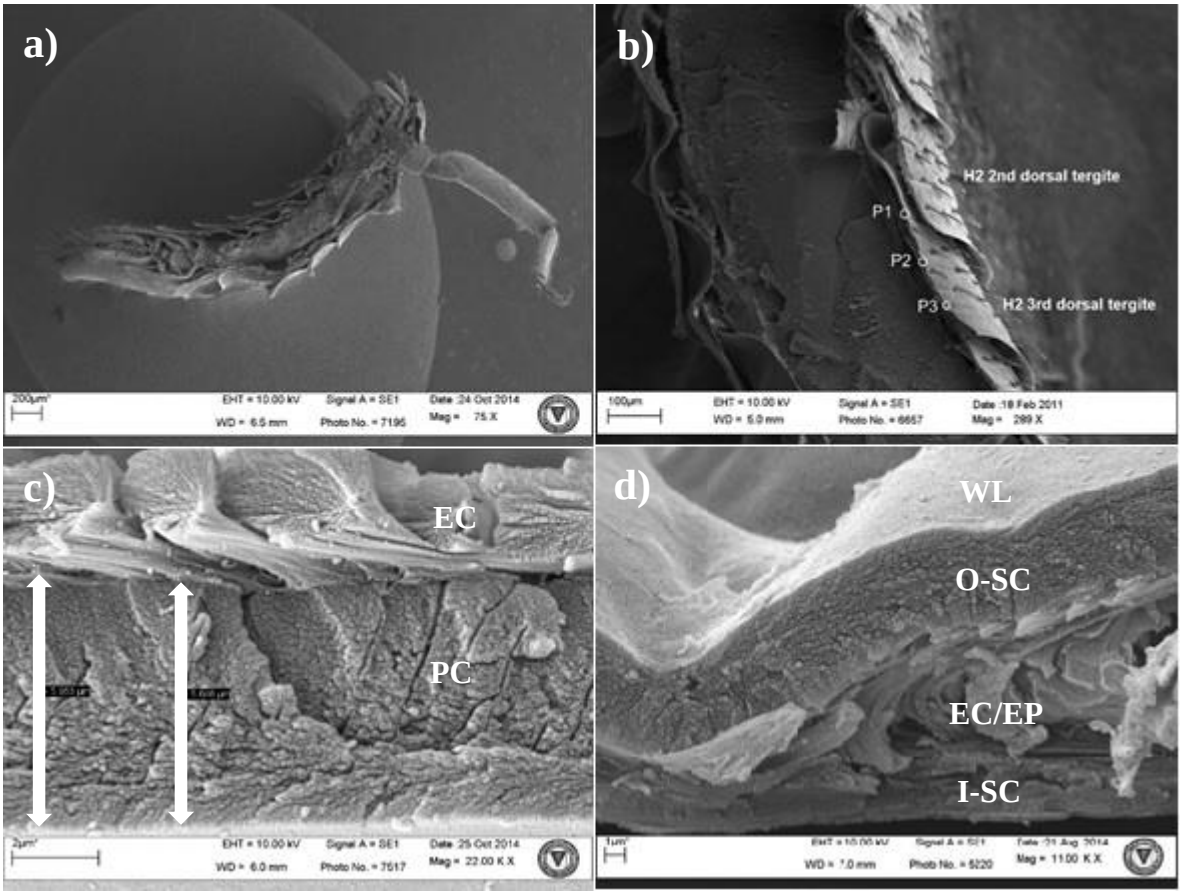

Figure 5.2 Scanning electron microscopy (SEM) images of a dorsoventrally sectioned adult male bed bug (7-days old) and points of measurements. **a)** Perpendicularly mounted, dorsoventrally sectioned (and decapitated) whole bed bug body in low magnification (x75). Sample image is represented using Royal Oaks sample No.5 (RO5). **b)** Measurement points (P1, P2, P3) shown from anterior to posterior ends of the 3rd dorsal (D) tergite (3D). The three measurement points (P1 – 3) of the 2nd D tergite (2D), 2nd ventral (V) sternite (2V), and 3rd V sternite (3V) were similarly positioned from anterior to posterior ends of the each segment. Sample image is represented using Harlan sample No. 2 (H2). **c)** Two of the three measurements are shown at P1 of the 2nd dorsal (D2P1) tergite segment of Royal Oaks sample No. 10 (RO10). Endo-cuticle (EC) is at the top part of the image, and pore canals (PC) are visible as lines appearing in the exo-cuticle. **d)** Cuticle structure of a bed bug: wax layer (WL, stripped); outer sclerotized cuticle (exocuticle, O-SC); endo-cuticle (un-sclerotized cuticle, EC/EP) or epidermis; inner sclerotized cuticle (exocuticle, I-SC). Sample image is represented using British sample No. 2 (B2 D2P3).

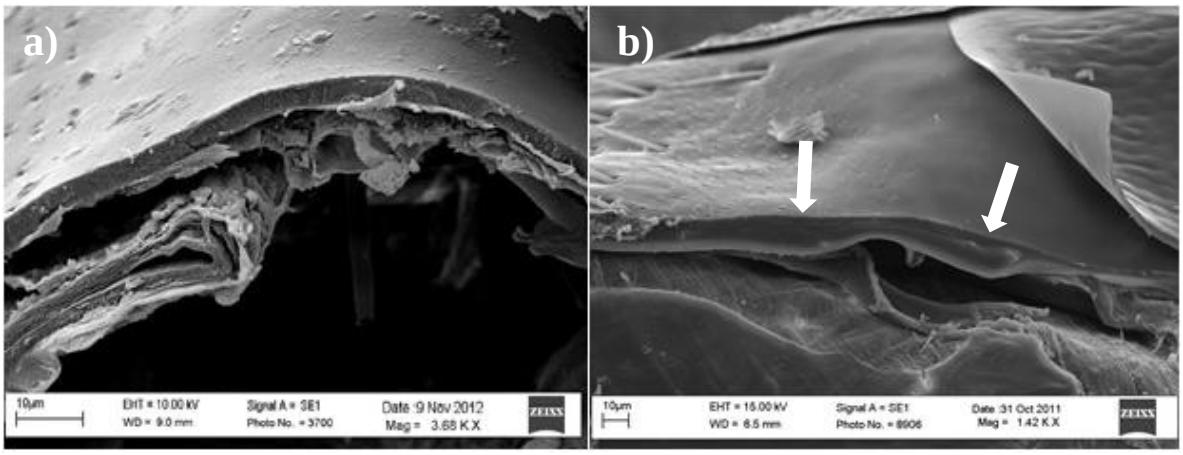

Figure 5.3 Sectioned bed bug cuticle exhibiting two different thicknesses from two different individuals. **a)** A bed bug cuticle exhibiting approx. average thickness. Sample image is represented using Epic Center sample No. 14, 3rd ventral sternite P1 (E14 V3P1). **b)** A bed bug cuticle exhibiting thicker than the average thickness. Arrows show the thickened part. Sample image is represented using Richmond sample No. 3 (R3 V3P1).

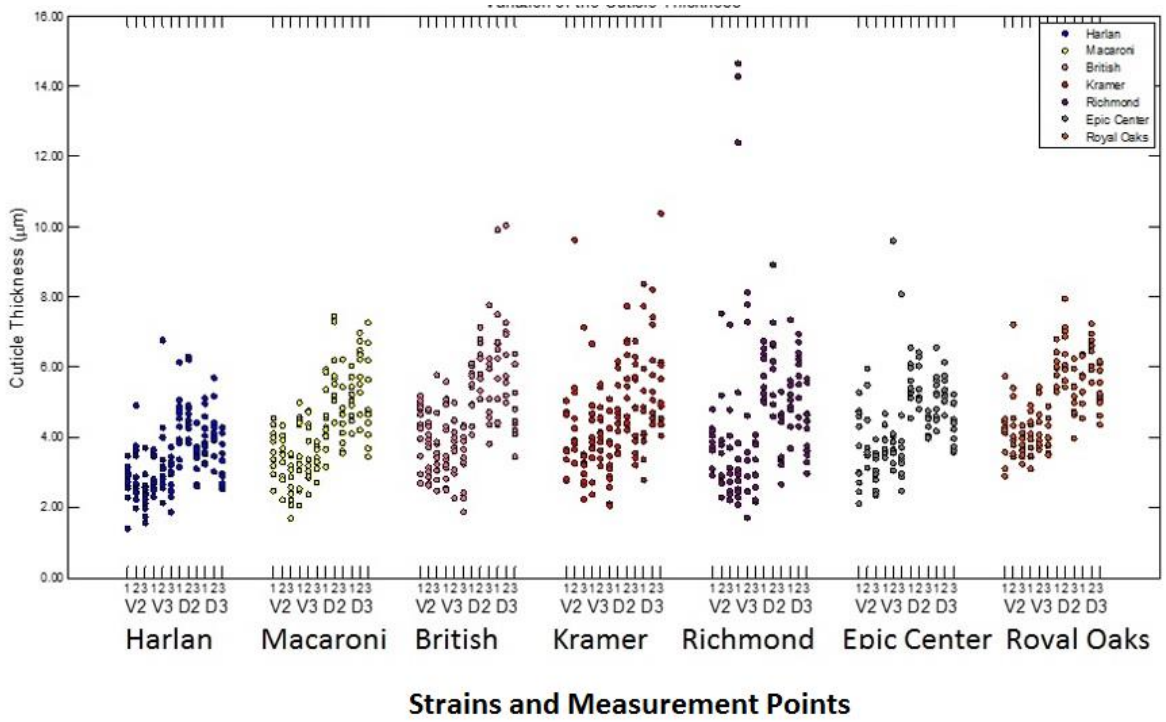

Figure 5.4 Cuticular thickness measurements (µm) from the four cuticular segments (2nd and 3rd sternites and tergites) across seven bed bug populations. Each segment were divided into three point positions (P1-P3), from anterior to posterior. Each point were measured three times, and the calculated average is represented by a dot. Statistical analysis using Wilcoxon Signed Rank Test revealed that the cuticular thickness measurements of the H susceptible laboratory strain were significantly lower than that of other strains in majority of the positions; however, the M susceptible field strain did not have statistically lower measurements.

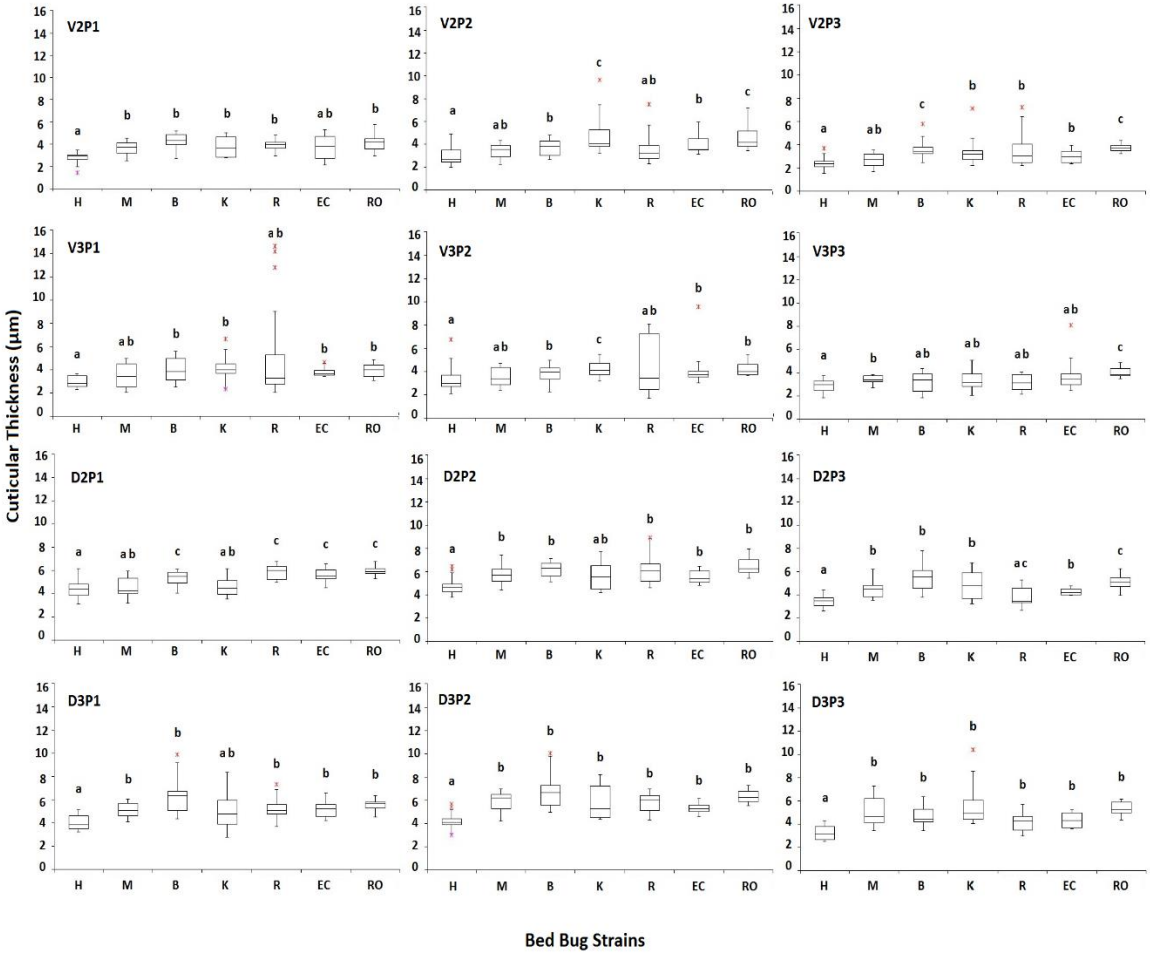

Figure 5.5 The median and quartile distributions of the cuticular thickness across the seven strains (H, M, B, K, R, EC, and RO) at each of the 12 point positions are represented based on the non-parametric one-way ANOVA Wilcoxon Signed-Rank Test based on H and M strains. The statistical differences across the strains are summarized by the lower-case alphabets (a, b, c) above each whisker representing 1.5 interquartile range (IQR). Outliers are shown in asterisks (minimum = pink, maximum = red).

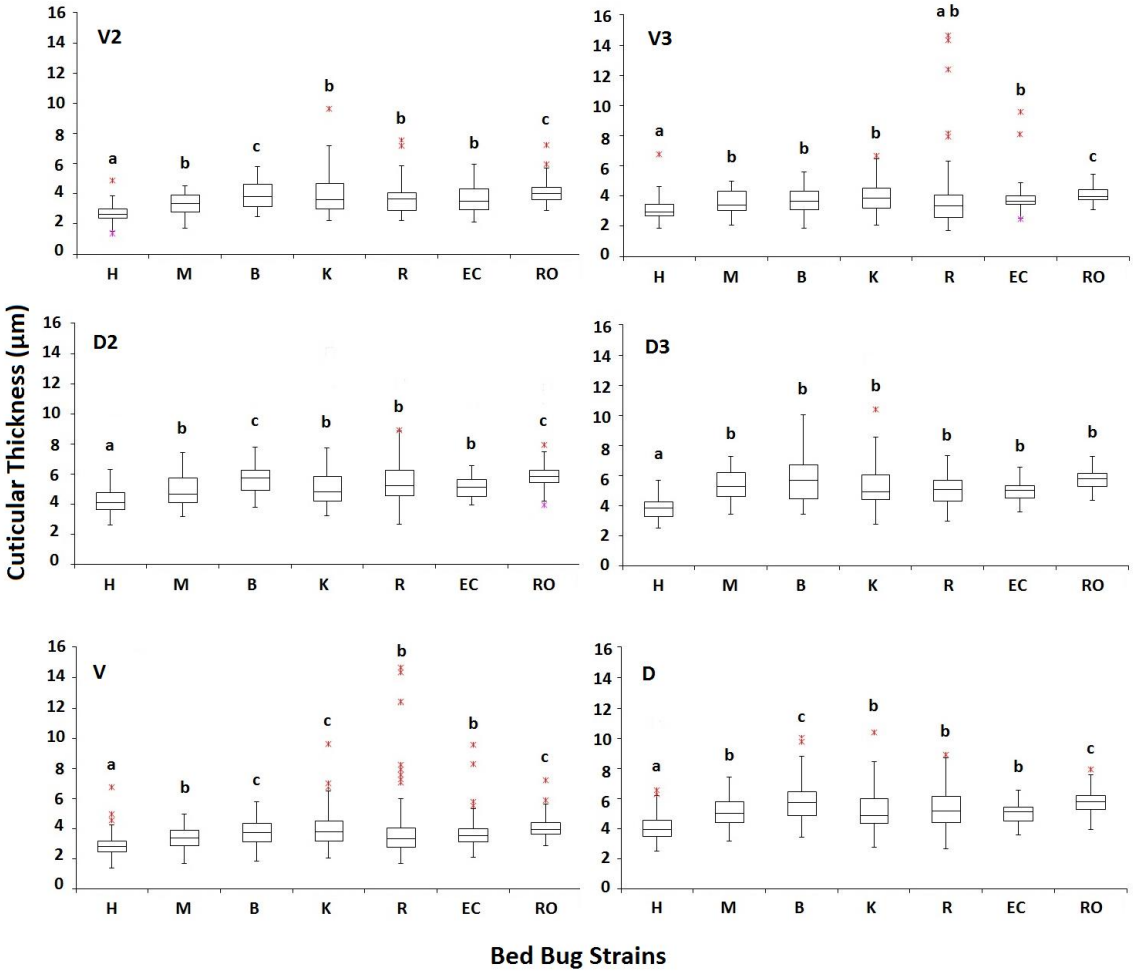

Figure 5.6 The median and quartile distributions of the cuticular thickness across the seven strains (H, M, B, K, R, EC, and RO) at 4 different segments (V2-3, D2-3) and 2 different surface sides (V or D) point positions are represented based on the non-parametric one-way ANOVA Wilcoxon Signed-Rank Test based on H and M strains. The statistical differences across the strains are summarized by the lower-case alphabets (a, b, c) above each whisker representing 1.5 interquartile range (IQR). Outliers are shown in asterisks (minimum = pink, maximum = red).

Chapter 6: Identification and description of the bed bug cuticular hydrocarbons using Gas Chromatography and Mass Spectrometry (GC-MS)

6.1 Introduction

The common bed bug, *Cimex lectularius* L., started to reappear in developed countries within the past fifteen years and quickly widespread worldwide (Boase 2001, Potter 2005 and 2006, Hwang et al. 2005, Doggett et al. 2011, Davies et al. 2012). Many hypotheses have been proposed to explain this worldwide resurgence (Boase 2001, Kells 2006, Potter 2005, Cooper 2011, Romero et al. 2007a and 2007b, Davies et al. 2012), but the direct cause is still unknown. The high levels of insecticide resistance commonly found across many of the field-collected population is believed to be the most convincing factor contributing to the bed bug resurgence (Alvaro field, Davies et al. 2012). The physiological mechanisms contributing to these high levels insecticide resistance are: increased production and activity of detoxification enzymes including cytochrome P450 and esterases (Adelman et al. 2011, Mamidala et al. 2012, Zhu et al. 2013); increased activities of ABC transporters (Mamidala et al. 2012, Zhu et al. 2013); imutations at the sodium channels target site causing *kdr* type resistance (Yoon et al. 2008); and reduced

cuticular penetration (Zhu et al. 2013, Koganemaru 2013b). Many modern populations of bed bugs have acquired multiple mechanisms of resistance within single population, which makes them exceptionally hard to control in the field (Adelman et al. 2011, Zhu et al. 2013).

Our laboratory determined that the cuticle of the insecticide resistant bed bug strains played a far greater role in protecting the bed bugs from insecticide exposure than the cuticle in suceptible strains (see Chapter 2). Our genetic evidence indicating the up-regulation of the cuticle-related genes also suggests that bed bug cuticle is very likely to be involved in insecticide resistance (Koganemaru et al. 2013b, Zhu et al. 2013).

The insect cuticle is protected by a wax layer at outer most surface (Wigglesworth 1933, Gullan and Cranston 2005, Blomquist and Bagneres 2010). The insect wax layer was first recognized by Dusham in 1918 as a barrier against moisture loss, similar conclusions were suggested by Ramsay in 1935 in a study examining cockroaches (Jackson and Baker 1970). Ramsay (1935) observed that the water transpiration rate was reduced by the insect cuticular surface lipids, thus preventing water loss (Blomquist and Bagneres 2010). The complex mixture of fatty and "waxy substances" (Blomquist and Bagneres 2010) was not proteinaceous, carbohydrate, or a simple fat; instead, the chemical properties

of the insect wax layer was similar to that found on the surface of plants (Wigglesworth 1933). Wigglesworth named it the "cuticulin" layer (1933). The insect cuticular waxes were formed in the cells of the internal lining of the cuticle. They were then transported to the outer surface of the cuticle through pore canals, as described by Locke (2001) using electron micrograph images.

Insect waxes are known to be predominantly comprised of long-chain fatty acids ranging in length from nC_{24-34} (Blomquist and Bagneres 2010). Some of the fatty acids have alcohol (-OH) or acid (–COOH) groups attached to the hydrocarbons (Chibnall et al. 1934). Baker et al. reported the first detailed insect hydrocarbon analysis in 1963 by extracting from American cockroaches and identifying the hydrocarbons using gas-liquid chromatography (GLC) (Blomquist and Bagneres 2010). Baker et al. (1963) extracted and identified *n*-pentacosane (nC_{25}), 3-methylpentacosane (methylated nC_{25}), and (Z,Z)-6,9-heptacosadiene (nC_{27} with two double-bonds). This study documented the first successful extraction of the three major classes of insect cuticular hydrocarbons (CHCs) known to date: *n*-alkanes, methyl-branched alkanes, and alkenes (Blomquist and Bagneres 2010).

Insect CHCs have now been compared among different families, genera, species, populations, and life stages since the 1950s. Therefore, many different species of

Blattodea, Diptera, Hymenoptera, Lepidoptera, Hemiptera, Orthoptera, etc. have been analyzed (Blomquist and Bagneres 2010). Early extraction methods used until around the 1960s involved grinding the whole insect body in a blender, and lipids were extracted using organic solvents such as chloroform or ether (Baker et al. 1963, Collins 1968, Jackson and Baker 1970). In later studies, polar and non-polar hydrocarbons (HCs) were extracted through a silica gel column, re-suspended in hexane, pentene, methanol, acetonitrile (ACN), etc., and separated through the GLC or gas-chromatography (GC) (Levinson et al. 1974, Carlson 1988). The method of hydrocarbon extraction has been improved since the 1970s. Today, insect samples are submerged into solvents, and then the insect CHCs components in the solvents are analyzed using gas-chromatography mass-spectrometry (GC-MS), liquid-chromatography and mass-spectrometry (LC-MS), or more recently, MALDI-TOF mass spectrometry (Blomquist and Bagneres 2010).

As the technology of the detection devices advanced and more insect CHCs were discovered, the CHCs were recognized to be useful for species identification around the 1980s (Carlson 1988, Blomquist and Bagneres 2010).

The role of insect CHCs as a cuticle protection has not been investigated extensively. Studies of the bed bug CHCs are no exceptions. While bed bug CHCs have

been investigated since the 1960s (Collins 1968), the majority of studies have been concentrated on chemical ecology, behavior, and physiology. The sex pheromones, aggregation pheromones, and alarm pheromones have been the focus of most bed bug CHCs research (Levinson et al. 1974a, Siljander et al. 2008, Ryne 2009, Olson et al. 2009, Domingue et al. 2010, Feldlaufer et al. 2010, Weeks et al. 2011, Feldlaufer and Blomquist 2011). Some electrophysiology has been used to study the bed bug olfactory system (Levinson et al. 1974b, Olson et al. 2009, Harraca et al. 2010). Therefore, the primary focus of the bed bug CHCs research have been to investigate: 1) how bed bugs recognize each other, or choose, and establish an aggregation site, 2) how bed bugs recognize a host and orient themselves toward the host, 3) how bed bugs navigate from the aggregation site to the host, and successfully return to the aggregation site after feeding.

Bed bugs only ingest blood as their primary water source, but survive extended period of starvation and desiccation, from few weeks to 140 days, depending on the life stage (Polanco et al. 2011). Benoit et al. (2007) found that bed bug cuticle was highly impermeable to water, efficiently conserving and retaining moisture resisting desiccation. In the absence of regular blood meals, bed bugs entered long-term quiescence, reduced transpiration rate, and formed aggregations, to enhance water conservation of the

population like other diapausing insects (Benoit et al. 2007). The impermeability of the bed bug cuticle (Benoit et al. 2007) may be, at least in part, related to an increased concentration of the wax layer (insect CHCs) covering the bed bug integument.

This study we investigated the bed bug CHC profiles and compared the quantities of each compound across several strains with different levels of insecticide resistance. We hypothesized that resistant strain bed bugs may have 1) different CHC profiles, 2) higher concentration of CHCs, or 3) additional compounds that might reduce their susceptibility to insecticides. We used four bed bug strains that were either pyrethroid susceptible or resistant. We compared the CHC composition both qualitatively and quantitatively using the GC-MS. This study will be the first to report bed bug CHC profiles (all of the *n*-alkanes, alkenes, and additional compounds) across different bed bug strains.

6.2 Material and Methods

6.2.1 Bed bug colonies

Collection and laboratory rearing of all the bed bugs strains, *C. lectularius* L., was conducted as described previously in Adelman et al. 2011. All bed bug populations in this study were fed defibrinated rabbit blood every 7 days using the artificial feeding system

described in chapter 2. Four different populations (strains) were used for this study: the Harlan (H), a pyrethroid susceptible laboratory strain; the British (B), a susceptible field strain; the Kramer (K), a field collected resistant strain; and the Richmond (R), a pyrethroid resistant strain (see Chapter 2 and 5 for more details).

6.2.2 Extraction and analysis of the bed bug cuticular hydrocarbons (CHCs) and additional compounds

Ten male bed bugs from each of the H, B, K, and R strains were selected at the 5th instar life stage few days after feeding, and transferred into new rearing containers. Each bed bug was removed from these rearing containers 7 days after the final (adult) molt, individually weighted, and transferred into 0.2 mL polypropylene microtubes. Cut filter paper with insect feces where the insects had rested for several hours to 24 hrs were also transferred into separate microtubes. To all microtubes, 50 µl of *n*-hexane (solvent) and 10 µl of undecane-d_{24} (3 ppm) in hexane (an internal standard; IS) were added. The microtubes were quickly mixed with a vortex mixer for few seconds. The bed bugs and cut filter paper with bed bug feces were submerged for 15 min to extract CHCs and additional compounds from the bed bug exoskeleton. After submerging, the microbutes

were centrifuged at 1,300 g (gravity) for 2 minutes to mix the CHCs and separate them from the bed bugs. Each supernatant containing the CHCs and additional compounds on the bed bug exoskeleton was transferred into an amber glass autosampler vial (1.8 mL; VWR 46610-726 ROBO, VWR International, LLC. Radnor, PA, USA) with a glass insert (Agilent Technologies, Santa Clara, CA) and a screw top cap (MicroSolv Technology Corporation, Eatontown, NJ, USA).

The gas chromatography-mass spectrometry (GC-MS) system was used to analyze the CHCs and additional bed bug compounds. The GC-MS components included a gas chromatograph 6890N, a quadrupole mass spectrometer 5973 inert, and an autosampler 7683B (Agilent Technologies, Santa Clara CA). The method of analysis was similar to the one used by Feldlaufer and Blomquist (2011). For the GC analysis, a column HP-5MS 5% Phenyl Methyl Siloxane column (30 m x 0.25 mm, 0.25µm film thickness) was used with helium as carrier gas at a constant flow rate of 1.0 ml/min. The injector was used in splitless mode as held at a temperature of 270 ºC. In order to analyze each sample, 1 µl from each autosampler vial was injected into the GC-MS injector. The oven temperature programming was set at 50 ºC for 2 min, increased to 280 ºC at a rate of 10 ºC/min, and then held constant at 280 ºC for 15 min. The temperature of the transfer line was set at

250 ºC. The ion source and the quadrupole temperatures were set at 230 ºC and 150 ºC, respectively. The mass spectra was acquired by electron impact ionization (70 eV) and scan mode was used.

The identification of the chromatogram peaks was completed using GC/MSD ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The retention times (RTs) and the mass fragmentations of the peaks were compared to that of commercial *n*-alkanes standards and to the library search results using NIST/EPA/NIH Mass Spectral Library Combined Main and Replicates Library data files (version 2002; Standard Reference Data Program, National Institute of Standards and Technology, Gaithersburg, MD, USA) in HP/Agilent ChemStation format.

For quantification of the saturated *n*-alkanes, the extracted ion chromatogram with a mass over charge ratio (*m/z*) of 85 was used; and for the IS, *m/z* of 180 was used. For the identification and quantification of the saturated *n*-alkanes, the average of three 1 µl injections results (3 replicates) for each peak that identified the *n*-alkanes was used for analysis. For quantification and identification of unsaturated hydrocarbons and additional compounds, only one 1 µl injection result (1 replicate) was used to represent each sample due to the restriction of time and labor to process the large quantity of data obtained. For

the analysis of unsaturated hydrocarbons and additional compounds, only the top 100 largest chromatogram peaks were selected, identified, and analyzed after the library search, and the remaining smaller chromatogram peaks were ignored. The small pieces of cut filter papers with insect feces where the insects had rested for several hours to 24 hrs were also tested and analyzed with two replicates. Each chromatogram peak was then manually confirmed and adjusted after they were automatically integrated by the software. The RT, mass spectra, and library search identification were also confirmed, while filtering the data by quality score less than 65. Compounds that had 65 or higher quality scores were used for the analysis. Similarly, each *n*-alkane peak on the chromatogram was manually confirmed and adjusted.

Based on the spectra generated by the GC-MS analysis of the bed bug samples, the final concentration of the *n*-alkanes in the bed bug cuticle was calculated by normalizing the data using individual insect body mass (g), the abundance of the IS (undecane-d_{24}) in each sample, and the function of the known concentration of the *n*-alkanes calibration standards. This normalization of the concentration was conducted using the following equation:

***n*-Alkane Concentration (ppb) = {[(raw abundance / IS abundance) + B]*[mass (g) /**

final volume (mL)]*1000} / A

Where A and B were the slope and the intercept of the function Y = A*X + B determined using each of the *n*-alkane calibration standards using known concentrations. The final volume of hexane that was used to extract the cuticular hydrocarbons and additional compounds was 0.05 mL.

Similarly, the relative abundance of the unsaturated hydrocarbons and additional compounds in each sample were calculated by normalizing the data using individual insect body mass (g) and the abundance of IS (undecane-d_{24}). However, since there are no standards (like the above equation) to use for the saturated *n*-alkanes to determine the slope and intercepts for each compound, the following equation was used to calculate the relative abundance of the unsaturated hydrocarbons and additional compounds in each sample:

Relative Abundance of Additional Compounds = [(raw abundance / IS abundance) / mass (g)]

For the compounds that were identified multiple times within a same sample, with multiple chromatogram peaks (approx. found <10% of the total chromatogram peaks), and had very similar RTs, the peak with higher quality score was selected and used for analysis. If the quality scores of the peaks were both high and similar in value with close RTs, then

the larger abundance chromatogram peak was selected for the statistical analyses. It was assumed that these compounds were most likely the isomers, thus were thought to be of identical chemical composition and have similar molecular structures.

6.2.3 Statistical Analysis

All statistical tests were performed using SAS® 9.4 (SAS Institute Inc., Cary, NC, USA) under the guidance of the Laboratory for Study Design and Statistical Analysis at the VA-MD Regional College of Veterinary Medicine (Virginia Tech, Blacksburg, VA). Due to the non-parametric distribution of the data, the Kruskal-Wallis, one-way ANOVA was used to determine the differences in the profiles and quantification of *n*-alkanes and additional compounds across all bed bug strains. The Dunn's Procedure of Multiple Comparisons was used to perform strain by strain comparisons for each compound. For the compounds that were significantly different in concentration ($P < 0.05$) based on the Kruskal-Wallis test among the strains, box and whisker plots for each compound were created to illustrate the median and quartile distributions of the data by strain.

6.3 Results

6.3.1 General patterns of the chromatogram peaks

Overall, the chromatogram peaks were concentrated around the RT of approximately 10.0 min to 20.0 min in all of the samples, including the samples of filter paper with bed bug feces. Several chromatogram peaks were still present and clustered at lower RTs of approximately 3.0 – 10.0 min. All the experimental samples (bed bug individuals) except for the filter paper with bed bug feces shared a chromatogram pattern of two distinctly large peaks that were identified as 2-hexenal, (E)- (RT of 5.509 min) and 2-octenal, (E)- (RT of 7.673 min) (Figure 6.1). These two compounds were by far the two most abundant in most bed bug samples, even though the abundance of these two compounds varied among individuals within each strain. The filter paper and feces samples were lacking these two large peaks of 2-hexenal, (E)- and 2-octenal, (E)-, making the chromatogram pattern distinctly different from those of insect samples.

While all of the insect samples shared the two large peaks of 2-hexenal, (E)- and 2-octenal, (E)-, there were two types of the chromatogram patterns observed among the four strains: one with the third and fourth large peaks (H2, B2, K2, and R2), and another type of the chromatogram lacking the third and fourth large peaks around RT of 15.0 min (H1, B1,

K1, and R1). The majority of the samples were the latter type, lacking the third and fourth large peaks. Only 3 samples from H, 4 from B, 2 from K, and 2 from R strain produced the third and fourth large peaks. For the majority of the samples, hexadecanamide was the third large peak right at before RT of 15.0 min. The fourth large peak right after RT of 15.0 min was 9-octadecanamide, (Z)- for the majority of the samples that showed the peak. The two exceptions of these were one R and one B sample. One R sample had 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester instead of hexadecanamide identified as the third large peak, and hexanedioic acid, bis(2-ethylhexyl) ester instead of 9-octadecanamide, (Z)- as the fourth large peak. In contrast, one B sample had benzene-1,4-diamine, N-(2-methyl-3-indolyl methylene)- identified as the third large peak, and squalene (or 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-) identified as the fourth large peak. Filter paper with bed bug feces had one large peak which was identified as cholesterol right before RT of 20.0 min, although they did not have either of the two large peaks of 2-hexenal, (E)- or 2-octenal, (E)-.

The lists of *n*-alkanes and additional compounds extracted are summarized in Table 6.1, 6.2, Figure 6.2, and 6.3. Additional compounds that were extracted yet have not been analyzed are summarized in Table 6.3. Compounds that were extracted from

insect feces and filter paper are listed in Table 6.4, excluding the *n*-alkanes and the compounds in Table 6.1 and 6.2. The concentration range of the *n*-alkanes and the relative abundance of the additional compounds varied dramatically among different compounds (Figure 6.2 and 6.3). There was great variability among the strains within each compound (Table 6.1 and 6.2). Likewise, the individual sample variances within each strain were moderate to high among different compounds. The great variation among individual samples was observed among nC_{27-32}, 2-hexenal, (E)-, and 2-octenal, (E)- (Fig 6.2 and 6.3). Details of the *n*-alkanes and additional compounds including individual variations are discussed in the following sections, 6.3.2 and 6.3.3.

6.3.2 Saturated cuticular hydrocarbons (n-alkanes)

All of the insect samples shared a general trend with high concentrations (median > 10,000 bbp) of nC_{27-31}, medium concentrations (2,000 – 10,000 ppb) of nC_{15-25} and nC_{32}, and low concentrations (< 2,000 ppb) of nC_{12} and nC_{14} (Table 6.1). Majority of the samples had zero to undetectable concentration of $nC_{8,}$ $nC_{13,}$ and $nC_{26,}$ except for a few individuals in H, B, and K strain with very limited concentrations (approx. 700 – 4,200 ppb). All samples from R strain had zero concentration of $nC_{8,}$ $nC_{13,}$ and nC_{26}. No

detectable concentrations of $nC_{7,}$ nC_{9-11}, or nC_{33-40} were observed in any samples from any of the four strains.

The statistically significant differences in *n*-alkane concentrations among the strains were observed in nC_{27-31}, indicated by the red asterisks in Figure 6.2 and bolded P-values in Table 6.1. However, the detailed strain by strain comparisons of the nC_{27-31} using the Dunn's Procedure of Multiple Comparisons revealed no distinct correlation between the concentration of the *n*-alkanes and the susceptibility to pyrethroid insecticides (Figure 6.4).

6.3.3 Unsaturated cuticular hydrocarbons and additional compounds

The lists of unsaturated CHCs and additional compounds extracted from insect samples are summarized in Table 6.2, 6.3, and Figure 6.3. The total of 87 compounds were extracted and identified from the insect samples, among which 26 were analyzed (Table 6.2 and Figure 6.3), and additional 61 compounds were identified (Table 6.3). Unlike *n*-alkanes, unsaturated CHCs and additional compounds clearly exposed several distinctive characteristics among the strains. Among the 26 compounds that have been analyzed, 2,4-octadienal, (E,E-) was the only compound consistently present in R samples,

but rarely found in H, B, and K samples; in fact it was present only in few individuals in H, B, and K strains ($P < 0.0003$; Table 6.2 and Figure 6.5). In contrast, decane 2,3,7-timethyl- was almost exclusively absent in R samples except for few individuals, but it was consistently present in most of the samples from H, B, and K strains (all other strains). The decane 2,3,7-timethyl- was found to be a significant contributor to the resistant strain but only at the 90% CL, not 95% CL ($P < 0.08$; Table 6.2). Interestingly, benzoic acid, 4-ethoxy-, ethyl ester and decane, 3,7-dimethyl- were absent in H samples except for few samples, but were more often present in other strains (B, K, and R). However, the relative abundance of these compounds widely varied between individuals, more than among the strains.

Remarkably, the abundance of the 14 unsaturated CHCs and additional compounds (> 50%) within the total of 26 compounds that have been analyzed were significantly different across the four strains (Table 6.2). Among which, six out of the 14 compounds (43%) were present in greater abundance only in R strain but not in other strains, based on the detailed analysis of the strain by strain comparisons (Figure 6.5). The six compounds were [A] 1,4-Hexadiene, 4-methyl-; [C] 2,4-Heptadiene, (E,E)-; [D] 2,4-Hexadienal, (E,E)-; [E] 2,4-Octadienal, (E,E-); [H] 2-Hexenal, (E)-; and [M] Furan, tetrahydro-2,5-

dimethyl- (Figure 6.5 and Table 6.2). Compounds such as [I] 2-Octenal, (E)-; [K] 5-Methyl-5-hexen-3-ol; [L] Cyclopentanol, 1-methyl-; and [N] Vinylfuran were also significantly greater in abundance in the R strain compared to other strains, except for one strain. Thus, the two major peaks that were always present in all the insect samples, [H] 2-hexenal, (E)- and [I] 2-Octenal, (E)-, were present in significantly greater abundance in R strain compared to other strains, especially [H] 2-hexenal, (E)-. The only exception was that the relative abundance of [I] 2-Octenal, (E)- in R strain were similar to that of B strain. Also, the relative abundance of many of above listed compounds from R strain was similar only to K strain with reguard to the abundance of [K] 5-Methyl-5-hexen-3-ol and [L] Cyclopentanol, 1-methyl-; and only to H strain for [N] Vinylfuran.

In contrast, the relative abundance of [B] 2,4-Dimethyl-1-heptene; [F] 2-Heptenal, (E)-; [G] 2-Hexanone; and [J] 3-Hexanone from R strain was statistically similar to that of other strains (Figure 6.5 and Table 6.2). Interestingly, H strain had a significantly lower relative abundance of [G] 2-Hexanone and [J] 3-Hexanone, while other three (B, K, and R) strains shared a similar and relatively higher abundance. In contrast, the abundance of [B] 2,4-Dimethyl-1-heptene was relatively higher in H strain than that of B, K, or R strain, were somewhat similar to that of K strain. The relative abundance of the compound [F] 2-

Heptenal, (E)- had great similarity among the four strains, but it was only significantly lower in B strain compared to that of R strain.

Variance among individuals within each strain and among the four strains ranged dramatically from small to large, depending on compounds and strains (Figure 6.3 and Table 6.2). The variance range was especially large among individuals for [H] 2-hexenal, (E)- and [I] 2-Octenal, (E)-, the two major peaks detected in all of the insect samples. Individuals that had the lowest relative abundance of these two compounds were observed from H strain for both compounds. In contrast, the lowest relative abundance of these two compounds was observed always greater in R strain than that of H strain. Variance among individuals in other compounds were smaller compared to the variance among individuals of [H] 2-hexenal, (E)- and [I] 2-Octenal, (E)-. The range of variance varied dependent on the compounds (Figure 6.5).

The total of 47 additional compounds were unique to insect samples among the 61 additional compounds that have been detected and identified (Table 6.3). Another 14 compounds within the 61 additional compounds found in samples were also identified from the filter papers and feces samples (Table 6.3 and 6.4). The five compounds that were consistently present from all four strains were: 2-n-Butyl furan; Tridecane, 1-iodo-;

Octadecane, 1-iodo-; 9-Octadecenamide, (Z)-; and Heptadecane, 9-octyl-. The six compounds that were present in both the filter papers with bed bug feces and insect samples from all four strains were: Tridecane, 1-iodo-; Octadecane, 1-iodo-; Heptadecane, 2,6,10,15-tetramethyl-; Pentadecane, 2,6,10-trimethyl-; Disulfide, di-tert-dodecyl; and Heptadecane, 9-octyl-. Within the 47 compounds unique to insect samples, nine compounds were found across all four strains: 2-n-Butyl furan; 2-Pentene, 4,4-dimethyl-, (Z)-; 1-Butene, 2,3,3-trimethyl-; Dodecane, 1-iodo-; Decane, 2-methyl-; Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester; Hexadecanamide; 9-Octadecenamide, (Z)-; and Hexadecane, 1-iodo- (Table 6.3). It is worth mentioning that many compounds were not found in R strain but were found in other strains, or found significantly less in R strain compared to other strains. These compounds were: Oxetane, 2,2,4-trimethyl-; Undecane, 4-methyl-; Dodecane, 2,6,11-trimethyl-; Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester; Nonahexacontanoic acid; Tetradecanamide; Disulfide, di-tert-dodecyl; Hexadecanamide; and Hexadecane, 1-iodo-.

The total of 15 compounds were exclusively found on filter papers with feces out of the 29 compounds identified from these samples (Table 6.4). The statistical analysis of the filter papers with bed bug feces samples could not be performed because data could not

be normalized by mass (g) as with the insect samples.

6.4 Discussion

To our surprise, the analysis of *n*-alkanes did not find a strong correlation between the *n*-alkane concentration and the levels of insecticide resistance, including the nC_{27-31} that did produce statistical differences among different strains. The general pattern of the *n*-alkanes concentration was similar to that of Feldlaufer and Blomquist (2011), showing higher concentration for the nC_{27-31} (Figure 6.2 and 6.4, Table 6.1). However, a link between the CHCs carbon chain length and reduced desiccation rate (which might be related to reduced cuticular penetration that increases the levels of insecticide resistance) described in other studies was not observed in our study. Our study did not detect any of the monomethyl- or trymethyl- *n*-alkanes that Feldlaufer and Blomquist (2011) reported ($2MeC_{26}$, $2MeC_{28}$, $2MeC_{30}$, $3MeC_{31}$, $2MeC_{32}$, or 11, 13, 15-methylhentriacontane, or 13, 15, 17-methyltritriacontane), nor 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal (Feldlaufer et al. 2010). These could be a result of different extraction methods, due to the fact that we used hexane instead of pentane which most of other scientists have used to extract the bed bug CHCs.

Although we did not find compounds identical to those of other studies, we did detect many other monomethyl-, dimethyl-, or trymethyl- *n*-alkanes, as well as many other additional compounds such as esters, aldehydes, acids, ketones, alcohols, amides, iodoalkanes, bromoalkanes, disulfide, butyl- and propyl-branched alkanes, furan, cyclopentene, and cyclohexadienes (Figure 6.3 and 6.5, Table 6.2 and 6.3). Many of these compounds still need quantification analyses (Table 6.3). However, we have successfully increased identified and extended the list of bed bug wax layer compounds, in addition to the Harlan strain across several populations. We have also successfully identified several compounds that might potentially be associated with the bed bug insecticide resistance.

Our quantification analysis of some unsaturated CHCs and additional compounds revealed that many of the compounds had strong positive or negative correlations between the relative abundance and the levels of insecticide resistance across the bed bug strains (Fighre 6.5). In our study, the six compounds that had a significantly greater relative abundance in pyrethroid resistant R strain relative to the susceptible strains included: [A] 1,4-Hexadiene, 4-methyl-; [C] 2,4-Heptadiene, (E,E)-; [D] 2,4-Hexadienal, (E,E)-; [E] 2,4-Octadienal, (E,E-); [H] 2-Hexenal, (E)-; and [M] Furan, tetrahydro-2,5-dimethyl- (Figure 6.5).

2-Hexenal, (E)- and 2-octenal, (E)- were the two major *n*-alkanes peaks detected across all samples from four bed bug strains in our study. These peaks were consistent with previous studies (Collins 1968, Levinson et al. 1974a and b, Siljander et al. 2008, Feldlaufer et al. 2010). These two compounds were commonly understood to function as the major bed bug aggregation and alarm pheromones (dose-dependent) (Levinson et al. 1974b, Siljander et al. 2008, Harraca et al. 2010). Interestingly, both compounds has a greater relative abundance in our pyrethroid resistant R strain samples (Figure 6.5 and Table 6.2).

Another compound, 2,4-Octadienal, (E,E-), that Siljander et al. (2008) had reported in their study was also consistently found in our pyrethroid resistant R samples but it was not found in pyrethroid susceptible H, B, or K samples, except for few samples. Similar to alcohols and ester modifications, unsaturated HCs usually have the lower melting point compared to that of saturated HCs. Though 2,4-Octadienal, (E,E-) was not detected in the susceptible H, B, or K samples, this additional HCs might be contributing to the total concentration of the resistant R strain CHCs, compared to that of H, B, or K samples. Note that the majority of other compounds such as 1,4-Hexadiene, 4-methyl-; 2,4-Heptadiene, (E,E)-; 2,4-Hexadienal, (E,E)-; 2-Hexenal, (E)-; and 2-Octenal, (E) -; 5-

Methyl-5-hexen-3-ol; Cyclopentanol, 1-methyl-; and Furan, tetrahydro-2,5-dimethyl- were also all increased in resistant R strain (Figure 6.5). In contrast, decane 2,3,7-trimethyl- was absent in almost all R strain samples.

The CHCs production is a part of the whole physiological process within a bed bug; therefore, the difference we have observed among the strains in many compounds might be related to other physiological processes and other mechanisms of insecticide resistance. For example, one of the mechanisms of insecticide resistance that have been recognized in earlier bed bug studies involves cytochrom P450 enzyme (Romero et al. 2009, Bai et al. 2011, Adelman et al. 2011). In bed bugs, the levels of insecticide resistance was decreased when the enzyme was inhibited by PBO pre-treatment (Romero et al. 2009). Also, the levels of cytochrome P450 gene transcripts were found to be increased in resistant strain bed bugs (Bai et al. 2011, Adelman et al. 2011). Since the final conversion step of the CHCs is controlled by the cytochrome P450, there might be some relationships between the cytochrome P450 which is involved in the CHCs production and detoxification of the insecticides. It might worth investigating which P450 genes are involved in the bed bug CHCs production, and see if silencing the gene will increase the susceptibility of the resistant strain bed bugs to insecticides.

Campbell et al. (2014) observed the bed bug eggs from resistant strains were already resistant from early stage of development, and that many of the resistant-related transcripts levels including cuticle-related genes were higher in the resistant strain eggs. In insects, the developing ovary was found to be able to initiate the production of insect HC (pheromone) (Blomquist and Bagnere 2010). The endocrine system is responsible for producing the insect HC, including sex pheromones (Blomquist and Bagnere 2010). For example, in cockroaches and beetles, juvenile hormone (JH) is responsible for initiating the production of sex pheromones (HCs) and initiating vitellogenesis (Blomquist and Bagnere 2010). Therefore, the greater abundances of some of the CHCs we observed in the resistant strain might be the result of increased hormonal activities (such as JH, ecdysteroids, etc.).

This study provided an initial step into the investigation of the bed bug CHCs across different levels of insecticide resistance. We have identified, quantified, and investigated each compound extracted from the bed bug cuticular wax layer. While we did not observe a direct correlation between the CHCs concentration with the bed bug insecticide resistance levels, we identified many additional compounds in the bed bug wax layer that have not been previously reported and may potentially contributing to the

reduced cuticular penetration. The quantification of additionally identified compounds still must be completed, among which include hexadecanamide and 9-octadecenamide, the 3rd and 4th large peaks we have detected in some samples across all the strains (Figure 6.1).

6.5 Acknowledgements

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Table 6.1 List of *n*-alkanes (C_nH_{n+2}; where the total number of carbons in the hydrocarbon chain is represented on the left column) with the concentration (ppb) median and the range in parentheses for each of the four strains. The number of individual insects tested are shown under the column (n). Statistically significant P-values ($P < 0.05$) are shown in bold numbers on the right column, based on the Kruskal-Wallis, one-way ANOVA. The results of Dunn's Procedure of Multiple Comparisons showing statistical significant differences among the strains are identified by the lower-case letter in superscript (a, b, c).

***n*-Alkane**	**Strain**	**n**	**Concentration Median (Range)**	**P-value**
7	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
8	Harlan	10	0.0 (0.0-3109.9)	0.5599
	British	10	0.0 (0.0-764.3)	
	Kramer	10	0.0 (0.0-3297.6)	
	Richmond	10	0.0 (0.0-0.0)	
9	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
10	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
11	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
12	Harlan	10	0.0 (0.0-1226.5)	0.1202
	British	10	586.2 (0.0-962.4)	
	Kramer	10	929.7 (0.0-1214.9)	
	Richmond	10	284.9 (0.0-1182.2)	

13	Harlan	10	0.0 (0.0-708.2)	0.495
	British	10	0.0 (0.0-1767.6)	
	Kramer	10	0.0 (0.0-695.9)	
	Richmond	10	0.0 (0.0-0.0)	
14	Harlan	10	1249.1 (0.0-4652.9)	0.1214
	British	10	1371.8 (926.4-1880.10)	
	Kramer	10	1614.4 (1388.9-2935.2)	
	Richmond	10	1283.8 (906.6-2365.7)	
15	Harlan	10	5233.5 (1509.1-16213.4)	0.0899
	British	10	4177.0 (2043.0-6860.2)	
	Kramer	10	5281.7 (4563.7-10724.6)	
	Richmond	10	3988.8 (1523.1-8898.0)	
16	Harlan	10	2821.0 (1289.5-7800.0)	0.2444
	British	10	2566.2 (1735.3-3705.4)	
	Kramer	10	3175.0 (2575.5-5601.5)	
	Richmond	10	2503.4 (1460.6-5562.9)	
17	Harlan	10	3756.2 (1561.7-9753.8)	0.5501
	British	10	3401.0 (2011.0-7227.6)	
	Kramer	10	3862.3 (3201.7-7048.4)	
	Richmond	10	3122.4 (1969.1-11583.0)	
18	Harlan	10	2375.0 (0.0-7118.3)	0.1291
	British	10	1935.3 (1305.4-3829.10)	
	Kramer	10	2770.2 (2189.4-6915.6)	
	Richmond	10	2300.8 (1776.9-8849.4)	
19	Harlan	10	3487.6 (1272.2-9363.4)	0.1686
	British	10	2531.0 (1694.4-6411.8)	
	Kramer	10	3473.1 (3134.2-8781.8)	
	Richmond	10	2863.5 (1517.8-13916.6)	
20	Harlan	10	8789.0 (0.0-19267.3)	0.1493
	British	10	6478.8 (2923.6-11388.1)	
	Kramer	10	7865.8 (6959.6-16440.7)	
	Richmond	10	6376.9 (0.0-15803.5)	
21	Harlan	10	3478.9 (0.0-10633.3)	0.4405

	British	10	2195.4 (0.0-5436.9)	
	Kramer	10	4115.6 (0.0-7771.9)	
	Richmond	10	2397.6 (0.0-10850.7)	
22	Harlan	10	6741.8 (0.0-13128.9)	0.0931
	British	10	4680.6 (2435.6-6986.9)	
	Kramer	10	5788.8 (4429.7-11822.1)	
	Richmond	10	4159.6 (0.0-14076.5)	
23	Harlan	10	5149.6 (1092.3-10406.8)	0.2502
	British	10	3856.7 (2004.8-5216.6)	
	Kramer	10	4741.6 (3239.4-7597.0)	
	Richmond	10	3392.3 (1528.7-11725.9)	
24	Harlan	10	4111.9 (0.0-8645.3)	0.2131
	British	10	2933.7 (1398.8-6013.7)	
	Kramer	10	3805.1 (2723.7-8504.0)	
	Richmond	10	3134.8 (0.0-20525.0)	
25	Harlan	10	4035.2 (0.0-9715.5)	0.3398
	British	10	3864.2 (2352.9-5650.2)	
	Kramer	10	4489.8 (3230.6-7135.9)	
	Richmond	10	3224.6 (0.0-7595.3)	
26	British	10	0.0 (0.0-0.0)	0.227
	Harlan	10	0.0 (0.0-4189.5)	
	Kramer	10	0.0 (0.0-3042.6)	
	Richmond	10	0.0 (0.0-0.0)	
27	Harlan	10	15421.0 (8008.2-26459.9) [a]	**0.006**
	British	10	10974.9 (6482.5-20915.1) [b]	
	Kramer	10	16587.6 (10952.8-28336.1) [a]	
	Richmond	10	11116.4 (6201.5-15084.5) [b]	
28	Harlan	10	9729.4 (5914.2-15064.5) [ab]	**0.0488**
	British	10	8179.2 (3950.7-11101.0) [a]	
	Kramer	10	13320.3 (6114.7-28353.5) [b]	
	Richmond	10	8944.6 (4804.7-13397.3) [a]	
29	Harlan	10	27698.8 (18119.7-34974.9) [ab]	**0.0087**
	British	10	21733.0 (17488.5-28209.8) [ac]	

	Kramer	10	36346.7 (15925.7-52449.7) [b]	
	Richmond	10	21300.3 (16165.3-33059.0) [c]	
30	Harlan	10	12517.6 (10455.3-15375.4) [ab]	**0.0433**
	British	10	11803.5 (9654.4-17130.3) [ab]	
	Kramer	10	19369.2 (7272.1-35374.0) [a]	
	Richmond	10	9619.3 (7106.1-14354.3) [b]	
31	Harlan	10	35728.1 (24435.5-41546.9) [ab]	**0.0299**
	British	10	41343.8 (31519.3-52357.8) [a]	
	Kramer	10	43889.5 (23820.5-87591.6) [a]	
	Richmond	10	31771.6 (22118.9-45711.2) [b]	
32	Harlan	10	8991.0 (0.0-13042.4)	0.7173
	British	10	7747.8 (0.0-13315.1)	
	Kramer	10	9192.0 (0.0-25534.7)	
	Richmond	10	7106.3 (0.0-12574.1)	
33	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
34	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
35	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
36	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
37	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	

	Richmond	10	0.0 (0.0-0.0)	
38	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
39	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	
40	Harlan	10	0.0 (0.0-0.0)	1
	British	10	0.0 (0.0-0.0)	
	Kramer	10	0.0 (0.0-0.0)	
	Richmond	10	0.0 (0.0-0.0)	

Table 6.2 List of additional compounds that were found through the NIST/EPA/NIH Mass Spectral Library search (quality score > 65) with the relative abundance median and range in parentheses. The number of individual insects tested are shown in column n. The P -values were calculated based on the Kruskal-Wallis, one-way ANOVA, and the statistically significant values ($P < 0.05$) are shown in bold. The results of Dunn's Procedure of Multiple Comparisons showing statistical significances are identified by the letter in superscript (a, b, c).

Compound	Strain	n	Relative Abundance Median (Range)	P -value
1,4-Hexadiene, 4-	Harlan	10	1444.7 (276.4-2981.4) [a]	**0.0098**
methyl-	British	10	759.5 (475.3-3281.6) [a]	
	Kramer	10	1185.8 (512.9-1727.7) [a]	
	Richmond	10	2838.0 (1073.8-4926.3) [b]	
2,4-Dimethyl-1-heptene	Harlan	10	696.2 (135.9-1309.9) [a]	**0.0362**
	British	10	452.9 (104.4-733.0) [b]	
	Kramer	10	595.7 (321.8-917.5) [ab]	
	Richmond	10	376.1 (169.7-634.3) [b]	
2,4-Heptadiene, (E,E)-	Harlan	10	1471.0 (314.7-2908.4) [a]	**0.0076**
	British	10	780.2 (540.1-3412.2) [a]	
	Kramer	10	1188.5 (517.5-1756.7) [a]	
	Richmond	10	2977.3 (1139.2-4954.8) [b]	
2,4-Hexadienal, (E,E)-	Harlan	10	1464.9 (263.9-2929.4) [a]	**0.0078**
	British	10	761.2 (524.4-3301.5) [a]	
	Kramer	10	1194.7 (581.6-1641.9) [a]	
	Richmond	10	2816.9 (1118.4-4943.7) [b]	
2,4-Octadienal, (E,E-)	Harlan	10	0.0 (0.0-1162.0) [a]	**0.0003**
	British	10	0.0 (0.0-902.7) [a]	
	Kramer	10	0.0 (0.0-0.0) [a]	
	Richmond	10	1132.6 (0.0-1520.7) [b]	
2,5-Cyclohexadiene-1,4-	Harlan	10	850.2 (0.0-5657.8)	0.203
dione, 2,6-bis(1,1-	British	10	1765.7 (0.0-4976.8)	
dimethylethyl)-	Kramer	10	1917.4 (1341.1-3657.8)	

	Richmond	10	1078.8 (0.0-8509.1)	
2-Heptenal, (E)-	Harlan	10	708.7 (0.0-1701.4) [ab]	**0.033**
	British	10	228.3 (0.0-1067.9) [a]	
	Kramer	10	660.1 (244.0-1289.6) [ab]	
	Richmond	10	1223.9 (201.5-2234.1) [b]	
2-Hexanone	Harlan	10	1790.6 (899.0-4226.5) [a]	**0.0071**
	British	10	2828.9 (1561.3-4644.6) [b]	
	Kramer	10	2928.1 (1928.2-3925.9) [b]	
	Richmond	10	2766.3 (2053.9-3866.2) [b]	
2-Hexenal, (E)-	Harlan	10	169591.5 (44398.2-302493.5) [a]	**0.0006**
	British	10	126601.1 (95529.0-193249.4) [b]	
	Kramer	10	148195.9 (114068.1-236644.9) [ab]	
	Richmond	10	254285.4 (163049.1-287522.1) [c]	
2-Octenal, (E)-	Harlan	10	144091.5 (42067.2-252290.6) [ac]	**0.0137**
	British	10	204284.5 (152776.6-243928.7) [b]	
	Kramer	10	131243.8 (103090.6-224936.8) [c]	
	Richmond	10	204954.7 (132328.1-304013.2) [b]	
3-Hexanol	Harlan	10	1980.1 (1022.9-4560.7)	0.2194
	British	10	2136.7 (1371.2-3312.1)	
	Kramer	10	2324.0 (1670.6-3275.6)	
	Richmond	10	2303.2 (1623.8-3483.5)	
3-Hexanone	Harlan	10	1391.8 (725.5-3284.0) [a]	**0.0034**
	British	10	2343.8 (1437.6-4057.1) [b]	
	Kramer	10	2205.5 (1501.4-3320.5) [b]	
	Richmond	10	2166.9 (1545.4-3108.9) [b]	
5-Methyl-5-hexen-3-ol	Harlan	10	393.1 (223.1-1008.5) [a]	**0**
	British	10	728.1 (453.7-1481.7) [ab]	
	Kramer	10	982.9 (744.9-1993.0) [bc]	
	Richmond	10	1477.8 (1004.6-2182.7) [c]	
Benzoic acid, 4-ethoxy-,	Harlan	10	0.0 (0.0-5990.8)	0.104
ethyl ester	British	10	1662.0 (0.0-4325.6)	
	Kramer	10	2739.3 (2177.7-4763.8)	
	Richmond	10	1859.8 (0.0-4660.4)	

Cyclopentane, 1,3-dimethyl-	Harlan	10	548.7 (304.2-1536.4)	0.1262
	British	10	440.8 (263.5-737.7)	
	Kramer	10	544.5 (371.0-627.7)	
	Richmond	10	198.4 (0.0-724.4)	
Cyclopentanol, 1-methyl-	Harlan	10	601.7 (0.0-1890.0) [a]	**0.0001**
	British	10	1149.6 (695.1-2253.3) [ab]	
	Kramer	10	1376.5 (0.0-3332.5) [bc]	
	Richmond	10	2346.6 (1708.3-3491.7) [c]	
Decane, 2,3,5,8-tetramethyl-	Harlan	10	744.8 (0.0-1850.3)	0.7276
	British	10	567.3 (0.0-3039.0)	
	Kramer	10	795.9 (0.0-983.4)	
	Richmond	10	595.8 (0.0-3144.2)	
Decane, 2,3,7-trimethyl-	Harlan	10	1585.2 (0.0-5482.9)	0.0842
	British	10	846.5 (0.0-1563.7)	
	Kramer	10	1088.5 (0.0-3629.2)	
	Richmond	10	0.0 (0.0-1275.9)	
Decane, 2,4,6-trimethyl-	Harlan	10	453.0 (0.0-5796.0)	0.5528
	British	10	411.1 (0.0-1332.9)	
	Kramer	10	1130.4 (0.0-3335.9)	
	Richmond	10	454.0 (0.0-1710.5)	
Decane, 3,6-dimethyl-	Harlan	10	568.3 (0.0-2708.0)	0.1091
	British	10	588.7 (0.0-1041.9)	
	Kramer	10	1000.6 (0.0-2085.6)	
	Richmond	10	449.6 (0.0-694.7)	
Decane, 3,7-dimethyl-	Harlan	10	0.0 (0.0-3550.0)	0.5556
	British	10	596.3 (0.0-2595.7)	
	Kramer	10	435.3 (0.0-3450.7)	
	Richmond	10	241.1 (0.0-846.3)	
Decane, 3,8-dimethyl-	Harlan	10	5620.9 (79.2-18699.8)	0.0918
	British	10	3380.6 (0.0-6995.8)	
	Kramer	10	5748.7 (976.2-11842.9)	
	Richmond	10	2183.6 (0.0-10360.0)	

Furan, tetrahydro-2,5-dimethyl-	Harlan	10	1471.0 (314.7-2908.4) [a]	**0.0076**
	British	10	780.2 (540.1-3412.2) [a]	
	Kramer	10	1188.5 (517.5-1756.7) [a]	
	Richmond	10	2977.3 (1139.2-4954.8) [b]	
Nonanal	Harlan	10	1466.2 (0.0-21098.2)	0.507
	British	10	681.0 (0.0-4174.4)	
	Kramer	10	1037.8 (0.0-16495.1)	
	Richmond	10	875.6 (0.0-1105.4)	
Squalene	Harlan	10	6693.9 (949.7-11877.4)	0.7872
	British	10	4409.9 (906.0-49529.4)	
	Kramer	10	6045.6 (2387.4-29880.1)	
	Richmond	10	4847.2 (1166.9-14289.2)	
Vinylfuran	Harlan	10	1421.8 (283.2-2655.1) [ab]	**0.0213**
	British	10	744.5 (395.7-3700.4) [a]	
	Kramer	10	816.8 (356.8-1418.9) [a]	
	Richmond	10	2021.6 (740.4-3628.9) [b]	

Table 6.3 List of additional compounds that were extracted from the bed bug samples and identified by the GC-MS through the NIST/EPA/NIH Mass Spectral Library search (quality > 65) that have not yet quantified. Approximate ratio per mass (g) of the bed bug from one sample, approximate retention time (RT) of the compound, and the number of samples and strain(s) are also shown. Verification of the chromatogram peaks and statistical analyses will be followed.

	Compound Name	Approx. RT (min)	Approx. Ratio/g (Max)	Comments
1	3-Methylpyridazine	3.756	2,144	only 1 R and 1 K.
2	Oxetane, 2,2,4-trimethyl-	3.885	2,907	only 1 K and 2 B, not from H or R.
3	1-Hexene, 5-methyl-	5.712	1,113	only 1 R.
4	2-n-Butyl furan	5.961	2,392	all 4 strains, many samples.
5	2-Heptenal, (Z)-	6.703	1,974	only 1 H and 1 R.
6	2-Pentene, 4,4-dimethyl-, (Z)-	6.801	2,400	all 4 strains, few samples from each. (Z)- and (E)-isomers, and 2-Pentene, 2,4-dimethyl- were detected.
7	1-Butene, 2,3,3-trimethyl-	6.807	2,248	all 4 strains, a few from each.
8	Undecane, 2,6-dimethyl-	7.242	1,025	only 1 H.
9	Undecane, 5,7-dimethyl-	8.036	1,054	only 1 B and 1 R.
10	Undecane, 5-methyl-	8.036	1,928	only 1 B.
11	Undecane, 3,9-dimethyl-	8.041	2,157	only 1 R and 1B.
12	Undecane, 4,7-dimethyl-	8.042	3,599	only 1 H and 1 K.
13	Dodecane, 2,6,10-trimethyl-	8.078	1,793	from 2 K, 1 H, 1R, not from B.
14	Octane, 3-ethyl-2,7-dimethyl-	8.083	1,745	only 1 H.
15	Undecane, 4-methyl-	8.151	2,095	from 1H, 4K, 1B, not from R.

16	Dodecane, 4,6-dimethyl-	9.230	1,717	only 1 H and 1 K.
17	1-Iodo-2-methylundecane	9.437	1,415	only 1 H and 1 R.
18	Octadecane, 5-methyl-	9.754	9,724	only 1 H.
19	Undecane, 3,7-dimethyl-	9.754	4,569	only 1 K.
20	Octane, 2,4,6-trimethyl-	9.821	2,107	only 1 K.
21	Dodecane, 1-iodo-	9.826	5,602	all 4 strains, a few from each.
22	Undecane, 4,8-dimethyl-	9.873	2,066	only 2 K.
23	Hexadecane, 2,6,11,15-tetramethyl-	10.714	1,199	only 1 K.
24	Undecane, 4,6-dimethyl-	10.724	2,938	only 1 R, 1 K, 2 H, not from B.
25	Undecane, 3,8-dimethyl-	10.724	2,149	only 1 K and 1 B.
26	Heptadecane, 8-methyl-	10.729	4,103	only 1 H, 1 B, 1 R, not from K.
27	Hexadecane, 7-methyl-	10.781	1,968	only 1 K.
28	Dodecane, 2,6,11-trimethyl-	11.150	9,820	only H, K, B, not in R.
29	Tridecane, 1-iodo-	11.150	8,158	all 4 strains, many samples.
30	5,5-Dibutylnonane	11.274	7,156	only 1 R and 1 K.
31	Decane, 2-methyl-	11.279	6,233	all 4 strains, but only in a few from each strain.
32	Tridecane, 5-propyl-	11.342	3,986	only 1 H.
33	Undecane, 2,9-dimethyl-	11.409	1,189	only 1 R.
34	Tridecane, 3-methyl-	11.419	1,974	only 1 H.
35	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	11.549	7,885	all 4 strains, several samples each but only 1 from R.
36	Octadecane, 1-chloro-	11.782	1,827	only 1 H.
37	Methoxyacetic acid, 2-tetradecyl ester	12.058	1,629	only 2 K.
38	Dodecane, 2-methyl-	12.161	1,634	only 1 H.

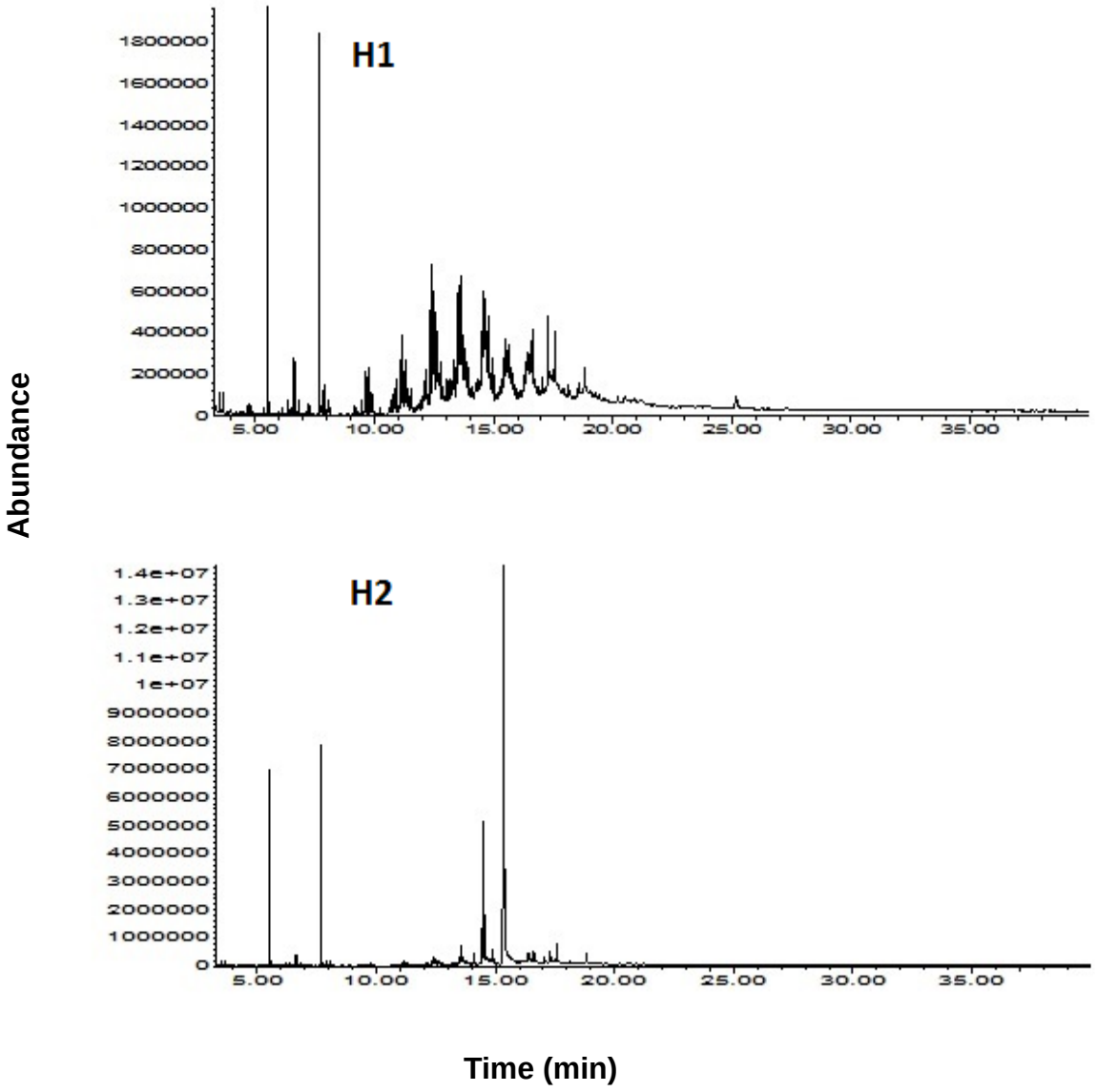
39	Octadecane, 1-iodo-	12.379	17,271	all 4 strains, many samples.
40	2-Bromo dodecane	12.395	4,898	most H, but only 1 R. Not from B or K.
41	Cyclopentane, 1-butyl-2-propyl-	12.478	1,577	only 1 H.
42	Nonahexacontanoic acid	12.525	9,240	only a few samples from each of the H, K, B, but none from R.
43	Heptadecane, 2,6,10,15-tetramethyl-	12.753	8,821	all 4 strains, at least a few samples from each.
44	6-Tetradecanesulfonic acid, butyl ester	13.121	3,673	only 2 K.
45	Tetradecanamide	13.536	17,534	only 3 H and 2 B, not from K or R.
46	Tridecanol, 2-ethyl-2-methyl-	13.562	8,371	only 3 K.
47	Pentadecane, 2,6,10-trimethyl-	13.573	11,885	all 4 strains at least a few samples from each strains, but only 1 R.
48	Disulfide, di-tert-dodecyl	13.599	5,906	all 4 strains, but only 3 R.
49	Octadecane, 2-methyl-	13.723	4,836	only 2 K and 1 R.
50	Heptadecanoic acid, 16-methyl-, methyl ester	14.237	4,277	only 2 H.
51	Hexadecane, 2-methyl-	14.486	3,560	only 2 K.
52	Nonadecane, 2-methyl-	14.486	4,360	from 2 K, 1 B, 1 R, not from H.
53	Hexadecanamide	14.517	137,474	several from H and B, but only 2 K and 1 R.
54	9-Octadecenamide, (Z)-	15.321	951,289	all 4 strains, many samples.
55	Octadecanamide	15.389	64,333	from 2 B, 1 K, 1 R, not from H.
56	Heptadecane, 9-octyl-	15.446	10,153	all 4 strains, many samples.
57	Heptadecane, 3-methyl-	16.603	6,686	from 2 H, 2 B, and 4 R, but not from K.
58	Hexadecane, 7,9-dimethyl-	16.873	2,266	only 2 B, 1 H, 1 R, not from K.

59	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	17.173	2,278	from 1 K, 2 B, 2 R, not in H.
60	Heptadecane, 2-methyl-	17.365	4,612	from 2 H, 2 K, 1 R, not from B.
61	Hexadecane, 1-iodo-	18.777	34,045	from 3 K, 2 B, 1 H and 1 R.

Table 6.4 List of compounds that were extracted from the bed bug feces on filter paper on which the insects had rested from several to 24 hrs. The compounds that were listed in Table 6.1, 6.2, and the compounds with less than 65 quality values were excluded from this table. Approximate quality and a comment if it was found in only one of two samples are also shown.

	Compound Name	Approx. Quality	Comments
1	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	76	only 1
2	10-Methylnonadecane	80	
3	1-Chloroeicosane	70	
4	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	99	only 1
5	2-Propenoic acid, 2-methyl-, hexylester	72	only 1
6	6-Tetradecanesulfonic acid, butylester	80	only 1
7	Cholesterol	99	
8	Dichloroacetic acid, 4-pentadecylester	76	only 1
9	Disulfide, di-tert-dodecyl	68	
10	Heptadecane, 2,6,10,15-tetramethyl-	91	only 1
11	Heptadecane, 8-methyl-	78	
12	Heptadecane, 9-octyl-	90	
13	Hexadecane, 2,6,11,15-tetramethyl-	80	only 1
14	Methoxyacetic acid, 2-octyl ester	72	only 1
15	Nonahexacontanoic acid	74	
16	Octadecane, 1-iodo-	72	
17	Octadecanoic acid, methyl ester	96	only 1
18	Oxetane, 2,2,4-trimethyl-	72	only 1
19	Pentadecane, 2,6,10-trimethyl-	83	
20	Tetradecane, 4-methyl-	86	only 1
21	Tetrapentacontane	80	only 1
22	Tetratetracontane	91	only 1
23	Tetratriacontane	87	
24	Tridecane, 1-iodo-	83	
25	Tridecane, 5-propyl-	80	only 1

26	Undecane, 4,6-dimethyl-	72	
27	Undecane, 4,7-dimethyl-	72	only 1
28	Undecane, 4-methyl-	80	only 1
29	Undecane, 5-methyl-	74	only 1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25.00
30.00
35.00
H2
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1.3e+07
1.2e+07
1.1e+07
1e+07
9000000
8000000
7000000
6000000
5000000
4000000
3000000
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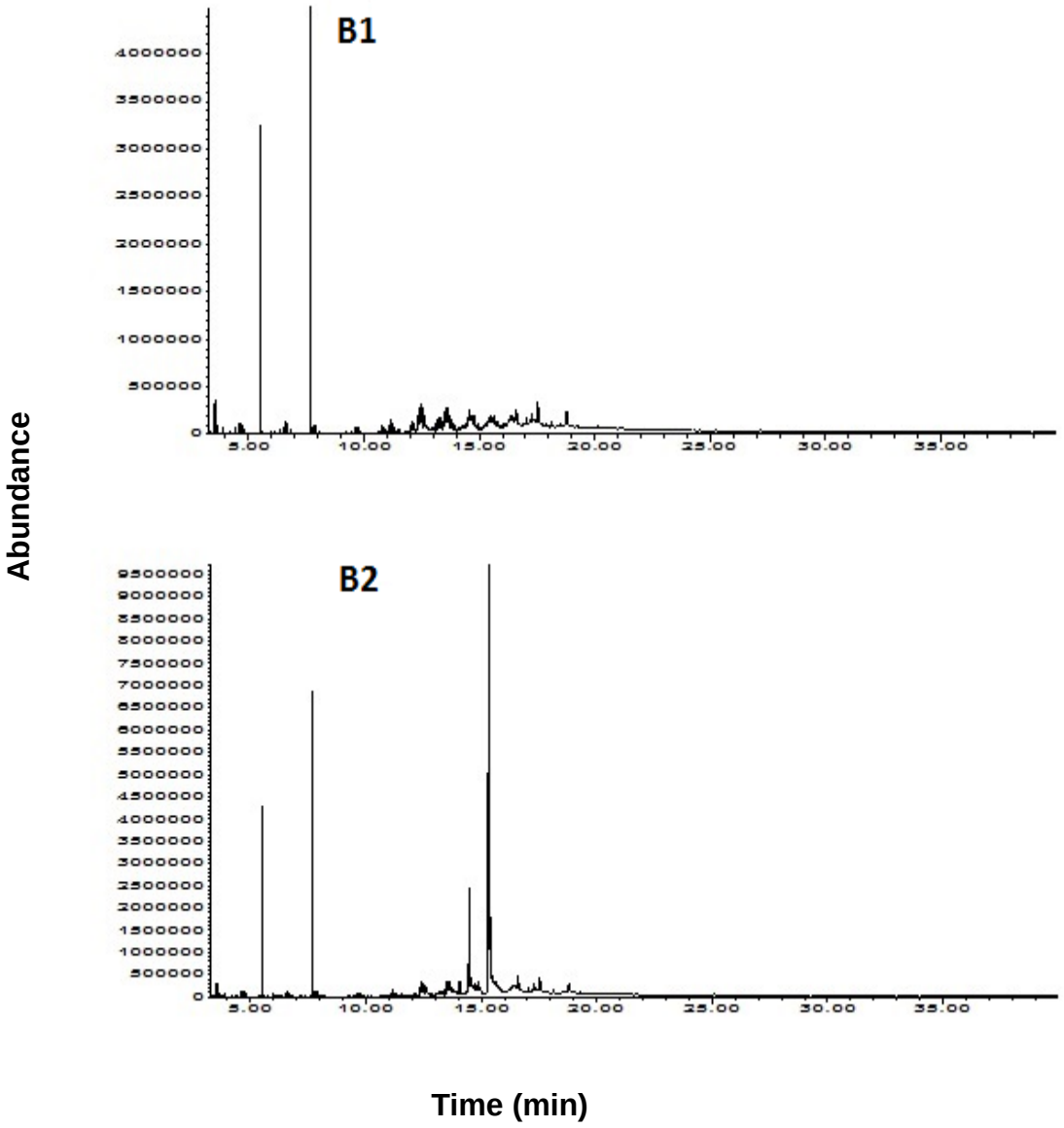
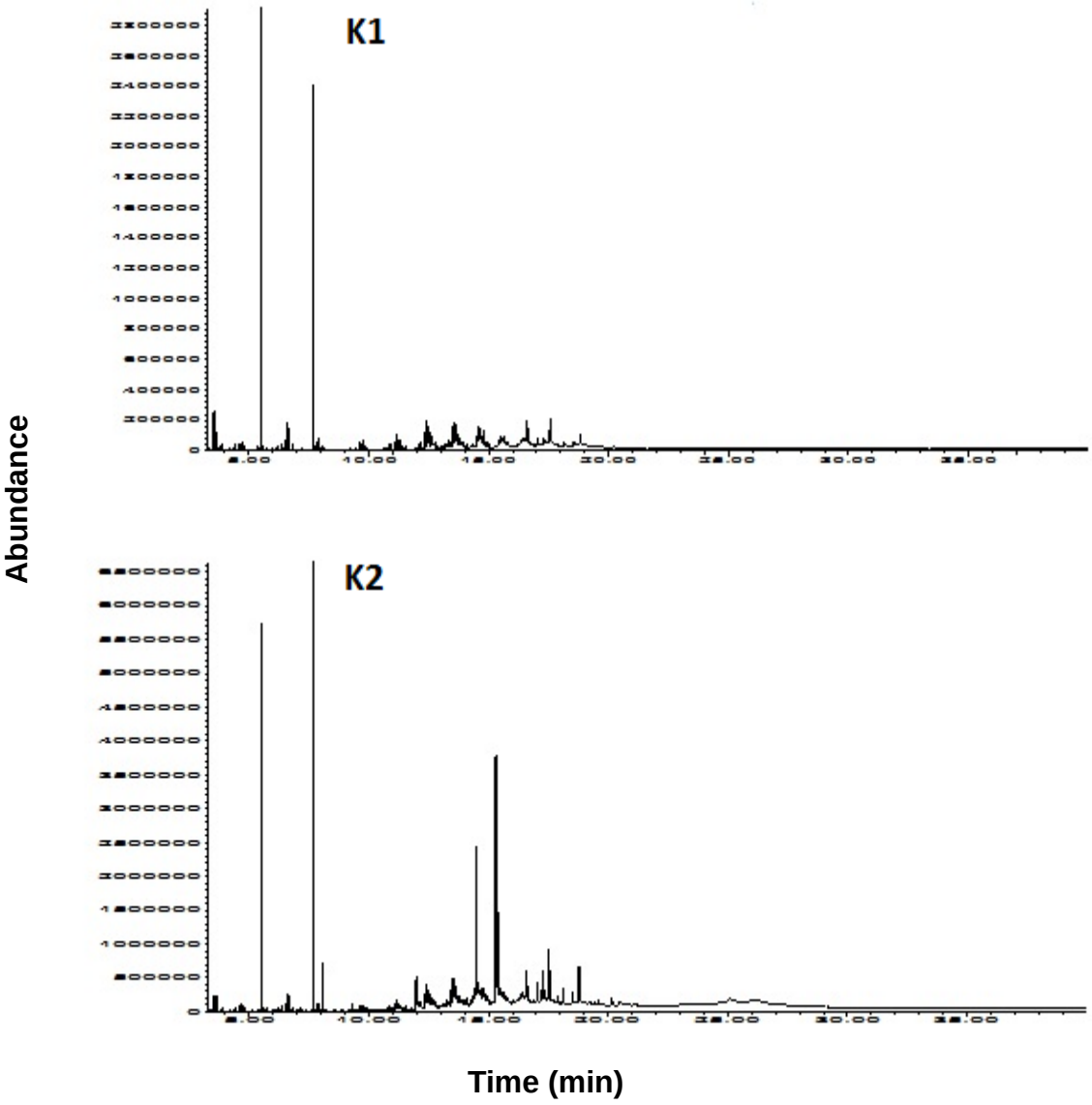
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Abundance
Time (min)


B1
B2
Abundance
Time (min)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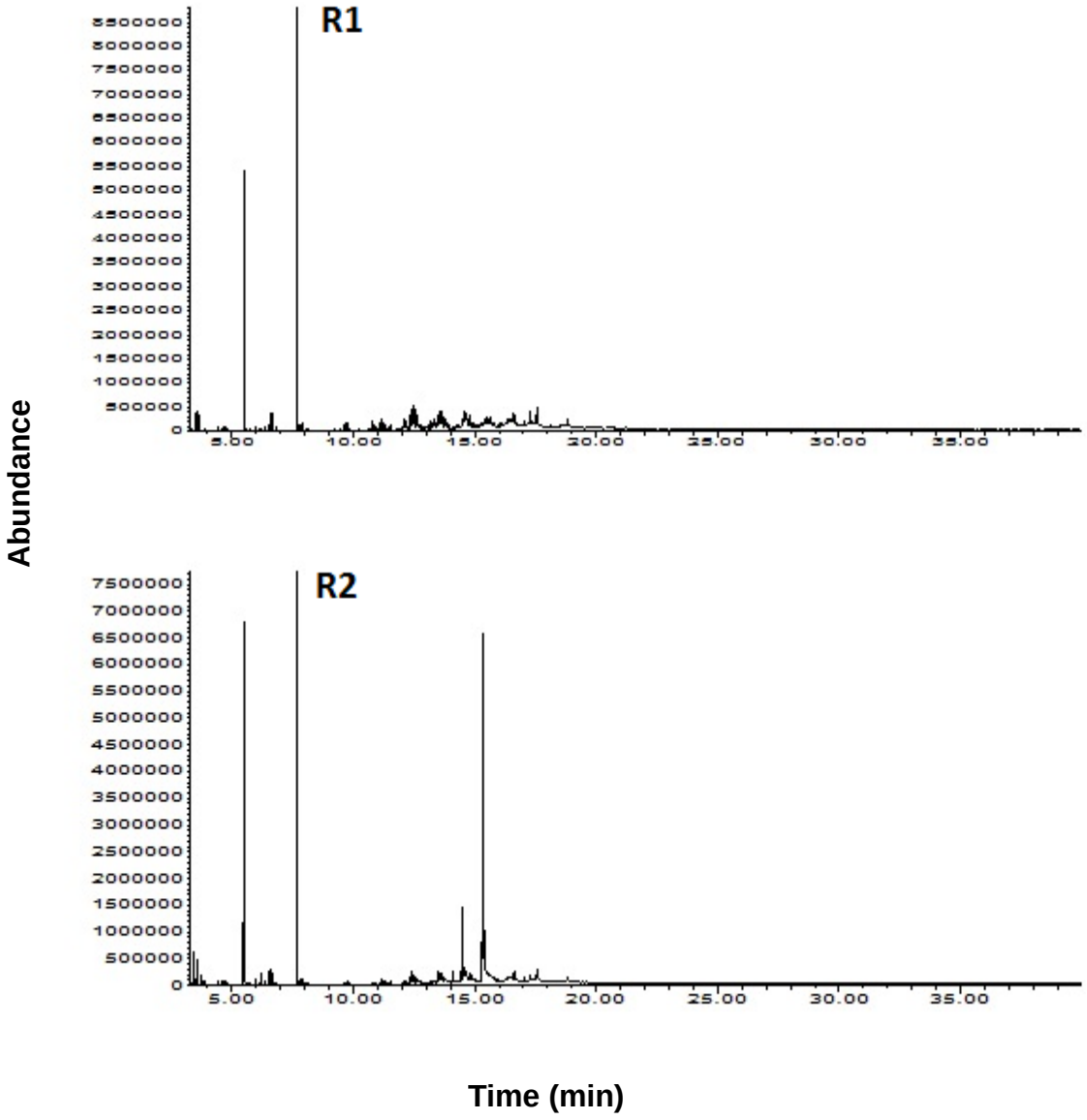
K2
Abundance
Time (min)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Abundance
Time (min)

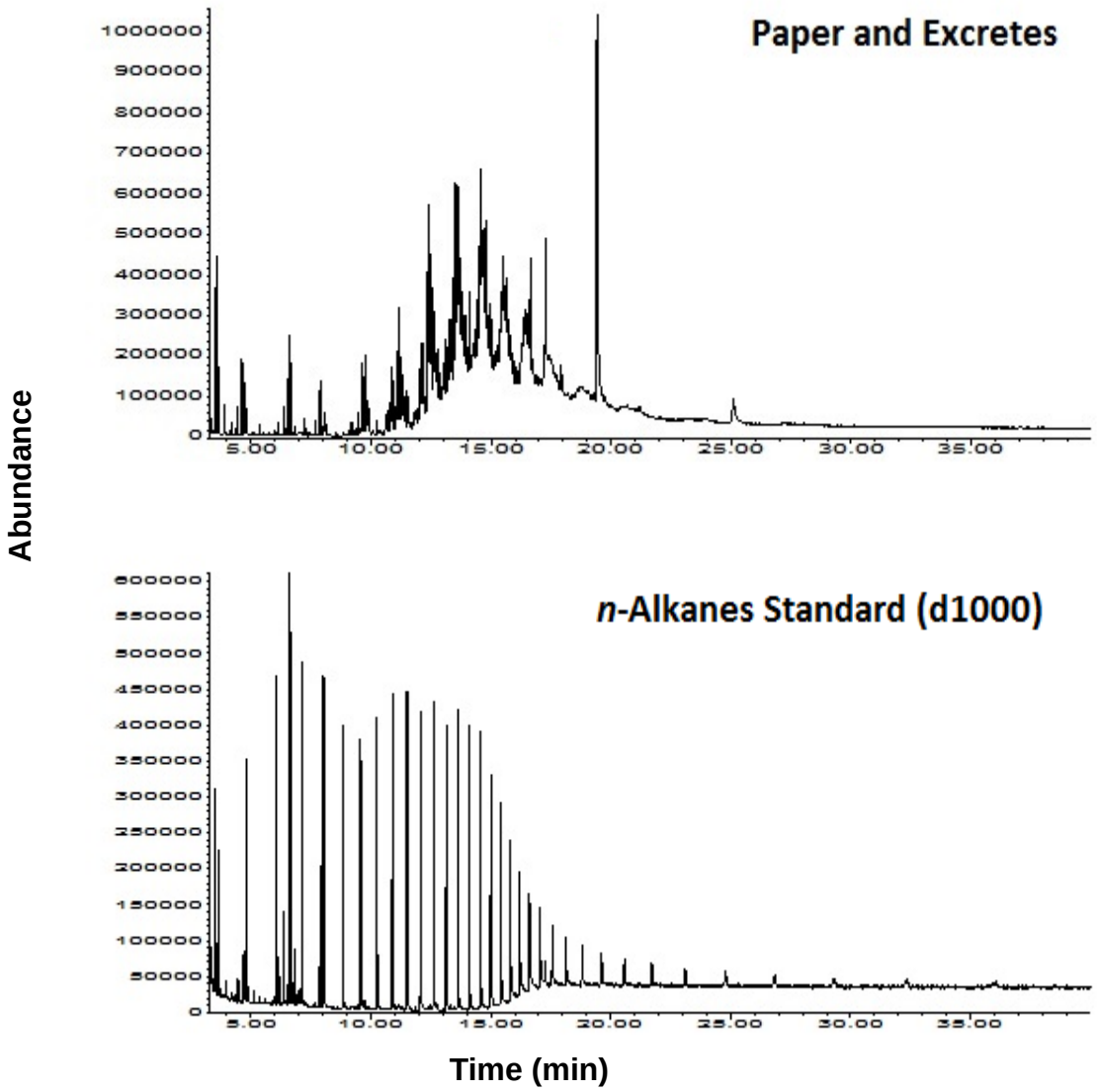

Figure 6.1 Representative chromatogram peaks of the H, B, K, and R strain CHCs and additional surface compounds, the filter papers that insects rested from several to 24 hrs with insect feces, and the *n*-alkanes standard diluted to 1/1000. All strains had two patterns of chromatogram peaks: ones without large peak(s) around 15.0 min (H1, B1, K1, R1), and others with the large peak(s) (H2, B2, K2, R2). Range of the abundance varied significantly among individuals, but overall patters of the chromatogram peaks were very similar among the four strains. The chromatogram peaks of the filter papers and bed bug feces were distinctly different from that of insect CHCs and additional compounds.

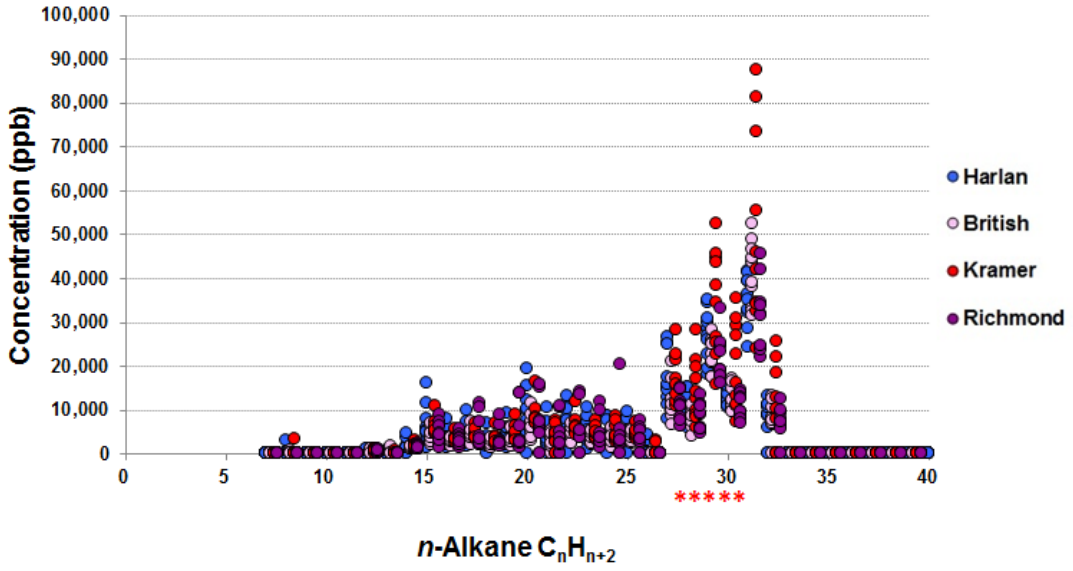

Figure 6.2 Comparison of *n*-alkane (C_nH_{n+2}) concentration among Harlan (H), British (B), Kramer (K), and Richmond (R) strains. Each dot represents the average concentration (ppb) of three separate GC-MS injections after normalizing the data by the individual insect body mass (g), the abundance of the IS (undecane-d_{24}, 3 ppm), and the function of the known concentration of the *n*-alkanes calibration standards. Statistically significant ($P < 0.05$) *n*-alkanes based on the Kruskal-Wallis, one-way ANOVA, across the strains were $nC_{27} - nC_{31,}$ that are indicated by the red asterisks below the x-axis.

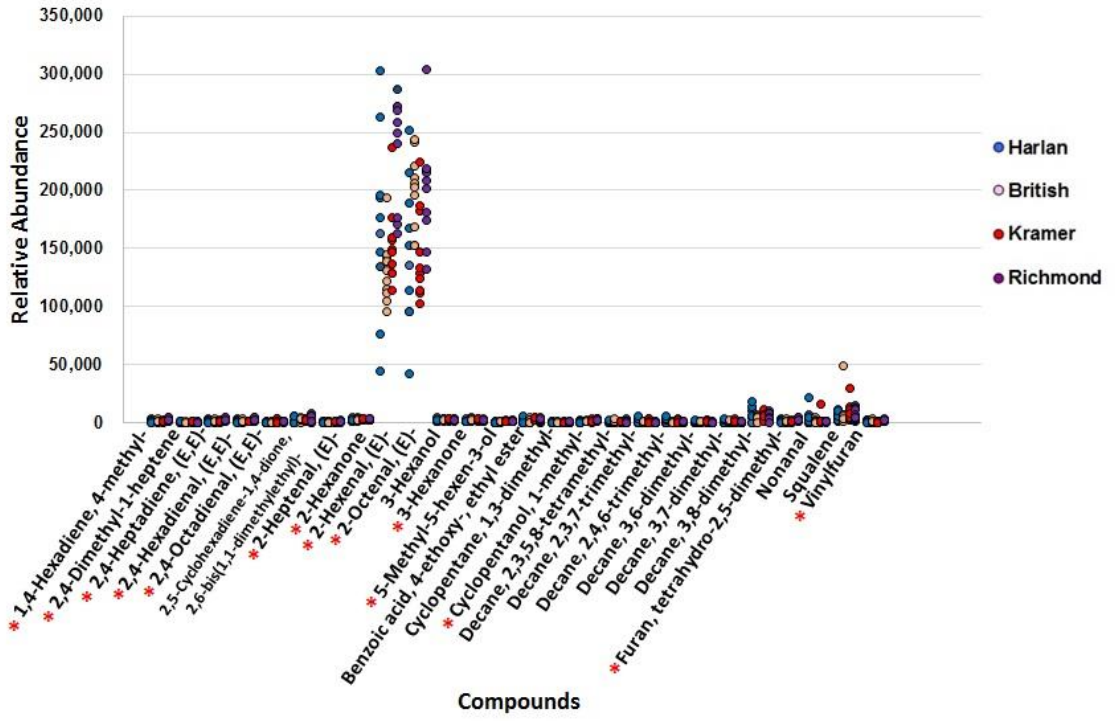

Figure 6.3 Comparison of additional compounds found after the NIST/EPA/NIH Mass Spectral Library search and their relative abundance among Harlan (H), British (B), Kramer (K), and Richmond (R) strains. Each dot represents relative abundance after normalizing the single measurement data by the individual insect body mass (g) and the abundance of the IS (undecane-d_{24}). Statistically significant ($P < 0.05$) compounds based on the Kruskal-Wallis, one-way ANOVA across the strains are marked with red asterisks next to the compound identifications on the x-axis.

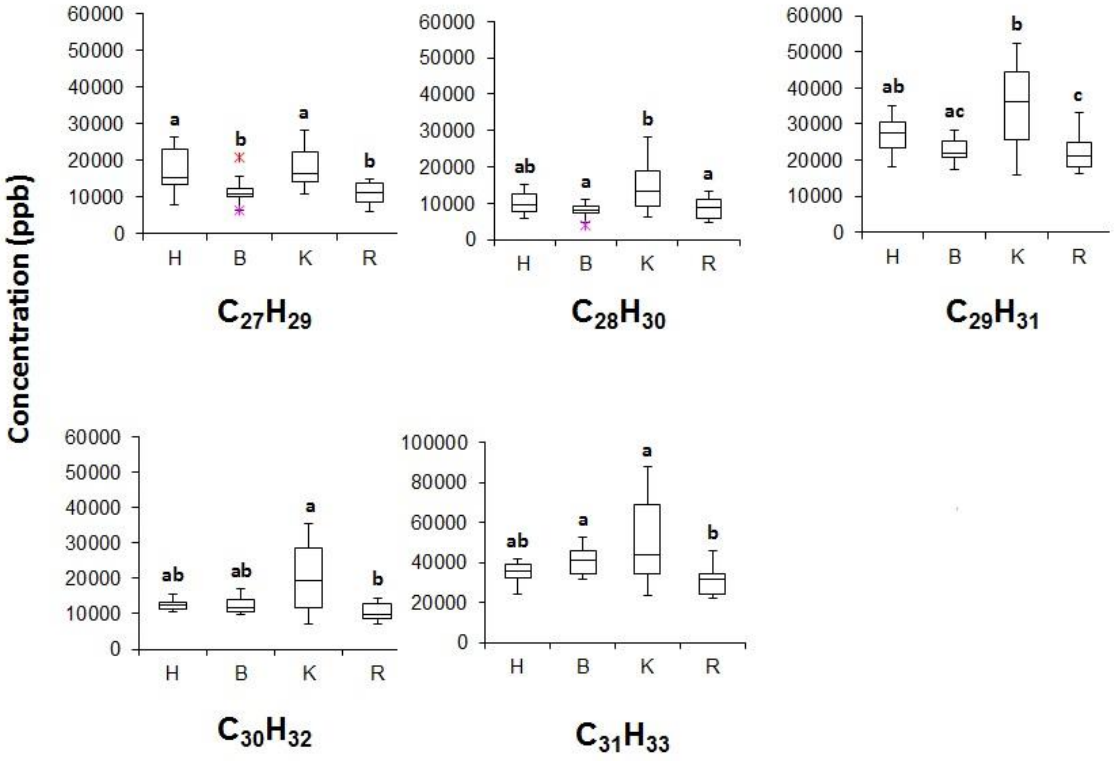

Figure 6.4 Concentration of the *n*-alkanes that were statistically significant across the strains (nC_{27-31}; $P < 0.05$) based on the Kruskal-Wallis, one-way ANOVA, showing the median and quartile distributions. The results of Dunn's Procedure of Multiple Comparisons showing statistical differences across the strains are summarized by the lower-case letter (a, b, c) above each whisker representing 1.5 interquartile range (IQR). The data was normalized by the individual insect body mass (g) and the abundance of the IS (undecane-d_{24}); then the slope of the *n*-alkane standards were used to determine the adjusted concentration of the *n*-alkanes. Outliers are shown in asterisks (minimum = pink, maximum = red).

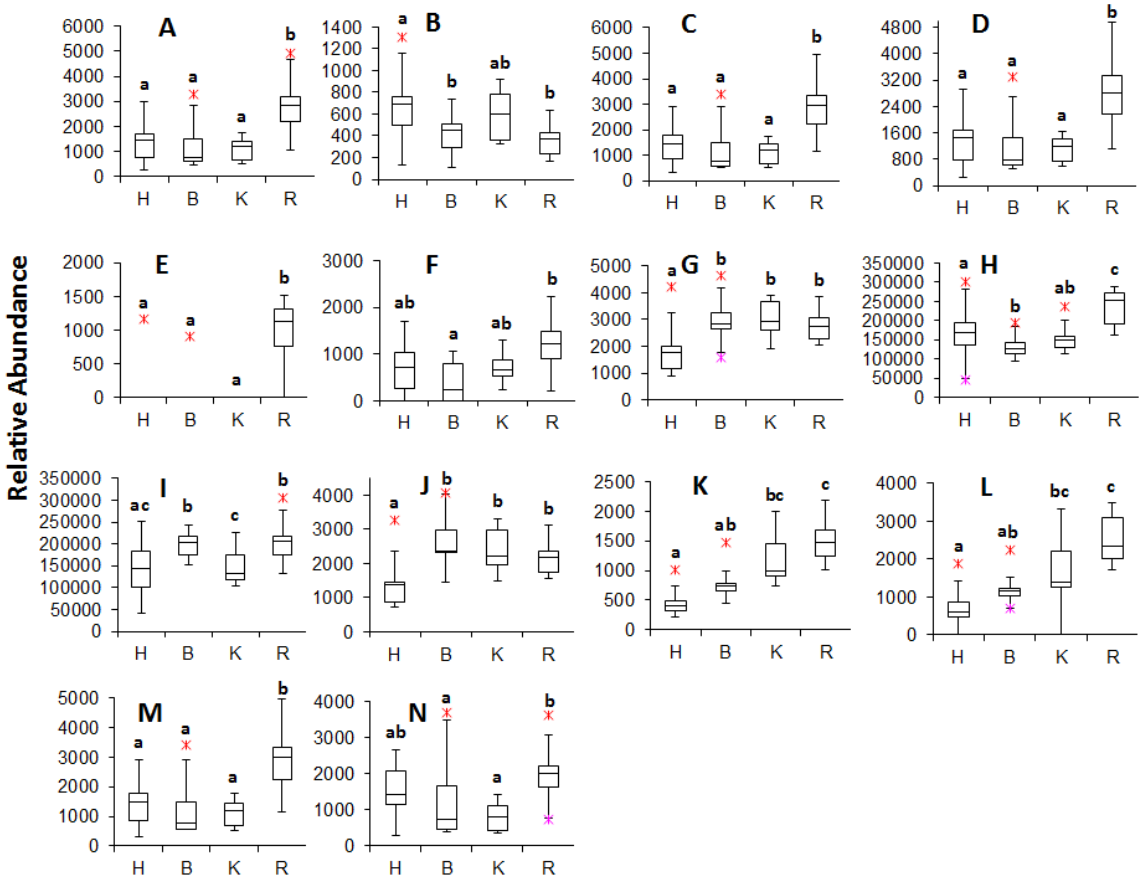

Figure 6.5 Relative abundance of the additional compounds that were statistically different ($P < 0.05$) across the strains based on the Kruskal-Wallis, one-way ANOVA, showing the median and quartile distributions. The compounds are: [**A**] 1,4-Hexadiene, 4-methyl-; [**B**] 2,4-Dimethyl-1-heptene; [**C**] 2,4-Heptadiene, (E,E)-; [**D**] 2,4-Hexadienal, (E,E)-; [**E**] 2,4-Octadienal, (E,E-); [**F**] 2-Heptenal, (E)-; [**G**] 2-Hexanone; [**H**] 2-Hexenal, (E)-; [**I**] 2-Octenal, (E)-; [**J**] 3-Hexanone; [**K**] 5-Methyl-5-hexen-3-ol; [**L**] Cyclopentanol, 1-methyl-; [**M**] Furan, tetrahydro-2,5-dimethyl-; and [**N**] Vinylfuran. The results of Dunn's Procedure of Multiple Comparisons showing statistical differences across the strains are summarized by the lower-case letter (a, b, c) above whisker representing 1.5 interquartile range (IQR). The data was normalized by the individual insect body mass (g) and the abundance of the IS (undecane-d_{24}), calculated by the sample abundance / IS abundance / g of the sample (individual insect). Outliers are shown in asterisks (minimum = pink, maximum = red).

Chapter 7: Summary

The Common bed bug, *Cimex lectularius* L., is an obligatory hematophagous human ectoparasite that has been recognized as the most difficult urban pest to control after the sudden world-wide resurgence in developed countries in the past 15 years. After the sudden world-wide resurgence of the bed bug populations around the late 1990s, field-collected population of bed bugs were found to be highly resistant to insecticides. The mechanisms of insecticide resistance that have been reported in bed bugs are target-site insensitivity (*kdr*), enhanced detoxification enzymes (P450, carboxyl esterases, etc.), and reduced cuticular penetration. The overall goal of this study was to investigate the mechanisms of the reduced cuticular penetration type insecticide resistance in field-collected resistant populations (strains) of bed bugs.

In Chapter 2, we determined the lethal dosage (LD_{50}) of pyrethroid insecticide necessary to kill pyrethroid-resistant field strain bed bugs using topical and injection application. We compared the LD_{50s} of two susceptible (Harlan and British) and two resistant (Richmond and Epic Center) strains. The resistant strain bed bugs had significantly greater RRs compared to that of the susceptible strains, in both topical and

injection application, verifying that they were highly resistant to pyrethroid insecticides. In addition, both resistant strains demonstrated significantly greater penetration resistance ratios compared to that of susceptible strains. This study provided the solid evidence that the resistant strain cuticle was much less permeable compared to that of susceptible strains, reducing and delaying the insecticide penetration.

In Chapter 3, we determined the levels of gene transcription specific to the development of the insect cuticle (CPR-type cuticle protein genes) in a pyrethroid-resistant field strain bed bugs collected from Richmond, VA, using real-time quantitative polymerase chain reaction (qRT-PCR). We merged our transcriptomic data with the data published by Mamidala et al. (2012), and identified 62 putative bed bug cuticle protein-encoding contigs based on the presence of the Chitin-binding 4 (CB4) domain of the Rebers and Riddiford Consensus (R&R Consensus; CPR family cuticular proteins). Based on the qRT-PCR analysis of the mRNAs, we found many of the putative cuticular protein genes were highly up-regulated in Richmond resistant strain compared to those of susceptible Harlan strain. This study suggested the possibility of the resistant strain bed bugs thickening the cuticle or increasing the quantity of cuticular proteins that may

contribute to reduced cuticular penetration of insecticides.

In Chapter 4, we identified and described the bed bug cuticular proteins using the matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry (MALDI-TOF/TOF). We collected bed bug cast skins (exoskeletons), ground them to form a powder-like pellets, washed several times with 50 mM DTT-SDS buffer, then digested with enzyme trypsin. Peptides were extracted and the amino acid sequences were determined. The total of 265 peptides were identified with 95% confidence level, among which 206 belonged to only one of 50 confidently identified proteins. In addition to the CPR family, we also identified the CPRL, CPF, CPFL, TWDL, and CPAP1 family proteins. We also compared the peak areas of each peptides, which identified 7 proteins that were significantly different between Richmond resistant and Harlan susceptible strain. Three proteins (14, 25, and 39; corresponded as CLEC004437-PA, CLEC002505-PA, and CLEC007768-PA genes; identified as a new protein, hypothetical secreted protein, and CPR 84) were statistically different between the resistant and susceptible strain when comparing both the peptide counts and peak area analysis. This study not only confirmed the presence of the

previously identified CPR protein genes expressed and produced in the bed bug cuticle, but also indicated the presence of many other families of cuticular proteins expressed in bed bugs. The profile of the cuticular proteins between the Richmond resistant and the Harlan susceptible strains were almost identical. Additional analyses using different digestion methods and tools are being investigated.

In Chapter 5, we determined and compared the cuticular thickness of resistant and susceptible bed bug strains using Scanning Electron Microscopy (SEM). To compare the cuticular thickness, bed bugs were bilaterally sectioned from the prothorax to the abdomen tip after submerging in a general purpose fixative, treated with series of increasing concentrations of ethanol, dried and mounted on the SEM mounting stub exposing the sectioned surface parallel to the mounting stub. A total of 3 point locations (Ps) were selected and measured on each of the 2nd and 3rd segments, both dorsal and ventral surface sides. We found statistical differences of the cuticular thickness among different strains (populations). However, correlation between the levels of insecticide resistance and cuticular thickness were not found. We concluded increasing the cuticular thickness of the sclerotized exocuticle may not be a mechanism of reduced cuticular penetration type

insecticide resistance in bed bugs. The thickness of the unsclerotized endocuticle and the density of the cuticle should be investigated and compared next across insecticide resistant and susceptible bed bug strains.

In Chapter 6, we identified and described bed bug cuticular hydrocarbon profiles using Gas-Chromatography and Mass-Spectrometry (GC-MS). Bed bug cuticular hydrocarbons and additional compounds that consist the bed bug wax layer were extracted by submerging the whole individual bed bug in hexane. The profile and concentration (abundance) of the saturated cuticular hydrocarbons (*n*-alkanes) and additional compounds (methylated alkanes, alkenes, esters, aldehydes, acids, ketones, alcohols, amides, etc.) were identified and compared using commercially available standards and through the library search. The total of 87 compounds in addition to *n*-alkanes were extracted and identified from the insect samples, among which 26 were analyzed, and additional 61 compounds were identified. We found bed bugs have increased amount of $C_{27} – C_{33}$ *n*-alkanes, however there were no correlation found with the concentration of the *n*-alkanes and the levels of insecticide resistance. In contrast, several compounds such as (non-alkanes) exhibited the correlation between the concentration of the compounds and the levels of

insecticide resistance. The compounds that might be associated with the insecticide resistance are: [A] 1,4-Hexadiene, 4-methyl-; [C] 2,4-Heptadiene, (E,E)-; [D] 2,4-Hexadienal, (E,E)-; [E] 2,4-Octadienal, (E,E-); [H] 2-Hexenal, (E)-; and [M] Furan, tetrahydro-2,5-dimethyl-; [I] 2-Octenal, (E)-; [K] 5-Methyl-5-hexen-3-ol; [L] Cyclopentanol, 1-methyl-; and [N] Vinylfuran. The two major peaks that were always present in all the insect samples, [H] 2-hexenal, (E)- and [I] 2-Octenal, (E)-, were present in significantly greater abundance in R strain compared to other strains, especially [H] 2-hexenal, (E)-. Also, the variance range among individuals were also large for these two major peaks of [H] 2-hexenal, (E)- and [I] 2-Octenal, (E)-. The total of 47 additional compounds were unique to insect samples among the 61 additional compounds that have been detected and identified. Another 14 compounds within the 61 additional compounds found in samples were also identified from the filter papers and feces samples. The total of 15 compounds were exclusively found on filter papers with feces out of the 29 compounds identified from these samples. This study indicated that the concentration of saturated *n*-alkanes in cuticular hydrocarbons were not associated with the levels of insecticide resistance, however many of the additional compounds that were also consisting

the bed bug wax layer might be contributing to the reduced cuticular penetration of insecticides.

Overall, we found three lines of evidence to support reduced cuticular penetration as a mechanism of insecticide resistance in some bed bug populations: the differences in LD_{50} values (penetration resistance ratios), the up-regulation of cuticle protein-associated genes, and the increased amount of some additional compounds in the wax layer. This study provides additional evidence of the reduced cuticular penetration type resistance in bed bugs. Not only this study reveals some mechanisms behind the reduced cuticular penetration type resistance, but also offers some useful knowledge to the industry to help develop better formulation of currently available insecticides. In this study, we were able to reveal some mechanisms behind the reduced cuticular penetration type insecticide resistance.

The future works of the bed bug cuticle investigation should be: 1) RNAi of the up-regulated cuticle protein genes to shut-down and evaluate which gene is responsible for reduced cuticular penetration; 2) immunolocalization of the proteins that were found increased in numbers of peptides copies in the resistant strain and identify the proteins

concentrated in the exoskeleton of the bed bug integument; 3) examining the cross-linking structure of the cuticular proteins; 4) extracting, identifying, and measuring the amount of metal content that might be hardening the cuticle or reducing the insecticide penetration; 5) identifying mutations in the pyrethroid-resistant bed bugs cuticular proteins; 6) measuring the density, the endocuticular thickness, and the numbers of pore canals within an area of the bed bug cuticle; and 7) detailed investigation of the additional compounds in the wax layer that might be associated with insecticide resistance. Any of above studies might provide deeper understanding into the mechanisms of reduced cuticular penetration in resistant population of bed bugs. Also, investigating further into the genomes, endocrine regulation of the cuticle formation, the association of P450s in cuticle synthesis, and finding links among them will most likely provide the better understanding of the mechanisms of insecticide resistance and some novel control methods.

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Appendix A. List of proteins and their peptides sequences extracted and identified from Harlan (H) susceptible and Richmond (R) resistant strains cast skin extracts. The peak area, average R/H ratios, the genome annotation, and the identified proteins (along with the type when available) are also shown.

Protein	Sequence	Theor m/z	Avg R/H	Std	n	p-value	Genome Annotation	Identified Protein	Type
1	VPVPVHHTVNVPVPHPVAVPVPRPVAVPVVR	3260.93	0.61	0.20	2	0.23	CLEC011347-PA, CLEC011348-PA, CLEC011350-PA, CLEC011352-PA	New	
1	VPVPVHHTVNVPVPHPVAVPVPR	2443.41	1.05	0.48	2	0.62			
2	RVPYSVPVPH	1150.64	1.04	0.24	3	0.89	CLEC011389-PA, CLEC011391-PA	New	
2	RVPYSVPVPHVVPVPTK	1871.09	1.05	0.59	3	0.86			
3	HVPVPVPHPVK	1205.72	0.88	0.09	3	0.14	CLEC011354-PA	Unclassified CPs (clusters)	
3	NVPVAVPHPVPVHK	1489.86	0.76	0.16	3	0.10			
3	VAVPVPQPYPVTVEK	1622.92	0.98	0.36	3	0.94			
3	VVKPYPVPVPKPVPYTVVK	2106.27							
3	AVPVHVPQPVPVTVPR	1692.00	1.10	0.34	3	0.63			
3	AVPVPQPYPVTVEK	1523.85							
3	AVPVPQPYPVTVTR	1523.86							
3	PVPVPVPQPVPVSVPR	1663.99	0.91	0.21	3	0.77			
3	PVPVSVPRPVPVPVPHPVPVSVPQPY	2736.56							
3	VVKPYPVPVPKPVPY	1678.99	0.80	0.17	2	0.29			

4	TVPVPVPHPVR	1197.71	0.98	0.20	3	0.85	CLEC011353-PA	New
4	VPVPHPVAVPVPKPVAVPVVK	2125.33	1.03	0.38	3	0.82		
4	PVPVPVDRPYPVPVER	1816.01	0.99	0.31	3	0.93		
4	TVPVPVPHPVRVPVD	1607.93	0.72	0.23	2	0.25		
5	TVSVPVPHPVAVPVPRPVAVPVVR	2468.49	0.33	0.21	3	0.12	CLEC011346-PA	New
5	VPVPVHR	803.49	0.40	0.28	2	0.43		
5	RVPVPVHR	959.59						
5	TVAVPVPHPVSVPVD	1512.84	2.41		1			
5	TVAVPVPHPVSVPVDRPYPVQVPR	2605.46	1.03	0.40	2	0.69		
5	TVSVPVPHPVAVPVPR	1650.97	0.41	0.09	3	0.11		
6	VPVPHPVAVPVPKPVAVPVVR	2153.33	1.19		1			
7	DAGGSGGGSGNFLFDVIR	1725.82	1.23	0.65	3	0.96	CLEC010982-PA	hypothetical secreted protein
7	EGHYGEGVPGPLTR	1468.72	1.03		1			
8	EVPVPVPQPYPVTVEK	1777.97	1.43	0.37	2	0.37	CLEC009177-PA, CLEC011344-PA	New
8	HVPVPVAQPVYVHK	1569.89	0.93	0.06	2	0.18		
8	TYVTEGVLK	1009.56						
8	VPVHVPVAHPVPVPVAKPYPVTVEK	2655.54						
8	AVDRPVPYPVK	1240.70	0.83		1			
8	GYVAAAPVVTK	1075.61	0.85		1			
8	NYLAAAPVVTK	1146.65	1.06	0.23	2	0.72		
8	NYVAAAPVVTK	1132.64						

8	SYVAAAPVVTK	1105.63						
8	SYVAAAPVVSK	1091.61						
9	QPVTVTQPELVYR	1529.83	1.30	0.38	3	0.32	CLEC006846-PA	new
9	VNVAPTQVNVVR	1295.74	1.25	0.40	3	0.67		
10	VAVPAVAPVAVHSTTVHHPSYLL	2365.30	0.82	0.48	2	0.56	CLEC006545-PA	NPLP3 (?)
10	VVLPLGGAVVR	1079.69	1.17	0.33	3	0.41		
10	VVLPLGGAVVRVPSLDSAVVK	2075.26	2.24		1			
11	KVPYSVPVPHVVPVPTK	1843.08	0.86	0.35	3	0.95	CLEC011386-PA, CLEC011388-PA	Unclassified CP
11	QETFPVVVPHPVPVPVTR	1998.12	1.62	0.48	3	0.15		New
11	TQPVPVPHPVPVDVPRPVK	2058.19	1.22	0.21	3	0.23		
11	VPVPVEKPYPVPVPQPYPVTVTR	2558.44	1.50	0.14	2	0.22		
12	VVAPVAVAHAAPVVAAPVAAVGVAHAAPVSYGLGYGR	3434.91					CLEC004440-PA	new
12	AAPVSYGLGYGR	1210.62	1.07	0.32	3	0.60		
12	AAPVVAAPVAAVGVAHAAPVSYGLGYGR	2591.41	0.77		1			
13	**TYGYSGLGYGR**	**1193.56**	**0.58**	**0.14**	**3**	**0.03**	**CLEC004439-PA**	**new**
13	AAPVAYGHGLGYGR	1388.71	1.00	0.33	3	0.88		
13	APVAVAHAAPVAVAH	1380.77	1.00	0.37	2	0.79		
13	ASHAVAAPVAAVGVAHAPVATSYANTYR	2722.41						
14	**AAPAPVAVAHAAPVATSYANTYR**	**2269.17**	**0.34**	**0.19**	**2**	**0.03**	**CLEC004437-PA**	
14	AAPVATSYANTYR	1384.69	0.33	0.16	3	0.13		
15	AAPAPVAVAHAAPVAAVGVAHAPVATSYANTYR	3141.66	1.15		1		CLEC004438-PA	
15	SVAQAPVATSYANTYR	1698.84						
15	SVAQAPVATSYSNTYR	1714.84						

16	YLGYGAGLGYSTLL	1447.75	0.93		1		CLEC004435-PA	new
16	YLGYGAGLGYSTLLH	1584.81	1.06		1			
17	TVGYSTHTVGVAAAPAVGYAAAPVVAAR	2627.39	1.05	0.07	3	0.38	CLEC004384-PA	new
17	TYGVAAGPAVGVAAAPAVGVVAAR	2095.17	1.13	0.13	2	0.33		
17	LATPLHTVTQQR	1364.76	0.72	0.16	3	0.10		
17	TPVVQSAPVVAAAPAVGVVAAR	2030.18	1.16	0.32	3	0.58		
17	TPVVSEVEVR	1114.61	1.14	0.07	2	0.37		
17	TYGVAAAPAVGVVAAK	1444.82	1.24	0.52	2	0.58		
17	TYGVAAAPAVGYATGYAAAPAVVAAK	2381.25	1.01	0.29	2	0.77		
17	TYGVAAAPAVVSHR	1398.75	0.93	0.19	3	0.66		
17	TYGVATAPAVGYAAAPVVAAR	1976.06						
17	TYGYAAAPAVVAAK	1352.72	4.19		1			
17	VAAVGRPVQTVVPHVTQVQPEVHVQR	2830.58						
17	VAVDVPVKTPVVSEVEVR	1922.10	0.75	0.17	3	0.13		
17	TYGVAAAPAFGYAAAPAVGYAAAPAVGVVAAR	2921.53	0.95	0.09	3	0.41		
18	LIGPLFSDGITAPR	1456.82	1.56	0.63	2	0.43	CLEC001985-PA	unknown
18	LTGVIDANTVYGITETFAR	2041.06	1.76		1			
18	PEVNPNVIDAFSAAAFR	1817.92						
19	QSVLGSQLPLPR	1294.75					CLEC002329-PA	Peroxidase
19	PTVNPNIIDAFSAAAFR	1803.94						
19	TPFNTLTGVIDGNTVYGVTEEFGR	2587.27	1.13	0.51	2	0.82		
20	FDNTGNYR	986.43	1.30	0.60	2	0.70	CLEC000231-PA	
20	GDFGGEYVPGFGGAYR	1648.74	0.99		1			
20	GDYGGGYYQSGYK	1414.59	1.28		1			

21	LSPQAQEQR	1056.54	2.21		1			unknown
21	QAAPQQLPPEYLSLLR	1824.00	1.29		1			
21	QLQASAVQQLQAQQAAQQQAR	2294.20						
21	VTPSPEQSGSEGQQPAYYR	2080.96	1.21		1			
22	LIDLLTAR	914.57					CLEC011522-PA	CPAP1-N, hypothetical secreted protein
22	QFYDIEER	1099.51						
22	SQLQQPSPQPSNVQQFQSGFGR	2445.19	0.50	0.03	2	0.06		
22	TAQVSNQER	1032.51	0.57		1			
22	TGPNHPANAQQFQSGFVR	1955.95	0.81		1			
22	TNIQPLR	841.49	0.40		1			
22	TVTNQPSNAQQFQSGFVR	2008.98	0.35	0.14	2	0.10		
23	TYAAPVATYAK	1155.60	0.79	0.27	3	0.32	CLEC002629-PA	CPF3b, CPFL
23	TYAAPVAAYAHAPAVATYAAPAYAK	2480.26	0.82	0.12	2	0.18		
23	TYAAAPVATYAHAAPLAYAAPAYAK	2494.28	0.82	0.03	2	0.22		
23	AYAAPAVATYAHAPAVATYAK	2078.07	1.04		1			
23	TYAAPAVATYAHAAPVAHAYAAPAVAK	2624.36						
24	DGDVVQGSYSLVEPDGSR	1879.87	0.45		1		CLEC000304-PA	
24	VVEYTADPVNGFNAVVHK	1959.00						

								hypothetica
25	GASFNHLGYSSGLGLSSGLGYGSGVR	2500.22	1.10		1		**CLEC002505-PA**	**l secreted**
								protein
25	GYAGDSVSQFTSNVQGAHSYSTNSFSR	2854.27	0.72	0.14	2	0.23		
25	NVTPAVSYSAPAVTTVHAAPAVAAVR	2520.36						
25	SSSYHGNGYTGVGYGAVAPAVSYAAPAVAAVR	3070.50	0.68	0.04	3	0.11		
25	SSTYHGNGYTGASYAAPAVSAVR	2287.07	0.55		1			
25	SSTYHGSGYTGAGYASAAPLVSGLR	2430.17	0.68	0.21	3	0.18		
25	TSSVSYSSPAVTTVHAAPAVAAVR	2329.21	0.92	0.18	3	0.65		
25	TTNGGVAVGAPAVAAVR	1510.83	0.69	0.16	3	0.18		
25	TVAPAVSYAAPAVASVR	1629.90	0.83	0.14	3	0.06		
25	**TVAPAVSYAAPAVSYAAPAVASVR**	**2289.22**	**0.72**	**0.07**	**3**	**0.04**		
25	TVAPAVSYAAPAVSYAAPAVSYAAPAVASVR	2948.55	1.27	0.36	3	0.40		
25	**TVGPAVSYATPAVAALR**	**1643.91**	**0.72**	**0.07**	**3**	**0.02**		
25	TVSTPAVSYAAPAVTTVHATPAFSYGAPAFR	3108.58	0.77	0.18	3	0.17		
25	TVTPAVSYAAPAVASVR	1659.91	1.38	0.26	3	0.13		
25	TYSTATPLFK	1128.59	0.83	0.22	2	0.48		
25	YGAGLGYGSGLR	1170.59	0.72	0.12	3	0.10		
25	YGSGLGYGSGLR	1186.59	0.71	0.19	3	0.15		
25	YGSGLGYGSGLSYGAGLGYGSGLR	2269.09	0.77		1			
26	**AAHFAAVAR**	**913.50**	**0.92**	**0.00**	**3**	**0.04**	**CLEC000744-PA, CLEC000747-PA**	
26	PVVHTPVVH	984.56	0.99	0.48	3	0.50		
27	VVGVDLEGVK	1014.58	1.27	0.26	3	0.18	CLEC011867-PA	New

27	EPPVGGGYSYSR	1268.59	0.98		1				
27	GAVLEQVR	871.50	0.74	0.13	3	0.32			
27	GVPSSSYGV	852.41	0.84		1				
28	AAHLVQQHNEAVR	1472.77	1.23	0.08	2	0.15	CLEC000748-PA	CPR 15	RR-2
28	NVHGVGSVPEFSPADAPDVALVK	2305.18	1.13	0.51	3	0.63			
28	PAVVAAAPAVVAAPAAVAAPAVVTSDLPEVVAAR	3091.75							
29	AAVAAPVVAK	896.56	1.04	0.23	3	0.95	CLEC004093-PA	CPR 31, 32	RR-2
29	AVVAAHAAPVLAK	1217.74	0.86	0.24	2	0.54			
29	DGDVVHGSYSLVEADGTR	1876.87	0.86	0.23	2	0.60			
29	TVDYTADPVNGFNAVVHK	1946.96	0.75	0.29	3	0.41			
29	VATPVAYAAHGYAAPAYYHH	2130.02	0.77		1				
29	AAHAAPVAYAAPVAK	1407.77	0.59	0.20	2	0.12			
29	DGDVVHGSYSLVEADGTRR	2032.97	0.80		1				
29	GVLSAPAVVAPGAPLAYGGYAR	2057.12							
29	GVVGAPAVVAPGAPLAYGGYAR	2013.09	0.62	0.15	3	0.19			
29	TVDYTADPVNGFNAVVHKEAAVVK	2544.31	1.44		1				
30	DGDVVQGSYSLVEPDGTR	1893.88	1.35		1		CLEC004096-PA	CPR33, 34, 35	RR-2
30	DGDVVQGSYSLVEPDGTRR	2049.98	0.47		1				
31	GSYSYVDPSGQR	1315.59	1.02	0.16	2	0.82	CLEC004802-PA	CPR 48, 49	
31	NAAILTEQR	1015.55							
32	TQPQQYPAPVQEYR	1704.83	0.75	0.41	3	0.42	CLEC004782-PA	CPR 48, 49	
32	AQPQYQPQPQYQPR	1728.85	0.92	0.25	3	0.53			
32	PQPQPQYQPQPQYQPQVPR	2304.15							

32	PQPQPQYQPR	1238.63	0.90	0.71	3	0.45			
33	ATPGGGAYYNPGPAYR	1611.76	0.54	0.03	2	0.20	CLEC006890-PA	CPR 54	RR-1
33	AVAQQVAEAR	1042.56	1.07	0.73	3	0.93			
34	EHNAHPQVVQK	1286.66	0.64		1		CLEC005785-PA		
34	VVEYTADPHNGFNAVVHR	2024.99							
35	EHNTHPVVQK	1188.61	0.43		1		CLEC005789-PA		
35	AHTGDVHSQSEER	1452.65							
35	VHSQSEER	971.45							
36	YQGEPQYPQQAGSSPGR	1849.85	1.09	0.78	3	0.87	CLEC005565-PA	CPR 79	CPR other
36	YQGEPQYPQQAGSSPG	1693.75	1.20		1				
37	QQGSSPGGR	873.42	0.53		1		CLEC005566-PA	CPR 80	CPR other
37	QTPSSQYGVPGAGAVTPSSR	1946.96							
37	SGFGTPSAQYGAPSAQLGPQSAR	2235.08	0.92	0.13	2	0.53			
37	SGFGTPSTQYGAPSAQLGPQSAR	2265.09							
37	SGFGTPSTQYGAPSGQGPQSAR	2137.99	0.64		1				
37	SGSPSTEYGTPSAFGASAR	1829.83	0.90	0.11	2	0.34			
37	TGNQGYQSGTGSR	1312.59	1.06	0.57	2	0.86			
38	AGGAAVNQGYSGTLGSR	1565.77	1.21		1		CLEC005568-PA		
38	GGNQYAGR	822.39							
38	SQGYSGVAGTQGYTGSSR	1762.80							
39	AQPQVYQVAGK	1188.64					**CLEC007768-PA**	**CPR 84**	
39	**DQVYQFQPK**	**1152.57**	**0.69**	**0.01**	**2**	**0.05**			

39	QQTAGSLPVGATAR	1356.72	0.66	0.03	2	0.23			
40	FAAPLAYAAPVAK	1289.73					CLEC008665-PA	CPR 88, 89	CPR other
40	VAAPLAYAAPVAK	1241.73	1.42		1				
41	VASPYGYAHY	1127.52	0.79	0.11	3	0.10			
42	EAGAHPVAK	879.47	1.06		1		**CLEC011465-PA**	**CPR 94**	
42	VAAPVAYAAAPAYAK	1433.78	0.74	0.26	3	0.35			
42	VAAPVAYAAAPAYATHAYAAPAYAK	2450.25	0.84		1				
42	**VAAPVAYASPAYGYHH**	**1673.81**	**0.78**	**0.07**	**3**	**0.03**			
42	VVAAPAYAK	889.51	1.00	0.24	2	0.89			
43	SAPAFAYSAGPAVK	1336.69	0.97		1		CLEC011415-PA	CPR 99	
43	YAAPAAEVR	947.49	0.89	0.09	2	0.21			
43	YAAPAVASYAAAPVVK	1548.84	0.94		1				
43	YAAPAVASYHQASSVK	1649.83	0.76		1				
43	GQYSLVEPDGSVR	1406.69	0.92	0.37	3	1.00			
43	DGDVVKGQYSLVEPDGSVR	2020.00	0.97	0.52	2	0.93			
44	APPVQTIR	881.52					CLEC011589-PA	CPR 104	CPR other
44	NFPVPSFAQLEEQVQPQVLR	2326.22	1.05	0.58	3	0.73			
45	TGNVQFR	821.43	1.28	0.18	3	0.26	CLEC012184-PA		
45	VVHYTADEYGYR	1472.68	1.28		1				
45	PKTGNVQFR	1046.57	0.69		1				
46	RPHQPPPPPQK	1278.71	0.97	0.06	3	0.54	CLEC002176-PA	TWDL 1-3	
46	LPAIAPPAEQK	1134.65							

47	ALNHGLPVAHAVAAPVVAAR	1934.11	0.94		1		CLEC000743-PA	CPRL 4, 5
47	NLLGGGVPLHLPADTPEVALAK	2182.22						
48	AVLVNPNALASPADTLEVALAK	2177.22	2.40	1.23	2	0.34	CLEC000743-PA	CPRL 4, 5
48	QAHAVAHENQK	1215.59	1.10	0.88	2	0.71		
49	NVLGGGVPLHLPADTHEVAVGK	2180.18	1.95	1.17	2	0.45	CLEC000743-PA	CPRL7, 8; CPRL 9, 10
49	SAVGLVGVVNPHSLPVPADTLHVALAK	2661.51						
50	SVWGVNPHSLPVPLDTLEVAVGK	2414.31					CLEC000743-PA	CPRL 9, 10
50	SLPVPLDTLEVAVGK	1537.88	0.91		1			
51	AYAAPAVATYAAPAVATYAHAAPVVAK	2586.37	0.86	0.14	3	0.33	CLEC002626-PA	CPF3b, CPFL
51	AYAAPAVATYAHAAPVVAK	1841.99						CPF1, 2, 3a
51	VSNPGYATYAAAPVAYAAPAVATYAH	2567.26	0.98		1			
51	VSNPGYATYAAAPVAYAAPAVATYAHAAPVVTK	3233.66	0.83	0.30	3	0.53		
51	AAPVAYAPAAVTSQSSNTLR	1975.02	0.96	0.33	2	0.76		
51	APAAVTSQSSNTLR	1402.73						
51	SFGNLGQVSTFHK	1421.72	0.88	0.26	3	0.47		
51	TPYSSVSK	868.44	1.07	0.15	2	0.77		
51	TVDTPYSSVSK	1183.58	0.78	0.28	3	0.34		
51	TVDTPYSSVSKSDVR	1640.81	0.77	0.07	2	0.39		
1,4,5,6	HVTVTHR	849.47	0.87	0.34	3	0.78		
1,5,6	PVAVPVVR	836.54	1.21	0.38	3	0.45		
1,6	TVPVPVPHPVSVPVD	1538.86	1.14	0.11	2	0.33		
1,6	TVPVPVPHPVSVPVDR	1694.96						

13,14,15	APVATSYANTYR	1313.65				
13,15	AAPVAAVGVAHAPVATSYANTYR	2257.17			2	0.34
14,15	AAPVAAVGVAHAAPVSYGYGLGYGR	2374.23	1.13		1	
14,15	AAPVSYGYGLGYGR	1430.71	1.05	0.29	3	0.68
2,11	PVSVPQPYPVTVTR	1539.85				
2,11	QETVAVPVPQPVPVTVTR	1917.08	1.16	0.50	3	0.76
2,11	QVPVPVPQPYPVSVPQPYPVTVTR	2644.45	0.99		1	
2,11	SVPVPHVVPVPTK	1355.80	1.24	0.58	3	0.95
2,11	TQPVPVPQPVPVD	1372.75	0.56		1	
2,11	TQPVPVPQPVPVDVPR	1724.97	0.99	0.34	3	0.73
2,11	TYPVPVTHQVPVPVPQPYPVSVPQPYPVTVTR	3538.91	1.00	0.33	3	0.95
2,11	VPYSVPVPH	994.54	2.02		1	
2,11	VPYSVPVPHVVPVPTK	1714.99	1.01	0.17	3	0.72
2,11	VVHPTPVVH	984.56	0.99	0.48	3	0.50
2,11	VVHPTPVVHAAPVVH	1558.89	1.14	0.41	3	0.50
2,3,11	PQPVPVTVTR	1093.64	1.00	0.27	3	0.94
2,3,11	PQPYPVTVTR	1157.63	0.90		1	
2,3,11	PVPQPVPVTVTR	1289.76	0.99	0.17	3	0.94
2,3,11	PVPVTVTR	868.53	1.04	0.26	3	0.91
2,3,11	PYPVTVTR	932.52	1.46	0.77	3	0.77
23,51	AAPVAYAAPAYAK	1263.67	0.58		1	
23,51	AYAAPAVATYAH	1205.59	1.22		1	
23,51	TYAAAPVATYAH	1235.61	1.40		1	
24,29,30,40,41	PVNGFNAVVHK	1181.64	1.20		1	

29,30	PHPQYSYAYDVQDALTGDSK	2255.03	0.82	0.13	2	0.42
29,30	TVVADEYDPHPQYSYAYDVQDALTGDSK	3147.41	0.81	0.41	2	0.69
29,34,35,40,41	VAYAAPVAK	889.51	1.00	0.24	2	0.89
30,40,41	TVEYTADPVNGFNAVVHK	1960.98				
34,35	VAYAAPVVQK	1045.60	0.94		1	
34,42	DGDVVHGSYSLVEPDGSKR	2016.96	0.65		1	
34,42	VAAPVAYAAPAAYAAPAYAK	1907.01	1.27	0.20	2	0.40
4,5,6	PQPVPVPVER	1117.64	1.00	0.18	3	0.78
4,5,6	PVPQPVPVPVER	1313.76	1.08	0.21	3	0.78
4,5,6	PVPVPVER	892.53	1.65	1.33	2	0.71
4,5,6	PYPVPVER	956.52	1.07	0.38	3	0.88
4,5,6	QPVPVPVER	1020.58				
4,5,6	VAVPVPQPVPVPVER	1582.93	1.04	0.27	3	0.92
4,5,6	VPQPVPVPVER	1216.70				
4,6	RVPVPVHHTVK	1268.76	0.83		1	
4,6	VPVPHPVAVPVPK	1335.81	1.01	0.19	2	0.95
4,6	VPVPVHHTVK	1112.66	0.81	0.31	3	0.34
40,41	SGDVVQGSYSLTDSDGTR	1843.83	0.86		1	
40,41	SGDVVQGSYSLTDSDGTRR	1999.93	0.62	0.04	2	0.33
40,41	TVEYTADPVNGFNAVVHKEPLVAK	2598.36				
40,41	VAAPFGYGAPVAK	1247.68	0.88	0.21	3	0.49
40,41	VAAPVAYAAPVAK	1227.71	0.84	0.10	3	0.14
47,48	QAQLVQQHTEGTR	1495.76	1.01	0.11	3	0.70

47,49	HAQLVQQHTEGTR	1504.76	1.06	0.65	2	0.82
47,49	QAQLVQQHSEGTR	1464.72	1.30	0.52	3	0.62
47,49	SLPVPLDTPYVAHAK	1607.88	0.72	0.23	2	0.25
47,49	SVLVNANALPVPADTLEVALAK	2205.25	0.93	0.47	2	0.56
47,49	SVWGVNPYSLPVPLDTPYVAHAK	2510.31				
47,49	TPYVAHAK	886.48				
49,50	QAHAVAHAR	960.51	1.33	0.48	3	0.35
5,6	PVPVPVERPYPVPVER	1830.03	1.21	0.24	3	0.30