Morphological and Physiological Characteristics that Contribute to Insecticide Resistance in Bed Bug (*Cimex lectularius* L.) Eggs

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ABSTRACT

Although bed bug eggs are a difficult life stage to control with our currently labeled insecticides, few studies have examined how bed bug egg morphology and physiology is potentially related to pesticide resistance in bed bug eggs. Bed bug egg morphological features were examined using scanning electron microscopy (SEM) and the chorion and respiration structures were identified. Scanning electron microscopy photographs and bed bug egg measurements indicated there were no morphological differences between different bed bug egg strains (susceptible and resistant). Bed bug egg respiration rates measured by the amount of oxygen consumed (standard metabolic rate; SMR) also indicated there was no difference in SMR between different bed bug egg strains. Water conservation during respiration is vital to terrestrial insects. Therefore, similar patterns would be expected between egg water loss and respiration rates. However, susceptible strain eggs lost more water than one resistant strain of bed bug eggs, which was dissimilar from the respiration results, indicating that bed bug egg water loss and respiration are not directly related. Dose- response bioassays using two insecticide formulations (Temprid; imidacloprid/β-cyfluthrin, and Transport; acetamiprid/bifenthrin) indicated that bed bug eggs collected from pyrethroid resistant adult bed bug strains are also highly resistant. RNA sequencing of bed bug eggs from two resistant strains indicated that egg resistance may be directly related to the overexpression of multiple genes associated with insecticide resistance.

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Dedication

To my family and friends

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Chapter 1. Introduction

The common bed bug, *Cimex lectularius* L., is a blood sucking ectoparasite of humans. Bed bugs must obtain multiple blood meals to reproduce and complete development. The bed bug life cycle consists of an egg stage and 5 nymphal stages before becoming an adult. Bed bugs must take a blood meal at each stage of the life cycle in order to molt into the next stage.

Bed bugs are currently not known to transmit disease. However, in 2010 the Environmental Protection Agency (EPA) and Centers for Disease Control (CDC) declared bed bugs to be public health pests because their bites sometimes cause allergic skin reactions, secondary infections due to the victim scratching and introducing bacteria into the bite site, or psychological distress in the victim (i.e. anxiety and sleeplessness). The recent resurgence of bed bugs across the United States has stimulated research on their biology, behavior and control methods.

Pest control companies in the United States have relied primarily on pyrethroid insecticide applications for bed bug treatments (Kaufman et al. 2006). The low cost and ease of applying pesticides compared to non-chemical methods continues to drive this current trend of bed bug control. The large majority of pesticides labeled for indoor use are pyrethroids, consequently, bed bugs have been frequently exposed to pyrethroid insecticides. The frequent exposure of bed bugs to pyrethroids has resulted in significant resistance to these active ingredients (Romero et al. 2007, Moore and Miller 2006, Yoon et al. 2008, Adelman et al. 2011). Different physiological mechanisms have been investigated as potential contributors to bed bug pyrethroid resistance. These mechanisms include target site mutations, enhanced enzyme detoxification, and reduced cuticular penetration.

Bed bug eggs present one of the greatest challenges in control because of the difficulty associated with locating and killing them. Bed bug eggs are difficult to locate because female bed bugs lay eggs singly instead of in groups, thus eggs may be scattered throughout the environment. Furthermore, the cryptic nature of bed bugs results in female bed bugs laying a few eggs in many different places that are usually hidden from obvious sight. Also complicating bed bug egg control is the fact that many insecticides that are effective in killing adult bed bugs do not have the same effectiveness in preventing bed bug egg hatch. Bed bug eggs from resistant strains are more difficult to kill than bed bug eggs from susceptible strains when treated with pyrethroid insecticides. For example, when eggs from a susceptible strain were treated with bifenthrin (0.05%), all eggs failed to hatch (Miller unpublished data). However, when eggs from a resistant strain were treated with the same formulation, 75% of eggs hatched successfully (Miller unpublished data).

Differences in mortality between bed bug eggs from susceptible and resistant strains could have two possible explanations; either the eggshell provides more protection to the embryo in resistant strains, or the developing embryo inside of the egg may have one or more resistance mechanisms. The eggshell may have modified morphological features in resistant strain eggs to limit insecticide exposure. For example, the eggshell in resistant strains could be thicker than that of susceptible eggs to reduce insecticide penetration, or the aeropyles (breathing structures) in bed bug eggs from resistant strains could be smaller or fewer in number. We originally hypothesized that if fewer or smaller aeropyles were present in resistant strain bed bug eggs, then we would expect there would be significant differences in respiration rates between susceptible and resistant eggs. However, our metabolic activity research indicated that respiratory differences were a function of bed bug strain differences and not insecticide resistance.

The purpose of this study was to quantify bed bug egg insecticide resistance and then determine physiological differences between susceptible and resistant bed bug eggs. Overall, there were four different objectives of this study: 1) to describe and quantify morphological features of the bed bug egg using scanning and transmission electron microscopy, 2) to determine and compare water loss and standard metabolic rates of susceptible and resistant bed bug eggs, 3) to determine the lethal concentration required to kill 50% of a bed bug eggs and first instars (LC_{50}) from three different bed bug strains, and 4) to compare transcript expression levels between susceptible and resistant bed bug eggs for potential mechanisms of resistance.

Chapter 2. Literature Review

Bed Bug Biology

The common bed bug, *Cimex lectularius* L., is thought to have evolved from the ancestral bat bug that dwells within caves. As humans formed civilizations and moved out of caves, they carried bed bugs with them (Usinger 1966). Archeologists (Panagiotakopulu and Buckland 1999) reported that bed bug fossils have been found dated as being over 3,500 years old in the remains of an Egyptian workmen's village. In modern times, bed bugs have continued to be transported across the globe with humans and have resurged in the United States, London and Australia (Reinhardt and Siva-Jothy 2007).

Cimex lectularius belongs to the Order Hemiptera and Family Cimicidae. The life cycle consists of an egg stage and five nymphal stages (instars) before becoming an adult. Each nymph must consume a blood meal in order to molt into successive life stages. Although bed bugs are ectoparasites of humans, there are no reports indicating that they can vector human disease (Goddard 2009). However, bed bug bites can cause allergic reactions, primarily consisting of red, itchy, rash-like patches at the bite site. The frequency of a bed bug feeding varies, and is dependent upon environmental temperatures and availability of a host, but laboratory populations feed on average every 7 days (Usinger 1966). Not surprisingly, increases in bed bug size requires consumption of larger blood meals (Usinger 1966, Johnson 1941). Bed bugs primarily feed while humans are asleep and are attracted to the steady supply of CO_2 humans provide while sleeping. Bed bugs are a cryptic species and after feeding return to their harborages in cracks and crevices.

Bed bugs mate by traumatic insemination, a process in which the male pierces the female's abdomen with his aedeagus and ejaculates sperm into a specialized female organ, the organ of Berlese. The sperm then migrates through the female's paragenital tract for egg

fertilization. As a result, male bed bugs never use the genital tract for insemination although females possess a reproductive tract that is similar to other insects (Reinhardt and Siva-Jothy 2007). Males focus on recently fed females for copulation, although it is unclear on how males locate these females (Reinhardt and Siva-Jothy 2007). A female bed bug is inseminated approximately 5 times after she consumes a blood meal (Stutt and Siva-Jothy 2001). However, repeated inseminations have been found to decrease the life span and number of eggs a female lays during her lifetime (Polanco et al. 2011).

Bed Bug Control Methods

Methods for controlling bed bugs in the mid-1800s and early 1900s included using mercury chloride, pyrethrum, gasoline, kerosene, benzene, alcohol, sulfur and hydrogen cyanide (Potter 2011). Many of these chemicals had to be directly applied on the bed bugs to kill them and were extremely dangerous to the applicator.

Dichloro-diphenyl trichloroethane (DDT) was first used for bed bug control in 1942 (Potter 2008). DDT was highly effective and bed bugs were essentially eliminated in the United States during the 1940s-50s due to the widespread use of DDT. However, within a decade bed bugs became resistant to DDT (Usinger 1966). Malathion was the insecticide that replaced DDT for control of resistant bed bugs. Unlike DDT, that targeted voltage gated sodium channels, malathion has a different mode of action and was effective for bed bug control when first introduced.

Malathion is an organophosphate insecticide that inhibits acetylcholine-esterase (Ware and Whitaker, 1989). The accumulation of acetylcholine at the neuromuscular junctions causes involuntary twitching of the insect and eventually paralysis. Malathion began to be used in the

1950s for bed bug control, but within a decade bed bugs also became resistant to malathion (Feroz 1971).

In modern times, not only are insecticides used for bed bug control, but other various non-chemical methods have also been implemented. Non-chemical control methods include various desiccant dusts (e.g. diatomaceous earth and silica dust), laundering, spot cold treatments (Cryonite), and heat treatments (whole home or containerized heat). Heat treatments could arguably be the most effective non-chemical control method. If heat treatments are done properly, bed bug infestations can be eliminated. Watanabe (2010) reported that 100% mortality of bed bugs could be achieved with heat application and by steam with temperatures > 40 °C. Pereira et al. (2009) demonstrated that elimination of bed bugs can be achieved using heat chambers constructed of polystyrene sheathing boards, oil filled electric heaters and box fans. 100% mortality of bed bugs was achieved inside of the chambers when the bed bugs were exposed to temperatures at or above 41°C at times ranging from 2-7h (Pereira et al. 2009). Bed bug mortality was not achieved if the bed bugs were placed into areas where the heat could not penetrate harborage sites (i.e. deep inside couches).

Desiccant dusts (i.e. diatomaceous earth and silica gels) have been used for decades as effective insecticides (Ebeling 1971). Wang et al. (2009) reported that applying diatomaceous earth in combination with steam treatments caused over a 97% reduction in bed bug populations after ten weeks. Benoit et al. (2009) determined that combining desiccant dusts with pheromones increased bed bug mortality, by causing an increase in bed bug movement and therefore increasing the amount of dust they walked in and accumulated on their bodies. Diatomaceous earth and silica dust work essentially the same way, by absorbing cuticular lipids and causing the insects to dehydrate (Appel et al. 1999).

Pyrethroid insecticides are synthetically derived from naturally occurring pyrethrum, which is produced from the flowers of chrysanthemum plants. Pyrethroids have a similar mode of action to DDT, causing ion leakages inside insect nerve cells (Ware and Whitaker, 1989). Currently, pyrethroid insecticides are the most commonly used insecticide for bed bug treatment in the United States. Almost all products labeled for bed bug control contain pyrethrins or pyrethroid active ingredients (Moore and Miller 2006). Because of their widespread use, bed bugs have become highly resistant to pyrethroid insecticides (Romero et al. 2007, Moore and Miller 2006).

Although pyrethroid insecticides are relatively inexpensive, bed bug treatments are extremely expensive due to the amount of labor required. The cost of a treatment for one bedroom is estimated at $400 US dollars (Harlan 2007). This treatment includes three hours of inspection, customer education, and one insecticide application by a pest control operator, all in a single visit (Harlan 2007). As is the case with almost all bed bug treatments, multiple visits are required for effective control. Therefore, costs associated with bed bug control can quickly escalate. Insecticide resistance is one of the main contributors to the difficulties and costs associated with bed bug control.

Pyrethroid Resistance in Bed Bugs

The bed bug resurgence in the United States during the 1990s is primarily attributed to pyrethroid resistance. Their resistance to deltamethrin, λ-cyhalothrin, bifenthrin and permethrin has been well documented (Romero et al. 2007, Moore and Miller 2006). Each of these active ingredients belong to the same pyrethroid class, therefore cross resistance is an issue because of the absence of different modes of action.

Three physiological mechanisms of resistance have been identified in pyrethroid resistant bed bug populations; these are target site mutations (*kdr* resistance), enhanced detoxification enzyme activity, and reduced cuticular penetration resistance. With regard to *kdr* resistance, two point mutations in the α-subunit gene in the voltage sensitive sodium channel were found in a New York bed bug population resistant to deltamethrin (Yoon et al. 2008). Frequencies of both of these mutations in another bed bug population were highly proportional to insecticide resistance, suggesting that these mutations are directly related to pyrethroid resistance (Seong et al. 2010). Adelman et al. (2011) discovered that certain genes were over expressed in a bed bug pyrethroid resistant strain collected from Richmond, VA, suggesting an increase in metabolic resistance. Koganemaru et al. (2013) found that genes associated with cuticle proteins were also highly expressed in the same Richond, VA strain of bed bugs. These studies established that bed bugs have various genetic mechanisms to enhance resistance to insecticides.

Although not associated with insecticide resistance, pyrethroid repellency can decrease the efficacy of insecticides because the insect is actively avoiding surfaces that are treated with insecticides. Repellency has been documented in many different pests, e.g. cockroaches, ants and termites (Ebeling et al. 1966, Knight and Rust 1990, Su and Scheffran 1990). Moore and Miller (2006) found that bed bugs are not repelled by pyrethroid insecticide formulations and that these pesticides do not cause bed bug movement into untreated harborages. However, Romero et al. (2009) found that bed bugs did not avoid filter papers treated with deltamethrin, but were unable to rest on the filter papers because they were too agitated by the resulting nerve excitation. Deltamethrin treated haborages containing feces and eggs did attract bed bugs (Romero et al. 2009). Bed bug behavioral responses to insecticides were influenced by a variety of factors, e.g.,

the amount of insecticide applied, insecticide susceptibility within a population, and stimuli within the environment (Romero et al. 2009).

Insect Egg Biology and Physiology

The number of insect eggs produced by a particular species may be influenced by environmental factors, including habitat climatic conditions and predator pressure. While adult insects have adapted to local conditions in ways that maximize their fecundity, the embryos themselves also have adapted mechanisms to enhance their survival. One of these mechanisms is the enclosure of the embryo within an egg shell.

The insect eggshell, also called the chorion, protects the embryo and prevents it from desiccation. The chorion consists of an outer chorion layer, an inner chorionic layer and a vitelline membrane (Wolf and Reid 2001, Wolf et al. 2002). The vitelline membrane is the thin, innermost layer of the chorion (Wolf et al., 2002). An inner waxy layer prevents water loss and is located between the inner chorionic layer and the vitelline membrane.

In the family Pentatomidae (stink bugs), the chorion is characterized by the surface structure, termed either "spinose" or "coarse" (Wolf et al. 2002). "Spinose" refers to insect eggs that have spike-like projections in patterns jutting from the surface. The term "coarse" refers to the eggshell having reticulated pit-like structures on the outer surface (Wolf et al. 2002).

The outer surfaces of the chorion in true bugs are often irregularly shaped, with external holes extended into a plastron network inside of the chorion. This network is part of the embryonic respiratory system that provides the embryo with atmospheric oxygen. The exterior holes that penetrate through the chorion are called aeropyles. The aeropyles serve to connect the exterior of the chorion with the plastron network. Within the chorion, most insect eggs have an

inner-air filled space that is referred to as the pillar region that connects with aeropyles that allow for gas exchange. Aeropyles of many terrestrial insects are located on the end of respiratory horns. Hinton (1969) suggested that these horns allow for more efficient uptake of oxygen when the egg is surrounded by a layer of water when it rains (Hinton 1969).

Once fully developed, the embryo inside of the eggshell hatches through the operculum, or egg cap region. Many larvae have a specialized spine, or egg burster, to assist them in hatching from the operculum. The egg burster is usually located on the larvae head. Larvae that do not have specialized egg bursters hatch from the eggshell by internal pressures that literally push them through the eggshell operculum.

Bed Bug Egg Biology

When the female bed bug oviposits eggs, she secretes a cement-like substance that adheres the egg to the substrate on which the egg is laid. The female does not produce a single batch of eggs, but oviposits a small number of eggs every day for 10-12 days after taking a blood meal. A blood meal is required for a female bed bug to begin ovipositing eggs. The amount of eggs a female is able to oviposit is dependent upon the number of matings, nutrient level, and her level and type of insecticide resistance (Polanco et al. 2011). Approximately 3 days after feeding, a mated female will begin to oviposit eggs (Usinger 1966). If she has no access to an additional blood meal, oviposition will stop at about 11 days (Usinger 1966). A female bed bug will oviposit between 1 to 12 eggs per day (Krueger 2000). Polanco et al. (2011) found that female bed bugs, on average, lay between 132 and 156 eggs during their lifetime. The eggs are highly viable since approximately 98% of those laid will hatch into live nymphs.

Relative humidity has been found to have little affect on egg hatch (Johnson 1941) and bed bug eggs will hatch in approximately one week when held at room temperature (approx. 25 °C). Johnson (1941) found that bed bug eggs failed to hatch at high temperatures (37 °C). Eggs also failed to hatch at temperatures below 13 °C (Johnson 1941). How et al. (2010) found that tropical bed bug eggs, *Cimex hemipterus*, hatched at temperatures between 20 °C and 35 °C. However, *C. hemipterus* eggs failed to hatch at above 38° C (How and Lee 2010). Kells and Goblirsch (2011) determined that the thermal death point for *C. lectularius* nymphs and adults was 46.1° C. However, the thermal death point for *C. lectularius* eggs was 54 °C.

Morphological characteristics of bed bug eggs have not been thoroughly described. Measurements of the egg chorion, and the subsequent description of the chorion characteristics, have been based on observations made using light microscopy. Davis (1956) measured the width of the bed bug egg chorion and found that it was 15 µm wide at the neck region and 10 µm wide in the posterior region (Davis 1956). Usinger (1966) stated that there was an air-filled space in the anterior portion of the bed bug eggshell and that the aeropyles do not reach the rim margin of the operculum but are found elsewhere. Usinger (1966) reported that there was an average of 150 aeropyles present on the chorion. Micropyles are small exterior holes also found on the chorionic outer surface that allow sperm to enter the egg for fertilization. Usinger (1966) stated that bed bug eggs do not have micropyles present on the chorion, because they disappeared before the chorion was secreted (Usinger 1966).

Insect Egg Microscopy

Insect egg studies have been accomplished using scanning electron microscopy (SEM) and/or transmission electron microscopy (TEM). Both microscopic techniques allow for more

detailed observations of the morphological characteristics of insect eggs when compared with light microscopy. SEM is primarily used to study the outer surface of the chorion, while TEM is used to study the inner layers of the chorion. SEM and TEM have both been useful tools in increasing knowledge of insect eggshell morphology and physiology.

Scanning electron microscopy uses reflected secondary electrons to form an image which allows visualization of the morphological organization of insect egg exterior surfaces, and has been used to characterize insect eggshell morphological features at a high resolution (Wolf et al. 2002). Observations of these minute morphological features has provided taxonomists with the ability to distinguish between eggs of the same genus. For example, Suman et al. (2011) found distinct morphological differences between two different species of mosquito eggs (*Aedes aegypti* and *Aedes albopictus*). Using scanning electron microscopy, Suman et al. (2011) analyzed 33 morphological features of both egg species (*Aedes aegypti and Aedes albopictus*) and indicated that the eggs differed significantly by 48%.

Scanning electron microscopy has also been used to study the chorionic layers to gain an understanding of how the chorion was formed. Ma et al. (2002) studied tarnished plant bug eggs (Hemiptera: Miridae) and determined that follicle cells not only secreted the vitelline membrane, but also deposited the inner chorionic layer and the scaffolding to form the inner air layer. The follicle cells also secrete the operculum and respiratory horns. Baker and Ma (1994) studied and measured *Neurocolpus nubilus* eggs (Hemiptera:Miridae) and found that the chorion had four distinct layers using TEM. The outer layer was the most electron dense and 0.23 μm wide. The second layer was the least electron dense and comprised most of the chorion. The third layer housed struts that separate cavities within the layer and the fourth, or innermost layer, was not

electron dense and was uniformly thick. These layers are characteristic in many hemipteran insect eggs.

In 2002, Ma et al. used transmission electron microscopy (TEM) to study the chorion of tarnished plant bug eggs (Hemiptera: Miridae). Similar to Baker and Ma (1994), it was further found that the chorionic layers of tarnished plant bug eggs could be distinguished into different zones according to their electron densities. The darkness of a layer was found to be directly correlated to increasing electron density. Chorionic layers were differentiated by the intensity of darkness the layers appeared in the TEM imagery.

Insect Respiration

Like all terrestrial animals, insects require oxygen to fuel respiratory activities. Insects exchange gas with the atmosphere via a respiratory system that consists of spiracles, tracheae and tracheoles. The spiracles are located on the outside of the insect and the spiracular openings are located within the cuticle and are most often found on the thorax and abdomen in adult insects. The spiracles lead to tracheae, which are tubes inside the insect's body, and are connected to tracheoles. Tracheoles extend to metabolically active cells providing oxygen directly to tissues. The rate and volume of insect respiration can be measured using specialized equipment (respirometer). Basic components of newer respirometers include both an oxygen and carbon dioxide analyzer connected to a computer programmed to record and analyze the data.

Respirometers measure the rate at which an organism consumes oxygen and produces carbon dioxide. Two systems may be utilized to measure respiration rates of insects; closed system respirometry and flow-through respirometry. Closed system respirometry works by pumping a known concentration of gas into the respirometry system, then any fluctuations in gas

concentrations attributed to the organism are measured. Closed system respirometry is more effective for measuring small amounts of gases compared to flow-through respirometry. During flow-through respirometry, gas changes emitted by an organism in an airstream are measured by gas analyzers (Lighton 2008).

The three most common insect respiration patterns have been identified as: cyclic, continuous and discontinuous gas exchange (DGC). The cyclic respiration pattern is characterized by bursts of CO_2 release where the spiracles never completely close (Contreras 2009). In the continuous phase, the spiracles remain open and gas exchange is maintained at a constant level. The DGC pattern is characterized by three distinct phases: open, closed, and the flutter phase. During the open phase, spiracles are continuously open to allow the constant exchange of gases. During the closed phase, the spiracles are shut tightly preventing any gas exchange and subsequent water loss. When the spiracles are closed, CO_2 accumulates within the insect and the spiracles begin to open and close repeatedly, referred to as the flutter phase. Cyclic, continuous and DGC are all respiration patterns found in adult insects. However, the embryo chorionic system must allow for efficient gas exchange while simultaneously limiting water loss. Insect egg respiratory patterns have not been widely studied. As a result, determining the rate and pattern of bed bug embryonic respiration may provide information on the physiology, respiratory and water balance of bed bug eggs.

Typically, insect embryos respire through openings of the chorion called aeropyles, which allow for efficient gas exchange and reduce water loss during respiratory activities. Since insect eggs are small, they have a large surface area to volume ratio, further increasing their need for water conservation. Terrestrial insect eggs usually do not obtain water from their environment and must conserve the moisture they are provided at the time of oviposition.

Woods (2010) studied the trade-offs of water loss and gas exchange in *Manduca sexta* eggs and found that eggs underwent dynamic changes in the metabolic rates during their development. At first, the embryonic metabolic rate was relatively low, but then the rates of gas exchange increased as the embryo developed and neared hatching. Insect embryo respiration rates are also affected by fluctuations in temperature. Woods and Hill (2004) discovered that oxygen uptake by insect eggs is temperature dependent and that moth eggs, *Manduca sexta*, were oxygen limited when temperatures were increased to 32-37 °C.

Insect Egg Water Loss

Aquatic insect eggs primarily respire with a plastron. The plastron is a gas filled air layer below the outer chorion of the egg shell. Some terrestrial insect eggs that are oviposited in environments flooded with water may also have a plastron. The plastron acts as physical gill that allows eggs to respire under water (Hinton 1970). Terrestrial eggs typically do not have a plastron, but do have a gas layer directly under the outer surface of the chorion and connected to the aeropyles. Many terrestrial insect eggs have small numbers of aeropyles (Hinton 1970). A reduction in aeropyle number may be an evolutionary mechanism to limit water loss of terrestrial insect eggs.

Terrestrial insect eggs are provided all of the water necessary for survival and development at the time of oviposition. Therefore, the embryos must conserve this limited amount of water during their development. Water loss occurs across the chorion and is correlated to oxygen consumption requirements of the embryo. The more gas exchange that occurs, the more vulnerable the insect embryo is to water loss. Environmental factors that can exacerbate water loss include elevated environmental temperature and low relative humidity, as it may relate

to their embryonic development and the density or number of eggshell chorionic layers. As insect embryos develop into larvae, the metabolic rates and water loss rates increase (Woods 2010). The inner waxy layer of the insect egg is the primary layer that provides protection from water loss. Woods (2010) used organic solvents to extract the waxy layer from *Manduca sexta* eggs and was able to document a significant increase in water loss.

The respiratory and water conservation physiology of the common bed bug embryo have been largely neglected. Benoit et al. (2007) quantified water requirements of the entire life cycle of the bed bug, except for the egg stage. Interestingly, it was found that *C. lectularius* had a low net transpiration rate similar to desert adapted insects. Bed bugs can tolerate losing 1/3 of their body weight to dehydration (Benoit et. al 2007). Bed bugs that had lost 20-30% of their body weight to dehydration did not actively consume water provided to them but instead clustered together. This resistance to dehydration is enhanced by bed bug aggregation and quiescence behaviors.

Survival and water loss of the tropical bed bug, *Cimex hemipterus* F., was found to be significantly influenced by temperature and humidity (How and Lee 2010). Tropical bed bug egg hatch failure was correlated with increases in temperatures. Between 90-100% eggs hatched at temperatures between 20 and 35˚C, but hatching stopped at their thermal lethal limit at 38˚C. *C. hemipterus* egg incubation period also shortened as temperatures increased.

Insect Cuticular and Chorionic Permeability

The cuticle is the primary protector of an insect's internal structures from environmental stressors. Desiccation is one of the most significant stressors that insects must cope with to survive. Insects can enhance their desiccation resistance by (1) increasing body water content (2)

decreasing their water loss or (3) increasing their water loss tolerance (Gibbs et al., 1997). Adult insects and some immature stages of insects can regulate water loss using different respiratory patterns, (i.e. discontinuous gas exchange), or by physiological changes in cuticular permeability. Water lost through the insect cuticle is widely believed to be the primary method of water loss for insects. For example, a study evaluating the mechanisms of water loss in drywood termites, found that nearly 93.5% of water loss was attributed to cuticular water loss, and this water loss was found to be 20 times greater than respiratory water loss (Shelton and Appel, 2000).

Epicuticular lipids play a large role in preventing water loss through the cuticle. When the lipid layer was removed, the rate of water loss in German cockroaches increased greatly (Appel and Tanley, 1999). *Drosophila melanogaster,* the vinegar fly, has been found to rapidly enhance desiccation resistance by hardening of the cuticle and decreasing the rate of water lost. Fruit fly cuticular water loss rates decreased significantly when the insects were exposed to a desiccation pre-treatment, opposed to insects that were not exposed to a previous desiccation treatment (Bazinet et al., 2010).

As mentioned earlier, the insect chorion is the protective barrier between the embryo and environmental stressors. The chorion is relatively impermeable because of the waxy layer that encompasses the vitelline membrane. Interactions of the waxy layer with the crystalline chorionic layer have also been suggested to improve desiccation resistance (Margaritas 1985). In addition, the structure and length of lipids in the waxy layer have also been shown to improve desiccation resistance (Gibbs 1998). Flies (*Drosophila melanogaster)* that had been selected for desiccation resistance had longer cuticular lipids (Gibbs 1998).

In Eastern Subterranean termite alates, cuticular permeability values were greater for male alates compared to female alates (Shelton and Appel, 2001). Female alates were larger in

mass compared to males but did not have significantly greater lipid content (Shelton and Appel 2001). Females are larger in size and body mass and therefore have a larger surface to volume ratio, which could enhance desiccation resistance.

Insect Ovicides

Insect eggs, like other insect stages, may vary in their susceptibility to insecticides, and this insecticide susceptibility changes during embryonic development (Smith and Salkeld 1966). Differences in susceptibility between insect egg species may be due to different adaptations in the chorion for facilitating the uptake of oxygen (Smith and Salkeld 1966). In addition to respiratory structures, insecticides may also enter the chorion through micropyles (Beament 1952).

Few studies have focused on insecticide resistance of eggs. Toloza et al. (2008) studied resistance patterns in Reduviid, *Triatoma infestans,* eggs. They found that insecticide resistance varied between eggs from different populations of *T. infestans* that were collected throughout Argentina and Bolivia. The eggs were selected later in embryonic development and treated topically with four different insecticides. Eggs from a resistant strain that were several days developed were found to be as resistant to deltamethrin as the first instars (Toloza et al. 2008). Also, eggs from the resistant strain were found to be resistant to lambda-cyhalothrin but susceptible to fipronil and fenitrothion. This pattern of resistance was also found in the resistant strain first instars.

Head lice, *Pediculus humanus capitis* (Phthiraptera: Pediculidae), have been shown to be highly resistant to pyrethroid insecticides. Cueto et al. (2008) studied eggs, nymphs and adults from three different resistant head louse populations. Eggs were found to be highly resistant to

permethrin in populations that had already demonstrated a high resistance to pyrethroid insecticides in adults and nymphs. Their study suggested that there were similar resistance mechanisms within head louse eggs and adults from the same population.

Summary

Insecticide resistance is the primary reason for the recent worldwide bed bug resurgence. Improving our knowledge of bed bug egg biology is crucial to develop treatments that are effective and to decrease control costs. Bed bug eggs create significant control challenges because they are small in size, difficult to locate and difficult to kill with our current insecticides. Because bed bug eggs are a source of reinfestation after treatment for nymphs and adults, they need to be a primary focus when developing new bed bug control strategies and technologies.

Bed bug eggs from resistant strains are more difficult to kill than eggs from susceptible strains (Miller unpublished data), and this resistance could be attributed to different physiological characteristics of the chorion, or the embryo itself. Alterations of micropyle and aeropyle size and number may be an enhanced physiological mechanism that results in reduced insecticide penetration. Also, insect eggs have been demonstrated to decrease the chorion permeability to reduce water loss. Decreasing chorionic permeability could be a mechanism that enhances insecticide resistance. The goal of this research was to determine bed bug egg and first instar insecticide resistance and to identify any physiological modifications that may enhance resistance.

Chapter 3. Morphological Description of Bed Bug Eggs (Hemiptera: Cimicidae) Using Scanning Electron Microscopy

Introduction

In the simplest form, insect eggshells are typically comprised of three layers (exochorion, endochorion [inner and outer] and vitelline membrane). The vitelline membrane is the innermost layer that surrounds the embryo. A few insect studies have further subdivided the eggshell layers into waxy layers and crystal chorionic layers, which most probably serve as the main functions of the eggshell against water loss. In addition to the eggshell layers, there are structures present on the eggshell for respiration (aeropyles) and fertilization (micropyles) and also inner eggshell structures for the movement of oxygen (pillars, sometimes also referred to as struts or columns). However, these structures and layers differ between different egg groups, families and species of insects dependent on their habitat and individual respiratory and water requirements.

Generally, insect eggshells are composed of a meshwork air filled layer and tiny pores (aeropyles) in the chorion that form the eggshell respiratory structure and allow the exchange of atmospheric gases (Hinton, 1970). Respiratory structures have been investigated in a number of hemipteran eggs using scanning electron and transmission electron microscopy. In the subfamily Harpactorinae, studied by Haridass (1986), the aeropyles are located on the collar (outer rim of anterior portion of eggshell) with pore canals in the operculum ("cap", where the embryo emerges from the eggshell). Similarly, in Triatominae eggs, the aeropyles are located in the rim (outer portion of "collar") and are covered by the operculum (Aldana et al. 2011, Haridass 1985). However, one exception is found in *Triatoma protracta*, because there is no defined rim on the eggshell and the aeropyles are found on the operculum border (Villalobos et. al. 2012).

Respiratory structures are modified in some hemipteran insects based on the habitat in which they are deposited. For example, Reduviidae eggs have an extended appendage on the operculum, termed the veil, where the aeropyles are found (Pikart et. al. 2012; Wolf and Reid, 2001). Furthermore, many insect eggs laid on plants have respiratory structures formed into horns, termed "respiratory horns". Respiratory horns are long extensions on the eggshell, thought to allow for oxygen exchange when the egg is flooded during periods of rain (Hinton, 1970, 1981). The majority of *Lygus lineloaris* eggs are embedded into plant material, leaving only the operculum and respiratory horns exposed. The aeropyles are located on the end of respiratory horns in *L. lineloaris* eggs (Ma et al., 2002). Conversely, *Neurocolpus nubilus* eggs are not embedded in plant material so the respiratory horns are absent (Baker and Ma, 1994).

Within the eggshell, various column-like structures appear to allow gas exchange through the eggshell layers to the embryo. *Lygus lineloaris* eggs, (Hemiptera: Miridae), contain an air layer within the chorion comprised of several columns, and these columns are referred to as "collanades" (Ma et al., 2002). The egg of *Neurocolpus nubilus*, (Hemiptera: Miridae) also contains an air layer with columns, referred to as "struts", located in the chorion (Baker and Ma, 1994). Chiappini and Reguzzi (1997) investigated four Nabis species (Hemiptera:Nabidae) (1997), and found that the chorion contained internal channels comprising the aeropyles, and the aeropyles started on the egg rim and were continuous with the pillar layer (located in the inner collar/rim).

Investigating bed bug egg structure is essential for bed bug control because eggs are a unique stage that has proven difficult to kill with conventional bed bug treatments. Eggshells are a protein structure comprised of a waxy layer that acts as a barrier restricting penetration of liquid formulated insecticides. Furthermore, there are few areas within the eggshell that

insecticides can fully penetrate. Most likely the openings on the outer eggshell, the aeropyles and micropyles, are potential entry points for ovicides and other chemicals to enter the eggshell (Smith and Salkeld, 1966). However, our study and previous studies have yet to identify any micropylar structures in bed bug eggs, further limiting areas insecticides can penetrate the bed bug eggshell. Although bed bug eggs are difficult to control, few studies have investigated bed bug egg structures.

Four studies have described bed bug eggs: Cobben (1968), Hinton (1981), Southwood (1956) and Baker et al. (2013). The earliest studies: [i.e. Cobben (1968) and Southwood (1956)] provide some detailed descriptions of bed bug eggs, however neither publication provided photographs of the descriptions. Likewise, Hinton (1981) published several scanning electron micrographs of various Cimicid species eggs but did not provide detailed descriptions. To date, Baker et al. (2013) provided the most detailed description on bed bug egg morphology including scanning and transmission micrographs. In this study, we expand upon what is currently known about bed bug egg morphology by providing a description of the bed bug egg respiratory structures. Specifically, we have examined bed bug eggshell structures and provide bed bug egg size comparisons from three different strains using scanning electron microscopy.

Materials and Methods

Bed bug eggs were collected from three bed bug strains (Harlan, Richmond and Royal Oaks). All bed bug strains had been maintained at the Dodson Urban Pest Management Laboratory (DUPML), located on the Virginia Tech Campus in Blacksburg, VA. The Harlan strain (pyrethroid susceptible) had been maintained at the DUPML since 2005. This strain was originally received from Dr. Harold Harlan (National Pest Management Association, Fairfax, VA) in 2005, with additional bed bugs sent to the DUMPL in 2011. Dr. Harlan collected this

strain from an army base in Fort Dix, NJ in 1978 and maintained them by feeding them on himself. The Royal Oaks strain (pyrethroid resistant) was collected in Royal Oaks, Michigan in 2006. The Richmond strain (pyrethroid resistant) was collected in an elderly group home in Richmond, VA in 2008.

All bed bug strains were maintained in an environmental chamber at 27 °C, 60% RH and a 12:12 light/dark photoperiod. Each strain was housed in plastic jars containing pieces of cardboard to provide harborage. The plastic jars were enclosed with mesh at one end to allow females to feed once weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) through the mesh on an artificial feeding system.

Following feeding, female bed bugs were exposed to males and allowed to mate for two days. After two days, five groups of ten mated females were placed into Petri dishes (Fisher Scientific Inc., 6 X 5 cm) and provided clean pieces of filter paper (Whatman # 1, 4.2 cm diameter) as an ovipositional substrate. The females were allowed to lay eggs for 2-3 days. Eggs were removed from the filter paper using soft tip forceps and placed into silicone capsules for SEM analysis or placed into Petri dishes for light microscopy studies.

For the SEM analyses, eggs were washed in sodium cacodylate buffer (0.1 M) for 15 minutes. The eggs were then fixed with osmium tetroxide (OsO_4; 1%) in sodium cacodylate buffer (0.1 M) and dehydrated in a series of ethanol solutions (15%, 30%, 50%, 70%, 95% and 100%). Bed bug eggs were then dried using a critical point dryer (LADD Research, Williston, VT) at 12°C for 30 min. to remove excess liquid. Following drying, bed bug eggs were attached to an aluminum stub with double-sided tape. The eggs were then sputter coated (Sputter Coater, SPI Supplies Inc., model # 11430) with a gold layer to make the eggs more conductive for the SEM to reduce charging effects of electron build up. All eggs were subsequently examined using

a Zeiss EVO®40 scanning electron microscope (Carl Zeiss AG; Jena, Germany). This microscope has the ability to magnify objects from 7 to 1,000,000 times.

For the light microscopy analysis, eggs (n = 5-7) were removed from Petri dishes and dipped into either food coloring alone or food coloring combined with neem oil for 20 seconds. Light microscopy images were taken using a Canon PowerShot camera (ELPH 130 IS; Canon images city, state) that was held to the eyepiece of a light microscope.

SEM images were used to measure the size (length and width) of 15 eggs from each strain. These images were also used for making detailed chorionic structure observations. Width measurements were taken at the widest portion of each egg and at the narrow collar region. Mean egg size (length and width ± SE) were compared between all three strains using analysis of variance, ANOVA (JMP® Pro 10.0 software; SAS institute 2012). Mean separation tests of length and width measurements were conducted with Tukey's HSD tests. *P* values of 0.05 were used to indicate significance.

Results

In describing bed bug egg morphological features, we used terminology previously published by Baker et al. (2013), Hinton (1981) and Margaritas (1985). Bed bug eggs are cylinder shaped and slightly ovoid at the operculum (Figure 3.1: 1A and 1B). The outer eggshell is comprised of many spike-like projections forming polygonal patterns along the length of the eggshell (Figure 3.1: 1D). Bed bug eggs are affixed to substrates with a cement-like substance that the female secretes during oviposition. This cement substance causes debris to adhere to the outer surface of the eggshell (Figure 3.1: 1A, 1B, and 1D).

First instar nymphs emerge from the egg through the operculum. The dorsal surface of the operculum is also composed of many polygon structures, however unlike the spike projections on the eggshell, these polygons are connected and uniform in shape, giving the surface of the operculum a honeycomb appearance (Figure 3.1: 1C and 3A). The interior layer surrounding the embryo (embryonic cuticle) was observed during emergence (Figure 3.1: 1A and 1B). The embryonic cuticle detaches from the first instar following emergence and remains attached to the eggshell (Figure 3.2: 2A).

As previously described by Baker et al. (2013), we also observed a collar structure on the bed bug eggshell that the operculum fits securely within. The outer region of the collar is referred to as the rim (Figure 3.1: 1D; and Figure 3.3: 3B and 3C). The collar is composed of multiple layers (exochorion, pillar region and endochorion) that comprise the columnar region (Figure 3.2: 2A, 2B, and 2C). Note that Baker et. al. (2013) called the layers the apical rim and separated the pillar region into both the palisade and ribbed layers.

We observed multiple small holes on the edge of the dorsal side of the operculum (Figure 3E). Similar small holes were also present on the ventral side of the operculum (Figure 3.3: 3B, 3C, and 3D). On the ventral side of the operculum we observed column structures (operculum column region) that were similar to the pillar region of the eggshell (Figure 3.3: 3B, 3C; and 3D).

Eggs that were dipped into a water-based dye (food coloring) for approximately 20 seconds became colored near the anterior portion of the egg but the color did not disseminate through the entire eggshell (Figure 3.4). The dye penetration through the columnar region of the eggshell from the operculum indicates that the egg cap is the site of gas exchange, as well as the site where potential contaminants can enter the eggshell. When we added an oil-based insecticide to the food coloring (Cirkil™), and dipped eggs for the same amount of time, the dye and

insecticide penetrated the entire eggshell. Interestingly, the dye did not penetrate the embryonic cuticle or the embryo, indicating that the eggshell, particulary the embryonic cuticle, may play some role in embryonic protection (Figure 3.5: A and B).

We observed no differences in the morphological features of bed bug eggs between strains. Structurally, all bed bug eggs were similar, with the same layers within the rim and projections on the eggshell and operculum. There were no significant differences between either the length of the bed bug eggs or the diameter of the eggs in relation to the posterior and neck region (Table 1). Bed bug eggs from all three strains ranged from 0.95- 0.98 (± 0.02) mm in mean length. At the widest posterior region of eggs from all three strains, eggs ranged between 0.41-0.43 (±0.04) mm in width and at the narrow neck region the eggs ranged from 0.27-0.28 (±0.00) mm in width.

Discussion

Bed bug eggs are approximately 1 mm in size (Table 1). Although the bed bug eggs we studied were from strains collected from different geographical locations, there were no differences between the size and morphological features we measured. All three strains differ in their susceptibility to insecticides but insecticide resistance didn't affect egg size or chorion structure.

Baker et al. (2013) provides the most recent and detailed description of bed bug eggs. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) photographs detailed the overall appearance of bed bug eggs and the eggshell layers. However, they did not suggest any physiological functions of the morphological features they described. Earlier publications (Usinger, 1966; Southwood, 1956; Hinton, 1981) provided some description of bed

bug (and closely related hemipteran) egg morphological features, but photographs and methodologies were not included. Usinger (1966) stated that the anterior portion of the bed bug chorion had a distinct air filled layer and there were 150 aeropyles on the collar. However, Usinger (1966) did not describe where the collar was located or provide any photographs of the structures described. Southwood (1956) briefly described Cimicomorpha eggs and suggested there were "canals" in the rim of the chorion, which Southwood termed micropyles and pseudomicropyles, but stated that it is unknown whether these structures were used for fertilization or gas exchange. While Hinton (1981) provided several photographs of *Cimex hemipterus* and *Cimex lectularius* eggs; Hinton did not provide any detailed descriptions of the morphological features or their potential functions.

Structurally, the outer chorion of the bed bug eggshell has multiple spines that form irregular polygon shapes. Similar chorionic spine structures, referred to as "spinose", have been well documented in the Pentatomidae (Hemiptera) family (Candan and Suludere, 2006; Kumar, 2002; Wolf and Reid 2001, Bundy and Mcpherson, 2000; Javahery, 1994; Lambden and Lu, 1984). Hinton (1981) provided scanning electron micrograph photographs for different Cimicidae species eggs. Spines can be observed on the eggshell of *Primicimex cavernis*, but appear to be absent on *Cimex hemipterus*. Instead, the *C. hemipterus* eggshell looks similar to the continuous polygonal structuring we observed on the *C. lectularius* operculum. The photographs provided for both *C. hemipterus* and *Hesperocimex sonorensis* indicate that the operculum structure is very similar to eggs of *Cimex lectularius* (Hinton, 1981).

Bed bug eggs appear to have respiratory structures similar to those in the various Hemipteran eggs. Generally, eggs of the infraorder Cimicomorpha contain a continuous inner chorionic meshwork that contains pillar-like struts (Cobben, 1968). We observed similar

meshwork in bed bug eggs, with the pillar region containing many columns or "struts". Bed bug egg respiration appears to take place through the operculum and within the collar. Gas exchange may occur through the small openings present on the ventral and dorsal side of the bed bug operculum. The edge of the dorsal and ventral side of the operculum is very porous and could also function for egg respiration. The pillar region occurs within the rim (collar). Many columns are present within the pillar region. Similar column structures have been observed in many Hemipteran eggs (Haridass, 1985, 1986; Cobben, 1968; Chiappinni and Reguzzi, 1997) and have been suggested as structures that function as respiratory structures. In the bed bug egg, the observed columns probably function as a passage for oxygen through the eggshell.

We observed respiratory structures similar to previously described Hemipteran eggs but were unable to find any structures that may function as a micropyle. Micropyles are the entry point for sperm to fertilize eggs (Hinton 1981). According to Hinton (1981), the micropyles are absent in the subfamily Cimicinae. Cobben (1968) believed that the micropyles were absent because bed bug eggs are fertilized before chorion formation.

Dipping bed bug eggs into food color confirmed that the operculum rim was the likely entry site of respiratory gas exchange between the chorionic interior and outer atmosphere. Specialized structures (aeropyles or micropyles) are entry points for ovicides and other chemicals to enter the eggshell (Smith and Salkeld, 1966). It is obvious from the fact that the food coloring (mixed only with water only) diffused partially through the anterior portion of the egg (Figure 3.4) that molecules enter the operculum and then diffuse through the eggshell posteriorly. However, when the food coloring was combined with an oil-based insecticide, we observed full penetration of the color throughout the eggshell. We believe this a result of the neem oils' interaction with the waxy components of the eggshell. Interestingly, the neem oil/food coloring

combination did not penetrate the embryonic cuticle (Figure 3.5: A and B). Thus, it would appear the embryonic cuticle provides the most protection for bed bug embryos from toxicants in the surrounding environment.

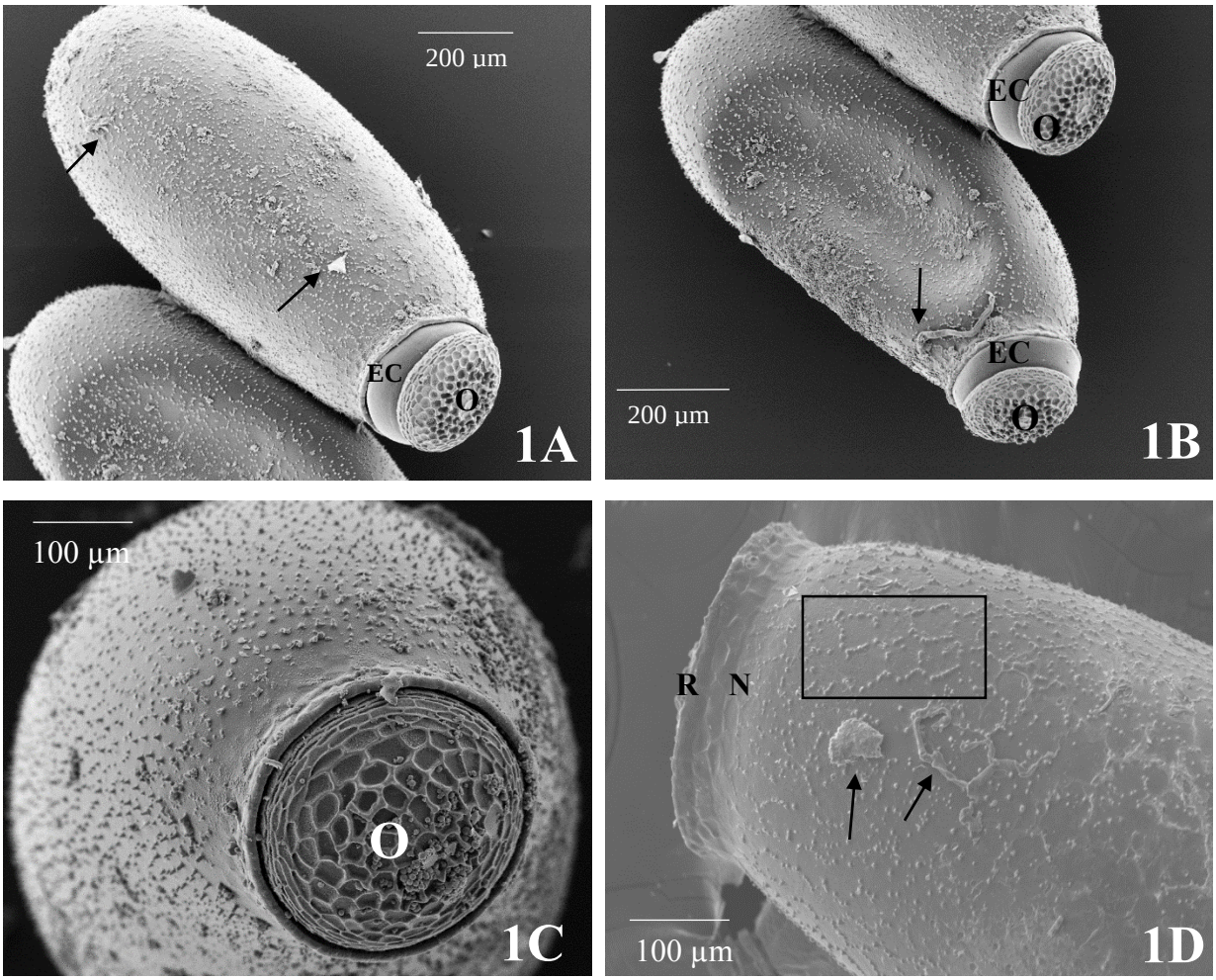

Figure 3.1: A-D. Scanning electron micrographs of Harlan susceptible strain bed bug eggs. Emergence of first instars occurs through the operculum (O) located on the anterior portion of the egg. Figures 1A and 1B: The embryonic cuticle (EC) covers the embryo during bed bug egg hatching. Figure 1C: anterior portion of the egg shows the polygonal structured operculum (O) and the spike-like projections that form the polygons on the outer eggshell. Figure 1D: A lateral view of spike projections that form polygons are enclosed within the box on the lateral side of the bed bug egg. (R) represents the rim region and (N) represents the neck region on the anterior portion of the egg. Arrows ( ) point to debris adhered to the exterior eggshell.

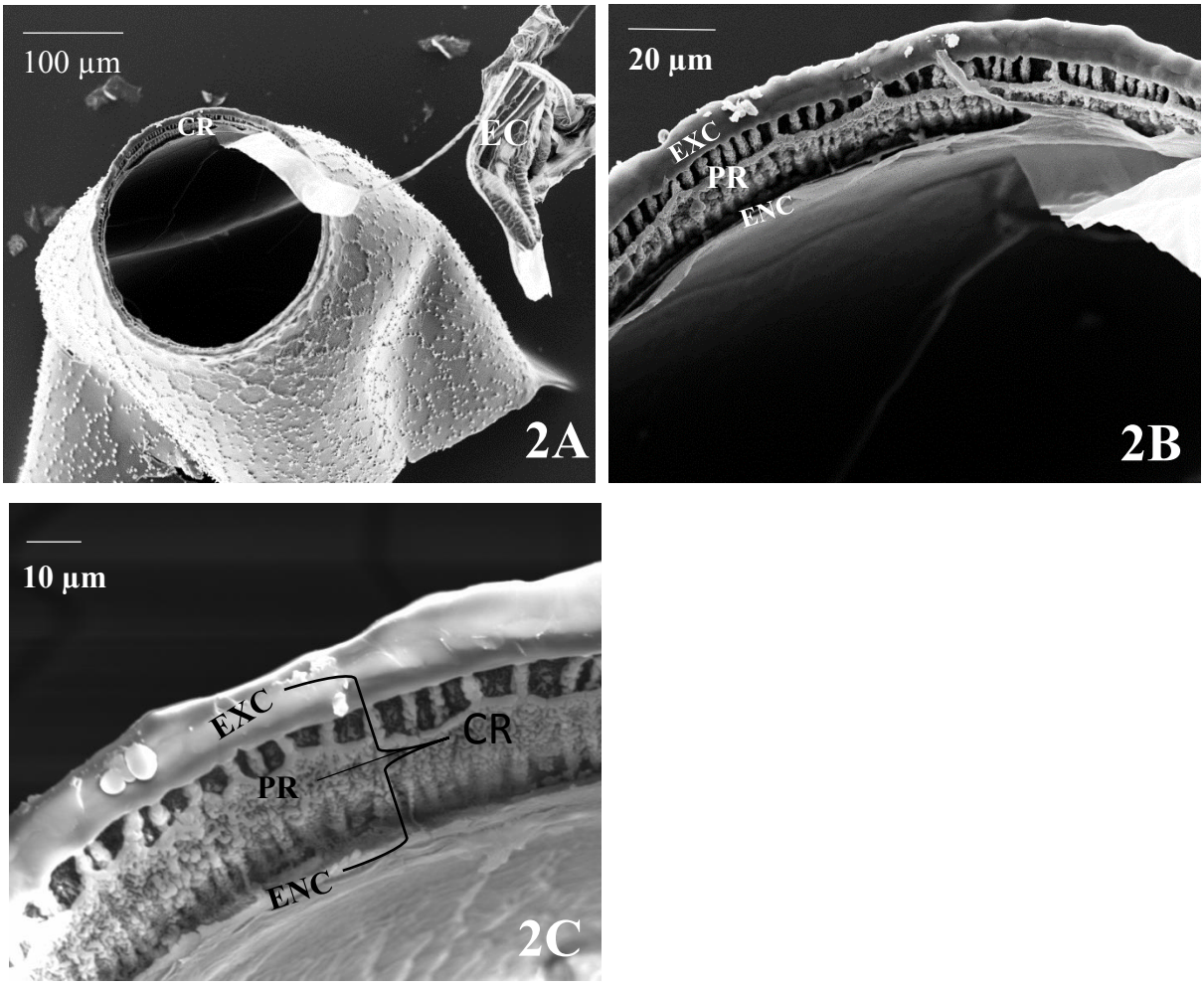

Figure 3.2: A-C. Scanning electron micrograph of hatched eggs with the operculum removed and the anterior layers exposed. Figure 2A: The embryonic cuticle (EC) is still attached to the egg after first instar emergence and the inner columnar region (CR) in the rim with layers exposed. Figure 2B: The different eggshell layers from exterior to interior: exochorion (EXC); pillar region (PR); and endochorion (ENC). Figure 2C: The eggshell layers magnified 2,700x to show the three distinct layers (EXC, PR and ENC) that comprise the columnar region (CR).

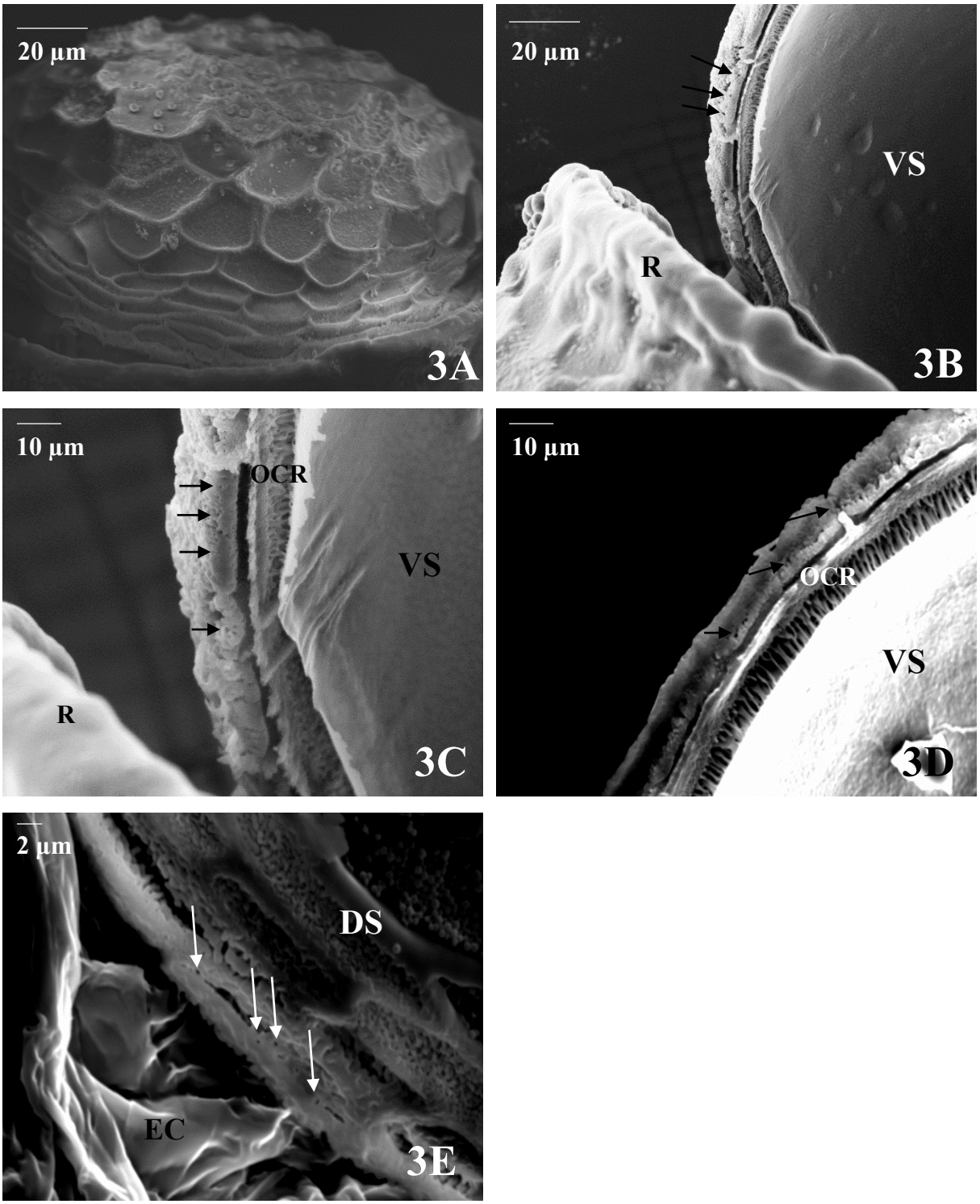

Figure 3.3: A-E. Scanning electron micrographs of the bed bug egg operculum. The arrows are pointing to the aeropyles on the ventral (VS) and dorsal side (DS) of the bed bug egg operculum. Figure 3A shows the dorsal side of the operculum. Figure 3B: The ventral side (VS) of the

operculum is exposed and the outer rim (R) above the neck of the eggshell is shown. Figures 3C & 3D: The ventral side (VS) of the operculum is exposed and shows the operculum columnar region (OCR). Figure 3E: The dorsal side (DS) of the operculum is shown with the embryonic cuticle (EC) after egg hatch.

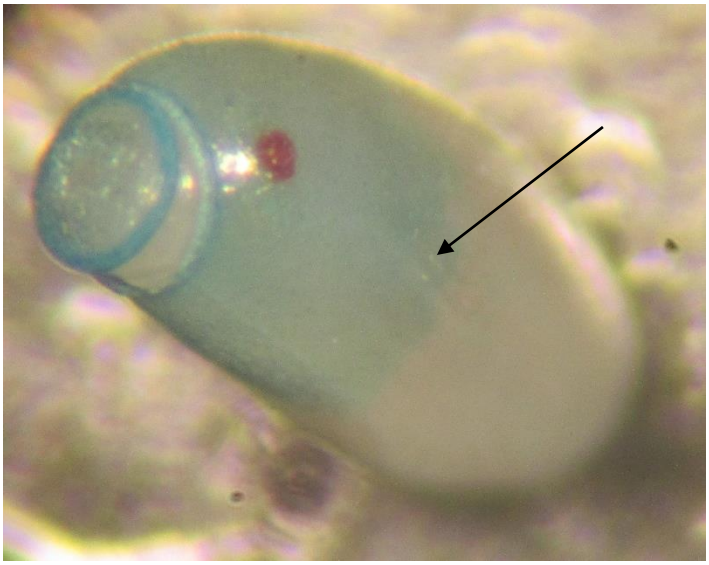

Figure 3.4 Demonstration of dye (food coloring) diffusion through the chorion of a Harlan strain bed bug egg after being dipped into the dye for 20 seconds. The operculum opened during the dipping process and the embryonic cuticle is exposed. The eye spot is also visible through the eggshell. The dye entered the anterior portion of the egg at the rim but did not disseminate throughout the entire chorion. The arrow indicates where the dye stopped moving through the eggshell.

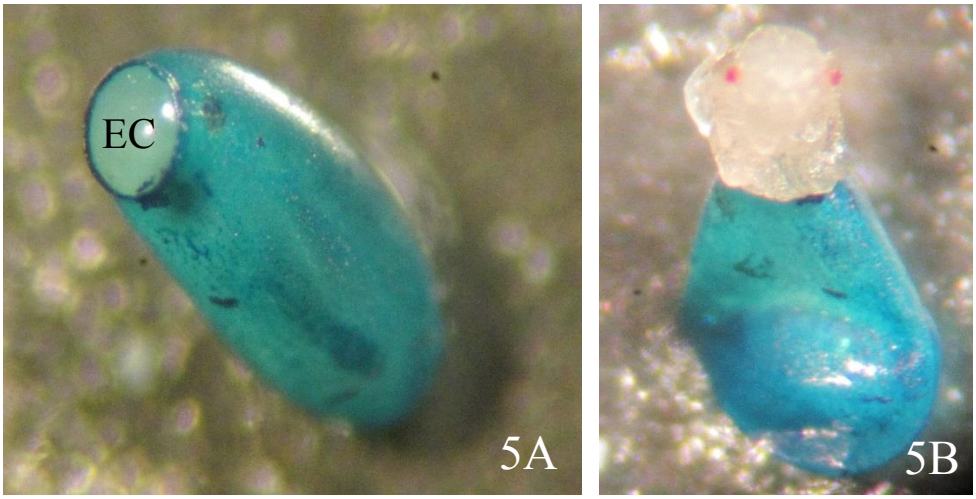

Figure 3.5: A and B. Full dissemination of blue dyed neem oil formulated insecticide (Cirkil; Terramera Inc., Vancouver, BC) through the entire eggshell of a bed bug egg. Figure 5A shows the bed bug eggshell with the cap removed and the embryonic cuticle (EC) is exposed. Figure 5B is the same egg with the first instar partially extracted. The dyed oil based insecticide did not penetrate the embryonic cuticle in figure 5A nor the embryo in figure 5B, however, the dyed insecticide disseminated throughout the entire eggshell after being dipped into the formulation for 20 seconds.

Table 3.1 Comparison of length and width measurements of three bed bug egg strains (Harlan susceptible, Richmond resistant and Royal Oaks resistant) obtained using SEM.

Strain	*n*	Length[1,2](mm)	Posterior width[1,3] (mm)	Neck width[1,4] (mm)
Harlan	15	0.98 ± 0.02 A	0.43 ± 0.04 A	0.28 ± 0.00 A
Richmond	15	0.95 ± 0.02 A	0.41 ± 0.00 A	0.27 ± 0.00 A
Royal Oaks	15	0.98 ± 0.02 A	0.44 ± 0.00 A	0.27 ± 0.00 A

[1]Mean ± SE. Mean values followed by the same letter are not significantly different (JMP 10.0; SAS; P = 0.05).

[2]Length measurements were based on the overall length of the egg, measuring from the operculum to the most posterior point of the egg.

[3]Posterior width measurements were obtained by measuring the width of the widest portion of the egg near the posterior end.

[4]Neck width refers to the region adjacent to the bed bug collar.

Chapter 4. Metabolic Activity and Water Loss in Bed Bug Eggs (*Cimex lectularius* L.)

Introduction

Most insect eggs do not obtain water from their surrounding environment, instead they contain all of the water required for development at the time of oviposition (Hinton 1981). Furthermore, insect eggs are small in size and consequently have high surface area to volume ratios (Hinton 1981). These characteristics make insect eggs particularly vulnerable to desiccation, complicating the balance of water conservation with respiration activity. The insect eggshell is comprised of a waxy layer and a crystalline structure designed to prevent water loss, but the different ways in which eggshell layers contribute to water conservation is not fully understood. Woods (2010) suggests that the thick outer chorion layer and the inner waxy and crystalline chorionic layers assist in gas flux and water loss resistance.

Several studies have evaluated the trade-off between water loss and respiratory activities in insect (*Manduca sexta*) eggs (Woods et al. 2005, Zrubrek and Woods 2006, Woods 2009). Woods (2009) found that *M. sexta* eggshell conductance increased as embryos developed in order to compensate for the higher metabolic demand. This increase in eggshell conductance resulted in increased water loss. Interestingly, *Manduca sexta* eggs lost more water when they were deprived of oxygen; however, when eggs were exposed to an excess of oxygen, there was no significant difference in metabolism or water loss (Zrubrek and Woods 2006). Zrubrek and Woods (2006) suggested that eggshell conductance could increase but was not capable of decreasing when the eggs experienced stressful oxygen conditions. Woods et al. (2005) investigated the role eggshell layers played in insect eggshell conductance and found that water loss increased significantly when the waxy layer of the eggshell was extracted using a combination of chloroform and methanol. This increased water loss suggested that the waxy

layer of the eggshell provided significant water loss reduction. Moreover, the crystalline chorionic layer beneath the waxy layer appeared to play a significant role in water-proofing capabilities of the eggshell (Woods et al. 2005). However, it is not fully understood how the eggshell modifies conductance when exposed to different oxygen levels. Woods (2009) suggested that when oxygen levels were low, that either the embryo signals a change in the eggshell or the eggshell layers themselves respond to hypoxic oxygen levels.

Standard metabolic rates can be measured by either oxygen consumption or carbon dioxide production in ectothermic animals when at rest. Metabolic rates (respiratory activity) have been determined for a number of insect species, although few studies have focused on the egg stage. Metabolic rates have been quantified for moth eggs (Woods and Hill 2004, Zrubek and Woods 2006), milkweed bug eggs, beetle eggs, grasshopper eggs, fly eggs (Richards 1964) and locust eggs (Kambule et al. 2011, Slama 2000). Most recently, Kambule et al. (2011) determined metabolic rates of diapause and non-diapause locust eggs (*Locustana pardalina*). Diapause eggs had low, stable metabolic rates, whereas non-diapause eggs had increased metabolic rates throughout their development (Kambule et al. 2011). Richards (1964) found that developmental stage and temperature influenced egg oxygen consumption rates in six different insect species. In general, all insect eggs that were studied consumed more oxygen as embryos developed (Richards 1964). Slama (2000) found that there was a 20-fold increase in oxygen consumed between freshly oviposited eggs compared to eggs nearing hatch in *Schistocerca gregaria*. Woods and Hill (2004) determined that oxygen availability affected the metabolic rates in moth eggs, thus influencing development time and subsequent survival. When eggs were exposed to higher temperatures, hyperoxic and hypoxic conditions greatly reduced survival.

Bed bug eggs are particularly vulnerable to desiccation because they are deposited within indoor human environments, which are typically characterized by warm temperatures and low relative humidity. Yet, the response of bed bug eggs to temperature fluctuations and humidity is unknown. The purpose of this study was to measure metabolic and water loss rates between bed bug eggs from susceptible and pyrethroid resistant strains, which allowed us to further investigate respiratory quotients (RQ) and chorionic permeability. For our study, standard metabolic rates were measured as the amount of oxygen consumed per egg mass over a given amount of time (ml^{-1} h^{-1} g^{-1}). Eggshell permeability values were also calculated to determine the amount of water lost through the eggshell.

Materials and Methods

Test Insects

Laboratory strain bed bugs were acquired from Dr. Harold Harlan (National Pest Management Association, Fairfax, VA) in February 2005. Dr. Harlan maintained this population since 1973 by feeding them on himself. In addition, three field strain populations (Richmond, Royal Oaks and Epic Center) of bed bugs were also evaluated. The Richmond field strain was collected from an elderly group home located in Richmond, VA in 2008. The Epic Center field strain was collected in 2008 in an apartment complex in Cincinnati, Ohio. The Royal Oaks (resistant) strain was collected from a field population in Royal Oaks, MI in 2006.

All bed bug strains were fed weekly with defibronated rabbit blood using an artificial feeding system (Hemostat, Dixon, CA). The bed bug strains were contained in plastic rearing jars enclosed with mesh at one end to allow for feeding through the mesh. Rearing jars contained pieces of cardboard to provide harborage and a substrate for the bed bugs to crawl up and feed

through the mesh. The plastic rearing jars containing all three bed bug strains were maintained in an environmental chamber at 27°C, 60% RH, and a 12:12 L:D photoperiod.

Resistance ratios were determined for each strain by calculating the LT_{50} values for adult bed bugs exposed to dried residues of deltamethrin (0.06%). Richmond, Epic Center and Royal Oaks strain bed bugs exposed to deltamethrin (0.06%) all had calculated resistance ratios greater than 400.

For all studies, recently fed females (30 groups of 10) were placed into Petri dishes (60x 15 mm, Fisher Scientific) and provided with a clean piece of filter paper (Whatman # 1) for oviposition. Captive females were provided with a clean piece of filter paper daily. Filter papers containing eggs that were 24 hours old were removed and allowed to age for 2 more days. Prior to the bioassay, eggs were gently removed from the filter papers using soft-tip forceps.

Bed Bug Egg Water Loss Over Time

Bed bug eggs from one susceptible (Harlan) and two resistant bed bug strains (Richmond and Royal Oaks) were used in all water loss assays. Groups of 5 eggs from each strain were weighed on a Cahn C-35 Microbalance (Thermo Fisher Scientific Inc., Waltham, MA) and then placed inside of an aluminum weigh boat. After the initial weight was recorded, groups of eggs were weighed again at 2, 4, 6, 8, 24, and 48 hours. Between each weighing period, aluminum weigh boats containing eggs were placed inside sealed plastic containers (Rubbermaid, Fairlawn, OH) and maintained in an environmental chamber at 25 °C. Containers were prepared for egg storage by adding magnesium perchlorate desiccant crystals (Thermo Fisher Scientific Inc., Waltham, MA) into the bottom of the containers in order to maintain a constant RH of 0% inside the containers. To minimize any container effects, each container held six egg replications, (two replications from each strain) in a randomized complete block design. Following the 48 hour

weighing period, each egg replication was dried in an Isotemp oven (Model 655F; Thermo Fisher Scientific Inc., Waltham, MA) to the point of desiccation. Dried eggs were re-weighed to determine biomass.

Similar to Appel et al. (1991), eggshell permeability values were quantified from water loss measurements taken between the first 2 and 4 hour period. Water loss measurements between 2 and 4 h of desiccation were used to determine eggshell permeability to avoid changes in eggshell shape that may occur rapidly in response to desiccation over time. Therefore, this early 2 hour period was selected as the best estimate of chorionic permeability. To calculate chorionic permeability (surface area x water loss x saturation deficit), we first had to determine the surface area of the egg. Since bed bug eggs are cylindrical in shape, the surface area of the egg was calculated using the formula determining the surface area of a cylinder ($2\pi r^2 + 2\pi rh$) where "r" was ½ the egg width and "h" was the egg lenth. A microscope fitted with a micrometer was used to measure mean egg length and width of 12 bed bug eggs from each bed bug strain. The saturation deficit (23.756 mmHg) was held constant because all experiments were conducted under the same humidity and temperature conditions.

Metabolic Quantification of Bed Bug Eggs

Groups of bed bug eggs (7-20) were placed into a syringe (3 ml; Becton, Dickinson and Company, Rutherford, NJ, USA). Two holes (1.4 mm diameter) were drilled into the syringe barrel at the interface of the plunger and barrel. Each syringe was then attached to a manifold (Figure 4.1) with each syringe plunger pulled up above the drilled holes (Figure 4.1) to allow dry, CO_2 free air to push through each syringe for 5 minutes to flush them of all ambient water vapor and CO_2. Following removal of water vapor and CO_2, each syringe was removed from the manifold and a needle (26 gauge intradermal bevel needle; Becton, Dickinson and Company,

Rutherford, NJ, USA) was attached to the syringe. Each syringe plunger was set so the volume inside the syringe barrel was 0.7 ml. Finally, the needle was inserted into a rubber stopper to prevent the exchange of atmospheric gases and water. The syringes containing bed bug eggs were then laid on a plastic tray and placed inside of an environmental chamber. Eggs inside syringes were incubated at one of 6 temperatures (10, 20, 25, 30, 35 and 39˚C) for different amounts of time (depending on the incubation temperature). The exact time of incubation was recorded, starting from the time the syringe was sealed. Each syringe was considered a replicate with a minimum of 7 eggs per syringe. A minimum of 10 replicates were used for each temperature with two control syringes. The control syringes contained no eggs but were subjected to the same procedures as the syringes containing eggs to adjust for leakage of any gasses within the experimental egg syringes.

Following incubation, a bed bug egg air sample (0.5 ml) from inside each syringe barrel was injected into the respirometry system for analysis. A Sable Systems TR-3 respirometry system (Sable Systems, Henderson, NV) was used to determine the amount of O_2 depletion and CO_2 production in each syringe. The system used atmospheric air in the room and forced it through a purge gas generator (Whatman Inc., Haverhill, MA) to remove CO_2 and H_2O. The atmospheric air is then equalized in large barrels to reduce the air pressure. After equalization, the atmospheric air was pulled through a Drierite-Ascarite-Drierite (Drierite-W. A. Hammond Drierite Co., LTD., Xenia, OH; Ascarite- Thomas Scientific, Swedesboro, NJ) column to further remove any traces of water or CO_2. Finally, atmospheric air was pulled through an injection port, where the syringe samples were also injected. Both the atmospheric air and the syringe sample were then drawn through a Li-6251 CO_2 analyzer (Li-COR Inc., Lincoln, NE) then Oxzilla II O_2 analyzer (Sable Systems, Henderson, NV). The Sable Systems oxygen analyzer compared the

clean atmospheric air to the sample taken from inside the syringe and quantified the differences in gas volumes between the syringe sample and atmospheric air. Specifically, the oxygen analyzer compared the amount of oxygen in the clean air sample to that of the syringe sample where oxygen depletion has occurred. A Sable Systems mass flow system MFS2 (Sable Systems, Henderson, NV) was used to maintain airflow flow through the system at the rate of 100 ml/min.

All syringe sample data was recorded using the Datacan V software (Sable Systems, Henderson, NV, USA). Data was analyzed by measuring the area below the peaks (representing gas volumes) to calculate O2 consumption and CO2 production within each syringe and gas volume data was then converted to ml/hr.

Note that two different studies were conducted that used oxygen consumption as the primary variable. First, oxygen consumption values were compared for Harlan susceptible strain eggs incubated at different temperatures. Secondly, oxygen consumption values were compared between all bed bug strains held at a constant temperature of 25°C.

Statistical Analysis

The amount of water loss measured over time was compared among all bed bug strains (Harlan, Richmond and Royal Oaks) using a Repeated Measures ANOVA (JMP SAS 9.0). Values of $\alpha \leq 0.05$ were used to indicate significance. First, we evaluated within strain water loss effects for each strain over time. Next, we analyzed water loss measurements between strains (Harlan, Richmond and Royal Oaks).

Metabolic rates were compared both between strains held at a constant temperature, and for a single strain held at multiple temperatures. Initially, both studies were analyzed using ANCOVA with mass as a covariate. However, mass was not found to be a significant factor in any of the tests, therefore ANCOVA was replaced with an ANOVA. Values of $P \leq 0.05$ were

used to indicate significance. When we compared the metabolic rates of eggs between strains held at a constant temperature, means were separated using Fisher's LSD. Respiratory rate for Harlan strain eggs at different temperatures were analyzed using linear regression analysis. An ANOVA was performed to determine the significance of the model ($P \leq 0.05$) and an R^2 value was calculated to determine how well the points fit the line. All statistical analyses were conducted using JMP® Pro 10.0.0 (SAS institute 2012).

Results

Water Loss Rates and Chorionic Permeability Values

All three strains of bed bug eggs lost water at similar rates over the 48 h test period (Harlan = 0.68 ± 0.1 μg/hr, Richmond = 0.96 ± 0.14 μg/hr, and Royal Oaks = 1.0 ± 0.15 μg/hr) (Figure 4.2). Although the Harlan strain had the lowest rate of water loss, the Repeated Measures ANOVA indicated that there were no significant differences in the amount of water lost at each time period (0, 2, 4, 6, 8, 24 and 48 hrs) between strains. However, chorionic permeability values (μg/hr/mm^2/mmHg) were significantly different between strains (F = 36.9, df = 2, 35; P = 0.0001), with values ranging from 64.5 ± 0.46 for the Harlan strain to 43.6 ± 0.45 for the Richmond strain. The mean separation test (Fisher's LSD) indicated that all three strains' chorionic permeability values were each significantly different from each other.

Temperature Effects on Respiratory Rates and RQ

The results of the ANOVA indicated that temperature had a significant impact on respiratory rate (F = 15.7, df = 4,68; P = 0.0001). The mean separation test (Fisher's LSD) showed there was a significant difference between Harlan bed bug egg respiratory rate at high temperatures (30 and 35°C), room temperature (25°C), and low temperatures (15 and 20°C) (Table 4.1). As expected, oxygen consumption increased as temperatures increased in Harlan

strain bed bug eggs (Figure 4.3). The linear regression model was significant ($P = 0.0004$) and a good fit ($F = 64$; $r^2 = 0.96$). Harlan bed bug egg respiratory quotient values (RQ = oxygen consumption/carbon dioxide production) ranged from 0.54 ± 0.06 to 0.67 ± 0.08 for eggs exposed to the 5 different temperatures.

Strain effects on respiratory rates

The rate of oxygen consumption of all three strains is shown in figure 4.4. The ANOVA indicated that the overall effect of strain on oxygen consumption was not significant at the 0.05 level ($P = 0.096$). However, the Fisher's LSD test suggested that the Harlan susceptible strain bed bug eggs (0.16 ± 0.01 O_2/hr/gr) consumed more oxygen than Epic Center resistant strain bed bug eggs (0.12 ± 0.02 $ml^{-1}\,hr^{-1}\,gr^{-1}$). There was no significant difference in oxygen consumption between Richmond strain bed bug eggs (0.15 ± 0.01 $ml^{-1}\,hr^{-1}\,gr^{-1}$) and the other two strains. RQ values for the three strains were (O_2 consumption/CO_2 production) were 0.69 ± 0.17 (Harlan), 0.58 ± 0.11 (Richmond) and 0.63 ± 0.15 (Epic Center).

Discussion

Bed bugs are capable of surviving several months without consuming a blood meal (Polanco et al. 2011) and are highly resistant to desiccation (Benoit et al. 2007). Benoit et al. (2007) compared water balance characteristics of all bed bug life stages with the exception of eggs. Benoit et al. (2007) found that first instar bed bug nymphs lost water more rapidly than all other bed bug stages. First instar bed bug net transpiration rates (water loss) were 0.402 ± 0.011%/h (Benoit et al. 2007). Water loss rates gradually decreased as young instars molted into subsequent stages, with adult males losing the least amount of water at 0.101 ± 0.0007%/h (Benoit et al. 2007).

Our study is the first to quantify water loss characteristics in bed bug eggs. We found no significant differences between resistant and susceptible bed bug eggs with regard to water loss rates. Royal Oaks eggs contained more water initially relative to the other strains, but lost water at rates similar to the two other bed bug strains over a 48 hour period. Interestingly, there were significant differences found between all three strains with regard to initial water loss across the chorion (within the first 4 hours). The Harlan strain lost significantly more water in relation to egg size and saturation deficit (chorionic permeability) (64.5 ± 0.46 $\mu g/hr/mm^2/mmHg$) during the first 2 hour desiccation period when compared to the two resistant strains (Richmond 43.6 ± 0.45 $\mu g/hr/mm^2/mmHg$, and Royal Oaks 57.7 ± 0.60 $\mu g/hr/mm^2/mmHg$).

In general, oxygen consumption increases with increasing temperatures, until the insect egg reaches a thermal limit (Slama 2000 and Richards 1964). In our study, Harlan bed bug eggs followed this trend, with their metabolic rates increasing with increased temperatures. However, Harlan egg metabolic rates decreased at 39°C (Table 4.1). The lethal temperature for bed bug eggs is 54.8°C (Kells and Goblirsch, 2011). We suspect that the observed low metabolic rate at the highest temperature tested (39°C) could have been a result of the embryo being stressed as temperatures approached the embryonic thermal lethal limit.

Metabolic rates can be used to determine respiratory quotient (RQ) values. Respiratory quotient values can be used to determine the substrate (protein, fat or carbohydrates) an embryo is oxidizing during development (Boell 1935). Bed bug eggs from all strains had similar RQ values (near 0.7) when measured at 25°C, suggesting that the bed bug embryo is utilizing lipids for embryogenesis. Bed bug egg RQ values were similar to desert locust embryo RQ values, which were 0.7 for all embryonic stages (Slama 2000). The RQ values we recorded for bed bug

eggs are higher than values previously reported for bed bug nymphs and adults (Devries et al. 2013). Devries et al. (2013) reported newly hatched nymphs of having RQ values of 0.53 ± 0.01. These different RQ values would suggest that newly hatched nymphs have different metabolic requirements after eggshell emergence than during embryonic development.

The metabolic differences we observed in bed bug eggs may be due only to strain differences and are not a function of insecticide resistance. Richmond resistant strain bed bug eggs exhibited similar metabolic rates to both Harlan susceptible and Epic Center resistant strain bed bug eggs (Table 4.2). However, Epic Center and Harlan strain bed bug egg metabolic rates were significantly different (Table 4.2). We had expected that resistant bed bug eggs would exhibit higher metabolic rates due to enhanced metabolic enzyme activity (Kramarz and Kafel 2003). Kramarz and Kafel (2003) found that beet armyworm pupae metabolic rates increased when they were continuously exposed to the toxin zinc. Kramerz and Kafel (2003) suggested that the observed increased respiration rates in beet armyworm pupae could be contributed to increased detoxification activity in individuals exposed to the metal zinc. However, Kramerz and Kafel (2003) only measured metabolic rates following beet armyworm pupae exposure to a toxin and their increased metabolic activity could result from pupae stress. Similar to our study, Dingha et al. (2009) found that metabolic rates of adult German cockroaches, *B. germanica*, were not significantly different between pyrethroid susceptible and resistant strains. The authors suggested that resistance reversion could have contributed to the absence of significant differences because the cockroaches were not continuously exposed to insecticides. Therefore, our bed bug eggs may also be experiencing resistance reversion due to the absence of constant insecticide exposure in the laboratory.

In conclusion, we found no difference in oxygen consumption between resistant and susceptible bed bug eggs. Future studies should evaluate bed bug egg respiratory behavior both during and after insecticide exposure. These respiration rates would be valuable to determine how bed bug eggs physiologically respond to insecticide exposure. Although we found significant differences in chorionic water loss between susceptible and resistant eggs in the early stages of desiccation; water loss rates were similar over a 48 h period between strains. Further studies would be necessary to determine if there are differences in water loss during embryo development and to determine if eggshell morphological features play a role in water loss prevention.

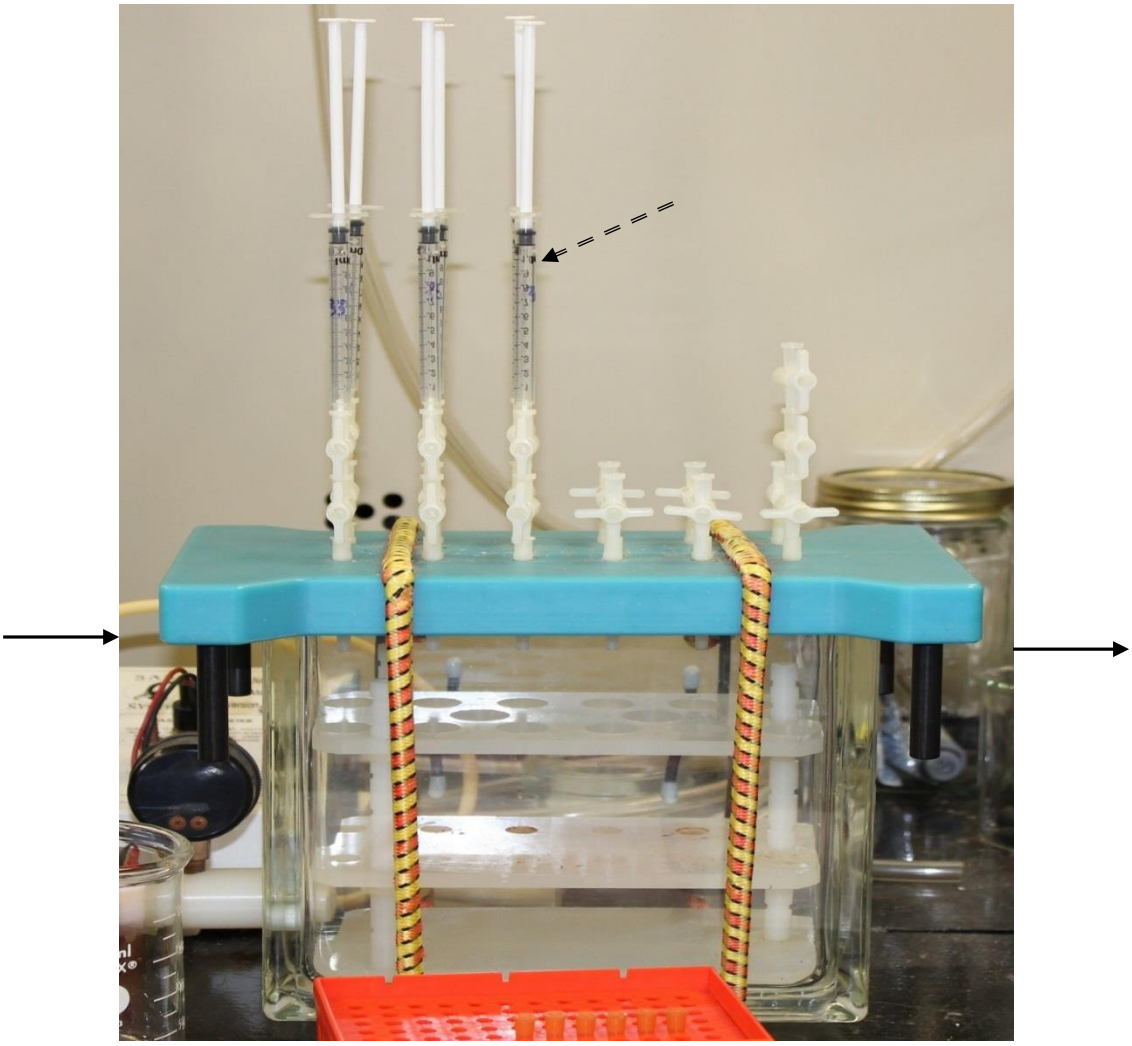

Figure 4.1 Photograph of syringes containing eggs attached to the manifold (not pictured is a purge-gas generator that provides dry, CO_2 free air to the manifold). The syringe plungers are pulled above the drilled holes to allow dry, CO_2 free air to be pushed through the syringe and out of the holes. The dry, CO_2 free air is pulled into the manifold through tubes and then is pulled out of the manifold on the other side (gas direction is indicated by the arrows [left to right]). The manifold is controlled by valves for air flow and the valves are open where the syringes are attached. The dashed arrow is pointing to the area where the holes were drilled in all of the syringe barrels.

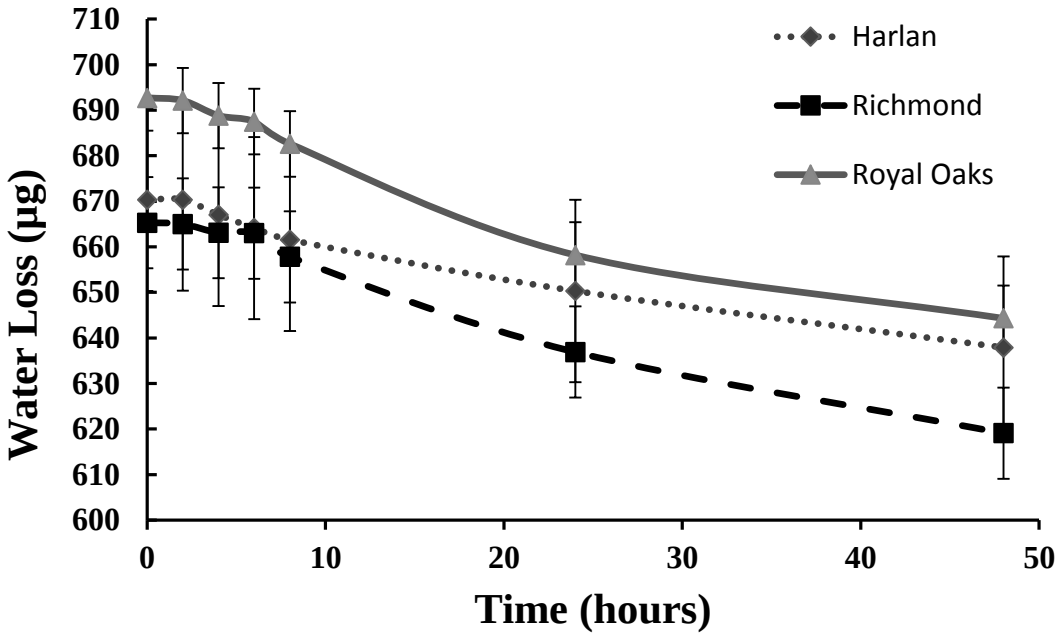

Figure 4.2 Water loss of a susceptible strain bed bug eggs (Harlan) and two pyrethroid resistant strain bed bug eggs (Royal Oaks and Richmond) over 48 hours. There were no significant differences between strains with regard to water loss, determined using a Repeated Measures ANOVA (P=0.6309; JMP Pro 10.0; SAS Institute 2012).

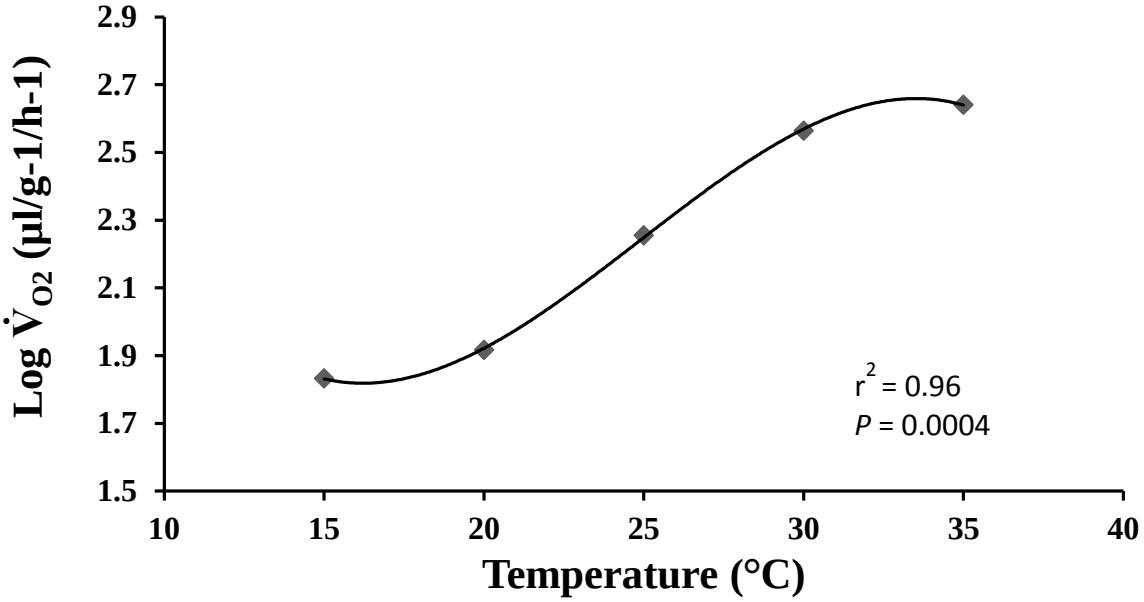

Figure 4.3 Harlan strain bed bug egg oxygen consumption ($\dot{V}_{O_2}$) at 5 temperatures (15, 20, 25, 30 and 35°C). Solid line represents the first-order regression of log transformed oxygen consumption on temperature. Linear regression analysis (JMP Pro 10.0: SAS Institute 2012) indicated that as temperatures increased, oxygen consumption of Harlan strain bed bug eggs increased and that temperature significantly impacted oxygen consumption ($P = 0.0004$).

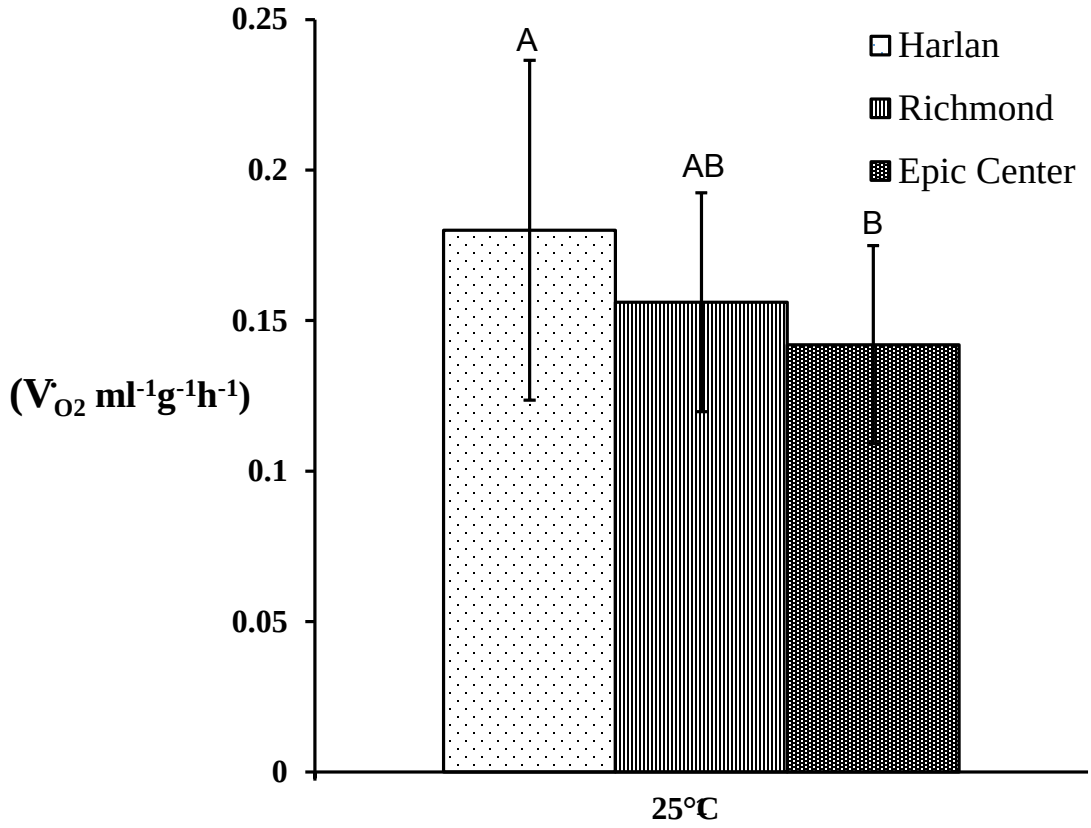

Figure 4.4 Rate of oxygen consumption ($\dot{V}_{O_2}$) for three strains of bed bug eggs (Harlan, Richmond, Epic Center) measured at 25°C. Harlan strain eggs consumed more oxgen than Epic Center strain bed bug eggs, while Richmond consumed similar amounts of oxygen as the other two tested strains (ANOVA; JMP Pro 10.0; SAS Institute 2012). Levels of significance were determined by P values ≤ 0.05.

Table 4.1 Comparison of mean mass specific oxygen consumption ($\dot{V}_{O_2}$), mean carbon dioxide production ($\dot{V}_{CO_2}$) and RQ values across 6 temperatures for the Harlan susceptible strain.

Temperature (℃)	*n*	Mean $\dot{V}_{O_2}$ ± SE	Mean $\dot{V}_{CO_2}$ ± SE	RQ Values
15	8	0.068 ± 0.017a	0.038 ± 0.026	0.56
20	12	0.083 ± 0.018a	0.052 ± 0.008	0.65
25	16	0.180 ± 0.056b	0.097 ± 0.031	0.54
30	16	0.367 ± 0.102c	0.213 ± 0.033	0.55
35	13	0.438 ± 0.087c	0.245 ± 0.031	0.57
39	8	0.315 ± 0.100	0.153 ± 0.026	0.5

Means followed by different letters are significantly different (ANOVA; JMP Pro 10.0; SAS institute 2012). Level of significance was determined for P values ≤ 0.05. As temperatures reached 39 °C, the bed bug eggs became too stressed and oxygen consumption decreased, therefore that temperature was excluded from the ANOVA analysis.

Table 4.2 Comparison of mean mass specific oxygen consumption ($\dot{V}_{O_2}$), mean carbon dioxide production ($\dot{V}_{CO_2}$), and RQ values for three bed bug egg strains (Harlan susceptible, Richmond resistant, and Epic Center resistant strains) at 25°C.

Strain	*n*	mass (mg ± SD)	$\dot{V}_{O_2}$ mean ± SE	Mean $\dot{V}_{CO_2}$ ± SE	RQ Values
Harlan	16	0.93986 ± 0.000193	0.18 ± 0.05a	0.097 ± 0.031	0.54
Richmond	14	0.95667 ± 0.00007	0.16 ± 0.04a	0.080 ± 0.021	0.52
Epic Center	5	0.985 ± 0.000	0.14 ± 0.03b	0.87 ± 0.014	0.57

Means followed by different letters are significantly different ($P \leq 0.05$; ANOVA; JMP; SAS Institute 2012).

Chapter 5. Insecticide Resistance in Bed Bug Eggs and First Instars (Hemiptera: Cimicidae)

Introduction

Although studies have documented that bed bugs can carry multiple pathogenic organisms on their bodies and in their excrement; they are not known to be successful at disease transmission (Delaunay et al. 2010, Burton 1963, Sabou et al. 2013). Subsequently, bed bugs do not have the public health status associated with other blood sucking insects, including mosquitoes, ticks and fleas. However, bed bug bites can result in allergic cutaneous reactions in humans with varied symptoms (Reinhardt et al. 2009, Leverkus et al. 2006, Fletcher et al. 2002, Goddard and deShazo 2009, Churchill 1930, Goddard et al. 2011) and if bed bug populations become large, frequent blood meals can result in anemia (Pritchard and Hwang 2009, Korinek et al. 2011). Aside from physiological complications brought upon by bed bug infestations, bed bugs also can cause psychological distress including depression, sleeplessness and anxiety (Goddard and deShazo 2012, Comack and Lyons 2011, Susser et al. 2012). Furthermore, bed bugs can be economically devastating because of the high costs associated with their control.

There are a number of factors that make bed bug infestations difficult to control. For example, bed bugs are a cryptic species and hide in household belongings (electronics, books, toys etc.) that can't be treated with conventional insecticides. Another factor making bed bugs difficult to control is their high resistance to many insecticides currently labeled for bed bug control. Bed bug insecticide resistance is a result of *kdr* mutations, enhanced enzyme

detoxification activity and cuticular penetration resistance. The cost of bed bug treatments further complicate bed bug control because many people can't pay for the labor intensive treatments required to eliminate bed bug infestations. Lastly, bed bug eggs contribute to the difficulties and costs associated with bed bug treatments.

Most conventional insecticides labeled for bed bug control are ineffective against bed bug eggs (Pinto et al. 2007). Conventional bed bug protocols require at least three treatments applied at two week intervals to allow bed bug eggs to hatch. Subsequently, pest control professionals are treating newly hatched nymphs rather than the eggs. Although bed bug eggs are difficult to control, there are few studies available that have evaluated insecticide efficacy for controlling bed bug eggs (Goddard 2013; Callaway and Musgrave 1939). To date, our study is the first to evaluate bed bug egg and first instar resistance.

Insecticide resistance in different species of insect eggs has been demonstrated in studies where resistance was already quantified in the adult or larval stages (Cueto et al. 2008; Leonard et al. 1991; Ho and Goh 1984). Insecticide resistance between eggs and first instars has been shown to be differentially expressed in *Triatoma infestans* (Toloza et al. 2008), suggesting that first instar resistance was not indicative of egg resistance. Insecticide resistance has also been selected for in insect eggs (*Musca domestica*) using diflubenzuron (Grosscurt 1980).

The purpose of this study was to determine insecticide resistance in bed bug egg and first instars. We conducted dose-response bioassays with two combination products commonly used for bed bug control, Temprid (imidacloprid [0.10%] / β-cyfluthrin [0.05%],) and Transport (acetamiprid [0.05%]/bifenthrin [0.06%]), to determine LC_{50} values and subsequent resistance ratios of bed bug eggs from three strains. We also assessed bed bug egg mortality with a pyrethroid insecticide (Suspend; deltamethrin [0.06%]). Deltamethrin has been used for several

years for bed bug control. Consequently, several papers have documented deltamethrin resistance in adult bed bugs (Moore and Miller, 2006; Romero et. al, 2007; Adelman et. al 2011; Seong et. al, 2010; Kilpinen et. al, 2011).

Materials and Methods

Experimental Insects

Three bed bug strains were used for this study, an insecticide susceptible strain (Harlan), and two insecticide resistant strains (Richmond and Epic Center). The Harlan susceptible strain was acquired from Dr. Harold Harlan (National Pest Management Association, Fairfax, VA) in February 2005. Dr. Harlan maintained this population since 1973 by feeding them on himself. The Richmond resistant strain was collected from an elderly group home located in Richmond, VA in 2008. The Epic Center resistant strain was collected in 2008 from an apartment complex in Cincinnati, Ohio.

All bed bug strains were fed weekly with defibronated rabbit blood on an artificial feeding system (Hemostat, Dixon, CA). The bed bug strains were maintained in plastic rearing jars enclosed with mesh at one end to allow for feeding. Rearing jars contained pieces of cardboard to provide harborage and a substrate for the bed bugs to walk up and feed through the mesh. The plastic rearing jars containing all bed bug strains are stored in an environmental chamber at 27°C, 60% RH, and a 12:12 L:D photoperiod.

Prior to the bioassay, recently fed and mated female bed bugs (30 groups of 10) were collected from all three strains and placed into plastic Petri dishes (Fisher Scientific Inc., 6 cm X 5 cm) each containing a piece of filter paper (Whatman # 1, 4.2 cm diameter) for oviposition. The females were provided a new piece of filter paper daily.

Egg Resistance Assessment

Bed bug eggs (4-5 days old) were removed from filter papers using soft-tip forceps. Egg removal caused no visible damage to the eggs and did not result in increased mortality compared to a control group of eggs that were not removed from filter papers. The selected age range (4-5 d. old) was chosen for the bioassay to allow maximum embryonic development while simultaneously avoiding hatch during the test.

Three insecticides were chosen for this resistance evaluation, (1) Temprid SC (imidacloprid [0.10%] / β-cyfluthrin [0.05%], Bayer CropScience, Research Triangle Park, NC), (2) Transport GHP (acetamiprid [0.05%]/bifenthrin [0.06%]; FMC Corp., Philadelphia, PA), and (3) Suspend SC (deltamethrin [0.05%], Bayer CropScience, Research Triangle Park, NC). All eggs were exposed to five concentrations of each insecticide formulated with water (Table 1). Control treatment eggs were dipped into water only.

Bed bug eggs (5 replications of 10) were dipped into each insecticide concentration using a centrifuge tube (Fisher Scientific Inc., 50 ml) that had been cut in half. A large hole was cut into the lid and covered with mesh (Figure 1). The eggs were placed onto the mesh closure of the centrifuge tube and immersed into each insecticide formulation for 5 seconds. The mesh, containing eggs, was then dried with a KimWipe (Kimtech, 11 cm X 21 cm) to remove excess insecticide. Using a paint brush, eggs were immediately removed from the mesh into a Petri dish containing a clean piece of filter paper. Egg hatch failure was recorded after 14 days.

First Instar Resistance Assessment

Harlan, Richmond, and Epic Center strain bed bug eggs were allowed to hatch within Petri dishes. Following hatch, unfed first instar bed bugs (7-10 days old) were collected using a

paint brush. Two insecticides were chosen for this study, Temprid SC (imidacloprid [0.10%]/β-cyfluthrin [0.05 %], Bayer CropScience, Research Triangle Park, NC) and Transport GHP (acetamiprid [0.05%]/ bifenthrin [0.06%], FMC Corp., Philadelphia, PA). First instars were exposed to five concentrations of each insecticide (Table 2). An aliquot (150 µl) of each insecticide concentration was applied to a filter paper disc (Whatman # 1; 4.2 cm diameter) and allowed to dry completely. The 150 µl aliquot of insecticide fully covered the filter paper but did not saturate the paper to the point of runoff. The treated filter papers were then placed on top of a hardboard panel (7 cm^2). Control treatments received only water.

First instars (5 replications of 5 insects) were released on top of the treated surface and contained by inverting the bottom of a petri dish on top of the treated filter paper. The Petri dish was smaller in diameter than the filter paper, therefore all of the first instars were continuously exposed to the treated surface. To ensure that first instars could not escape, small bolts (weights) were placed on top of the plastic Petri dishes. Mortality was recorded after 24 hours and was defined by individuals that did not move after prodding with a paint brush after 24 hours.

Statistical Analysis

The LC_{50} values (concentration that kills 50% of individuals) were calculated for eggs from each strain exposed to each insecticide using PoloPlus (Version 1.0; LeOra Software). Bed bug egg deltamethrin LC_{50} values were not calculated because there was little bed bug egg mortality at the highest tested concentrations. It was impractical to test higher concentrations because the formulation would no longer stay into suspension.

The LC_{50} values (concentration that kills 50% of individuals) were determined for first instars from each strain exposed to each insecticide using Prism (GraphPad Software, Inc.).

Based on bed bug egg mortality with Deltamethrin (0.06%), we did not test first instars using Deltamethrin.

Significant differences between LC_{50} values of eggs and first instars from each strain exposed to each insecticide were determined by the failure of the confidence intervals (CI) to overlap. Resistance ratios were calculated by dividing the egg LC_{50} value of the resistant strain by the egg LC_{50} value of the field strain and were calculated for first instars by dividing the LC_{50} value of the lab strain by the LC_{50} value of each field strain. To further evaluate differences between egg and first instar resistance, we calculated stage resistance ratios. These stage resistance ratios were determined by dividing the largest LC_{50} value of either stage (egg or first instar) by the smallest LC_{50} value of either stage.

Results

Egg Resistance Assessment

As expected**,** Harlan strain susceptible eggs died at lower concentrations than the other two populations tested when exposed to imidacloprid/β-cyfluthrin (LC_{50} = 0.409 µl/ml) and acetamiprid/bifenthrin (LC_{50} = 0.000022 µg/ml). Richmond and Epic Center eggs were not highly resistant to imidacloprid/β-cyfluthrin (Richmond RR = 3.01; Epic Center RR = 5.13), although the LC_{50} values of both strains were significantly greater than that of the Harlan strain (Table 3). However, Richmond and Epic Center eggs were much more resistant to acetamiprid/bifenthrin, indicated by relatively high resistance ratio values (Richmond RR = 35.45; Epic Center RR =936.36). The LC_{50} value was significantly greater than Richmond when exposed to acetamiprid/bifenthrin, as indicated by the failure of the confidence intervals to overlap. LC_{50} values could not be calculated for the eggs treated with deltamethrin because we could not formulate a concentration high enough for 80% mortality. Richmond and Epic Center

egg percent mortality was lower when exposed to deltamethrin than Harlan strain eggs at all tested concentrations (Table 6).

First Instar Resistance Assessment

The LC_{50} values for Harlan strain first instars treated with imidacloprid/β-cyfluthrin was 0.041 µl/ml. The LC_{50} value for Harlan first instars was significantly lower (0.0000067 µg/ml) when exposed to acetamiprid/bifenthrin. The LC50 values calculated for Richmond and Epic Center first instars were significantly greater than that of the Harlan strain eggs when exposed to imidacloprid/β-cyfluthrin, but were not significantly different from eachother as indicated by the overlapping confidence intervals. Note however, that the calculated resistance ratios for Epic Center eggs exposed to imidacloprid/β-cyfluthrin was at least 4-fold greater than that of the Richmond strain eggs (Table 4).

The LC_{50} values of first instars exposed to acetamiprid/bifenthrin concentrations were all significantly lower than those exposed to imidacloprid/β-cyfluthrin in all three strains. However, the LC_{50} values were significantly different in all three strains; Harlan < Richmond < Epic Center (Table 4). Similar to the imidacloprid/β-cyfluthrin evaluations, the Epic Center strain resistance ratio values calculated for first instars exposed to acetamiprid/bifenthrin was 20 fold greater than that of Richmond strain eggs.

Stage Resistance Comparisons

Harlan eggs were slightly less susceptible than Harlan first instars (Stage resistance ratio [SR] = 3.28) when treated with acetamiprid/bifenthrin. However, Harlan eggs were even less susceptible than first instars when treated with imidacloprid/ β-cyfluthrin (SR = 9.98). Richmond first instars were less susceptible than Richmond eggs to imidacloprid/β-cyfluthrin (SR = 3.91).

Epic Center first instars were also less susceptible to imidacloprid/β-cyfluthrin than Epic Center eggs (RR = 9.4). There was relatively no difference between Richmond and Epic Center eggs and first instars exposed to acetamiprid/bifenthrin, indicated by the stage resistance ratios close to 1 (Table 5).

Discussion

Most research evaluating pyrethroid insecticide efficacy in bed bugs has been focused on third instars and subsequent life stages (Moore and Miller 2006, Romero et al. 2007, Adelman et al. 2011, Seong et al. 2010, Kilpinen et al. 2011). Goddard (2013) evaluated the efficacy of several insecticide products on bed bug eggs but did not evaluate egg resistance (Goddard 2013). Bed bug egg mortality can be achieved with some pressurized aerosol insecticides (Goddard 2013) but the same active ingredients formulated in water lacked efficacy against bed bug eggs. Surprisingly, when we tested deltamethrin (0.06%) at 10x the label rate against susceptible strain bed bug eggs, we did not achieve 100% mortality. The lack of efficacy of deltamethrin, similar to the Goddard et al. 2013 study, could be a result of the waxy components of the eggshell preventing water formulated products from permeating the eggshell.

Richmond and Epic Center strains are known to be resistant to pyrethroid insecticides (Moore and Miller 2006, Adelman et al. 2011, Miller and McCoy unpublished data). The Epic Center strain adult bed bugs exposed to dried residues of deltamethrin (0.06%) were found to be 418 times less susceptible compared to the Harlan susceptible strain (Miller and McCoy unpublished data). Comparisons of bed bug egg resistance ratios to adult resistance ratios would be ideal, but we could not achieve enough egg mortality at even the highest tested concentration of deltamethrin to calculate LC_{50} values. However, Richmond and Epic Center strain bed bug eggs both had lower mortality compared to Harlan susceptible strain bed bug eggs when exposed

to the same concentrations of deltamethrin, indicating that the Epic Center and Richmond eggs may be deltamethrin resistant.

New insecticide products have combined a pyrethroid insecticide with a neonicotinoid in attempts to circumvent the widespread resistance to pyrethroid products. Pest control operators in the United States surveyed in 2011 routinely used the combination pyrethroid/neonicotinoid products, Temprid and Transport, for bed bug treatments (Potter et al. 2011). Potter et al. (2012) compared the efficacy of Temprid and Transport to Suspend (deltamethrin; 0.06%), and found that both combination products were more effective against adult bed bugs than deltamethrin. Therefore, we chose both of these combination products for our bed bug egg and first instar resistance studies.

Overall, bed bug eggs and first instars from Richmond and Epic Center strains were somewhat resistant to the imidacloprid/β-cyfluthrin combination product but were more resistant to the acetamiprid/bifenthrin combination product, with the exception of Richmond first instars. Richmond first instars had a resistance ratio (RR) of 117 when exposed to imidacloprid/β-cyfluthrin compared to a RR of 102 to acetamiprid/bifenthrin. There is no way of knowing if the observed resistance is to the neonicotinoid or the pyrethroid because we tested combination products, however, we assume the observed resistance is to the pyrethroid component as documented in the adults of each strain. In 2008, when these bed bug populations were collected in the field, pest control companies were primarily using only pyrethroid products for chemical control. Temprid SC was not even labeled for bed use until 2010 (bed bug label amendment Jan. 14, 2010; EPA registration no. 432-1483). Although FMC registered Transport GHP in 2008 it is unlikely that these bed bug populations (Richmond and Epic Center) had been exposed to Transport GHP.

Gordon et al. (2014) documented resistance to both combination products we tested, imidacloprid/β-cyfluthrin and acetamiprid/bifenthrin, in bed bug populations. These bed bug populations varied in their levels of susceptibility. Insecticide resistance has been documented to be highly variable between bed bug populations (Gordon et al. 2014, Romero et al. 2007). In our study, Epic Center eggs and first instars were more resistant than eggs and first instars of the Richmond strain. Richmond and Epic Center strains were collected from different areas within the United States. Therefore, differences between resistance in Richmond and Epic Center eggs could be a result of previous insecticide exposure and selection pressure.

Interestingly, comparisons of stage resistance ratios indicated that there was little difference in resistance between eggs and first instars. We assume that the eggshell provides protection from insecticides but is not the determinant factor in egg resistance. In the Harlan susceptible strain, the eggs were less susceptible to imidacloprid/β-cyfluthrin and acetamiprid/bifenthrin compared to the first instars. We assume that because the Harlan susceptible strain has not been exposed to insecticides for over 30 years that the first instars should have little (or no) resistance mechanisms developed. Therefore, in the susceptible strain only the eggshell is providing protection against insecticides.

Treating first instars with insecticides following bed egg hatch may not be a practical answer for controlling bed bug infestations. This research indicates that first instars are equally as resistant to insecticides as bed bug eggs. The eggshell is probably providing a barrier for insecticide penetration but the embryo inside of the egg may also have similar resistance mechanisms as documented in adult bed bugs. Therefore, the two week interval may be insignificant with regard to killing emerging nymphs. However, multiple insecticide applications

are required because of the lack of residual activity of many insecticides against resistant bed bugs.

Table 5.1 Treatment concentration ranges for bed bug eggs dipped into Temprid SC ™ (imidacloprid [0.10%]/ β-cyfluthrin [0.05%]) and Transport GHP (acetamiprid [0.05%]/bifenthrin [0.06%] for one pyrethroid susceptible strain (Harlan) and two pyrethroid resistant strains (Richmond and Epic Center).

Treatment	Concentration range[4] (a.i./ml H_2O)
Harlan	
Temprid[1,2]	0.21-4.2 µl/ml
Transport[1,3]	0.0044-0.075 ng/ml
Richmond	
Temprid [1,2]	0.42-21 µl/ml
Transport [1,3]	0.32-22.5 ng/ml
Epic Center	
Temprid [1,2]	0.42-21 µl/ml
Transport [1,3]	0.45-33.75 ng/ml

[1]Formulations are based on label directions for Temprid SC and Transport GHP.

[2]Temprid SC label rate recommended for bed bug use is 16 milliliters per one gallon of water that results in the combined active ingredient concentration (0.15%), containing 0.10% imidacloprid and 0.05% β-cyfluthrin.

[3]Transport SC label rate recommended for bed bug use is 1 water soluble packet (0.3 oz) per 1 gallon of water, resulting in 0.11% combined active ingredients, containing 0.05% acetamiprid and 0.06% bifenthrin.

[4]All bed bug eggs were exposed 5 concentrations of each insecticide formulated with water.

Table 5.2 Treatment concentration ranges for bed bug first instars exposed to Temprid SC ™ (imidacloprid [0.10%]/ β-cyfluthrin [0.05%]) and Transport GHP (acetamiprid [0.05%]/bifenthrin [0.06%] for one pyrethroid susceptible strain (Harlan) and two pyrethroid resistant strains (Richmond and Epic Center).

Treatment	Concentration range[4] (a.i./ ml H_20)
Harlan	
Temprid[1,2]	0.0066-0.105 µl/ml
Transport[1,3]	0.0035-0.019 ng/ml
Richmond	
Temprid[1,2]	0.425-84 µl/ml
Transport[1,3]	0.32-22.5 ng/ml
Epic Center	
Temprid[1,2]	0.425-21 µl/ml
Transport[1,3]	1.5-33.75 ng/ml

[1]Formulations are based on label directions for Temprid SC and Transport GHP.

[2]Temprid SC label rate recommended for bed bug use is 16 milliliters per one gallon of water that results in the combined active ingredient concentration (0.15%), containing 0.10% imidacloprid and 0.05% β-cyfluthrin.

[3]Transport SC label rate recommended for bed bug use is 1 water soluble packet (0.3 oz) per 1 gallon of water, resulting in 0.11% combined active ingredients, containing 0.05% acetamiprid and 0.06% bifenthrin.

[4]All bed bug eggs were exposed 5 concentrations of each insecticide formulated with water.

Table 5.3 Comparison of bed bug egg LC_{50} values when exposed to 5 different concentrations of Temprid (imidacloprid/ β-cyfluthrin) and Transport (acetamiprid/bifenthrin) for a pyrethroid susceptible strain (Harlan) and two pyrethroid resistant strains (Richmond and Epic Center).

Treatment	*n*	LC_{50} (95% CI)	Slope ± SE	X^2 (df)	RR
Temprid					
Harlan	250	0.409 µl/ml a (0.276-0.548)	1.86 ± 0.24	33.42 (23)	
Richmond	320	1.23 µl/ml b (0.59-2.10)	1.13 ± 0.14	82.57 (30)	3.01
Epic Center	400	2.098 µl/ml b (1.049-4.587)	0.95 ± 0.10	149.91 (38)	5.13
Transport					
Harlan	250	0.022 ng/ml a (0.018-0.028)	2.334 ± 0.253	26.90 (23)	
Richmond	310	0.78 ng/ml b (0.00037-0.00144)	0.575 ± 0.098	29.17 (29)	35.45
Epic Center	240	20.6 ng/ml c (0.0064-0.0513)	0.481 ± 0.092	25.71 (22)	936.36

LC_{50} values followed by different letters are significantly different determined by the failure of the confidence intervals to overlap (PoloPlus 2004). Resistance ratios were determined by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain. Concentrations were determined from adjusting label rate formulations with information presented in Table 5.1. Five concentrations of each insecticide were used to determine LC_{50} values. LC_{50} values were calculated using PoloPlus (2004). Eggs were dipped into each concentration for 5 seconds and mortality was recorded after 14 days.

Table 5.4 Comparison of bed bug first instar LC_{50} values when exposed to 5 different concentrations of Temprid (imidacloprid/ β-cyfluthrin) and Transport (acetamiprid/bifenthrin) for a pyrethroid susceptible strain (Harlan) and two pyrethroid resistant strains (Richmond and Epic Center).

Treatment	*n*	LC_{50} (95% CI)	Slope ± SE	X^2 (df)	RR
Imidacloprid/ β-cyfluthrin					
Harlan	150	0.041 µl/ml a (0.030-0.063)	2.155 ± 0.335	38.17 (28)	
Richmond	195	4.81 µl/ml b (1.94-10.26)	0.663 ± 0.119	45.87 (37)	117.32
Epic Center	190	19.72 µl/ml b (8.18-184.48)	0.747 ± 0.169	45.39 (36)	480.98
Acetamiprid/bifenthrin					
Harlan	155	0.0067 ng/ml a (0.0054-0.0080)	3.292 ± 0.482	33.95 (29)	
Richmond	125	0.69 ng/ml b (0.21-1.43)	0.935 ± 0.187	29.91 (23)	102.99
Epic Center	115	13.6 ng/ml c (3.9-1215.8)	0.501 ± 0.132	23.31 (21)	2029.85

LC_{50} values followed by different letters are significantly different determined by the failure of the confidence intervals to overlap (PoloPlus 2004). Resistance ratios were determined by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain. Concentrations were determined from adjusting label rate formulations with information presented in Table 5.2. Five concentrations of each insecticide were used to determine LC_{50} values. LC_{50} values were calculated using PoloPlus (2004). First instars were placed onto treated filter papers of each concentration and mortality was recorded after 24 hours.

Table 5.5 Comparison of LC_{50} values between eggs and first instars within strain (Harlan pyrethroid susceptible, Richmond pyrethroid resistant and Epic Center pyrethroid resistant). Eggs and first instars were treated with either Temprid (imidacloprid/β-cyfluthrin) or Transport (acetamiprid/bifenthrin.

Treatment	Egg LC_{50} (95% CI)	1st Instar LC_{50} (95% CI)	$>LC_{50}$	Stage RR
Temprid				
Harlan	0.409 µl/ml (0.276-0.548)	0.041 µl/ml (0.030-0.063)	egg	9.98
Richmond	1.23 µl/ml (0.59-2.10)	4.81 µl/ml (1.94-10.26)	1st instar	3.91
Epic Center	2.098 µl/ml (1.049-4.587)	19.72 µl/ml (8.18-184.48)	1st instar	9.4
Transport				
Harlan	0.022 ng/ml (0.018-0.028)	0.0067 ng/ml (0.0054-0.0080)	egg	3.28
Richmond	0.78 ng/ml (0.37-1.44)	0.69 ng/ml (0.21-1.43)	egg	1.13
Epic Center	20.6 ng/ml (6.4-51.3)	13.6 ng/ml (3.9-1215.8)	egg	1.51

Stage resistance ratios were determined by dividing the largest LC_{50} value of either stage (egg or 1st instar) by the smallest LC_{50} value of either stage. The stage (egg or first instar) with the greater LC_{50} value is indicated in the $>LC_{50}$ value column. LC_{50} value information is provided in Tables 5.3 and 5.4.

Table 5.6 Bed bug egg mortality recorded after treatment with deltamethrin.

Concentration	Strain	n	% mortality
12 µl/ml			
	Harlan	50	46 %
	Richmond	50	20 %
	Epic Center	50	8 %
58 µl/ml			
	Harlan	50	68 %
	Richmond	50	14 %
	Epic Center	50	10 %
115 µl/ml			
	Harlan	50	60 %
	Richmond	50	32 %
	Epic Center	50	30 %

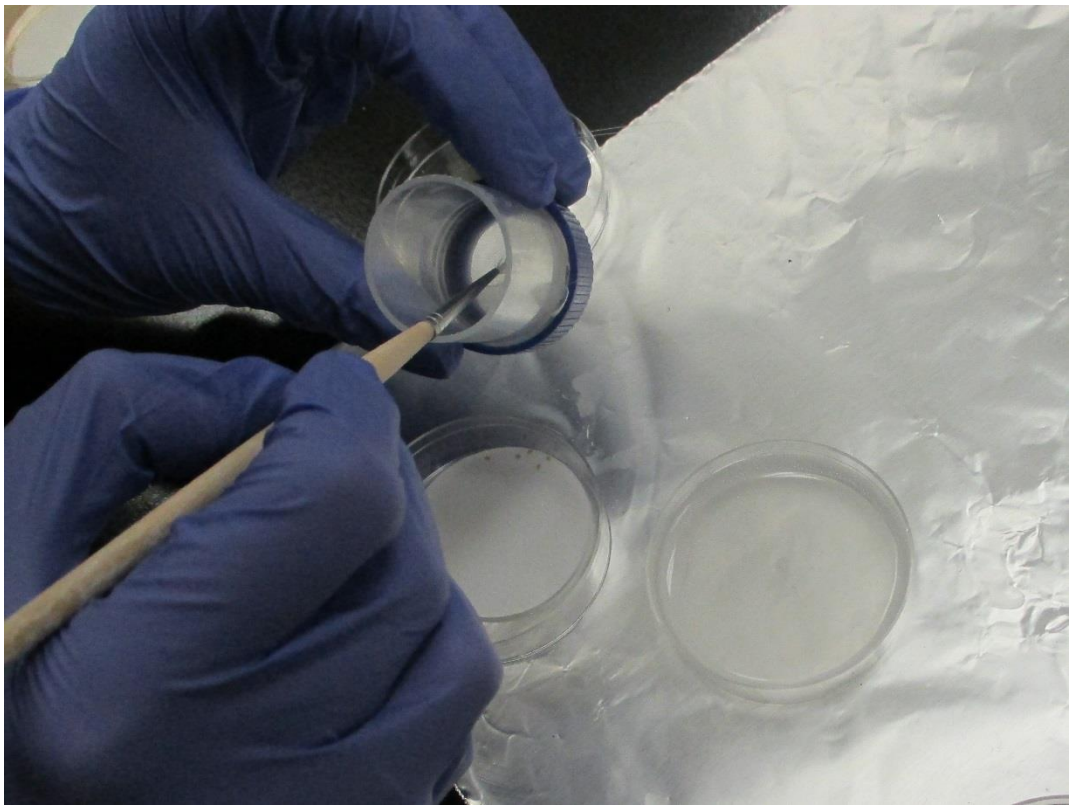

Figure 5.1 Centrifuge tube modified to dip bed bug eggs into insecticides.

Chapter 6. Pyrethroid Susceptible and Resistant Bed Bug Egg Transcript Expression Levels Quantified by RNA-Seq

Introduction

The common bed bug, *Cimex lectularius* L., was nearly eradicated in the United States due to widespread utilization of DDT and other synthetic insecticides during the mid-19th century (Ebeling 1975). Although DDT was highly effective against bed bugs, DDT resistance in bed bugs was documented in the 1950s (Busvine 1958). More recently, the most commonly used class of indoor insecticides in the United States have been pyrethroid insecticides (Kaufman 2006). The bed bug resurgence that began in the 1990s in the US may be partially due to cross resistance that may have occurred between chlorinated hydrocarbons (DDT) and pyrethroid insecticides as a result of similar modes of action; targeting voltage-gated sodium channels. Therefore, the rapid resistance documented to pyrethroid insecticides could have been a result of selection initiated by the use of DDT in the 1940's (Zhu et al. 2010). Consequently, the overuse of pyrethroid insecticides and the subsequent development of resistance has caused a recent exponential increase in the number of bed bug infestations in Europe, Australia and North America.

Bed bugs have been frequently exposed to pyrethroid insecticides and therefore have significant resistance to these active ingredients (Romero et al. 2007, Moore and Miller 2006). Multiple physiological mechanisms of pyrethroid insecticide resistance have been identified in bed bug populations across the United States. These mechanisms include target site mutations (Yoon et al. 2008, Zhu et al. 2010, Adelman et al. 2011), enhanced detoxification enzyme activity (Adelman et al. 2011, Bai et al. 2011), and reduced cuticular penetration (Koganemaru et al. 2013).

Knockdown resistance (*kdr*) in bed bugs has been attributed to mutations (V419L and L925I) associated with amino acid substitutions in the α-subunit of the voltage-gated sodium channel, first described in a New York bed bug population (Yoon et al. 2008). The same mutations(s) have also been found in a bed bug population collected from Richmond, VA (Adelman et al. 2011) and additional bed bug populations across the United States (Zhu et al. 2010), suggesting that *kdr* mutation(s) may be a widespread insecticide resistance mechanism in bed bugs.

Another insecticide resistance mechanism documented in bed bugs is enhanced metabolic activity aided by cytochrome P450 and esterase enzyme activity. Elevated transcript levels of a cytochrome P450 (CYP9) were identified in a bed bug population collected from Columbus, Ohio (Bai et al. 2011). A bed bug population collected from Richmond, VA had higher transcription levels of cytochrome P450 enzymes and carboxylesterases in conjunction with one *kdr* mutation (Adelman et al. 2011).

More recently, the Richmond, VA bed bug strain has also been documented to have reduced cuticular penetration type resistance to limit insecticide exposure (Koganemaru et al. 2013). Resistance ratios were several magnitudes higher in Richmond, VA bed bugs when injected with deltamethrin compared to topical applications, suggesting cuticular resistance (Koganemaru et al. 2013). Furthermore, cuticle protein (CPR) type transcripts were also found to be highly upregulated (20 fold) in the Richmond resistant bed bugs when compared with the Harlan susceptible strain (Koganemaru et al. 2013).

RNA-Seq techniques have been used to identify differentially expressed genes in both pesticide resistant and susceptible bed bug populations (Mamidala et al. 2012). Approximately 15,000 genes were differentially expressed between resistant and susceptible bed bug

populations with several genes identified that contribute to resistance (cytochrome P450s, carboxylesterases, cuticular proteins, antioxidant genes, ABC transporters, GSTs, and acetyl cholinesterase) (Mamidala et al. 2012). In this study, we used RNA-Seq to identify genes possibly involved with insecticide resistance in a pyrethroid susceptible and two resistant strains of bed bug eggs.

Materials and Methods

Bed Bug Egg Collection and Maintenance

Bed bug eggs were collected from one susceptible laboratory strain and two pyrethroid resistant field strains. Harlan susceptible strain bed bugs were acquired from Dr. Harold Harlan (National Pest Management Association, Fairfax, VA) in February 2005. Dr. Harlan maintained this population since 1973 by feeding the strain on himself. Two field strain populations (Richmond and Epic Center) were also evaluated for this study. The Richmond resistant strain was collected from an elderly group home located in Richmond, VA in 2008. The Epic Center resistant strain was collected in 2008 in an apartment complex in Cincinnati, Ohio.

All bed bug strains were fed weekly with defibronated rabbit blood using an artificial feeding system (Hemostat, Dixon, CA). The bed bugs strains were contained in plastic rearing jars enclosed with mesh at one end to allow for feeding through the mesh. Rearing jars contained pieces of cardboard to provide harborage and a substrate for the bed bugs to crawl up and feed through the mesh. The plastic rearing jars containing all bed bug strains were stored in an environmental chamber at 27°C, 60% RH, and a 12:12 L:D photoperiod.

RNA Isolation and Illumina Paired End Library Prepartion and Sequencing

Bed bug eggs (50 per replicate, 3 replicates per strain) were homogenized and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), which was then DNase treated (DNase I, New England BioLabs, Inc.) to remove any contaminating genomic DNA. RNA concentrations

were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) prior to being sent to the Virginia Bioinformatics Institute for library preparation (Virginia Tech, Blacksburg, VA). Briefly, the Virginia Bioinformatics Institute prepared the libraries using an IntegenX Apollo 324 Robot system. Then, one microgram of RNA was used for the PrepX PolyA mRNA isolation kit (Catalogue Number 40047) along with the PrepX RNA Seq Kit for Illumina sequencing (Catalogue Number 400046). Libraries were amplified using PCR (16 cycles). The library was quantified using Qubit and size verified using a bioanalyzer with final quantification performed with qPCR. The eight libraries were sequenced on one lane of the Illumina HiSeq 2500.

Bioinformatic Analysis

Illumina reads were mapped to the draft bed bug genome using TopHat (Trapnell et al. 2012). Cufflinks (Trapnell et al. 2012) was used to quantify transcript abundance and differentially expressed transcripts. BLASTx was used in the BLAST2GO (BioBam Bioinformatics S.L., Valencia, Spain) software that compared our bed bug egg nucleotide sequences to protein sequences to obtain gene annotation and gene ontology terms. Differentially expressed transcripts were analyzed using the ORF predictor (Min et al. 2005) to determine the open reading frame peptide lengths. Peptide lengths below 100 amino acids were not used for further analysis.

Results

The RNA-Seq analysis identified 387 transcripts as being significantly differentially expressed between the three bed bug egg strains (Appendix A). From the total 387 transcripts, 228 transcripts had open reading frames with peptide lengths containing less than 100 amino acids, many of which were identified as transposable elements (Appendix B).

Transcripts Differentially Expressed Between Epic Center Resistant Strain and Harlan Susceptible Strain Bed Bug Eggs

From the total differentially expressed transcripts, 52 transcripts were identified with peptide lengths exceeding or equal to 100 amino acids (Appendix A). A total of nine transcripts associated with cuticle structure or sclerotization were accumulated at higher levels in Epic Center resistant strain bed bug eggs compared to the Harlan strain. Other transcripts that were expressed at higher levels in Epic Center resistant strain eggs compared to Harlan eggs were TCONS00044876 [carboxylesterase], TCONS00035977 [heat shock protein], TCONS00036748 [zinc carboxypeptidase], and TCONS00016409 [serine protease].

Transcripts Differentially Expressed Between Harlan susceptible and Richmond Resistant Bed Bug Eggs

When comparing Harlan susceptible and Richmond resistant eggs, 64 differentially expressed transcripts were identified to have greater than 100 amino acids (Appendix A). A total of 8 transcripts associated with the cuticle were accumulated at higher levels in Harlan susceptible strain bed bug eggs when compared with Richmond resistant strain bed bug eggs. Transcripts that are commonly associated with insecticide resistance were accumulated at higher levels (<10 fold) in the Harlan strain susceptible bed bug eggs compared to Richmond resistant bed bug eggs, including, TCONS00011313 [peroxidase-like], TCONS00040167 [cholinesterase activity], and TCONS00042174 [cytochrome P450]. However, one transcript, TCONS00044876 [esterase], was more highly accumulated in Richmond resistant bed bug eggs (19 fold difference).

Transcripts Differentially Expressed Between Richmond Resistant and Epic Center Bed Bug Eggs

There were a total of 100 transcripts differentially expressed between Richmond resistant strain bed bug eggs and Epic Center resistant strain bed bug eggs after excluding transcripts with peptide lengths lower than 100 amino acids and transposons (Appendix A). Of those 100 transcripts, 24 transcripts associated with cuticle proteins were accumulated at higher levels in Epic Center eggs. Four additional transcripts associated with the cuticle or peritrophic membrane were accumulated at higher levels in Epic Center eggs than Richmond eggs. These transcripts included TCONS00000478 [protein yellow-like], TCONS00031459 [chitin binding peritrophin], TCONS00047692 [chitin binding] and TCONS00043700 [laccase]. Other transcripts differentially expressed encoded with metabolic processes or cellular transport were TCONS00008319 [bifunctional atp-dependent dihydroxyacetone kinase fad-amp lyase], TCONS00026983 [alpha-n-acetylgalactosaminidase-like], TCONS00034514 [acyl-delta-14 desaturase], TCONS00034797 [probable adenylate kinase isoenzyme], TCONS00000495 [serine protease like], and TCONS00029798 [gtp binding protein].

Association of Significantly Differentially Expressed Transcripts with Genes and Cuticular Proteins Identified in Adult Bed Bugs

A BLAST comparison of our significantly differentially expressed bed bug egg transcripts to adult bed bug genes identified in Adelman et al. (2011) and Koganemaru et al. (2013) resulted in 18 matches (Table 6.1). Of those, 15 transcripts were associated with cuticle proteins and one transcript each was associated with a carboxylesterase, acetylcholinesterase, and cytochrome P450 gene (Table 6.1).

The carboxylesterase (TCONS00044876) was accumulated to a higher extent in both the Richmond (19 fold) and Epic Center resistant eggs (30 fold) compared to the Harlan susceptible strain eggs. Similarly, adult Richmond resistant bed bugs were found to have a 6.8 fold increase

of this carboxylesterase gene compared to adult susceptible bed bugs (Adelman et al. 2011). The cytochrome P450 monooxygenase we identified (TCONS00042174) was down regulated in our Richmond resistant bed bug eggs compared to the Harlan susceptible strain. Adelman et al. (2011) found no difference in the expression of the same P450 gene between Richmond resistant and Harlan susceptible adult bed bugs. The metabolic enzyme acetylcholinesterase (TCONS00040167) was also down regulated in Richmond resistant bed bug eggs compared to Harlan susceptible bed bug eggs. However, in adult bed bugs this gene was accumulated in higher levels in Richmond resistant adult bed bugs (≤ 2 fold) compared to Harlan susceptible adult bed bugs (Adelman et al. 2011).

Discussion

Our RNA-Seq analysis of a pyrethroid susceptible strain and two pyrethroid resistant strains of bed bug eggs identified 387 transcripts as significantly differentially expressed when mapped to the bed bug genome. However, 228 transcripts differentially expressed were comprised of lower than 100 amino acids, several of which identified as transposable elements using BLAST2GO, and thus were probably not indicative of proteins. Unannotated transcripts were observed to have little protein-coding capacity (i.e. lower than 100 amino acids) (Kapranov et al. 2002).

Cytochrome P450s, esterases and glutathione-S transferases are widely known to metabolize pesticides in insects. Although adult resistant bed bugs have been found with overexpressed levels of multiple cytochrome P450s (Mamidala et al. 2012, Adelman et al. 2011), cytochrome P450 doesn't appear to play a role in bed bug egg resistance. We observed only one transcript encoding for a cytochrome P450 enzyme [TCONS00042174] and it was down-regulated in Richmond resistant strain bed bug eggs. Another metabolic enzyme,

acetylcholinesterase, was also down regulated in Richmond resistant strain bed bug eggs. However, one metabolic enzyme, a carboxylesterase, was highly up-regulated in both Richmond resistant and Epic Center resistant strain bed bug eggs. Therefore, the overexpression of this gene in both resistant strain bed bug eggs suggests that this carboxylesterase might contribute to insecticide resistance. Transcript expression differences among eggs from both of our resistant strain are probably a result of differences in their previous insecticide exposure.

The insect cuticle is comprised of a chitinous matrix that is bound together with proteins. Insect cuticle proteins can be classified by a conserved domain of amino acids using the R&R consensus (Rebers and Riddiford, 1988) and are classified as RR-1 (soft, flexible cuticle), RR-2 (hard cuticle) or RR-3 (unclassified function). Using qPCR, Koganemaru et al. (2013) revealed 70% of 62 cuticle protein (CPR) encoding contigs were overexpressed in Richmond strain adult male bed bugs. Furthermore, when the 62 CPRs were classified into the RR consensus, 14 contigs encoded as RR-1 type, 31 to RR-2 type and a single RR-3 type (Koganemaru et al. 2013). The authors suggested that the differences observed between topical and injection applications of deltamethrin in combination with the overexpression of cuticular proteins provides evidence for decreased cuticular insecticide penetration in at least one population of adult bed bugs (Richmond resistant strain).

We found a large number of transcripts (24) that were up-regulated in Epic Center resistant strain bed bug eggs encoded for cuticular proteins and structural components of the cuticle. Interestingly, 8 transcripts associated with cuticle proteins were down regulated in Richmond strain bed bug eggs compared with Harlan strain bed bug eggs. Similarly, Mamidala et al. (2012) found 8 transcripts down regulated in their pyrethroid resistant strain adult bed bugs. The presence of cuticular proteins overexpressed in Epic Center resistant strain bed bug eggs

suggests that cuticular penetration resistance might develop early during development. Although not a cuticle protein, similar results were found in the cytochrome P450 (CYP9) (Bai et al. 2011). This cytochrome P450 was demonstrated to be accumulated at higher levels in early instar bed bugs compared to later stages (Bai et al. 2011).

While none of our transcripts were identified as eggshell proteins (chorion proteins), it is likely that some novel genes that remain unidentified may be components of the eggshell. Various proteins (chorion and vitelline membrane components) and enzymes (phenoloxidase, peroxidase and laccase) involved in chorion structuring have been identified in *Anopheles gambiae* using proteomics (Amenya et al. 2010). We identified two transcripts in bed bug eggs that were associated with cuticle structure that have bene previously identified as genes associated with chorion structuring in *Anopheles gambiae* mosquito eggs, TCONS00011313 [peroxidase-like] and TCONS00043700 [laccase], however, further analyses would need to be conducted to determine if these enzymes are functioning in chorion or cuticle structuring.

Heat shock proteins are instrumental in maintaining or returning proteins to their previous functional state when exposed to stressful environmental factors. We identified one transcript associated with a heat shock protein, TCONS00035977, which was elevated at higher levels in Epic Center resistant strain bed bug eggs compared to Harlan susceptible strain bed bug eggs. Two heat shock proteins (Hsp) have been identified in adult bed bugs (Hsp70 and Hsp90) and were elevated in response to heat stress, cold stress, and during dehydration and rehydration (Benoit et al. 2009).

We have identified multiple transcripts associated with pyrethroid resistance in bed bug eggs that are similar to those identified in adult bed bugs. Our research indicates that insecticide resistance occurs early in bed bug development and may further complicate bed bug control

strategies. Metabolic resistance may be occurring by at least one overexpressed carboxylesterase identified in both resistant strains. Also, the overexpression of cuticular proteins suggests decreased insecticide penetration in bed bug embryos. Furthermore, heat shock proteins were overexpressed in Epic Center resistant strain eggs and are another indication of gene expression as a result of environmental stressors (including pesticides). The observed accumulation of bed bug egg genes in resistant populations have probably been selected for due to insecticide exposure.

Table 6.1 BLAST comparison of our bed bug egg transcripts that were previously identified in adult bed bugs. Cuticular proteins identified as RR1 and RR2 was demonstrated by Koganemaru et al. (2013).

Egg Transcripts	Adult Genes	Adult Bed Bug Genes Identified in Previous Literature
TCONS_00007675	Contig24800 (RR-1)	Koganemaru et al. 2013
TCONS_00014802	Contig5191 (RR-2)	Koganemaru et al. 2013
TCONS_00014803	Contig5191 (RR-2)	Koganemaru et al. 2013
TCONS_00018445	Contig8021 (RR-1)	Koganemaru et al. 2013
TCONS_00025086	Contig10305 (RR-2 resilin-like)	Koganemaru et al. 2013
TCONS_00025088	Contig8456 (RR-2 resilin-like)	Koganemaru et al. 2013
TCONS_00029404	Contig10887 (RR-2)	Koganemaru et al. 2013
TCONS_00044876	CE3959	Adelman et al. 2013
TCONS_00042174	CYP398A1	Adelman et al. 2013
TCONS_00018447	Contig10008 (RR-1)	Koganemaru et al. 2013
TCONS_00041409	Contig24230 (RR-2)	Koganemaru et al. 2013
TCONS_00018444	Contig329 (RR-1)	Koganemaru et al. 2013
TCONS_00040317	Contig5182	Koganemaru et al. 2013
TCONS_00018440	Contig820 (RR-1)	Koganemaru et al. 2013
TCONS_00040167	CE20922	Adelman et al. 2013
TCONS_00016130	Contig1800 (RR-2)	Koganemaru et al. 2013
TCONS_00040201	Contig469 (RR-2)	Koganemaru et al. 2013
TCONS_00017900	Contig2471 (RR-2)	Koganemaru et al. 2013

Table 6.2 Top 30 differentially expressed transcripts between Harlan susceptible strain bed bug eggs and Epic Center resistant strain eggs.

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00018435	237	endocuticle structural glycoprotein bd-1	Harlan	Epic	6.18372	461.348	6.22124
TCONS_00044876	248	esterase [Pyrrhocoris apterus]	Harlan	Epic	10.5723	295.909	4.80679
TCONS_00008272	295	upf0439 protein c9orf30-like protein	Harlan	Epic	12.0697	195.29	4.01615
TCONS_00036851	204	cg2150 cg2150-pa; protein_coding_gene	Harlan	Epic	17.1907	215.627	3.64883
TCONS_00000246	488	cg12164 cg12164-pa; Ferritin-like superfamily.	Harlan	Epic	13.5242	155.512	3.52342
TCONS_00047881	311	gcr cg5812-pa; proteinaceous extracellular matrix	Harlan	Epic	92.1401	998.742	3.43821
TCONS_00041409	139	pupal cuticle protein	Harlan	Epic	9.74982	88.0829	3.17541
TCONS_00014495	161	hypothetical protein Phum_PHUM506450 [Pediculus humanus corporis]	Harlan	Epic	11.7275	105.464	3.16879
TCONS_00025086	407	cuticle protein	Harlan	Epic	3.79158	33.3837	3.13827
TCONS_00037308	215	cuticular protein 4	Harlan	Epic	45.8634	332.08	2.85612
TCONS_00022728	647	isoform a	Harlan	Epic	5.85253	39.3368	2.74875
TCONS_00040201	185	Tpa: cuticle protein;structural constituent of cuticle	Harlan	Epic	173.785	1113.25	2.67941
TCONS_00008533	184	NA	Harlan	Epic	78.7558	322	2.0316
TCONS_00047692	675	chitin binding;chitin metabolic process;extracellular region	Harlan	Epic	17.0623	67.2541	1.97881
TCONS_00048502	144	cg31997 cg31997-pa; extracellular space	Harlan	Epic	114.184	442.53	1.95441
TCONS_00007675	147	tpa: cuticle protein	Harlan	Epic	45.1659	151.743	1.74833
TCONS_00004470	112	NA	Harlan	Epic	42.8435	140.766	1.71615
TCONS_00039739	656	NA	Harlan	Epic	22.0634	72.1717	1.70978
TCONS_00045417	457	protein odr-4 homolog [Nasonia vitripennis]	Harlan	Epic	7.30474	22.6639	1.63349
TCONS_00025192	227	c-type lectin 27kd	Harlan	Epic	29.7317	83.24	1.48527
TCONS_00037526	142	NA	Harlan	Epic	1675.4	4628.34	1.46599
TCONS_00035977	398	heat shock protein	Harlan	Epic	35.5098	93.8691	1.40243
TCONS_00002025	379	NA	Harlan	Epic	49.6349	129.67	1.38541
TCONS_00016409	436	serine protease h164	Harlan	Epic	18.7873	47.6462	1.3426
TCONS_00002944	200	ccaat enhancer-binding	Harlan	Epic	19.6669	49.026	1.31777

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00043700	744	laccase-like multicopper oxidase 1	Harlan	Epic	17.165	42.0423	1.29237
TCONS_00009212	190	similar to GA22033-PA [Tribolium castaneum]	Harlan	Epic	35.2651	85.7977	1.2827
TCONS_00021557	191	Uncharacterized protein ART2 [Camponotus floridanus]	Harlan	Epic	264.059	630.363	1.25532
TCONS_00036748	645	zinc carboxypeptidase	Harlan	Epic	79.4176	184.912	1.2193
TCONS_00017236	100	isoform a	Harlan	Epic	229.249	480.287	1.06698

Table 6.3 Top 30 differentially expressed transcripts between Richmond resistant strain eggs and Epic Center resistant strain eggs.

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00018435	237	endocuticle structural glycoprotein bd-1	Richmond	Epic	3.98677	461.348	6.85449
TCONS_00040201	185	Tpa: cuticle protein;structural constituent of cuticle	Richmond	Epic	38.1459	1113.25	4.86711
TCONS_00036851	204	cg2150 cg2150-pa; protein_coding_gene	Richmond	Epic	10.1041	215.627	4.41552
TCONS_00047881	311	gcr cg5812-pa; proteinaceous extracellular matrix	Richmond	Epic	51.9735	998.742	4.26426
TCONS_00000246	488	cg12164 cg12164-pa; Ferritin-like superfamily.	Richmond	Epic	9.93933	155.512	3.96774
TCONS_00031453	232	cuticular protein analogous to peritrophins 3-d1	Richmond	Epic	7.52577	86.955	3.53036
TCONS_00025192	227	c-type lectin 27kd	Richmond	Epic	10.3858	83.24	3.00266
TCONS_00037308	215	cuticular protein 4	Richmond	Epic	42.0303	332.08	2.98203
TCONS_00048502	144	cg31997 cg31997-pa; extracellular space	Richmond	Epic	74.6761	442.53	2.56706
TCONS_00002025	379	uncharacterized protein LOC102670836 [Apis dorsata]	Richmond	Epic	23.4773	129.67	2.4655
TCONS_00032452	210	myosin light chain 2	Richmond	Epic	145.624	781.282	2.4236
TCONS_00022728	647	isoform a	Richmond	Epic	7.48696	39.3368	2.39343
TCONS_00029404	305	structural constituent of cuticle	Richmond	Epic	25.008	129.633	2.37398
TCONS_00017236	100	isoform a	Richmond	Epic	94.3287	480.287	2.34813
TCONS_00018432	625	structural constituent of cuticle	Richmond	Epic	8.92201	43.6374	2.29012
TCONS_00031459	277	chitin binding peritrophin-	Richmond	Epic	36.7694	171.215	2.21923
TCONS_00007966	134	r2 protein	Richmond	Epic	577.681	2534.72	2.13348
TCONS_00001092	888	long form-like myosin complex	Richmond	Epic	12.3923	52.2672	2.07646
TCONS_00021359	127	NA	Richmond	Epic	419.423	1713.2	2.03021
TCONS_00039003	880	zinc finger ccch domain-containing protein 13- partial-chitin binding	Richmond	Epic	4.0125	15.5518	1.9545
TCONS_00047692	675	chitin binding;chitin metabolic process;extracellular region	Richmond	Epic	19.3277	67.2541	1.79895
TCONS_00004470	112	NA	Richmond	Epic	41.2338	140.766	1.7714
TCONS_00005195	462	hypothetical protein LOC100679659 isoform 1 [Nasonia vitripennis]	Richmond	Epic	18.0523	61.1809	1.7609
TCONS_00030655	191	NA	Richmond	Epic	16.833	55.0588	1.70968
TCONS_00015343	252	similar to CG14661 CG14661-PA [Tribolium castaneum]	Richmond	Epic	42.3725	129.108	1.60737

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00031130	288	tropomyosin 2	Richmond	Epic	60.543	176.12	1.54053
TCONS_00043700	744	laccase-like multicopper oxidase 1	Richmond	Epic	15.2878	42.0423	1.45946
TCONS_00018341	216	structural constituent of cuticle	Richmond	Epic	63.8118	143.749	1.17166
TCONS_00039739	656	NA	Richmond	Epic	32.4929	72.1717	1.15131
TCONS_00013266	167	NA	Richmond	Epic	82.5627	172.278	1.06117

Table 6.4 Top 26 differentially expressed transcripts between Harlan susceptible strain eggs and Richmond resistant strain eggs.

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00044876	248	esterase [Pyrrhocoris apterus]	Harlan	Richmond	10.5723	185.659	4.13429
TCONS_00008272	295	upf0439 protein c9orf30-like protein	Harlan	Richmond	12.0697	197.676	4.03367
TCONS_00023452	722	rho gtpase-activating protein 6	Harlan	Richmond	3.34151	17.1503	2.35966
TCONS_00036033	186	heat shock protein	Harlan	Richmond	25.0064	81.143	1.69817
TCONS_00022962	122	NA	Harlan	Richmond	617.175	1682.08	1.4465
TCONS_00001465	172	integral component of membrane	Harlan	Richmond	171.451	416.384	1.28012
TCONS_00008347	300	NA	Harlan	Richmond	57.5876	128.372	1.1565
TCONS_00005065	270	hypothetical protein LOC100159700 [Acyrthosiphon pisum]	Harlan	Richmond	453.389	949.219	1.06599
TCONS_00015560	188	g patch domain-containing protein 4	Harlan	Richmond	49.7302	103.663	1.05971
TCONS_00037526	142	NA	Harlan	Richmond	1675.4	3490.36	1.05887
TCONS_00018945	893	spindle pole;microtubule organizing center	Harlan	Richmond	25.0629	51.5112	1.03933
TCONS_00009279	132	NA	Harlan	Richmond	62.3674	113.794	0.86756
TCONS_00016069	130	NA	Harlan	Richmond	3493.46	1594.17	-1.13185
TCONS_00021359	127	NA	Harlan	Richmond	981.039	419.423	-1.2259
TCONS_00020874	222	mitochondrial import inner membrane translocase subunit tim8-like	Harlan	Richmond	113.891	48.0712	-1.24441
TCONS_00017236	100	isoform a	Harlan	Richmond	229.249	94.3287	-1.28115
TCONS_00001092	888	long form-like myosin complex	Harlan	Richmond	35.2769	12.3923	-1.50928
TCONS_00000524	156	NA	Harlan	Richmond	29.6046	8.88662	-1.73612
TCONS_00024539	456	colmedin	Harlan	Richmond	145.799	40.8733	-1.83475
TCONS_00032452	210	myosin light chain 2	Harlan	Richmond	527.761	145.624	-1.85764
TCONS_00042174	500	cytochrome p450	Harlan	Richmond	20.4073	4.94696	-2.04447
TCONS_00007966	134	r2 protein	Harlan	Richmond	2391.27	577.681	-2.04943
TCONS_00040201	185	Tpa: cuticle protein;structural constituent of cuticle	Harlan	Richmond	173.785	38.1459	-2.1877
TCONS_00016801	366	sodium- and chloride-dependent gaba transporter 1-like	Harlan	Richmond	28.9531	6.30806	-2.19845
TCONS_00014989	365	NA	Harlan	Richmond	30.6601	6.56982	-2.22244
TCONS_00010600	183	odorant-binding protein partial	Harlan	Richmond	51.8274	10.7607	-2.26795

Chapter 7. Summary

Bed bug infestations have rapidly increased and spread in the United States since a resurgence that began in the 1990s. As of 2010, bed bugs have been documented in all 50 states. Bed bug infestations have exponentially increased in lower income housing facilities. The increase is partly due to the high costs of bed bug treatments and the lack of insecticide efficacy as a result of insecticide resistance. Bed bug eggs further complicate treatment efforts because liquid formulation insecticides are less efficacious against eggs than other bed bug life stages. Although bed bug eggs present a unique challenge to bed bug control, few studies have focused on the egg stage.

In this study, bed bug egg morphological features were characterized using scanning electron microscopy. No morphological differences were found between susceptible and resistant strain eggs, however, detailed chorionic and respiratory structures were observed for all egg strains. The outer bed bug eggshell is characterized with spike projections that form polygonal patterns. Similar structuring was observed on the egg operculum. The operculum is located on the anterior portion of the egg and fits within the egg collar. Within the collar, multiple layers were observed (exochorion, pillar region and endochorion) that comprise the columnar region. Similar column and pillar region structures have been observed in other hemipteran eggs and have been suggested to function in egg respiration. Small holes, possibly aeropyles, were observed on the dorsal and ventral side of the operculum rim which is most likely the site of gas exchange and an entry point for environmental toxicants. Eggs dipped into dyed water suggested that the operculum was the site of gas exchange because the water entered the operculum and disseminated throughout the eggshell posteriorly.

Insect egg water loss is usually directly related to respiration activity. Therefore, the patterns of water loss and respiration in a given insect species would be expected to be similar. However, we did not see this trend in bed bug eggs. There were no significant differences between strains with regard to water loss but there were significant differences in oxygen consumption between one susceptible strain of eggs (Harlan) and one resistant strain of eggs (Epic Center). Interestingly, initial (first 4 hrs) water loss across the chorion (chorionic permeability) was significantly different between all three strains. As expected, Harlan susceptible strain egg metabolic rates increased as temperatures increased, until the eggs reached their thermal limit at 39 °C. The eggs we measured were not continuously exposed to any toxins, therefore we suspect that any differences in oxygen consumption are a result of strain differences and not associated with insecticide response.

Comparisons of resistance ratios between eggs and first instars indicated there was little difference in resistance between both stages. Our research is the first to suggest that treating newly hatched nymphs with insecticides may not be any more effective than treating bed bug eggs. The eggshell may provide some protection, but most likely, the embryo inside of the egg has developed resistance mechanisms. Resistance was documented in both Richmond and Epic Center strain eggs and first instars, with Epic Center strain eggs being the most resistant to both imidacloprid/β-cyfluthrin and acetamiprid/bifenthrin. Resistance differences between egg strains are probably a result of previous insecticide exposure.

We compared differentially expressed transcripts to identify genes associated with insecticide resistance between susceptible and resistant strain eggs. There were 387 transcripts differentially expressed in all three strains, however, 228 of those transcripts were predicted to encode proteins less than 100 amino acids and thus were probably non-coding. Multiple

transcripts associated with cuticular proteins and cuticle structure were identified and highly expressed in Epic Center resistant eggs, indicating that insecticide penetration resistance may develop early in bed bug development. Also, one carboxylesterase transcript was highly upregulated in both resistant strain eggs (Richmond and Epic Center), indicating that this metabolic enzyme may play a role in insecticide resistance. Multiple other transcripts associated with cell transport and other activities in the cell were expressed at higher levels in resistant strain eggs, and the over expression of these genes in resistant strains may be a result of insecticide exposure.

This study is the first to document insecticide resistance in bed bug eggs and first instars. This study is also the first to characterize morphological, physiological and molecular differences between eggs from susceptible and resistant bed bug strains. Bed bug eggs are a challenge for bed bug treatments and this research provides much needed knowledge of bed bug egg biology.

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Appendix A. Transcripts identified and expressed in three bed bug egg strains (Harlan, Richmond and Epic Center).

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00028118	NA	NA	Harlan	Epic	5.35831	0	_
TCONS_00029603	NA	NA	Richmond	Epic	1135.73	0	_
TCONS_00011313	756	Peroxidase like	Harlan	Richmond	8.09977	0	_
TCONS_00014324	589	pupal cuticle protein	Harlan	Richmond	6.64929	0	_
TCONS_00048963	569	hypothetical protein EAI_09843 [Harpegnathos saltator]	Harlan	Richmond	7.09822	0	_
TCONS_00041427	424	NA	Harlan	Richmond	78.9913	0	_
TCONS_00040167	374	Cholinesterase activity	Harlan	Richmond	3.41787	0	_
TCONS_00007366	353	NA	Harlan	Richmond	5.30321	0	_
TCONS_00036109	313	NA	Harlan	Richmond	9.17454	0	_
TCONS_00008319	308	bifunctional atp-dependent dihydroxyacetone kinase fad-amp lyase	Richmond	Epic	4.8191	0	_
TCONS_00034369	280	clotting factor b-like	Harlan	Richmond	4.80515	0	_
TCONS_00038706	246	NA	Harlan	Richmond	9.53279	0	_
TCONS_00025088	245	isoform a	Harlan	Richmond	19.7106	0	_
TCONS_00015628	242	NA	Harlan	Richmond	8.5527	0	_
TCONS_00018447	232	structural constituent of cuticle	Harlan	Richmond	25.7801	0	_
TCONS_00006060	230	unknown partial	Harlan	Richmond	4.28455	0	_
TCONS_00014802	230	pupal cuticle protein	Harlan	Richmond	90.768	0	_
TCONS_00041425	229	hypothetical protein LOTGIDRAFT_71141, partial [Lottia gigantea]	Harlan	Richmond	72.4529	0	_
TCONS_00006910	226	NA	Harlan	Epic	4.58616	0	_
TCONS_00006910	226	NA	Richmond	Epic	7.50512	0	_
TCONS_00011028	222	RNA-directed DNA polymerase activity	Harlan	Richmond	14.8839	0	_
TCONS_00011028	222	RNA-directed DNA polymerase activity	Harlan	Epic	14.8839	0	_
TCONS_00012195	218	defective proboscis extension response	Harlan	Richmond	3.3961	0	_
TCONS_00017898	210	cuticular protein 62bc cg1919-pa	Harlan	Richmond	6.93839	0	_
TCONS_00009872	205	cg34114 cg34114-pb;CD80-like, immunoglobulin C2-set;	Richmond	Epic	4.84256	0	_
TCONS_00043404	196	cd63 antigen-like	Harlan	Epic	4.25353	0	_
TCONS_00036366	188	NA	Harlan	Richmond	42.02	0	_
TCONS_00017900	186	similar to Cuticular protein 62Bc CG1919-PA [Tribolium castaneum]	Harlan	Richmond	6.41968	0	_
TCONS_00008533	184	NA	Harlan	Richmond	78.7558	0	_
TCONS_00035772	171	NA	Harlan	Richmond	28.5935	0	_
TCONS_00019750	169	NA	Richmond	Epic	7.55258	0	_
TCONS_00000629	168	neurotransmitter transport	Harlan	Richmond	3.11303	0	_
TCONS_00025390	167	NA	Harlan	Richmond	5.96965	0	_
TCONS_00049006	157	NA	Richmond	Epic	9.08757	0	_
TCONS_00001219	156	NA	Harlan	Richmond	14.0043	0	_
TCONS_00018552	156		Harlan	Richmond	144.136	0	_
TCONS_00049007	153	NA	Richmond	Epic	14.6351	0	_
TCONS_00000373	151	structural constituent of cuticle	Harlan	Richmond	14.8053	0	_
TCONS_00018444	151	endocuticle structural glycoprotein abd-4-like	Harlan	Richmond	12.13	0	_
TCONS_00035929	151	NA	Harlan	Richmond	6.04298	0	_
TCONS_00011698	148	NA	Harlan	Richmond	6.35674	0	_
TCONS_00011698	148	NA	Harlan	Epic	6.35674	0	_
TCONS_00012698	144	NA	Harlan	Epic	4.71578	0	_
TCONS_00012698	144	NA	Richmond	Epic	6.31775	0	_
TCONS_00008279	138	NA	Harlan	Richmond	6.64268	0	_
TCONS_00043037	138	NA	Harlan	Epic	6.71512	0	_
TCONS_00043037	138	NA	Richmond	Epic	4.54891	0	_
TCONS_00040536	137	NA	Harlan	Richmond	9.5771	0	_
TCONS_00040536	137	NA	Harlan	Epic	9.5771	0	_
TCONS_00038056	134	NA	Harlan	Epic	8.60383	0	_

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00026972	131	NA	Harlan	Richmond	5.31467	0	_
TCONS_00013717	127	hemicentin-1-like isoform x1	Harlan	Richmond	3.95826	0	_
TCONS_00030438	121	NA	Harlan	Epic	5.76485	0	_
TCONS_00027397	116	NA	Harlan	Richmond	7.77021	0	_
TCONS_00042593	107	NA	Harlan	Richmond	78.7627	0	_
TCONS_00024886	106	NA	Harlan	Epic	9.53092	0	_
TCONS_00038217	106	NA	Richmond	Epic	11.1966	0	_
TCONS_00024487	105	NA	Richmond	Epic	12.6397	0	_
TCONS_00030480	102	NA	Richmond	Epic	12.1891	0	_
TCONS_00029603	NA	NA	Harlan	Richmond	0	1135.73	_
TCONS_00014324	589	pupal cuticle protein	Richmond	Epic	0	7.08629	_
TCONS_00048963	569	hypothetical protein EAI_09843 [Harpegnathos saltator]	Richmond	Epic	0	14.3693	_
TCONS_00014394	556	structural constituent of cuticle	Richmond	Epic	0	9.28726	_
TCONS_00041427	424	NA	Richmond	Epic	0	90.7041	_
TCONS_00000478	417	protein yellow-like [Acyrthosiphon pisum]	Richmond	Epic	0	6.46992	_
TCONS_00007366	353	NA	Richmond	Epic	0	12.5205	_
TCONS_00036109	313	NA	Richmond	Epic	0	15.5454	_
TCONS_00016431	252	cuticle protein	Richmond	Epic	0	43.9409	_
TCONS_00005981	248	putative nuclease HARBI1-like [Acyrthosiphon pisum]	Harlan	Epic	0	5.44507	_
TCONS_00038706	246	NA	Richmond	Epic	0	15.0458	_
TCONS_00025088	245	isoform a	Richmond	Epic	0	49.381	_
TCONS_00015628	242	NA	Richmond	Epic	0	20.3569	_
TCONS_00018447	232	structural constituent of cuticle	Richmond	Epic	0	49.8801	_
TCONS_00014802	230	pupal cuticle protein	Richmond	Epic	0	87.5825	_
TCONS_00016433	230	larval cuticle protein a3a-like	Richmond	Epic	0	9.53113	_
TCONS_00041425	229	hypothetical protein LOTGIDRAFT_71141, partial [Lottia gigantea]	Richmond	Epic	0	97.8449	_
TCONS_00046876	228	unknown secreted protein	Richmond	Epic	0	5.44467	_
TCONS_00016130	227	larval cuticle protein a3a	Richmond	Epic	0	76.3135	_
TCONS_00026983	221	alpha-n-acetylgalactosaminidase-like	Richmond	Epic	0	8.04419	_
TCONS_00017898	210	cuticular protein 62bc cg1919-pa	Richmond	Epic	0	13.904	_
TCONS_00040532	197	NA	Richmond	Epic	0	14.1925	_
TCONS_00036366	188	NA	Richmond	Epic	0	6.07399	_
TCONS_00017900	186	similar to Cuticular protein 62Bc CG1919-PA [Tribolium castaneum]	Richmond	Epic	0	16.5813	_
TCONS_00023767	185	NA	Richmond	Epic	0	6.12871	_
TCONS_00008533	184	NA	Richmond	Epic	0	322	_
TCONS_00034514	182	acyl- delta-14 desaturase	Richmond	Epic	0	21.691	_
TCONS_00027298	181	unknown secreted protein	Richmond	Epic	0	7.65472	_
TCONS_00046569	180	NA	Richmond	Epic	0	10.132	_
TCONS_00008539	176	NA	Richmond	Epic	0	80.0952	_
TCONS_00035772	171	NA	Richmond	Epic	0	31.7863	_
TCONS_00018440	169	cuticular protein rr-1 motif 32	Richmond	Epic	0	28.2021	_
TCONS_00040399	168	odorant-binding protein 6	Richmond	Epic	0	16.0236	_
TCONS_00025390	167	NA	Richmond	Epic	0	25.542	_
TCONS_00001219	156	NA	Richmond	Epic	0	104.388	_
TCONS_00018552	156		Richmond	Epic	0	126.551	_
TCONS_00002168	153	larval cuticle protein a3a-like	Richmond	Epic	0	16.9237	_
TCONS_00033442	153		Richmond	Epic	0	5.99318	_
TCONS_00040489	153	cuticular protein analogous to peritrophins 1-a	Richmond	Epic	0	31.6816	_
TCONS_00000373	151	structural constituent of cuticle	Richmond	Epic	0	54.9192	_
TCONS_00018444	151	endocuticle structural glycoprotein abd-4-like	Richmond	Epic	0	102.607	_
TCONS_00035929	151	NA	Richmond	Epic	0	6.53361	_
TCONS_00000198	147	NA	Richmond	Epic	0	7.44016	_

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00034797	136	probable adenylate kinase isoenzyme	Richmond	Epic	0	5.01649	_
TCONS_00040490	133	cuticular protein analogous to peritrophins 1-a	Richmond	Epic	0	11.8537	_
TCONS_00004841	130	NA	Richmond	Epic	0	5.32051	_
TCONS_00006119	127	NA	Richmond	Epic	0	6.67862	_
TCONS_00006303	125	NA	Harlan	Richmond	0	8.16975	_
TCONS_00006303	125	NA	Harlan	Epic	0	15.7814	_
TCONS_00040317	120	pupal cuticle	Richmond	Epic	0	10.8722	_
TCONS_00009707	119	NA	Richmond	Epic	0	4.96666	_
TCONS_00000495	115	serine protease -like	Richmond	Epic	0	12.1262	_
TCONS_00042593	107	NA	Richmond	Epic	0	127.135	_
TCONS_00028796	105	cysteine rich secreted protein	Richmond	Epic	0	9.44623	_
TCONS_00046570	105	NA	Richmond	Epic	0	15.6551	_
TCONS_00003418	101	NA	Harlan	Richmond	0	10.7162	_
TCONS_00048871	101	NA	Richmond	Epic	0	14.6957	_
TCONS_00018435	237	endocuticle structural glycoprotein bd-1	Richmond	Epic	3.98677	461.348	6.85449
TCONS_00018435	237	endocuticle structural glycoprotein bd-1	Harlan	Epic	6.18372	461.348	6.22124
TCONS_00040201	185	Tpa: cuticle protein;structural constituent of cuticle	Richmond	Epic	38.1459	1113.25	4.86711
TCONS_00044876	248	esterase [Pyrrhocoris apterus]	Harlan	Epic	10.5723	295.909	4.80679
TCONS_00036851	204	cg2150 cg2150-pa; protein_coding_gene	Richmond	Epic	10.1041	215.627	4.41552
TCONS_00047881	311	gcr cg5812-pa; proteinaceous extracellular matrix	Richmond	Epic	51.9735	998.742	4.26426
TCONS_00044876	248	esterase [Pyrrhocoris apterus]	Harlan	Richmond	10.5723	185.659	4.13429
TCONS_00008272	295	upf0439 protein c9orf30-like protein	Harlan	Richmond	12.0697	197.676	4.03367
TCONS_00008272	295	upf0439 protein c9orf30-like protein	Harlan	Epic	12.0697	195.29	4.01615
TCONS_00000246	488	cg12164 cg12164-pa; Ferritin-like superfamily.	Richmond	Epic	9.93933	155.512	3.96774
TCONS_00036851	204	cg2150 cg2150-pa; protein_coding_gene	Harlan	Epic	17.1907	215.627	3.64883
TCONS_00031453	232	cuticular protein analogous to peritrophins 3-d1	Richmond	Epic	7.52577	86.955	3.53036
TCONS_00000246	488	cg12164 cg12164-pa; Ferritin-like superfamily.	Harlan	Epic	13.5242	155.512	3.52342
TCONS_00047881	311	gcr cg5812-pa; proteinaceous extracellular matrix	Harlan	Epic	92.1401	998.742	3.43821
TCONS_00041409	139	pupal cuticle protein	Harlan	Epic	9.74982	88.0829	3.17541
TCONS_00014495	161	hypothetical protein Phum_PHUM506450 [Pediculus humanus corporis]	Harlan	Epic	11.7275	105.464	3.16879
TCONS_00025086	407	cuticle protein	Harlan	Epic	3.79158	33.3837	3.13827
TCONS_00025192	227	c-type lectin 27kd	Richmond	Epic	10.3858	83.24	3.00266
TCONS_00037308	215	cuticular protein 4	Richmond	Epic	42.0303	332.08	2.98203
TCONS_00037308	215	cuticular protein 4	Harlan	Epic	45.8634	332.08	2.85612
TCONS_00022728	647	isoform a	Harlan	Epic	5.85253	39.3368	2.74875
TCONS_00040201	185	Tpa: cuticle protein;structural constituent of cuticle	Harlan	Epic	173.785	1113.25	2.67941
TCONS_00048502	144	cg31997 cg31997-pa; extracellular space	Richmond	Epic	74.6761	442.53	2.56706
TCONS_00002025	379	uncharacterized protein LOC102670836 [Apis dorsata]	Richmond	Epic	23.4773	129.67	2.4655
TCONS_00032452	210	myosin light chain 2	Richmond	Epic	145.624	781.282	2.4236
TCONS_00022728	647	isoform a	Richmond	Epic	7.48696	39.3368	2.39343
TCONS_00029404	305	structural constituent of cuticle	Richmond	Epic	25.008	129.633	2.37398
TCONS_00023452	722	rho gtpase-activating protein 6	Harlan	Richmond	3.34151	17.1503	2.35966
TCONS_00017236	100	isoform a	Richmond	Epic	94.3287	480.287	2.34813
TCONS_00018432	625	structural constituent of cuticle	Richmond	Epic	8.92201	43.6374	2.29012
TCONS_00031459	277	chitin binding peritrophin-	Richmond	Epic	36.7694	171.215	2.21923
TCONS_00007966	134	r2 protein	Richmond	Epic	577.681	2534.72	2.13348
TCONS_00001092	888	long form-like myosin complex	Richmond	Epic	12.3923	52.2672	2.07646
TCONS_00008533	184	NA	Harlan	Epic	78.7558	322	2.0316
TCONS_00021359	127	NA	Richmond	Epic	419.423	1713.2	2.03021
TCONS_00047692	675	chitin binding;chitin metabolic process;extracellular region	Harlan	Epic	17.0623	67.2541	1.97881
TCONS_00039003	880	zinc finger ccch domain-containing protein 13- partial	Richmond	Epic	4.0125	15.5518	1.9545
TCONS_00048502	144	cg31997 cg31997-pa; extracellular space	Harlan	Epic	114.184	442.53	1.95441

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00004470	112	NA	Richmond	Epic	41.2338	140.766	1.7714
TCONS_00005195	462	hypothetical protein LOC100679659 isoform 1 [Nasonia vitripennis]	Richmond	Epic	18.0523	61.1809	1.7609
TCONS_00007675	147	tpa: cuticle protein	Harlan	Epic	45.1659	151.743	1.74833
TCONS_00004470	112	NA	Harlan	Epic	42.8435	140.766	1.71615
TCONS_00039739	656	NA	Harlan	Epic	22.0634	72.1717	1.70978
TCONS_00030655	191	NA	Richmond	Epic	16.833	55.0588	1.70968
TCONS_00036033	186	heat shock protein	Harlan	Richmond	25.0064	81.143	1.69817
TCONS_00045417	457	protein odr-4 homolog [Nasonia vitripennis]	Harlan	Epic	7.30474	22.6639	1.63349
TCONS_00015343	252	similar to CG14661 CG14661-PA [Tribolium castaneum]	Richmond	Epic	42.3725	129.108	1.60737
TCONS_00031130	288	tropomyosin 2	Richmond	Epic	60.543	176.12	1.54053
TCONS_00025192	227	c-type lectin 27kd	Harlan	Epic	29.7317	83.24	1.48527
TCONS_00037526	142	NA	Harlan	Epic	1675.4	4628.34	1.46599
TCONS_00043700	744	laccase-like multicopper oxidase 1	Richmond	Epic	15.2878	42.0423	1.45946
TCONS_00022962	122	NA	Harlan	Richmond	617.175	1682.08	1.4465
TCONS_00035977	398	heat shock protein	Harlan	Epic	35.5098	93.8691	1.40243
TCONS_00002025	379	NA	Harlan	Epic	49.6349	129.67	1.38541
TCONS_00016409	436	serine protease h164	Harlan	Epic	18.7873	47.6462	1.3426
TCONS_00002944	200	ccaat enhancer-binding	Harlan	Epic	19.6669	49.026	1.31777
TCONS_00043700	744	laccase-like multicopper oxidase 1	Harlan	Epic	17.165	42.0423	1.29237
TCONS_00009212	190	similar to GA22033-PA [Tribolium castaneum]	Harlan	Epic	35.2651	85.7977	1.2827
TCONS_00001465	172	integral component of membrane	Harlan	Richmond	171.451	416.384	1.28012
TCONS_00021557	191	Uncharacterized protein ART2 [Camponotus floridanus]	Harlan	Epic	264.059	630.363	1.25532
TCONS_00036748	645	zinc carboxypeptidase	Harlan	Epic	79.4176	184.912	1.2193
TCONS_00018341	216	structural constituent of cuticle	Richmond	Epic	63.8118	143.749	1.17166
TCONS_00008347	300	NA	Harlan	Richmond	57.5876	128.372	1.1565
TCONS_00039739	656	NA	Richmond	Epic	32.4929	72.1717	1.15131
TCONS_00017236	100	isoform a	Harlan	Epic	229.249	480.287	1.06698
TCONS_00005065	270	hypothetical protein LOC100159700 [Acyrthosiphon pisum]	Harlan	Richmond	453.389	949.219	1.06599
TCONS_00013266	167	NA	Richmond	Epic	82.5627	172.278	1.06117
TCONS_00015560	188	g patch domain-containing protein 4	Harlan	Richmond	49.7302	103.663	1.05971
TCONS_00037526	142	NA	Harlan	Richmond	1675.4	3490.36	1.05887
TCONS_00015962	810	e3 ubiquitin-protein ligase uhrf1	Harlan	Epic	22.9457	47.616	1.05322
TCONS_00018945	893	spindle pole;microtubule organizing center	Harlan	Richmond	25.0629	51.5112	1.03933
TCONS_00018341	216	cuticle protein-like precursor [Acyrthosiphon pisum]	Harlan	Epic	71.2287	143.749	1.01303
TCONS_00016125	247	NA	Harlan	Epic	107.224	211.605	0.980754
TCONS_00031458	317	chondroitin proteoglycan-2-like	Harlan	Epic	82.0946	159.528	0.958448
TCONS_00009279	132	NA	Harlan	Richmond	62.3674	113.794	0.86756
TCONS_00007993	506	alpha-L-fucosidase activity	Harlan	Epic	17.5089	9.09983	-0.944174
TCONS_00021633	561	replicase polyprotein 1a	Richmond	Epic	224.029	111.44	-1.00742
TCONS_00016069	130	NA	Harlan	Richmond	3493.46	1594.17	-1.13185
TCONS_00021359	127	NA	Harlan	Richmond	981.039	419.423	-1.2259
TCONS_00020874	222	mitochondrial import inner membrane translocase subunit tim8-like	Harlan	Richmond	113.891	48.0712	-1.24441
TCONS_00005065	270	hypothetical protein LOC100159700 [Acyrthosiphon pisum]	Richmond	Epic	949.219	390.598	-1.28106
TCONS_00017236	100	isoform a	Harlan	Richmond	229.249	94.3287	-1.28115
TCONS_00026320	NA	NA	Richmond	Epic	222.065	80.1174	-1.4708
TCONS_00001092	888	long form-like myosin complex	Harlan	Richmond	35.2769	12.3923	-1.50928
TCONS_00000524	156	NA	Harlan	Richmond	29.6046	8.88662	-1.73612
TCONS_00024539	456	colmedin	Harlan	Richmond	145.799	40.8733	-1.83475
TCONS_00032452	210	myosin light chain 2	Harlan	Richmond	527.761	145.624	-1.85764
TCONS_00029798	196	gtp-binding protein sar1	Richmond	Epic	107.631	29.45	-1.86975
TCONS_00042174	500	cytochrome p450	Harlan	Richmond	20.4073	4.94696	-2.04447
TCONS_00007966	134	r2 protein	Harlan	Richmond	2391.27	577.681	-2.04943

Transcript	Peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00016801	366	sodium- and chloride-dependent gaba transporter 1-like	Harlan	Richmond	28.9531	6.30806	-2.19845
TCONS_00014989	365	NA	Harlan	Richmond	30.6601	6.56982	-2.22244
TCONS_00000524	156	NA	Harlan	Epic	29.6046	6.14855	-2.2675
TCONS_00010600	183	odorant-binding protein partial	Harlan	Richmond	51.8274	10.7607	-2.26795
TCONS_00024539	456	colmedin	Harlan	Epic	145.799	27.4891	-2.40705
TCONS_00035212	529	enzymatic polyprotein endonuclease reverse	Richmond	Epic	26.8388	4.61306	-2.54053
TCONS_00048747	232	uncharacterized protein LOC100871677 [Apis florea]	Harlan	Epic	71.9259	11.8358	-2.60335
TCONS_00003364	228	hypothetical protein EAG_08288 [Camponotus floridanus]	Harlan	Epic	60.0624	8.63246	-2.79862
TCONS_00030040	152	nucleic acid binding;RNA-DNA hybrid ribonuclease activity	Richmond	Epic	39.9	4.07956	-3.28991

Appendix B. Bed bug egg transcripts from three strains (Harlan, Richmond and Epic Center) with peptide lengths lower than 100 amino acids.

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00006302	99	pol polyprotein DNA integration	Harlan	Epic	0	20.0803	-
TCONS_00006302	99	pol polyprotein DNA integration	Richmond	Epic	0	20.0803	-
TCONS_00018553	98	NA	Harlan	Richmond	11.9659	0	-
TCONS_00018553	98	NA	Richmond	Epic	0	20.9258	-
TCONS_00030759	97	NA	Richmond	Epic	27.6155	0	-
TCONS_00037075	97	NA	Richmond	Epic	118.084	322.692	1.45034
TCONS_00019243	96	NA	Richmond	Epic	0	5.24967	-
TCONS_00031133	96	tropomyosin 2	Richmond	Epic	133.407	689.432	2.36958
TCONS_00025188	95		Richmond	Epic	0	11.1728	-
TCONS_00008158	94		Harlan	Richmond	12.0459	0	-
TCONS_00008158	94		Richmond	Epic	0	25.1551	-
TCONS_00008944	94		Harlan	Richmond	0	13.4838	-
TCONS_00020388	94		Richmond	Epic	0	5.74603	-
TCONS_00043757	93		Harlan	Epic	591.053	104.819	-2.49539
TCONS_00043757	93		Richmond	Epic	612.894	104.819	-2.54774
TCONS_00017372	92		Richmond	Epic	0	16.3047	-
TCONS_00032395	92	NA	Richmond	Epic	0	5.58803	-
TCONS_00043063	92		Harlan	Richmond	0	27.8092	-
TCONS_00043063	92		Richmond	Epic	27.8092	0	-
TCONS_00016979	91		Harlan	Richmond	0	20.6557	-
TCONS_00015721	90		Richmond	Epic	0	6.61739	-
TCONS_00029602	90		Harlan	Richmond	0	8.27195	-
TCONS_00029602	90		Richmond	Epic	8.27195	0	-
TCONS_00013627	89		Harlan	Epic	101.152	338.078	1.74082
TCONS_00049225	89		Richmond	Epic	19.1876	0	-
TCONS_00011699	88		Harlan	Epic	39.2332	0	-
TCONS_00013399	88		Richmond	Epic	0	19.6229	-
TCONS_00006814	87		Harlan	Epic	50.7954	26.476	-0.940014
TCONS_00049010	87		Harlan	Epic	0	23.3464	-
TCONS_00009754	86	structural constituent of ribosome;ribosome;translation	Harlan	Richmond	196.316	68.5237	-1.5185
TCONS_00029069	86	extracellular region;chitin binding;chitin metabolic process	Harlan	Epic	7.02346	0	-
TCONS_00012796	85		Richmond	Epic	13.2864	0	-
TCONS_00023489	85		Harlan	Richmond	0	25.7043	-
TCONS_00023489	85		Richmond	Epic	25.7043	0	-
TCONS_00033753	83		Harlan	Epic	16.0503	6.0241	-1.41378
TCONS_00042142	83		Harlan	Richmond	0	23.9492	-
TCONS_00009192	80		Harlan	Richmond	0	15.6545	-
TCONS_00009192	80		Richmond	Epic	15.6545	0	-
TCONS_00012520	80		Harlan	Richmond	0	12.961	-
TCONS_00012520	80		Richmond	Epic	12.961	0	-
TCONS_00020429	80		Harlan	Epic	0	30.9227	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00004231	79	pol polyprotein peptidase activity	Richmond	Epic	13.8713	0	-
TCONS_00035788	79	NA	Richmond	Epic	0	23.7002	-
TCONS_00017777	78		Harlan	Richmond	41417.1	4816.37	-3.10421
TCONS_00017777	78		Harlan	Epic	41417.1	11538.9	-1.84372
TCONS_00017777	78		Richmond	Epic	4816.37	11538.9	1.26049
TCONS_00028862	78	longitudinals lacking isoforms a b d l	Harlan	Richmond	45.183	224.841	2.31506
TCONS_00028048	77		Harlan	Richmond	4.98583	30.1082	2.59425
TCONS_00037718	77	rna-directed dna polymerase	Harlan	Richmond	5.52895	0	-
TCONS_00026258	76		Richmond	Epic	9.56886	0	-
TCONS_00030443	76		Richmond	Epic	8.78882	0	-
TCONS_00043883	76		Harlan	Epic	17.9981	7.25593	-1.31061
TCONS_00033640	75		Richmond	Epic	42.5391	0	-
TCONS_00044626	75		Harlan	Epic	15.6186	241.815	3.95257
TCONS_00044626	75		Richmond	Epic	78.693	241.815	1.6196
TCONS_00044945	75		Harlan	Epic	42.9344	19.9575	-1.10521
TCONS_00003785	74		Richmond	Epic	14.0529	0	-
TCONS_00010853	72		Harlan	Epic	18.0168	6.73178	-1.42028
TCONS_00048453	72		Harlan	Epic	0	10.1055	-
TCONS_00015779	71		Richmond	Epic	55.2844	0	-
TCONS_00024885	71		Harlan	Richmond	32.4164	0	-
TCONS_00024885	71		Richmond	Epic	0	31.5132	-
TCONS_00030913	71		Richmond	Epic	0	4.86114	-
TCONS_00033551	71		Richmond	Epic	60.0647	0	-
TCONS_00019836	70		Harlan	Epic	0	44.2441	-
TCONS_00006112	69		Harlan	Richmond	3.83007	0	-
TCONS_00040721	69	bardet-biedl syndrome 2 protein homolog	Richmond	Epic	0	28.0393	-
TCONS_00002310	68		Richmond	Epic	5.62238	0	-
TCONS_00031675	68	NA	Richmond	Epic	0	65.7617	-
TCONS_00031725	68	NA	Harlan	Richmond	0	13.279	-
TCONS_00031725	68		Richmond	Epic	13.279	0	-
TCONS_00043036	68		Harlan	Epic	5.75995	0	-
TCONS_00043036	68		Richmond	Epic	7.51578	0	-
TCONS_00018445	67		Harlan	Epic	32.8928	161.1	2.29211
TCONS_00018445	67		Richmond	Epic	3.44196	161.1	5.54858
TCONS_00011110	66		Harlan	Richmond	102.394	261.574	1.35309
TCONS_00011110	66		Harlan	Epic	102.394	697.175	2.76739
TCONS_00011110	66		Richmond	Epic	261.574	697.175	1.4143
TCONS_00012618	66		Harlan	Richmond	0	18.8891	-
TCONS_00012618	66		Richmond	Epic	18.8891	0	-
TCONS_00013038	66		Richmond	Epic	10.0576	0	-
TCONS_00038890	66		Harlan	Richmond	0	13.9285	-
TCONS_00038890	66		Richmond	Epic	13.9285	0	-
TCONS_00025930	65		Harlan	Epic	0	63.1777	-
TCONS_00025930	65		Richmond	Epic	0	63.1777	-
TCONS_00028323	65		Richmond	Epic	10.8724	0	-
TCONS_00035700	65	NA	Harlan	Epic	31.2626	0	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00035787	65	NA	Harlan	Richmond	6196.96	2179.04	-1.50787
TCONS_00000548	64	atp synthase subunit mitochondrial-like isoform x2	Harlan	Epic	307.767	143.924	-1.09653
TCONS_00014803	64	adult cuticle	Harlan	Richmond	92.1414	0	-
TCONS_00001714	63		Richmond	Epic	11.7996	0	-
TCONS_00017152	63		Richmond	Epic	11.3499	0	-
TCONS_00019962	63		Richmond	Epic	0	8.80191	-
TCONS_00021116	63		Harlan	Richmond	0	18.9622	-
TCONS_00021116	63		Richmond	Epic	18.9622	0	-
TCONS_00001720	62		Harlan	Richmond	0	46.7527	-
TCONS_00007245	62		Richmond	Epic	92.1354	0	-
TCONS_00034204	62	reverse transcriptase	Richmond	Epic	0	19.692	-
TCONS_00035638	62	NA	Harlan	Epic	200.877	92.7764	-1.11448
TCONS_00039589	62		Harlan	Epic	4.71417	0	-
TCONS_00039589	62		Richmond	Epic	6.1239	0	-
TCONS_00005604	61		Harlan	Richmond	0	9.44766	-
TCONS_00015696	61		Harlan	Richmond	0	81.6019	-
TCONS_00015696	61		Richmond	Epic	81.6019	0	-
TCONS_00026344	61		Harlan	Epic	0	80.9132	-
TCONS_00026344	61		Richmond	Epic	0	80.9132	-
TCONS_00036695	61	NA	Harlan	Richmond	4.22726	0	-
TCONS_00041077	61		Richmond	Epic	62.5246	0	-
TCONS_00001271	60	NA	Harlan	Richmond	0	72.7916	-
TCONS_00013576	60		Harlan	Richmond	0	6.01882	-
TCONS_00013576	60		Richmond	Epic	6.01882	0	-
TCONS_00014730	60		Harlan	Richmond	0	22.9014	-
TCONS_00030382	60		Richmond	Epic	10.5672	0	-
TCONS_00039789	60		Richmond	Epic	14.3128	0	-
TCONS_00002055	59		Harlan	Richmond	0	24.4538	-
TCONS_00002055	59		Richmond	Epic	24.4538	0	-
TCONS_00002253	59		Harlan	Richmond	0	5.26745	-
TCONS_00002253	59		Richmond	Epic	5.26745	0	-
TCONS_00009050	59	RNA-DNA hybrid ribonuclease activity;nucleic acid binding	Richmond	Epic	4.57918	0	-
TCONS_00009319	59		Harlan	Epic	14.4107	0	-
TCONS_00045134	59		Richmond	Epic	23.0671	0	-
TCONS_00048077	59		Richmond	Epic	48.2015	0	-
TCONS_00002101	58		Harlan	Epic	0	20.1983	-
TCONS_00002101	58		Richmond	Epic	0	20.1983	-
TCONS_00025539	58		Harlan	Epic	35.5013	6.43893	-2.46298
TCONS_00041043	58		Richmond	Epic	0	15.9633	-
TCONS_00045842	58		Richmond	Epic	11.462	0	-
TCONS_00006109	57		Harlan	Epic	0	5.96964	-
TCONS_00025882	57		Harlan	Richmond	0	14.0778	-
TCONS_00029752	57		Harlan	Richmond	0	14.4216	-
TCONS_00020462	56		Richmond	Epic	12.8813	0	-
TCONS_00022950	56		Harlan	Richmond	0	9.19878	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00032049	56		Harlan	Richmond	0	44.2369	-
TCONS_00040164	56		Richmond	Epic	27.0858	0	-
TCONS_00034931	55		Richmond	Epic	0	99.021	-
TCONS_00003668	54		Richmond	Epic	183.624	0	-
TCONS_00004207	54		Richmond	Epic	21.4183	0	-
TCONS_00006524	54		Richmond	Epic	35.4093	0	-
TCONS_00018315	54		Richmond	Epic	8.91674	0	-
TCONS_00018412	54		Richmond	Epic	27.7084	0	-
TCONS_00041143	54		Harlan	Richmond	0	50.7762	-
TCONS_00041143	54		Richmond	Epic	50.7762	0	-
TCONS_00003790	53		Harlan	Richmond	0	36.5597	-
TCONS_00003790	53		Richmond	Epic	36.5597	0	-
TCONS_00011972	53		Richmond	Epic	107.734	0	-
TCONS_00013393	53		Richmond	Epic	0	10.9617	-
TCONS_00020405	53		Harlan	Epic	7.75282	0	-
TCONS_00020499	53		Harlan	Richmond	2398.39	758.892	-1.6601
TCONS_00020499	53		Harlan	Epic	2398.39	717.652	-1.74071
TCONS_00030910	53		Harlan	Richmond	0	169.453	-
TCONS_00033776	53		Richmond	Epic	27.455	0	-
TCONS_00035205	53		Richmond	Epic	383.144	0	-
TCONS_00025056	52		Richmond	Epic	6.56449	0	-
TCONS_00035140	52		Richmond	Epic	0	17.8902	-
TCONS_00039974	52		Richmond	Epic	17.3304	0	-
TCONS_00017120	51		Harlan	Richmond	4.47456	0	-
TCONS_00018954	51		Richmond	Epic	9.4789	0	-
TCONS_00020459	51		Harlan	Epic	7.90175	0	-
TCONS_00020459	51		Richmond	Epic	19.1545	0	-
TCONS_00034276	51		Harlan	Richmond	0	67.3053	-
TCONS_00034276	51		Richmond	Epic	67.3053	0	-
TCONS_00034821	51		Harlan	Epic	0	4.78347	-
TCONS_00004789	50		Richmond	Epic	61.356	0	-
TCONS_00038902	50		Richmond	Epic	27.9659	0	-
TCONS_00042511	50		Richmond	Epic	0	8.049	-
TCONS_00010931	49		Harlan	Epic	4.68872	0	-
TCONS_00044491	49		Richmond	Epic	4.75067	0	-
TCONS_00015660	48		Harlan	Epic	9.16337	0	-
TCONS_00005403	47		Richmond	Epic	0	25.2023	-
TCONS_00019155	47		Harlan	Richmond	0	49.3784	-
TCONS_00028140	47		Harlan	Epic	201.116	0	-
TCONS_00033863	47		Richmond	Epic	20.8618	0	-
TCONS_00039810	47		Richmond	Epic	5.97228	0	-
TCONS_00040954	47		Harlan	Epic	9.45948	0	-
TCONS_00040954	47		Richmond	Epic	4.05765	0	-
TCONS_00045525	47		Harlan	Epic	4.68211	0	-
TCONS_00002257	46		Richmond	Epic	20.23	0	-
TCONS_00014656	46		Harlan	Richmond	7.78125	0	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00030355	46		Richmond	Epic	0	7.39945	-
TCONS_00012922	45		Richmond	Epic	14.9756	0	-
TCONS_00017784	45		Richmond	Epic	22.642	0	-
TCONS_00047194	45		Richmond	Epic	0	8.27663	-
TCONS_00002707	43		Harlan	Richmond	11.8625	0	-
TCONS_00005534	43		Richmond	Epic	13.693	0	-
TCONS_00007257	43		Harlan	Richmond	19.3318	0	-
TCONS_00011678	43		Richmond	Epic	13.5197	0	-
TCONS_00028142	43		Richmond	Epic	33.9368	0	-
TCONS_00030516	43		Harlan	Richmond	0	40.4105	-
TCONS_00030545	43		Harlan	Epic	10.0553	0	-
TCONS_00030545	43		Richmond	Epic	4.66021	0	-
TCONS_00037950	43		Richmond	Epic	0	6.05498	-
TCONS_00003026	42		Harlan	Richmond	673.441	3803.51	-
TCONS_00003026	42		Richmond	Epic	3803.51	906.054	-
TCONS_00015784	42		Richmond	Epic	21.0402	0	-
TCONS_00025054	42		Harlan	Epic	0	48.5229	-
TCONS_00025054	42		Richmond	Epic	0	48.5229	-
TCONS_00028071	42		Harlan	Epic	4.29891	0	-
TCONS_00033671	42		Harlan	Epic	41.4346	0	-
TCONS_00039828	42		Harlan	Epic	1003.35	0	-
TCONS_00047480	42		Harlan	Richmond	0	12.8597	-
TCONS_00047480	42		Richmond	Epic	12.8597	0	-
TCONS_00034609	41		Harlan	Richmond	0	2048.43	-
TCONS_00034609	41		Richmond	Epic	2048.43	0	-
TCONS_00042519	41		Richmond	Epic	18.5828	0	-
TCONS_00045550	41		Richmond	Epic	0	11.6453	-
TCONS_00004067	40		Richmond	Epic	0	4.41599	-
TCONS_00012967	40		Harlan	Richmond	0	43.3799	-
TCONS_00012967	40		Richmond	Epic	43.3799	0	-
TCONS_00019835	40		Richmond	Epic	0	58.6977	-
TCONS_00020999	40		Harlan	Richmond	0	1998.6	-
TCONS_00015056	39		Harlan	Epic	0	28.5736	-
TCONS_00015056	39		Richmond	Epic	0	28.5736	-
TCONS_00016640	39		Harlan	Epic	10.0874	0	-
TCONS_00016640	39		Richmond	Epic	10.4599	0	-
TCONS_00019815	39		Harlan	Epic	15.7324	0	-
TCONS_00019815	39		Richmond	Epic	25.4766	0	-
TCONS_00029605	39		Harlan	Richmond	0	15.7464	-
TCONS_00029605	39		Richmond	Epic	15.7464	0	-
TCONS_00004824	38		Harlan	Epic	7.12772	0	-
TCONS_00030939	38		Harlan	Epic	29.7379	0	-
TCONS_00034255	38		Richmond	Epic	0	4.7211	-
TCONS_00034465	38		Richmond	Epic	0	79.4413	-
TCONS_00036641	38	NA	Harlan	Epic	7.66572	0	-
TCONS_00036869	38	NA	Richmond	Epic	0	29.1795	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00002205	37		Richmond	Epic	0	33.4734	-
TCONS_00009087	37		Richmond	Epic	0	28.6203	-
TCONS_00030978	37		Richmond	Epic	15.7905	0	-
TCONS_00037862	37		Harlan	Richmond	4.25618	0	-
TCONS_00042558	37		Richmond	Epic	0	62.8552	-
TCONS_00043218	37		Richmond	Epic	17318.3	0	-
TCONS_00017215	36		Richmond	Epic	72.193	0	-
TCONS_00026345	36		Richmond	Epic	0	16.9937	-
TCONS_00037915	36		Richmond	Epic	0	9.87058	-
TCONS_00041106	36		Harlan	Richmond	0	13256.8	-
TCONS_00041106	36		Richmond	Epic	13256.8	0	-
TCONS_00005401	35		Richmond	Epic	0	19.1661	-
TCONS_00014607	35		Richmond	Epic	0	28.6325	-
TCONS_00018736	35		Richmond	Epic	16.7661	0	-
TCONS_00021826	35		Harlan	Richmond	0	201.68	-
TCONS_00025894	35		Richmond	Epic	12.4206	0	-
TCONS_00047392	35		Harlan	Epic	0	22.5212	-
TCONS_00001694	34		Richmond	Epic	11.1673	0	-
TCONS_00014879	34		Harlan	Richmond	0	4.11092	-
TCONS_00014879	34		Richmond	Epic	4.11092	0	-
TCONS_00023552	34		Harlan	Richmond	9.9807	0	-
TCONS_00023552	34		Harlan	Epic	9.9807	0	-
TCONS_00049210	34		Harlan	Richmond	11.2311	0	-
TCONS_00002166	33		Richmond	Epic	15.9554	0	-
TCONS_00002390	33		Richmond	Epic	13.5581	0	-
TCONS_00008934	33		Richmond	Epic	0	18.6863	-
TCONS_00025890	33		Richmond	Epic	283.718	0	-
TCONS_00028963	33		Harlan	Richmond	6.74333	0	-
TCONS_00028991	33		Richmond	Epic	5.41052	0	-
TCONS_00001871	32		Richmond	Epic	1374.05	0	-
TCONS_00024128	32		Richmond	Epic	0	272.816	-
TCONS_00002448	31		Richmond	Epic	23.7939	0	-
TCONS_00024003	31		Harlan	Richmond	29.9947	0	-
TCONS_00031668	31		Harlan	Richmond	0	232.695	-
TCONS_00031668	31		Richmond	Epic	232.695	0	-
TCONS_00030544	28		Harlan	Epic	9.73307	0	-
TCONS_00038665	28		Harlan	Richmond	0	22.0773	-
TCONS_00001924	25		Harlan	Richmond	0	13.8564	-
TCONS_00005629	24		Richmond	Epic	7000.17	0	-
TCONS_00017785	24		Harlan	Richmond	7.15586	0	-
TCONS_00027178	24		Richmond	Epic	95.4673	0	-
TCONS_00001883	23		Richmond	Epic	0	74.0794	-
TCONS_00002523	23		Harlan	Richmond	7.58816	0	-
TCONS_00019749	23		Richmond	Epic	7.64799	0	-
TCONS_00036877	22	NA	Richmond	Epic	35.2129	0	-
TCONS_00025452	20		Harlan	Richmond	22.4812	0	-

Transcript	Orf peptide lengths	Annotation	Strain 1	Strain 2	Strain 1 expression	Strain 2 expression	log2(fold_change)
TCONS_00025452	20		Richmond	Epic	0	42.5546	-
TCONS_00028260	15		Richmond	Epic	10.1123	0	-