Evaluation of Alternative Control Methods for Eliminating Insecticide-Resistant Bed Bugs (*Cimex lectularius* L.)

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Abstract

The common bed bug, *Cimex lectularius* L, has been a persistent pest of humans. Conventional pyrethroid-formulation insecticides are known to be ineffective for controlling modern bed bug populations. This study evaluates alternative treatment methods such as whole-home heat, a biological control agent (*Beauveria bassiana*), and the fumigant sulfuryl fluoride for control of insecticide-resistant bed bugs. Three heat systems with different energy sources (propane, electric, and glycol) were evaluated to determine attributes contributing to heat treatment efficacy. The glycol system produced the most mortality of the three systems, killing all nymphs, eggs, and most adults. Heat treatment duration and achieving lethal temperatures in complex environments were found to be the most important factors for treatment efficacy. These factors were directly correlated with technician diligence, specifically regarding monitoring surface temperatures and repositioning equipment. A formulation of *B. bassiana* was evaluated in the laboratory to determine its ability to infect bed bugs under varying conditions of temperature (15°C, 21°C, and 32°C) and humidity (30%, 50%, and 70%). It was found that humidity conditions (30%-50%) at ≈21°C produced the greatest bed bug mortality and the shortest bed bug median survival time. The fumigant sulfuryl fluoride was evaluated for its ability to eliminate bed bugs from motor vehicles and cargo trailers filled to 85% capacity. This study was the first to document that sulfuryl fluoride fumigation at the 1.9X dosage factor can kill all pyrethroid-resistant bed bug life stages (including eggs) in motor vehicles as well as in chambers filled with personal items.

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General Audience Abstract

Since the world-wide bed bug (*Cimex lectularius* L.) resurgence began in the late 1990s, populations of the common bed bug have been evaluated, but most have been found to be highly resistant to pyrethroid insecticides. This resistance has been particularly troublesome due to the fact that the 1996 US Food Quality Protection Act has eliminated the use of many existing chemistries from indoor use and has inadvertently limited the development of new active ingredients for indoor use due to the cost of the required animal testing to document the No Observable Effects Level (NOEL). Due to the lack of novel chemistry for addressing modern bed bug infestations, pest management professionals have had to identify new methods for applying existing products (chemical and non-chemical) for bed bug control. This study evaluated gaseous, mechanical, and biological control methods for bed bug elimination to determine which factors contribute the most to their efficacy, as well as how these methods might be applied in novel ways for control of bed bugs in homes, personal belongings, and even vehicles.

At the turn of the 20th century heat treatments were used for controlling bed bug infestations in homes and other structures. Today, mechanical heating systems are again being used to control bed bug infestations in homes and apartments. This study investigated the utility of three commercial heating systems for their ability to control existing infestations in apartment units. The three heat systems utilized different energy sources, different types of delivery equipment, and required different set up and take down procedures in apartments of different cubic footage and clutter levels. Overall, the Assault glycol heating system was found to be the

most effective in its ability to get (almost) all of our hidden (in hard to heat locations) temperature sensors up to bed bug lethal temperature. Interestingly, our statistical analysis determined that getting the hard to heat locations up to lethal temperature was directly correlated with heat technician activity. The more times that the technician entered the home to monitor the treatment and adjust the equipment positions, the greater the chance of getting the hidden sensors up to bed bug lethal temperature (and killing the bed bugs). However, it was also found that none of the heat systems tested killed all of the sentinel bed bugs in every replicate. Therefore, we learned heat treatments cannot be expected to eliminate each and every bed bug in a home, and that supplemental control products such as desiccants dusts should be applied after every heat treatment. When attempting to determine which heat system was the most effective for killing bed bugs, it was determined that regardless of which heat systems was being used, the attention and activity (monitoring temperatures and adjusting equipment) of the heat treatment technician was the most important factor contributing to bed bug mortality as well as the achievement of bed bug lethal temperatures in cracks and crevices for all three systems.

Our second study examined the environmental factors that influenced fungal growth after bed bug exposure to the insecticidal product Aprehend® (active ingredient *Beauveria bassiana*). Over the last several decades, this fungus has been widely used to control multiple insect pests. Recently, it has been labelled for bed bug control in indoor environments. This second study was intended to determine the atmospheric conditions (temperature and humidity) under which Aprehend sporulation was most effective for killing bed bugs. It was found that humidity conditions of 30-50% combined with temperatures of ~21°C produced the highest frequency of fungal infection and the shortest median bed bug survival time. This result was surprising because it was originally hypothesized that the 70% humidity condition would be equally

effective for promoting fungal growth. However, at all of the temperatures tested, bed bug fungal infection rates at 70% humidity were not as high as those observed when the humidity was within the 30-50% range. Therefore, this study was able to document that the temperatures and humidity combinations that would typically be found within human homes (21^{o}C at 30-50% humidity) were the most effective for producing fungal infections when bed bugs were exposed to the Aprehend product.

The final study addressing novel methods for controlling insecticide resistant bed bugs investigated the efficacy of using Vikane® gas fumigant (sulfuryl fluoride) at the 1.9× dosage rate for eliminating bed bugs in two challenging infestation situations: personal vehicles, and confined spaces densely packed with personal belongings. The vehicles used in this study were large minivans with seating that folded into the floor. The confined spaces were cargo trailers filled to 85% capacity with books, furniture, and other household items. Each van and trailer was equipped with ~90 sentinel bed bugs consisting of three groups of 9-11 bed bug eggs, 10 nymphs, and 10 adults. The Vikane Fumiguide calculator was used to determine the target dosage (g-h/m3) to apply in each replicate (e.g., one van or trailer). Sulfuryl fluoride concentrations were measured throughout the fumigation process using a Spectros SF-ReportIR. Concentration readings were input into the Fumiguide to determine when the accumulated dosage (g-h/m3) was achieved, and when aeration should be initiated. After aeration was complete, the sentinel bed bugs were removed from the replicates and bed bug nymph and adult mortality was recorded. Bed bug eggs were monitored for 23 d to determine latent mortality. Fumigated bed bug mortality for each replication was 100% regardless of life stage. Latent mortality was observed in a single bed bug egg, where the nymph never fully hatched. This study determined that fumigation with sulfuryl fluoride at the 1.9× dosage factor is an effective method

for eliminating insecticide resistant bed bugs from vehicles and personal belongings in densely packed situations.

Overall, it was found that the *Beauveria bassiana* product was most effective when applied under atmospheric conditions that are typically found in indoor home environments. This discovery was very reassuring, because the Aprehend product is one of very few that actually have residual activity with regard to bed bug control. This study also found that whole home heat treatments require rigorous pest technician attention and monitoring to achieve the best results. It was also determined that heat treatments cannot be assumed to be 100% effective on their own, and that they should be supplemented with additional (residual) treatment applications. Not surprisingly, fumigation with sulfuryl fluoride was determined to be 100% effective for eliminating bed bugs in personal belongings that had been packed into treatment chambers. The fumigation process also proved to be 100% effective for eliminating bed bug infestations in transport vehicles which cannot not be adequately treated and are at risk for having electronic components damaged if treated with heat.

In dedication to my family and my closest friends

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Chapter One:

Introduction

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), is a flightless hematophagous ectoparasite that has been a pest of human environments for thousands of years (Panagiotakopulu and Buckland 1999). The evolutionary process that is thought to have brought these hemipterans into contact with humans is based on the fact that Cimicid bugs are known to feed on bats. Therefore, the hypothesis has been that the ancestral bed bugs were ectoparasites of bats that existed in caves in the Middle East. When humans moved into the caves, approximately 37,000 years ago, they were thought to have displaced the bat populations leaving the bugs with nothing to feed on. Insects that altered their day/night cycle began to feed on humans were successful at reproducing and have remained ectoparasites of humans ever since. Therefore, when humans moved out of the caves, and started their agricultural societies they are thought to have taken the bed bugs with them (Usinger 1966). Thus, the bed bugs have co-existed with humans ever since.

Interestingly, a recent study evaluating DNA from live bed bugs collected around the world and even museum specimens, has concluded that bed bugs actually evolved 100 million years ago (during the time of the dinosaurs), which was 50 million years earlier than their assumed original bat hosts. This study determined that bed bug evolution is more complex than previously thought, and that the origin of their blood feeding behavior (as opposed to feeding on plants like other Hemipterans) must have been the result of very unique evolutionary selection pressures (Roth et al. 2019).

While the early details of bed bug evolution are still being discovered, it is well documented that bed bugs have had a very long and somewhat disturbing relationship with their human hosts over the more recent centuries. Bed bug remains have been found in ancient archaeological sites dating as far back as 1352 BC within an ancient Egyptian city (Panagiotakopulu and Buckland 1999). Ancient writers such as Aristotle and Aristophanes lamented the presence of bed bugs, while others such as Dioscorides praised the bed bug for their supposed medicinal properties. In more recent history, bed bug remains were found in a 17th century Canadian structure in Quebec City (Bain *et al.* 2009). Swedish botanist Pehr Kalm had visited North America in the 18th century and claimed that Canada was as infested with bed bugs as the old world. Interestingly, Pehr Kalm was told by a commander at Fort Frederick that Native Americans had no knowledge of bed bugs (Pehr Kalm 1749 Vol I: 319).

Numerous methods, some more unique than others, were used to combat bed bug infestations. Early exterminators in the 18th century offered advice on preventative inspections, warned against bringing infested personal belongings into new environments, and suggested that beds be separate from the surrounding woodwork to make bed bugs easily accessible (Southall 1730). While this advice was logically sound, other individuals suggested more extreme measures. *The Compleat Vermin-Killer* suggested filling cracks and crevices with gunpowder and lighting it on fire, as well as creating a boiled urine and plant solution that is spread on the joints of bed frames (Anonymous 1777).

During the 19th century, emphasis was placed on indoor cleanliness, and pyrethrum powder was used between bed sheets to protect occupants from bed bugs (Potter 2011). In the early 20th century, advancements of indoor heating allowed bed bug populations to thrive all year around, which brought about a bed bug population boost (Johnson 1941). To combat the bed bug

population boom, fumigations using hydrogen cyanide, sulfur dioxide, and ethylene oxide were conducted on homes (Ministry of Health 1934). Cleansing stations were also erected to steam treat the clothes and bedding (Potter 2018). These methods were heavily used in the early 20th century until Paul Muller discovered the insecticidal properties of a synthetic organochloride named dichloro-diphenyl trichloroethane (DDT) in 1939 (Potter 2018).

In the late 1930s, DDT entered the pest control market and was deployed for control of mosquitoes, agricultural pests, and bed bugs (Potter 2018). Bed bugs were essentially eradicated in developed nations due its liberal usage on any household item and even people. DDT was hailed as a "silver bullet", but this title was short-lived as within five years of its use there were already signs of bed bug resistance to DDT (Hawaii in 1947; Johnson and Hill 1948). Due to the development of resistance, organophosphate and carbamate insecticides replaced DDT and were used from 1958 to 1968 (Busvine 1958; Potter 2011). While resistance occurred with these insecticides, bed bug infestations had become so reduced that they were not considered an issue until the late 1990s when bed bug populations surged across the world in Africa, the Americas, Australia, Asia, and Europe (Doggett et al. 2012).

The modern bed bug will infest any human environment, but they are known to frequently infest lower-income housing, poverty-stricken communities, and elderly facilities. Financial constraints, resident turnover, and highly infested apartments contribute to the high bed bug introduction rates in these areas. Lower-income communities can be burdened by financial constraints, which limit their ability to hire effective pest control to eliminate bed bug infestations. High resident turnover poses the risk of dispersing bed bug populations within and between buildings, and highly infested apartments can act as reservoirs that bed bugs could disperse from (Wang 2016). In Wong's 2013 study, bed bug infestations occurred more frequently within elderly

and disabled facilities than in multi-family housing facilities. Not only are bed bugs found in structures, they are also found in transportation systems and rideshare vehicles which include Ubers, Lyfts, and taxis. In Dallas, Texas, a pest control operator stated that he treats between 5 and 10 “rideshare” vehicles per week. This operator also stated that treatment of rideshare vehicles for bed bugs is becoming more common (Howerton 2020).

The most common method used for controlling household pests is to spray them with insecticide formulations (Anonymous 2020a). These formulations typically contain pyrethroid active ingredients such as permethrin, deltamethrin, and lambda-cyhalothrin. More recently, these pyrethroids have been formulated in combination with another active ingredient chemistry known as neonicotinoids. While these active ingredients were originally combined to enhance the efficacy of pyrethroid formulations, bed bugs rapidly developed resistance to these formulations (Gordon et al. 2014). The mixed spray formulations have become only marginally effective for controlling modern bed bug populations, particularly their eggs. In fact, the bed bug resurgence is largely attributed to the development of resistance to insecticides (Romero et al. 2007, Zhu et al. 2010, Davies et al. 2012). Modern bed bug populations have been shown to be resistant to pyrethroids such as deltamethrin and cyhalothrin (Romero et al. 2007). They are also known to be resistant to neonicotinoids such as imidacloprid, acetamiprid, dinotefuran, and thiamethoxam (Romero and Anderson 2016).

There are several different genetic mechanisms of bed bugs resistance. Each of these mechanisms prevent the bed bugs from being susceptible to the insecticide in a different physiological way. The bed bug mechanisms of resistance include reduced cuticular penetration, increased metabolic detoxification via increased cytochrome P450 production and esterase activity, and target-site insensitivity (alpha subunit point mutations; kdr-type). Reduced cuticular

penetration refers to the thickening of the cuticle, which reduces and/or slows down the amount of insecticide that enters the bed bug's body (Romero 2018). Bed bugs with increased metabolic detoxification enzyme activity have an overexpression of enzyme-production genes, by which increased levels of detoxifying enzymes will help break down insecticides within a bed bug's body before they reach their target sites (Dang et al. 2017). Bed bugs with kdr-type resistance have one or more point mutations at the voltage-gated sodium channel alpha subunit, which can block insecticidal mode of action (Zhu et al. 2010). Unfortunately, modern bed bugs populations have been known to possess all three of these genetic mechanisms, which have severely reduced their overall susceptibility to our existing pesticide formulations. (Lilly et al. 2016a, b; Romero and Anderson 2016, Dang et al. 2017).

One particular resistance mechanism that has made the management of modern bed bugs almost impossible is the reduced cuticular penetration type resistance. Reduced cuticular penetration makes it very difficult for spray pesticide residues to penetrate the bed bug exoskeleton. Therefore, the only possible way of killing these individuals with a spray formulation is to spray the wet insecticide directly on their bodies. If a spray formulation is simply applied to cracks and crevices, and other bed bug harborage locations in the home, bed bug elimination cannot be expected. This is because there is no way for the insecticide to enter the bed bug's body by their simply walking over the insecticide residues, particularly once they are dry. Therefore, the reduced cuticular penetration type resistance has all but eliminated any residual activity that current insecticide formulations are supposed to have (Romero et al. 2007, Zhu et al. 2010, 2013; Davies et al. 2012, Lee et al. 2018).

Spray formulations insecticides are also limited in their ability to contact bed bugs that are hiding in cracks, crevices, and other hard-to-access locations. When high levels of clutter are

present in a home, bed bugs, and their eggs, are provided with hundreds if not thousands of harborage locations. The more traditional harborage locations for bed bugs include mattresses, couches, dressers, wall paneling, electric outlets, bags of clothes, toys, and electronic devices. However, household clutter such as piles of books, piles of clothing, shoes, framed family photographs, rolls of toilet paper, record albums, antique dolls, etc. can also provide locations for bed bugs and eggs to harbor, and many of these items cannot be treated with spray with insecticide formulations.

In order to address these insecticide resistant bed bug infestations, there has been an upsurge of novel technologies and methodologies over the last decade. Many novel technologies have been primarily focused on using heat to kill resistant bed bug infestations. There have been several commercial heating methods that have been marketed for bed bug control. The first and still one of the most widely used has been the application of steam (Anonymous 2020a). This is where a hand-held steaming device is moved along infested furniture or other bed bug aggregation sites to kill harboring bed bugs by exposing them to temperatures that are typically above their thermal death point of 55°C. However, steaming can be a very slow process when you consider that the applicator should only move the steamer at a rate of one inch per second to make sure the bed bugs and their eggs are adequately exposed.

Another heat treatment method that has yet to become widely used has been the development of portable heat chambers. These chambers are designed to be filled with heat tolerant items such as clothing, furniture, and books, family keepsakes, etc. that are known to harbor bed bugs. The chambers are filled with infested personal belongings, sealed, heated, and monitored over time to determine when all temperature sensors located included in the chamber reach bed bug lethal temperature. An important factor for effective heat treatment in these chambers is the

circulation of air, which allows heat to be evenly distributed and reach difficult-to-heat areas (Pereira 2009). It is established that eggs are more heat resistant and can survive higher temperatures than adults and nymphs (Kells and Goblirsch 2011). Sufficient hold times at lethal temperatures are also necessary to ensure the death of bed bugs and their eggs. It was determined that holding a temperature of >50°C for 0.0 minutes or 48°C for 71.5 minutes (Kells and Goblirsch 2011) was necessary for killing bed bugs. The necessity of having to make sure that all items in the heat chamber reach bed bug lethal temperature does require patience. Chamber heat treatments (depending on the size of the chamber) can take anywhere from 3 – 6 hours. This lengthy monitoring period has limited the interest in pest management professionals using this methodology due to the fact that labor time required complete these treatments reduces their overall productivity.

Whole home heat treatments have become a widely used method for addressing bed bug infestations although some heat systems have a much better track record than others when it comes to bed bug elimination (Miller 2022, personal communication). Whole home heat treatments involve the use of electric, propane, or glycol heaters to distribute heated air into the home. Fans are typically used to circulate the air, and the temperatures are monitored throughout the home to determine when all surfaces (and hard to heat locations) reach 55°C. While most (not all) whole home heat systems have the heating capacity to get the home up to bed bug egg lethal temperature, the heat technician must exert a large amount of monitoring effort and system manipulation.

Another more recent alternative to the application of spray formulation insecticides has been the application of fungal spores. Fungal spores are the first biopesticide to have been labelled for control of bed bugs. The Aprehend product (Conidiotec LLC, Center Hall, Pennsylvania), contains *Beauveria bassiana* conidia immersed in an oil solution. This solution is applied in 2-

inch bands on furniture, walls, and other surfaces that bed bugs will cross when attempting to forage. In the process of walking across these bands, the bed bugs will pick up conidia that will adhere to their cuticles. The conidia will germinate and ultimately penetrate the cuticle. Eventually, the germinated conidia will reach the hemocoel of the insect where it will release hyphal bodies to further spread the infection, resulting in host death.

Another methodology that has recently gained popularity for eliminating resistant bed bug infestations has been sulfuryl fluoride fumigation. While bed bug fumigation using sulfur dioxide and hydrogen cyanide has history of use in the United States, bed bug fumigation all but disappeared with the development of DDT. With the modern bed bug resurgence, researchers have been re-evaluating the utility of fumigation for bed bug control in structures as well as infested vehicles and personal belongings (Todd et al. 2021).

The purpose of this thesis was to evaluate the efficacy and efficiency of novel technologies for the control of insecticide-resistant bed bug populations. My research objectives were three-fold:

1. Determine the fundamental factors and efficacy of different whole home heat treatment systems
2. Determine the environmental conditions that promote the sporulation of an oil-based formulation of *Beauvaria bassiana*
3. Verify the ability of sulfuryl fluoride to eliminate bed bugs in motor vehicles and chambers filled to 85% capacity with furniture and personal belongings.

Chapter Two:

Literature Review

2.1 Bed Bug Morphology

The common bed bug is part of the Cimicidae family of insects in the order Hemiptera. The Cimicids are commonly known to be ectoparasites of birds and bats. However, there are at least 91 species in this family (Pinto et al. 2021), many of which have not been studied. Bed bugs are in the suborder of Heteroptera and are known to be closely related to plant feeding bugs. These plant feeders (which include stink bugs and boxelder bugs), possess piercing-sucking mouth parts that are used for extracting liquids out of plants. Bed bugs have piercing-sucking mouthparts but have evolved to feed on animal hosts. Bed bugs use their piercing-sucking mouthparts to pierce the skin and extract blood out of birds, bats, and humans, all of which are inherently capable of killing bed bugs during the feeding process.

Bed bugs are also unique in the fact that they have adapted to feed during the time when their hosts are the least conscious of their presence. Bat bugs originally adapted to feed during the day when their hosts were at rest. Those that later evolved to feed on cave dwelling humans had to adapt to a nocturnal feeding regimen in order to feed when the humans (and birds) were most vulnerable (Romero and Haynes 2010).

The Cimicid bug morphology has no doubt been a contributing factor to their feeding success. Their small size, and dorsoventrally flattened bodies allow them to forage undetected on sleeping humans, bats, and between feathers of sleeping birds. After feeding, they leave the host to hide in small cracks and crevices where they are relatively inaccessible, even when several are aggregating together. It is well known that a small, recently introduced population of the

common bed bug can be difficult to detect in a person's home. This is due to the fact that the bed bugs are so small and every fold, crack, and crevice in the home must be carefully inspected in the hopes of finding a live bed bug or egg. It is for this reason that pest managers must be intimately familiar with bed bug morphology and other bed bug evidence, if they hope to detect an incipient population.

Adults. The adult bed bug is typically reddish-brown in color and dorsoventrally flattened when unfed. Adults range in size from ~5.5 to 7.0 mm in length and are less than 3 mm wide (Usinger 1966, Krinsky 2019, Pinto et al 2021). It is for this reason that adult bed bugs are often described as being the size and color of an apple seed. Bed bugs have a 3-segmented rostrum that is held in a groove under the head and thorax when the bed bug is not feeding (Pinto et al. 2021). The rostrum is composed of a bendable labium, a pair of mandibular stylets, and a pair of maxillary stylets (Krinsky 2019). The bed bug antennae have 4-segments, and like other Hemipterans, the bed bug head is clearly visible above the pronotum. The common bed bug has an abundance of short-golden hairs on the body that appear combed towards the posterior. While the adult bugs do not have wings, they do have vestigial wing pads, which conceal the metanotum (Harlan 2006, Krinsky 2019). Adult female bed bugs are typically a bit larger in size when compared to males and tend to be more rounded at the end of their abdomen. Male bed bugs have a pointed end on their abdomen due to the presence of their paramere (Harlan 2006).

Another distinct feature of the adult female bed bug's anatomy is a cleft that is present on the hind margin of the 5th abdominal tergite. This is known as the spermalege. The adult male bed bug does not mate with the female in her genital area (where her eggs will emerge). Instead, the male uses his paramere to puncture the female spemalege. This will wound the female during the mating process but will functionally impregnate her. It is this wounding of the female during

the impregnation process that has resulted in bed bug mating being termed “traumatic insemination” (Krinsky 2019, Pinto et al. 2021).

Nymphs. Immature bed bugs typically appear as smaller, less hairy, yellow-colored versions of adults, given their hemimetabolous development (Krinsky 2019). Unlike the adults, nymphs lack vestigial hemelytral wing-pads, and they have underdeveloped reproductive organs (Usinger 1966). The Common bed bug has five nymphal life stages prior to reaching maturity (Usinger 1966, Harlan 2006, Krinsky 2019, Pinto et al. 2021). Each life stage can last between 3 to 10 days depending on the availability of a bloodmeal, which is necessary for each molt (Krinsky 2019). From the *Monograph of Cimicidae* by Usinger (1966), the developmental time of the bed bug from egg to adulthood can range from 24 days to 128 days depending on temperature conditions, with 30°C being optimal for development.

Eggs. Eggs of the common bed bug are pearly white, 1 mm long, and ovular in shape. Common bed bug eggs are also laid on their side upon the “long-convex” region of the egg (Usinger 1966). They are laid singly, or sometimes deposited in small groups by a female bed bug. Eggs are usually laid within the harborage location or near it but sometimes a wandering female can lay an egg in a distant location. All eggs are anchored to surfaces by a translucent substance that coats them. The red eye spots of the developing embryo can typically be seen when the egg reaches five days old. Depending on temperature, a first instar nymph will emerge from the egg within 4 - 12 days (Krinsky 2019, Pinto et al. 2021).

2.2 Bed Bug Biology and Behavior

Bed bugs are typically nocturnal and are known to hide away in cracks and crevices during the daylight hours (Usinger 1966, Harlan 2006). Some researchers have claimed that bed bugs prefer to aggregate on rough surfaces, fabric, or dry substrates that allow them hold on using their tarsal claws (Krinsky 2019). Bed bugs in human homes prefer textured harborages such as wood, plaster, and paper instead of metal or stone. However, bed bugs will aggregate on many surfaces including metal bed frames if that is where the host typically rests. In homes where bed bug infestations have been allowed to proliferate for several months or years, bed bugs can be found harboring in almost any location (e.g., on bathroom toilets, inside of books, painted bed room walls, ceiling wall junctions, picture frames and all types of personal belongings). Established bed bug harborage locations are typically identified by the presence of their light and dark fecal spots, and sometimes there is also a sweet odor that is produced by the bed bug's ventral metathoracic glands (Harlan 2006).

Feeding. Bed bugs will feed on humans, other mammals (in areas where bare skin is available), and birds to obtain their bloodmeal (Harlan 2006). Bed bugs are negatively phototactic and feed mostly in the dark or in subdued light (Krinsky 2019), but they will feed at any time of day or night if they are starved and there is a host available (Harlan 2006). The feeding process is well described by Krinsky (2019) and Usinger (1966). Prior to feeding the bed bug will approach the host with its antennae outstretched and the rostrum positioned at approximately a 90° angle. Once the bed bug has reached a desired location on the host's skin, the bed bug will pull the antennae backwards, and push against the skin with its rostrum. This pushing motion will drive the stylets into the skin, and the labium will become bent as stylets are injected into the skin's surface. The labium, according to Usinger (1966), aids in the stability of

the stylets as they penetrate the skin while also limiting the depth of their penetration. The pair of mandibular stylets alternate in penetrating the skin, which forms a passage that the maxillary stylets use to locate a suitable blood vessel. If a blood vessel is not found during the initial insertion, the bed bug will often move slightly and insert the rostrum again. This feeding process can result in multiple bites occurring in the same location. Overall, a bed bug's blood vessel search and ultimate feeding can take between 5 to 20 minutes to complete (Usinger 1966).

Given that the bed bug is a hematophagous insect, bloodmeals are required in order for the bed bug to develop, molt, and reproduce. For a nymph to molt into the next life stage, it requires at least one bloodmeal (Harlan 2006). Female bed bugs will need to feed and mate to lay viable eggs, and then continue to feed every 10 days post-mating to continue the egg laying process (Polanco et al. 2011a). While blood is an effective source of nutrients that bed bugs are obligated to feed on, they cannot acquire all necessary nutrients from blood alone. A key component of the bed bug's digestive system is the presence of *Wolbachia* symbionts that are localized in an organ attached to the gonads (male and female) called the bacteriome (Usinger 1966). The symbionts help synthesize the micronutrients riboflavin (vitamin B2) and biotin (vitamin B7), which the bed bugs cannot obtain from a bloodmeal alone. (Evison et al. 2018).

Reproduction. As described previously, bed bugs have a unique method of reproduction that has been termed "traumatic insemination." This process involves the male bed bug penetrating the fifth abdominal cleft of the female with his sclerotized paramere in order to inseminate her (Carayon 1966). Female bed bugs have a unique paragenital reproductive system, in which the sperm will move through to reach the ovaries. After the male deposits the sperm in the female mesospermalege, the sperm will migrate to the seminal conceptacles, where they will be stored. The stored sperm will eventually move through the oviducts in order to fertilize any

eggs that develop within the ovaries (Carayon 1966). While the female paragenital tract is adapted to move sperm during traumatic insemination, the genital tract is used solely for oviposition.

This traumatic copulatory method is resource-expensive for the female bed bug, given that the male causes injury to the site where insemination occurs. This results in nutritional energy being directed to repairing the wound site as well an immune response to help heal the injury done to the female (Reinhardt et al. 2005). This mating recovery process takes energy away from the female that could be used for egg production. It has been documented that adult female bed bugs that have access to regular blood meals but are only mated once produce 25% more eggs than females that are mated repeatedly (Polanco et al. 2011b).

2.3 Taxonomy and Geographic Origins

The bed bug family Cimicidae is currently distributed across the globe. Cimicidae has 24 known genera and 110 described species that are systemically categorized within six subfamilies (Henry 2009). Interestingly, Cimicidae is related to the predacious Hemipteran family Anthocoridae and the bat parasitizing family Polyctenidae (Krinsky 2019). Two Cimicid species within the *Cimex* genus, *Cimex lectularius* and *Cimex hemipterus* are known to be the most prominent ectoparasites of humans. Both of these species originated in the Old World, and *Cimex lectularius* is believed to have originated in the Middle East (Usinger 1966, Krinsky 2019).

2.4 The Bed Bug Relationship with Humans and Bats

Bed bugs have been a well-documented and persistent pest throughout human history. There have been a number of historical transcripts referencing their ability to infest human

homes, and the annoyance they cause their host who ultimately suffers from their itching red bites. However, ancestral bed bugs have not always been a human pest. It is thought that they originally fed on other organisms such as bats, that sheltered in caves. In fact, it is currently hypothesized that the original bed bugs most likely became human pests when ancient humans moved into the caves and either lived with, or removed the bats (Usinger 1966, Booth et al. 2015).

Booth et al. (2015) identified two distinct genetic groups from *C. lectularius* bed bugs that were collected separately from bat roosts and human dwellings. The Booth et al. (2015) data supports the notion that the ancestral hosts of the common bed bug were bats, and that current bat-associated bugs diverged when humans stopped using caves as shelters. However, it is now known that the biological evolution of the bed bug family Cimicidae stretches farther back in evolutionary history, pre-dating both humans and bats (Roth et al. 2019).

Roth et al. (2019) reconstructed the Cimicidae phylogenetic tree based on fossil evidence. Roth et al. (2019) estimated that the stem of the Cimicidae phylogenetic tree was 115 million years old. This made the age of the Cimicid family a minimum age of 100 million years old. The Cimicidae crown group origin (93.8 million years ago) was placed 30-50 million years prior to the earliest known bat, and 20 million years before the earliest inferred bats (73 million years ago). Based on evolutionary information between bed bug lineages, the paper suggests that bed bug stem species evolved 115-122 million years ago. It is interesting to note that Roth et al. (2019) stated that the ancestral host that bed bugs fed on prior to parasitizing bats is unknown.

The Roth et al. (2019) study provides strong evidence that the Cimicid bed bug family is very robust after having survived and evolved for potentially millions of years. When ancient humans took shelter within the same caves that old world bats were occupying, there were likely

opportunistic bat-associated bed bugs that took advantage of the arrival of the new warm-blooded hosts. When humans departed from caves and began to develop their agricultural societies, they brought the newly acquired parasites with them. Since that time, humans and bed bugs have maintained an intimate relationship that continues to flourish even today (See section 2.8).

2.5 Medical Importance

From an evolutionary perspective, it is not advantageous for an ectoparasite to have deleterious effects on the health of its host. At this time, many blood-feeding arthropods such as some mosquitoes, triatomine bugs, and ticks are known to be significant vectors of human diseases. Fear of these arthropods has contributed to the suspicion that today's modern bed bugs may be vectors of disease. Usinger (1966) has shown that human pathogens such as viruses and bacteria are capable of surviving in the bed bug for varying amounts of time. However, there is little to no evidence that has ever incriminated the bed bug as a disease vector. Doggett et al. (2012) reviewed existing scientific literature that evaluated the common bed bug's ability to transmit infectious diseases (Chagas; hepatitis; human immunodeficiency virus; etc.). Overall, these studies have concluded that bed bugs seem to be incapable of transmitting these diseases. In fact, bed bugs appear to carry "neutralizing factors" that prevent disease spread, such as the saliva containing potentially antimicrobial lysozymes and peptides (Doggett et al. 2012, Doggett 2018a). Another factor appears to be the immunological properties of the male and female bed bug reproductive systems, where the male ejaculate exhibits antibacterial properties and the female expresses genes in the spermalege that produce antimicrobial peptides. It is currently unknown if these genes are expressed in other parts of the bed bug body outside of the reproductive organs. Additionally, a physiological factor that inhibits bed bug disease

transmission potential is their host-specificity. Unlike ticks and mosquitoes, modern bed bugs do not actively feed on wildlife. This severely limits their ability to consume pathogenic organisms that might be dangerous if transferred to their human hosts.

While humans can be grateful that bed bugs do not vector pathogens that cause disease, individuals who have had to live with bed bugs can experience other physiological and psychological consequences associated with bed bug infestations. For example, during the bed bug feeding process, the bed bugs inject their saliva into the human skin. The saliva contains several proteins that have different functions. These functions include vasodilation (Valenzuela et al. 1995), platelet function inhibition (Valenzuela et al. 1996a), and anti-coagulation (Valenzuela et al. 1996b). Although the saliva contains proteins, there is no evidence that bed bug saliva contains anesthetics (Doggett et al. 2012). The bed bug saliva that is injected into the skin can induce cutaneous reactions, and these reactions often vary in severity. Most commonly, bites can appear as a 2-5 mm wide lesion with a central hemorrhagic punctum that can manifest into a wheal (Hwang et al. 2018). These cutaneous reactions can occur immediately, but there can also be delayed reactions that appear hours, days, or even weeks after the bite (Leverkus et al. 2006, Doggett et al. 2012, Bernardeschi et al. 2013). In some cases, individuals may not have any reactions to bed bug bites, although this lack of reaction is primarily attributed to the elderly, due to their reduced immune response (Potter et al. 2010). While the itching reactions to bed bug bites tend to resolve within a week or two, some individuals do develop secondary infections due to repeatedly scratching the feeding sites (Doggett et al. 2012, Hwang et al. 2018)

In addition to the dermatological impacts, bed bug infestations can also have an effect on the host's mental health. Bed bug infestations in a home can cause sleep disturbances and depravation to individuals (Potter et al. 2010, Dogget et al. 2012). Infestations can also cause

distress and increased anxiety (Potter et al. 2010). People that are predisposed to mental health problems may be at a higher risk of developing psychiatric conditions because of an infestation (Perron et al. 2018). Other issues that have been observed in people living with a bed bug infestation are self-inflicted social isolation, shame, and embarrassment. These reactions often contribute to the victim suffering symptoms of depression, while also dealing with the stress associated with the financial costs of bed bug eradication efforts (Comack and Lyons 2011).

In addition to the bites and anxiety caused by bed bug infestations, there has been some recently conducted field research that suggests that bed bugs may also be responsible for producing allergens in the home. At this time, there is evidence documenting that household arthropods often produce asthma-inducing allergens on their bodies, in their shed skins, and particularly in their fecal deposits (Doggett 2018b). It is well known that insect debris becomes intermixed with household dust where the allergen particulates can be inhaled by the human hosts living in the infested environment (Doggett 2018b). A study by Sternberg (1929) reported that a bed bug victim who was prone to summer asthma attacks, had those attacks cease when their bedding was cleaned frequently. While those attacks may have been caused by another insect's allergens, a later study in 1935 with a different bed bug victim, produced similar results (Jimenez-Diaz and Cuenca 1935). The 1935 bed bug host also suffered from seasonal allergies. This second study was able to document that this same sufferer also had strong allergic reactions when exposed to extracts of the common bed bug. A 1997 Chinese study by Wanzhen and Kaisheng was described by Doggett (2018). This study tested bed bug allergens on asthmatic and non-asthmatic patients to observe if allergic reactions would occur. They found that 63% of asthmatic patients reacted to the allergens, while only 11% of non-asthmatic patients had a reaction.

Environmental histamines are known to cause allergic reactions in humans, and bed bugs are known to produce histamines in their feces. A study by DeVries et al. (2018) revealed that histamine levels in bed bug infested homes were highly elevated in comparison to homes without infestations. DeVries also demonstrated that histamine levels in household dust would remain elevated even after heat treatments eradicated bed bug infestations. Given that bed bug infestations produce high levels of histamines, and humans are known to develop allergic reactions to environmental histamines, it is reasonable to suggest that bed bug infestations may present a respiratory health risk to some of their hosts.

2.6 Bed Bug and Human History

Humans have been lamenting the presence of bed bugs in written records dating as far back as 3rd-century BC. Writers such as Aristotle (*Historia Animalium* 556) and Aristophanes (*The Clouds* 634 cf. 710-742; *The Plutus* 541; *The Frogs* 439) included bed bug complaints in their records and plays. In Aristophanes' plays, characters sought out lodgings with "the fewest bugs" (*The Frogs*) and lamented about a "bum-bailiff from the mattress" biting them at night (*The Clouds*). Other writers however, had a more positive outlook on bed bugs, such as Pliny the Elder (24-79 AD) and Pedanius Dioscorides (40-90 AD) who wrote about the medicinal properties of bed bugs if they were ground up and mixed together with other substances to cure snake bites, urinary infections and health issues (Pliny *Natural History* XXIX. 61, 62, 63; Dioscorides *De Materia Medica* II. 36).

In addition to the ancient literature, Cimicids have actually been identified at archeological sites. In Egypt, specimens of *Cimex lectularius* L. were recovered from the Workman's Village in Amarna. The city of Amarna was founded by Akhenaten who reigned between the years 1352 BC and 1336 BC (Panagiotakopulu and Buckland 1999). This Cimicid

discovery confirmed that bed bugs have been harassing humans for at least 3000 years, and likely much longer than that. Cimicid remains have also been found in the Paisley Five Mile Point Caves in western North America. Human artifacts and bat remains were also found in these caves indicating that bats and humans cohabitated sporadically over the last 11,000 years. Given the opportunistic behavior of some cimicids, it is possible that even non-*lectularius* cimicids may have fed on the humans that occupied these caves (Adams and Jenkins 2017).

In more recent history, citizens of the Roman empire were known to have suffered from bed bug infestations. A Cimicid pronotum was recovered from a Roman site in Alcester, Warwickshire in modern England. While the pronotum belonged to a Cimicid bug, it was not certain whether the pronotum belonged to the common bed bug (*Cimex lectularius* L.), the pigeon bug (*Cimex columbarius)*, or some other species (Osborne 1971). Given that Roman occupants in the British Isles suffered bed bug infestations, it is likely that they cohabitated with bed bugs in the Mediterranean region as well. This idea can be supported by the Greek and Roman philosophers' and playwrights' alluding to bed bug infestations in their literature.

Across the Atlantic Ocean, bed bugs were known to have been found in colonial infrastructure as well. In Quebec City, bed bug remains were found in the Intendant's Palace. The Intendant's Palace (1675-1713) was built to accommodate the intendant, whose function was to oversee the administration of justice and the economy. Interestingly, bed bug remains were found in the palace's latrine (Bain *et al.* 2009).

In 18th century Canada, Swedish botanist Pehr Kalm claimed that Canada was just as infested with bed bugs as the Old World. Pehr Kalm observed bed bugs in Quebec after his 1749 visit of the upper New York state and stated that bed bugs had disturbed him all night (Pehr Kalm 1749). He also noted that bed bugs were thriving in rural regions just as much as they were

in the cities (Pehr Kalm 1749 , Bain 2004). Southward down the US eastern coast in Jamestown, what are believed to be *Cimex lectularius* L. specimens were recovered from, oddly, a colonial well in James Fort (King *et al*. 2013). It is highly likely that the Old World bed bugs were brought to the North American colonies aboard ships, and introduced in the continent where human-parasitizing bed bugs had not previously existed. Pehr Kalm noted that he did not see bed bugs among Native Americans, and that a commander at Fort Frederick had said that the Native American tribe members knew nothing about them (Pehr Kalm 1749 Vol I: 319). In not knowing about bed bugs, Native Americans lacked a word for the insect in their own language (Usinger 1966).

2.7 Historical Control Methods

A variety of evidence indicates that bed bugs have been plaguing humanity for a very long time. While the more ancient historians made it clear that every toxic and destructive method available was used by the civilians to rid themselves of this pest, it was well known that entire populations were rarely eliminated. In more recent history, however, humanity began to make more focused and organized efforts when attempting to eliminate bed bugs. The earliest record of an extermination business was in the 1690s, under the name of 'Tiffin and Son of London,' whose goal was to rid the nobility of bed bugs (Cowan 1865). In the early 1700's, *A Treatise of Buggs* was published by an early exterminator named John Southall. *A Treatise of Buggs* was a manual that contained bed bug behavioral observations and offered advice in how to actually eliminate infestations. Some advice involved preventative inspections and warned against bringing infested personal belongings into new environments (Southall 1730). Part of Southall's recommendations for beds were to make the beds plain and as free from of woodwork as possible. Elimination of wooden bed frames made the bed bugs easier to access when attempting to achieve complete

elimination. While *A Treatise of Buggs* attempted to offer more science-based and logical advice, other literature, such as *The Compleat Vermin-Killer*, offered more extreme advice. *The Compleat Vermin-Killer's* advice included filling bed crack and crevices with gunpowder and lighting it on fire, or boiling urine with plant matter and spreading the resulting solution along the joints of the bed frame (Anonymous 1777).

As one might expect, the first European colonists to become established in North America brought their bed bug cohabitants with them. In an effort to deter these introduced infestations, the colonists made bed frames from sassafras wood that had been soaked in a combination of boiled water, arsenic, and sulfur. Another method used to manage bed bug infestations in mid-1800s American neighborhoods included dusting pyrethrum powder between bed sheets to protect the occupant from the "voracious hotel bug" (Potter 2011).

In the mid-1800s and into the 1900s, an emphasis began to be placed on indoor cleanliness. In the mid-1800s, poor and overcrowded locations had become notorious for bed bug their infestations, while wealthy households had housecleaners that would keep infestations more or less in check (Potter 2011). Several control methods were recommended in a report by the Commissioner of Agriculture in 1875, but the "great remedy" for bed bugs was considered to be cleanliness with constant care and vigilance (inspection and crushing of live bugs) "every few days" (USDA 1875).

In the 1900s, bed bugs saw a great population boost with the development of indoor heating. The introduction of cast iron radiators at the turn of the century kept residents warm during colder seasons, and the development of electricity, fans, and forced air heating further assisted in spreading the heat throughout the living space (Potter 2011). The development of

indoor heating was also a boon to bed bug infestations. The warm indoor temperature allowed bed bug populations to thrive all year-round instead of fluctuating seasonally (Johnson 1941).

According to Great Britain's Ministry of Health bed bug report in the early 1900s, cities in Europe were extremely infested, to the extent that every urban authority was troubled with bed bug infestations regardless of the differences in social class (Ministry of Health 1934). Bed bugs infested dressing rooms, restaurants, furniture shops, theaters, coat rooms, school lockers, and laundries (Hartnack 1939, Mallis 1945, Potter 2011). Not only were bed bugs found in homes and furniture, but they were also being found in vehicles. According to M.F. Potter (2011), a 1930's Swedish survey inspecting 3000 moving vans found that 47% of those vans had bed bugs in them. This survey seemingly foretold of some of the problems that society faces today with bed bugs infesting rideshare vehicles (Howerton 2020).

To combat bed bugs in the mid-1900s, a number of control measures were instituted. One measure was the construction of "cleansing stations" so that families could heat their clothes and bedding using steam disinfectors to kill the bed bugs (Potter 2018). At the same time, fumigations using hydrogen cyanide were conducted regularly in vans loaded with furniture and personal belongings. In addition to hydrogen cyanide, fumigants such as sulfur dioxide and ethylene oxide were used to conduct home fumigations (Ministry of Health 1934). In addition to the insect control methods, public education came on board as a method of preventing the potential spread of bed bugs. The Department of Health in Scotland developed the "Glasgow System". The purpose of the Glasgow System was to educate new tenants about bed bug behavior and how household cleanliness could limit the proliferation of indoor pests, particularly bed bugs (Ministry of Health 1934).

In the early 1900s not only did bed bugs plague civilians, they were also a nuisance pest of soldiers in times of war. In World War I, during the East African campaign, there were accounts of bed bugs having invaded the cork lining of soldiers' helmets. At that time, soldiers were in the habit of placing all their helmets in a single pile each night before going to sleep. Needless to say, all of the helmets became infested within a short period of time (Matheson 1950). During World War II, bed bugs became a morale issue for the U.S. military. Bed bugs had become a major problem in U.S. military bases and the families of the soldiers urged their congressmen to find a solution. A measure that was taken to manage the military base infestations was fumigation with hydrogen cyanide (Potter 2011). While this fumigation method was very effective, it was also quite dangerous. Consequently, hydrogen cyanide fumigation required a great deal of containment and security efforts to prevent any non-target exposure.

It was not long after the widespread use of fumigation that dichloro-diphenyl-trichloroethane (DDT) was developed. DDT application rapidly became the preferred method for bed bug population control. DDT was considered far safer than fumigation, given that it was applied to items that humans regularly made contact with (e.g. entire mattresses, bed frames, and pillows; Potter 2018). In addition, DDT was much less expensive to use than fumigation. DDT was very effective for eliminating bed bug infestations and had a long period of residual activity (Madden et al. 1944, 1945; Stenburg 1947).

DDT was a synthetic organochloride that had also been widely used for the control of mosquitoes, flies, lice, and mites during World War II (WHO 1979). The discovery and synthesis of DDT is attributed to an unknown German chemistry student in 1874. However, the use of DDT became popular when Paul Muller discovered its insecticidal abilities in 1939 (Potter 2018). The use of DDT brought about a massive reduction in bed bug populations that had

previously infested human homes (for centuries), personal belongings, and machinery in the first world.

DDT works by binding to the insect's voltage-gated sodium channels and altering their function along the nerve membranes. This causes a constant sodium influx and continued depolarization within an insect's nervous system that leads to their death (Dong et al. 2014). DDT is also known to have long-lasting residual properties, where the lethal effects could last for six months (Madden et al. 1944, 1945), or even up to three years (Mallis 1954).

Although DDT applications had been initially focused on the elimination of lice, flies, and mosquitoes in and around military bases after 1942, DDT was most widely used to combat and eliminate bed bug infestations during World War II (Potter 2018). In 1945, the pest control market gained access to DDT and it was quickly found to be an effective means for eliminating bed bugs in homes (Potter 2011). After this discovery, DDT was used quite liberally, and was applied to almost any type of household item. DDT was applied to beds, incorporated into wallpaper, and formulated into "bombs" that could treat entire rooms, and even applied on humans (Potter 2018).

DDT was considered one of the most effective bed bug products ever known. However, bed bug resistance to DDT was identified within five years of its use (Hawaii in 1947; Johnson and Hill 1948). Within the following five years, bed bug resistance to DDT was confirmed and a recommendation from the National Pest Management Association resulted in a move away from the use of DDT. The use of DDT was replaced with the application of organophosphate and carbamate insecticides. These chemistries were used for bed bug control in the United States from 1958 -1968 (Busvine 1958; Potter 2011).

Although bed bugs had once been a prolific pest across the globe, the widespread use of DDT essentially eliminated bed bug populations in first-world nations. In many of those same nations, this "elimination" lasted for more than 40 years. This is why much of the younger populace of these nations never knew bed bugs existed, or at least thought they were gone for good. It came as quite a surprise when half a century later, bed bug populations began to reappear across the globe (Doggett et al. 2012)

2.8 Resurgence and Resistance

Over the last decade there has been a great deal of speculation as to the factors that have contributed to the bed bug global resurgence. Most entomologists agree that one of the major factors contributing to the bed bug resurgence has been the widespread selection of pesticide (primarily pyrethroid) resistant bed bug populations (Romero et al. 2007, Davies et al. 2012). One specific topic that is often mentioned when discussing the resistant bed bug resurgence is the use of pyrethroid impregnated bed nets. In the 1950s, bed bug resistance to DDT and dieldrin was recorded in West, North, and East Africa (Fourie and Crafford 2018). In the mid-1980s, these impregnated bed nets came into use in African nations to combat mosquitoes that were known to vector malaria. It has been hypothesized that the existing bed bug populations in Africa developed pyrethroid resistance from a combination of past DDT exposure and pyrethroid-impregnated bed nets that came into widespread use between 1995 and 1996. Many research studies published after 2001 mention correlations between the bed bug resurgence in Africa, the global bed bug resurgence, and the documentation of pyrethroid insecticide resistance in African and global bed bug populations (Davies et al. 2012).

Several other factors are thought to have contributed to the modern bed bug resurgence. These include the frequency of international and domestic air travel. The World Bank estimates

that domestic and international air travel was used by ≈1.67 billion customers in the year 2000 (World Bank 2022). The World Bank estimates that ≈4.56 billion customers used domestic and international air travel (prior to the COVID-19 pandemic) in 2019 (World Bank 2022), a near 300% increase in air travel in almost 20 years (World Bank 2022).

Certainly, the increasing human population density in urbanized locations since the 1940s is also thought to have contributed to the modern bed bugs' population increase and spread. Corresponding with the human population increase was a decrease in bed bug knowledge and familiarity. With the near eradication of bed bug populations in the 1940s and 50s, the average citizen, and even the pest control industry knew very little about bed bug biology and behavior. Professional pest managers were unaware of bed bug insecticide resistance and did not anticipate the modern bed bugs' ability to survive insecticide treatments. This lack of knowledge resulted in large cities with high populations densities such as New York and Chicago being among the first in the United States to experience and report the bed bug resurgence (Miller 2018).

Over the past twenty years, a variety of sources have provided insight into the severity of the bed bug resurgence within the United States. Cindy Mannes, a former director of public affairs for the National Pest Management Association, was quoted by *WebMD*'s Daniel DeNoon (2003) stating that pest control firms were receiving an increasing number of bed bug related calls between 1999 and 2003. DeNoon also stated that the Orkin Pest Management Company had predicted that there would be a 30% increase in bed bug calls between 1999 and 2004. However, just between 2001 and 2003 the number of bed bug calls increased by over 500%. In DeNoon's 2003 article, Mannes stated that bed bugs were present in all but eight states, but by 2006 bed bugs had been confirmed in all 50 states (Gangloff-Kaufmann et al. 2006). An article by Whitford (2017) quotes the founder and president of Specialty Consultants, Mr. Gary Curl, who

estimated that a total of 815,000 bed bugs treatments were conducted in 2015. Mr. Gary Curl also estimated that 907,875 bed bug jobs had been conducted in in 2016, a 11.4% increase in bed bug jobs between 2015 and 2016.

The NPMA regularly performs surveys called the "*Bed Bugs without Borders*" for the pest control industry to understand how bed bug trends change over time. In 2011, a *Bed Bugs without Borders* survey indicated that bed bugs were being reported across the United States (Potter 2011). The *Bed Bugs without Borders* surveys were intended to quantify the severity in the growth of the global bed bug resurgence. These surveys are conducted online, and the bed bug queries were sent to pest control companies in the United States as well as international companies. The survey questions asked about customer misidentification (for example, a customer confusing bed bugs with fleas or other pests; National Pest Management Association 2018); bed bug infestation treatment frequency; where bed bugs were located in the home; management methods used by the company; and business practices (e.g. warranties, guarantees, etc.; Potter et al. 2010). According to Potter's 2011 survey, 99 percent of the pest manager respondents had encountered actual bed bug infestations, whereas ten years prior, only 11 percent of respondents reported even receiving bed bug calls.

In a more recent (2018) *Bed Bugs without Borders* survey, it was found that bed bugs in the United States were commonly found in single-family homes, apartments, and hotels. Bed bugs were also commonly occurring in nursing homes, schools, day care centers, and a variety of other places such as medical facilities and public transportation centers (NPMA 2018). According to this 2018 survey, 97% of pest management professionals had treated for bed bugs during the previous year. The survey also found that people often confused bed bugs with other pests. Seventy-one percent of pest management professionals had been called to treat for fleas

when upon arrival to the customer's home, the pest problem was found to be a bed bug infestation. This survey revealed that even as late as 2018, customers still had a lack of familiarity with bed bugs even though they had become a widely publicized pest in the United States since the early 2000s.

As discussed previously, one of the hypothesized causes of the modern bed bug resurgence is the widespread development of insecticide resistance among modern populations. Insecticide resistance develops within an insect population in response to selective environmental pressures such as repeated insecticide applications. The consequence of these applications is that the insecticide kills off those individuals in the population that are susceptible to the insecticide, but fails to kill those individuals that are genetically not susceptible. Repeated insecticide exposures have selected for resistant individuals in the modern populations, while the susceptible bed bugs have been eliminated (Staunton et al. 2008, Dang et al. 2017). This has resulted in the resistant bed bugs passing their genetics on to their offspring, so that within a few generations, all individuals within the population are genetically resistant to insecticides. At this time, modern bed bug populations are known to have three key mechanisms of resistance. These resistance mechanisms are: 1. target site insensitivity (*kdr* resistance); 2. enhanced detoxification enzyme activity, and 3. reduced cuticular penetration.

Target-site insensitivity (*kdr*) resistance is a key mechanism for resistant bed bugs surviving pyrethroid insecticide treatment. Both DDT and pyrethroids exert their toxic effect by binding to voltage-gated sodium channels (VGSC) in the insect nervous system. This causes a constant influx of sodium through nerve membranes that lead to insect paralysis and death (Yu 2008). Amino acid point mutations, dubbed "*kdr*" mutations, within the VGSC make it difficult for pyrethroids and DDT to bind to the intended target site (Romero 2018).

The first "*kdr*" target site resistance mutation in bed bugs was discovered in the alpha-subunit after the analysis of the VGSC in bed bugs collected from the state of New York (Yoon et al. 2008). The hypothesis that pyrethroid resistance was caused by this mutation in the New York bed bugs was supported by low mortality responses of these bed bugs during insecticide exposure bioassays (Yoon et al. 2008). Based on this discovery, Zhu et al. (2010) evaluated 110 bed bug populations from across the United States to determine the extent of bed bug *kdr*-mutations. Five populations, two with pyrethroid-resistance and three with no resistance, showed no mutations. Twelve populations that were assayed showed one or two VGSC target-site mutations (L925I and V419L). Zhu et al. (2010) also discovered that 88% of 93 populations that were not assayed for pyrethroid susceptibility possessed a least one of the two mutations known to cause *kdr* resistance. This discovery was particularly important when we consider that the majority of insecticide spray formulations that are allowed to be used indoors due to the Food Quality Protection Act of 1996, are pyrethroids. The alpha subunit mutation is specific for blocking the DDT and pyrethroid insecticide mode of action. Therefore, the *kdr* mutation severely limits the efficacy of the few products that we have that are labelled for bed bug control in the United States and across the globe.

A second mechanism of resistance that has been found to be prolific in modern bed bug populations is enhanced detoxification enzyme activity. Enzymes inside the insect body are responsible for the metabolism of nutrients as well as the catabolism of harmful substances. Monooxygenases such as cytochrome P450 (P450s) have long been known as detoxification enzymes that insects use to break down the insecticides that get inside their bodies. Insect populations that have been repeatedly exposed to insecticides have been selected for those individuals that had the largest numbers of enzymes. Those individuals that genetically have

higher concentrations of enzymes survive the insecticide applications via detoxification, and live to pass their genetics on to their offspring (Romero 2018). Romero et al. (2009b) conducted an insecticide synergism study where deltamethrin was combined with piperonyl butoxide (PBO). This formulation was then applied to a resistant bed bug strain. PBO is known to inhibit the mixed function oxidase system in insects. Therefore, when it was discovered that the PBO greatly reduced the resistance ratio of deltamethrin-resistant bed bugs, this indicated that the cytochrome P450s were the major contributors to the bed bug strain's resistance. Another study by Lilly et al. (2016a) also found that large numbers of detoxifying enzymes such as hydrolytic esterases and microsomal oxidases were present in a bed bug strain that was known for having a multi-generational history of insecticide resistance.

A study by Romero and Anderson (2016) discovered that bed bugs can develop resistance to neonicotinoids. Neonicotinoids are a chemistry that has recently been paired with pyrethroids in some of the more novel formulations currently registered for bed bug control. A pyrethroid-resistant bed bug strain that had been collected prior to the introduction of neonicotinoid-pyrethroid combination products, was found to have resistance to neonicotinoids. This study indicated that mechanisms of resistance to pyrethroid insecticides (possibly enhanced detoxification enzyme activity) may also confer resistance to neonicotinoid insecticides. However, at this time, the specific mechanisms of neonicotinoid-resistance in bed bugs are unknown.

The third mechanism of resistance that has been documented in household bed bug populations is reduced cuticular penetration. Insects that have reduced cuticular penetration type resistance are known to have thicker exoskeletons than those of susceptible individuals (Koganemaru 2015, Lilly et al. 2016b). Having a thicker cuticle serves to block pesticide

residues from entering the insect's body and eliminating any lethal effects. Thus, the resistant insects may contact insecticide (typically dried) residues during their activities, but the thicker cuticle prevents them from picking up a lethal dose. With regard to bed bugs, reduced cuticular penetration and cuticular biochemical modifications have all but eliminated any of the residual activity that has been associated with spray formulation insecticide products (Romero 2018). A study conducted by Lilly et al. (2016b) compared the cuticles of bed bugs that had been knocked down after exposure to *lambda*-cyhalothrin for two hours and four hours, with those that had appeared to be unaffected after twenty-four hours of exposure. The study found that bed bugs unaffected by *lambda*-cyhalothrin after 24 hours had significantly thicker cuticles than those that were more susceptible to the pyrethroid. A similar study conducted by Koganemaru (2015) also documented measurably thicker exoskeletons in resistant bed bug strains. A study by Zhu *et al.* (2013) compared cuticular protein expressions in pyrethroid-resistant bed bugs with those of susceptible bed bug strains. Zhu et al (2013) found that pyrethroid-resistant bed bugs had an overexpression of cuticular proteins, and that when gene expression of the proteins (cytochrome P450s and ATP-binding cassette (*Abc*) transporters) were downregulated, the resistant bed bugs became more susceptible to insecticides (Zhu *et al.* 2013). This means that in addition to bed bugs being selected for physically thicker cuticles, they are also being selected for enhanced cuticular protein expression that further increases resistance to pyrethroid insecticides.

There is no question that the selection pressure of spray formulation insecticide applications has allowed only those bed bugs with the most favorable cuticular modifications to survive. The consequence of selecting for this particular type of resistance mechanism, however, has greatly hindered the pest manager's ability to eliminate these "thick-skinned" populations. While pyrethroid-based insecticides still may be able to kill bed bugs that are sprayed directly

with the wet product, we can no longer rely on dried pesticide residues to kill any remaining bed bugs in a home after the initial application has been completed.

A very compelling question with regard to bed bug resistance has been "why aren't the chemical manufacturers coming up with new active ingredients to combat these resistant bed bugs?" It is important to understand that one of the key barriers to the development of novel insecticides to combat resistant bed bugs is the Food Quality Protection Act of 1996 (FQPA) (EPA 1996). The FQPA was intended to amend the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), which required that any new active ingredient that was to be used indoors, must be subjected to extensive animal testing to prove that the product has no acute, chronic, mutagenic, or teratogenic effects (EPA 1996). This animal testing takes approximately ten years to complete and costs approximately hundreds of millions of dollars (Lee et al. 2018a). This investment would also require that the chemical manufacturer make back this money in sales prior to the product going off patent and being available to their competitors who had not made that initial investment. Another issue that has hindered the development of new active ingredients for bed bugs, is the ten-fold safety factor (dilution) required for indoor insecticides where children are likely to be exposed (EPA 1996). In some cases, new active ingredients might not be as effective for killing bed bugs when diluted to 10-times below the "no observable effect level". Another consideration of the FQPA that should be considered is the size of the indoor market when it comes to insecticide applications. When applying a pesticide inside an apartment, a couple of ounces of the spray formulation may be all that is applied. Within those fluid ounces, are maybe a few grams of the active ingredient. Therefore, only a small quantity of the new $350 million-dollar active ingredient might be applied in an entire building. Compare this with an agricultural field where a new insecticide might be applied over hundreds of acres.

It is certainly in any chemical company's best interest to focus their financial investments on developing new insecticide products for agricultural use as opposed to the indoor market. This is because in an agricultural field, hundreds of gallons of new insecticide product might be applied to a crop of corn or alfalfa that does not require a 10-fold dilution of the active ingredient.

In 1996, the FQPA instituted stricter pesticide safety regulations overall. These regulations included new safety standards; 15-year pesticide registration reviews, a tenfold safety factor for children; and the quantification of the aggregate pesticide risk that might come from varied sources (i.e., food, water, and residential sources; EPA 1996) to fill the "Risk Cup". Pyrethroids are due to begin the re-registration process in the 2020s. Note that the original passing of the FQPA resulted in the EPA terminating the registrations of multiple insecticides previously labelled for indoor and agricultural uses (EPA 1996). Some of the insecticides that had their indoor registrations terminated included organophosphate (with exception of Nuvan strips which use dichlorvos) and carbamate formulations, leaving the pest control industry with only a small arsenal of pesticide tools (pyrethroids) to control bed bug populations. We might expect that future re-registration efforts may reduce our bed bug product options even further.

2.9 Current Control Methods

Since 1990, there have been ~12 classes of insecticide products evaluated for control of the common bed bug (Lee et al. 2018). These classes have included chlorinated hydrocarbons, pyrethroids, organophosphates, carbamates, neonicotinoids, pyrroles, insect growth regulators (IGRs), spinosyn, oxidiazolone, phenyl pyrazoles, botanicals, desiccant dusts (inorganics), insecticide mixtures (e.g. pyrethroid-neonicotinoids), and others (e.g. fumigants and repellents). Some of these compounds, such as organophosphates and carbamates, are currently restricted from indoor usage in the United States. However, there are also a number of non-chemical

options currently in use for bed bug control which include the use of heat and entomopathogenic fungi. The different types of chemical and non-chemical bed bug control options currently available will be described in this section.

2.9.1 Chemical Controls for Bed Bugs

Pyrethroids are the most common chemistry formulated in pesticide products that are labelled for bed bug control (Dang et al. 2017). Pyrethroids are synthetic compounds that mimic pyrethrum, a natural insecticide compound extracted from dried *Chrysanthemum* flowers (Yu 2008). Deltamethrin and permethrin are some of the more common pyrethroid compounds used in insecticide formulations, but with the onset of insecticide resistance other synthetic pyrethroids such as cyfluthrin, bifenthrin, and *lambda*-cyhalothrin have been developed as well. Pyrethroids have been formulated into several different types of products including sprays, pressurized aerosols, dusts, and even impregnated mattress liners and bed nets (Lee et al. 2018). As discussed previously, the pyrethroid mode of action is to bind to the alpha sub-unit in the VGSCs which increases the flow of sodium ions into the insect cells. The constant influx of sodium ions results in neural over-excitation (excessive firing), which leads to the paralysis and death of the target insect (Yu 2008). Over the last decade, the chemical manufacturing industry has been pairing another chemical group, the neonicotinoids, with the pyrethroids to control resistant bed bugs.

Neonicotinoids are analogs of nicotine, and agonistic mimics of acetylcholine which activate the nicotinic acetylcholine receptors (Yu 2008, Lee et al. 2018). Neonicotinoids will bind to the receptors but will not be destroyed by acetylcholinesterase, which hydrolyzes acetylcholine under normal circumstances. This binding activates and continues activating the receptor, which results in overstimulation of the cholinergic synapses that cause hyperexcitation,

convulsions, paralysis, and ultimately insect death (Yu 2008). In modern bed bug control products, pyrethroids are frequently paired with neonicotinoids like imidacloprid, acetamiprid, thiomethoxam, or clothianidin in the form of liquid sprays (Lee et al. 2018). While these combination sprays have been effective, there is already evidence of resistance to some of the pyrethroid-neonicotinoid products in field collected bed bug populations (Romero and Anderson 2016).

One of the few non-pyrethroid-neonicotinoid spray formulations that was initially popular for bed bug control, was Phantom aerosol. The active ingredient in Phantom was chlorfenapyr which is a pyrrole insecticide. Pyrroles work by disrupting the ability of the insect to synthesize ATP and produce energy in their mitochondria. This is done by an active metabolite of the pyrrole disrupting the proton gradient across the mitochondrial membrane (Yu 2008). While the pyrrole mode of action would appear to be effective for controlling any household insect, the effectiveness of chlorfenapyr turned out to be highly variable when it came to bed bug control (Lee et al. 2018). In some situations, the product was highly effective in eliminating bed bugs but in other situations (particularly when applied as a residual) it was completely ineffective.

Insect Growth Regulators (IGRs) were initially thought of as a logical method for controlling resistant bed bug populations (Goodman et al. 2013). This was because there is no known resistance to IGRs, and because there were already several products registered for indoor use at the time of the bed bug resurgence. There are currently two widely used types of IGRs that have very different modes of action, but both function by disrupting the insect physiology and eliminating their reproductive success. One type of IGR is known as a Juvenile Hormone Analogue (JHA). This type of IGR does not kill the insect outright but mimics their natural

hormones so that they do not develop into reproductive adults (Yu 2008, Lee et al. 2018). The second type of IGRs are the Chitin Synthesis Inhibitors (CSIs). CSIs inhibit the function of chitin synthetase, an enzyme that allows the newly formed exoskeleton on a molting nymph to sclerotize successfully. Inhibition of chitin synthetase disrupts the molting process of the insect, so that the nymph cannot shed the old exoskeleton properly and it dies during the molting process (Yu 2008; Lee et al. 2018). In German cockroaches, CSI exposed nymphs molt to adulthood but have twisted wings and other deformities (Demark and Bennett 1989). The most significant effect of the CSI is on adult female cockroaches who become incapable of producing viable egg cases (Demark and Bennett 1990).

Over the last decade, several IGRs have been evaluated for controlling bed bug populations. In the United Kingdom a JHA, S-methoprene, was found to be effective at eliminating reproduction in both susceptible and resistant bed bug strains under laboratory conditions (Naylor and Boase 2008). However, in the United States, another JHA (Hydroprene) was evaluated and found to have no significant effect on both bed bug development and bed bug fecundity when applied at the label rate. Hydroprene was capable of impacting development and fecundity, but it required an application rate that was 10-fold greater than the label specification (Goodman et al. 2013).

A problem that Doggett *et al.* (2012) presented with regard to using CSIs as a primary bed bug control method, was the necessity of the bed bug to obtain a blood meal in order for the product to work. The ethical implications of this blood meal requirement means that, the customer had to be fed upon in order for the bed bug to begin the molting process and die. Doggett *et al.* (2012) suggested that the use of CSIs, and requiring customers to be bitten, might present a litigation risk for pest management professionals.

2.9.2 Dusts

A variety of insecticidal dusts have been applied in homes and other locations for the prevention and control of bed bug populations. Many of the original dust products were formulations of pyrethroids, such as cyfluthrin, deltamethrin, and permethrin (Lee et al. 2018). However, since the bed bug resurgence, other dust products, often referred to as desiccant dusts, have entered the market. These products included diatomaceous earth (DE), silicon dioxide dusts, and even limestone dusts (Romero *et al.* 2009a, Potter *et al*. 2014, Singh *et al.* 2016). Desiccant dusts have proven to be the most effective for bed bug control because they act by absorbing the waxes on the bed bug epicuticles, which causes the insect to slowly lose their internal moisture and eventually succumb to dehydration (Yu 2008).

A problem that arises with some desiccant dusts is that they are slow acting and do not cause instant bed bug mortality. Diatomaceous earth is known to be slow acting (1-2 days) and brief exposures do not necessarily result in mortality (Benoit *et al.* 2009, Potter *et al*. 2014, Singh *et al.* 2016). This has resulted in some customer dissatisfaction with the product. However, silicon dioxide has been shown to be more efficacious than DE, killing bed bugs within 24 hours or less (Potter *et al*. 2014, Singh *et al.* 2016).

2.9.3 Heat and Cold Treatments

One of the most widely used non-chemical bed bug control methods has been heat. Heat treatments for bed bug have been applied in a variety of ways. One of the earliest methods used for treating modern bed bugs, was to apply steam to infested surfaces. Steam treatments at ≈150°F have been quite effective when used properly (Puckett et al. 2013). Puckett et al. (2013) determined the application parameters of steam treatment, which specify an application time of

10 seconds per 30.50 cm of surface area. To generate significant mortality, the bed bugs must be located directly under the steamer head because bed bugs that are in a position lateral to the steamer-head may escape treatment (Puckett et al 2013). Also, if the technician moves the steamer too quickly, the steamer may not deliver enough heat to kill bed bugs. Certain fabrics, such as leather (Wang et al 2018), are known to protect the bed bugs hiding under them from the heat. Thus, it is important to note that the effectiveness of any steam treatment is dependent on the vigilance of the technician.

Another method of bed bug heat treatment is the use of portable heat chambers. Heat chambers typically consist of portable tents that can be taken into a home and filled with infested furniture (depending on the size of the chamber) and belongings. The items in the heat chambers are heated to 130°F and monitored for several hours while the rest of the home is typically treated with spray formulation insecticides. As of 2020, heat chambers are starting to be used a method for treating bed bug infested vehicles (Howerton 2020).

In 2011, Kells and Goblirsch determined that the thermal death point for adult and nymph bed bugs was 117°F, while bed bug eggs required exposure to 120°F in order to die. This information turned out to be very important to the pest management industry. Prior to the Kells and Goblirsch (2011) study, many pest management companies had purchased portable heat systems that had no means of getting an entire apartment or home up to the bed bug thermal death point. So many of the initial attempts to eliminate bed bugs using whole home heat utterly failed.

The use of whole home heat systems has been a widespread alternative to spray formulation applications since the bed bug resurgence. Bed bugs are known to harbor in hard-to-reach locations within a home, and similar to fumigation, heat treatments do not require that

every bed bug harborage be located prior to treatment. Therefore, to the average consumer, whole home heat systems sound like a safe and ideal way of eliminating bed bugs throughout the house.

However, the efficacy of using whole home heat treatments for bed bug control can vary depending on the heating capacity of a particular system. Many of the heating systems that were marketed for bed bug control soon after the resurgence did not have the capacity to kill bed bugs in three-bedroom, two story, cinder block apartments during a standardized six-hour heating period. In addition, many companies that initially sold whole home heat treatments did not have to employ certified pest control professionals because they did not apply pesticides. Needless to say, many of the early heat treatments failed to eliminate bed bug infestations. Fortunately, many of the amateurs have left the heat treatment business, and only those pest management companies that can afford to purchase effective heating systems remain in the bed bug heat treatment business (Miller 2022, personal communication).

On the other end of the temperature spectrum, freezing bed bugs as a method of control has also been explored. Bed bugs have been known to die within an hour when exposed to very frigid conditions (-16°C, Benoit 2009). Wang and Cooper (2011) suggested that household freezers that reach temperatures of -14°C to -15°C could completely kill bed bugs after 4 days of exposure. While static freezing methods like putting bed bugs in a freezer is not a practical method of control, the use of portable instantaneous freezing equipment has been marketed as a method for bed bug control. The single marketed bed bug freezing device is the Cryonite® applicator. This applicator converts liquid carbon dioxide into particles of dry ice. The Cryonite® applicator then delivers a jet of dry ice from the nozzle at a temperature of -78°C. This temperature can kill bed bugs on the surfaces of furniture and walls as long as they are in the

direct line of contact with the dry ice. However, laboratory demonstrations have shown that bed bugs hiding beneath those bed bugs that received a direct Cryonite application, were able to survive. Another issue that was quickly discovered in the field, was that adults, nymphs, and bed bug eggs could be non-lethally ejected from the treatment area by the rapid air displacement caused by the Cryonite applicator device (Doggett 2013). It is for this reason that the Cryonite system was not encouraged for use in Doggett's (2013) Bed Bug Code of Practice in Australia.

2.9.4 Entomopathogenic Fungi

The application of entomopathogenic fungi as a biological control method for bed bug elimination has recently increased in popularity. Two fungal species that have been evaluated for their ability to eliminate bed bugs include *Beauveria bassiana* (Barbarin *et al*. 2012) and *Metarhizium anisopliae* (Ulrich *et al*. 2014). Of the two fungi, *B. bassiana* was shown to be more effective when applied in locations with lower humidity (50%) than *M. anisopliae* (98%). Thus, *B. bassiana* is thought to have a superior viability in homes. In addition, bed bugs were also shown to have potential defensive pheromones which may inhibit infection by *M. anisopliae* (Ulrich *et al.* 2015) making this fungal product less effective for bed bug control.

Currently, Aprehend® (ConidioTec LLC, Centre Hall, PA) is the only fungal product on the market that is labeled for the control of bed bugs. Aprehend is an oil-based formulation that uses suspended *B. bassiana* fungal spores as its active ingredient. *B. bassiana* is an entomopathogenic fungus that is known to infect a number of insect pests such as mosquitoes, house flies, Colorado potato beetles, triatomine bugs, and even German cockroaches (Lecuona 2001, Blanford et al. 2005, Wraight et al. 2009, Acharya et al. 2015, Davari et al. 2015, Ramírez et al. 2020).

In 2012, *B. bassiana* was evaluated for its potential as a biological agent to control bed bug populations. It was found to be highly infectious to bed bugs after only a short-term exposure with mortality occurring within three to five days (Barbarin et al. 2012). It was also found that pyrethroid resistant bed bug strains were susceptible to *B. bassiana* infections (Barbarin et al. 2017). It was originally thought that the reduced cuticular penetration resistance mechanism, which is widespread in the bed bug populations, might hinder fungal infection. However, a study by Pedrini et al. (2009) documented that pyrethroid-resistant kissing bugs, *Triatoma infestans*, were still susceptible to *B. bassiana* even when they had thickened cuticles and greater quantities of cuticular lipids (Pedrini et al. 2009). The Pedrini et al study (2009) suggests that bed bugs with reduced cuticular penetration resistance should also be susceptible to *Beauveria bassiana* infections.

The fact that laboratory and field collected resistant bed bug strains have both been shown to be susceptible to *B. bassiana* in laboratory assays (Barbarin et al. 2012; Barbarin et al 2017) indicates that the Aprehend product has great potential for bed bug population elimination in the field. In addition, the Aprehend product is known to have 30+ days of residual activity. This residual activity, and the fact that Aprehend has greater efficacy than several of the more widely used pyrethroid products, suggests that this fungal spore product may soon replace spray formulations pyrethroids as the primary bed bug control product in the future.

2.9.5 Fumigation

Fumigation refers the process of using gaseous insecticides to control insect populations. These processes can be conducted in entire structures, small containers, and vehicles (Doggett *et al.* 2012, Todd *et al.* 2021). Given that fumigants are generally highly toxic to all living things, all nontarget organisms (i.e., pets, plants, and humans) must be removed from the fumigated

space prior to fumigation. Pest control technicians are also required to undergo specialized training before being allowed to conduct fumigations (Doggett *et al.* 2012).

Bed bug fumigations have occurred since the early 20th century, chiefly for controlling heavy infestations of bed bugs (Potter 2018). Sulfur and hydrogen cyanide fumigations saw use for control of bed bugs in homes and personal belongings. These fumigants were used to eliminate bed bugs from household items prior to them being moved into human living spaces (Potter 2011).

Sulfur was used in what has been referred to as the "fire and brimstone" method of fumigation, which involves the burning of sulfur to fumigate a space (Matheson 1950, Potter 2018). In some instances, alcohol was added to enhance both ignition and burning of sulfur. Sulfur treated spaces were confined using flour-paste coated newspaper to block off cracks around windows and doors. Fireplaces were sealed by covering the chimney with blankets. Smaller vents, like keyholes, were stuffed using rags. Some drawbacks of sulfur fumigations were that fabrics and wallpaper would be bleached and damaged by the fumes. Another risk included the tarnishing and corroding of metal fixtures, which needed to either be removed or covered in Vaseline. A third drawback was the presence of a strong stench that occurred from burning the sulfur. The procedure was simple and cheap, and while the sulfur fumes were lethal to bed bug eggs, nymphs, and adults, the gases tended to have poor penetration and sometimes procedures had to be repeated.

Hydrogen cyanide was considered the best fumigant in the early 1900s (Potter 2018). Fumigations with hydrogen cyanide were conducted in transportation vehicles, buildings, and chambers to eliminate bed bugs from furniture, clothing, and other belongings (Mallis 1945). While hydrogen cyanide was highly effective, it was also costly and deadlier than sulfur.

Hydrogen cyanide fumigations were best conducted by professionals. But non-professionals also conducted fumigations that lead to deaths and serious injury (Potter 2011, Potter 2018). Given the dangers of hydrogen cyanide use and the strict safety requirements to avoid nontarget mortality, the advent of DDT presented a highly desirable alternative. DDT rapidly replaced hydrogen cyanide fumigation due to its lower cost, no human health risk, long residual activity, and effectiveness in eliminating bed bugs (Madden et al. 1944, 1945; Potter 2011, Stenburg 1947).

While fumigation has not been thought of as bed bug control method for several decades, fumigation is still a widely used in the United States and other locations for the control of drywood termites in structures, and stored products pests in food storage facilities. Sulfuryl fluoride is one of the most widely used fumigant for these processes because it is well known for its ability to penetrate through cracks, crevices, wood, and packed seed in stored grain siloes (Scheffrahn et al. 1992). It is also a nonreactive gas and does not leave toxic residues upon surfaces (Kenaga 1957, Derrick et al. 1990, Nead-Nylander 2013). The current sulfuryl fluoride (SF) products that are labelled for structural pest control are Vikane (99.8% SF, Douglas Products) and Zythor (99.3% SF, Ensystex).

Sulfuryl fluoride kills by entering the insect body through the spiracles, or aeropyles in the insect eggshell, where it is broken down into sulfate and fluoride. The fluoride acts as the primary toxin and disrupts the glycolysis cycle within the insect's cells. With glycolysis inhibited, insects can only metabolize proteins and amino acids for energy, which is insufficient for maintaining the target insect's metabolic rate, thus the insect dies (Meikle et al. 1963; Outram 1970; Todd et al. 2021).

Although sulfuryl fluoride (SF) products have been labelled for bed bug control since the 1960s, bed bugs were not a major pest issue at that time. The initial 3X dosage requirement for bed bug control was very high. Note that the SF dosage factor is based on the susceptibility of a specific pest species to SF relative to the susceptibility of drywood termites. The drywood termite dosage rate for SF is 1X. However, the bed bug resurgence, and the more recent research indicating the bed bugs populations (including the eggs) could be eliminated at a 1.9X rate (Phillips et al. 2014), has made SF fumigation (using Vikane®) a more practical and desirable method for controlling bed bugs in homes, personal belongings, and even vehicles. Current research examining the practical use of SF for bed bug elimination is being marketed to the pest management industry, so we expect to see more SF fumigation efforts for bed bug control in the future.

In the early 2000s a lesser-known fumigant product using the active ingredient dichlorvos (a volatile organophosphate) was formulated into a plastic resin strip (Nuvan Prostrips®, Amvac Chemical Corp., Los Angeles, CA, USA), and registered for use as a bed bug control method. These strips were intended to be placed into plastic bags, closets, and other confined spaces to kill bed bugs that had infested personal items (i.e. luggage, clothing, electronics) (AMVAC Chemical Corporation 2014). Lehnert et al. in 2011 explored the use of the dichlorvos strip as a whole room fumigant. It was found that the resin strips took one week to eliminate beds bugs in a room, but that time could be reduced to 3 days with the addition of a fan to move the air. The addition of a heat source, and a fan was found to reduce the bed bug elimination time to 36 hours, because the heat increased the volatility of dichlorvos. Unfortunately, the US EPA quickly noted that the use of a fan or heat was not on the insecticide label, and that the increased heated air flow released more active ingredient than was allowable. The publicized EPA restrictions

greatly reduced the product's popularity for use in the professional pest control market and the ProStrips are no longer offered as a professional control method. At this time, the Nuvan ProStrips are almost exclusively sold on "do-it-yourself" websites (DoYourOwn.com).

2.10 Summary

The common bed bug is an incredibly resilient and successful household pest. The bed bug has plagued humans for thousands of years, and it was only in the last century that humans have been able to significantly reduce their populations. Yet, over the last several decades the bed bug has massively resurged around the world. Their rebound is greatly attributed to their developing resistance to commonly used pesticides that target their nervous system (pyrethroids). In addition, to the bed bug resistance issue, human population density, and the ease of human transport combined with the limitations of new insecticide product development by the FQPA of 1996, has aided the bed bug populations in increasing their prevalence across the globe.

At this time, the most widely used conventional pesticides use a mixture of pyrethroids, neonicotinoids, and/or synergists in their formulations. However, bed bugs are already known to be incredibly resistant to pyrethroids and have also developed some resistance to neonicotinoids. While using a combination of these insecticides has been helpful in controlling large numbers of modern bed bug infestations, there are still individual bed bugs that continue to survive treatments. Failure to eliminate these resistant bed bugs will only result in reinfestations by even more resistant bed bugs in the future.

The resurgence of the modern bed bug and their inherent insecticide resistance emphasizes a need to find more effective, non-conventional control methods. A variety of non-conventional methods exist on the market in the form of whole home heat systems, fumigants,

and biological controls. This research study examines the efficacy and utility of sulfuryl fluoride fumigations, whole-home heat treatments, and a biological control product (Aprehend; *Beauveria bassiana*), as non-conventional control methods for eliminating modern bed bug infestations in homes, vehicles, belongings, and under different environmental conditions. The results of this research are intended to aid in the decision making of pest management professionals who are seeking out alternative methods to replace or supplement their current control strategies.

Chapter Three:

Evaluation and comparison of whole-home heat systems against bed bugs (*Cimex lectularius* L.) in public housing

Introduction

Throughout history, the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), has been a well-known pest of the human environment (Usinger 1966). Because of the bed bug's reliance on human blood for growth and reproduction, the common bed bug has adapted to cohabitate with their hosts, even as the human habitation has evolved from rural agricultural villages into today's cosmopolitan cities. In Rukke's research article (2018), the author considers modern human homes to be the "natural habitat" of bed bugs and describes bed bugs as being true anthropochores. The bed bugs' ability to adapt to all human environments has resulted in their becoming established pests on every continent in the world except for Antarctica. The modern bed bug's success can be attributed to several factors, including lack of predator pressure, their cryptic behaviors, and their malleable genetics which has allowed them to develop a variety of pesticide resistance mechanisms (Usinger 1966, Dang et al. 2017, Krinsky 2019).

Bed bugs were flourishing in the United States prior to the 1940's (Ministry of Health 1934, Hartnack 1939, Mallis 1945, Potter 2011). However, their populations were severely reduced in the mid 1940's by the widespread use of dichlorodiphenyltrichloroethane (DDT) and other broad-spectrum insecticides (Potter et al. 2010, Potter 2011). However, bed bug populations were known to have developed resistance to DDT within five years of its use (Hawaii in 1947; Johnson and Hill 1948). Between the years 1958 and 1968, organophosphates and carbamates replaced the use of DDT (Rafatja 1971, Potter 2018). Not surprisingly, the bed

bugs began to develop resistance to organophosphates and carbamates as well. However, by this time (Johnson and Hill 1948, Busvine 1958), bed bug populations had been almost completely eliminated from developed nations, and many people assumed that bed bugs had been eliminated for good.

The resurgence of modern bed bugs (the common bed bug, *Cimex lectularius* L. and tropical bed bug, *Cimex hemipterus* F.) that began in the 1990's caught many people by surprise. At first, the reason for the resurgence was completely unknown. But the resurgence is thought to be the result of remnant bed bug populations in Africa being exposed repeatedly to pyrethroid impregnated bed nets that were being used as mosquito control devices to reduce malaria transmission (Curtis et al. 2003, Kweka et al. 2009, Davies, T.G. et al. 2012).

Also contributing to the bed bug resurgence, the Environmental Protection Agency's (EPA) 1996 issuance of the Food Quality Protection Act (FQPA) resulted in pyrethroid chemistries being one of few remaining chemical classes labelled for indoor use in the United States. This left professionals and consumers with few chemical options to address the modern bed bug resurgence (Romero et al. 2007, Davies et al. 2012).

The modern bed bug is known to have multiple resistance mechanisms that allow them to survive exposure to pyrethroids. These resistance mechanisms include target site insensitivity, increased metabolic detoxification, and reduced cuticular penetration (Lilly et al. 2016a, b; Romero and Anderson 2016; Dang et al. 2017). The ever-increasing levels of resistance to insecticides has forced the pest management industry, in the United States and throughout the world, to seek out and evaluate alternatives to chemical spray formulations. Alternatives to spray insecticides have included the use of fumigation, steam heat, heat chambers, whole-home heat, frozen carbon dioxide, and entomopathogenic fungi (Kells and Goblirsch 2011, Olsen et al.

2013, Phillips et al. 2014, Barbarin et al. 2017, Kells 2018, Wang et al. 2018). One of the most widely used non-chemical methods of modern bed bug control has been the use of heat (Anonymous 2020a).

Heat treatments for bed bug control have been applied in a variety of ways. One of the earliest methods used for treating modern bed bugs was to apply steam to infested surfaces. Steam treatments are quite effective when used properly. Puckett et al. (2013) determined application parameters for steam treatment, which specify an application rate of 10 seconds per 30.5 cm of surface area. To generate significant mortality, the bed bugs must be located directly under the steamer head. Bed bugs that are in a position lateral to the steamer-head may survive the treatment (Puckett et al 2013). Also, if the technician moves too quickly, the steamer may not deliver enough heat to kill the bed bugs. Also, certain fabrics, such as leather (Wang et al 2018), are known to protect the bed bugs hiding under them from heat treatment. Thus, the effectiveness of any steam treatment is dependent on the diligence of the technician.

Another method of bed bug heat treatment is the use of portable heat chambers. Heat chambers typically consist of portable tents that can be taken into a home and filled with infested furniture (depending on the size of the chamber) and belongings. The items in the heat chambers are heated and monitored for several hours while the rest of the home is typically treated with conventional insecticides. As of 2020, heat chambers (portable trailers) have been used as a novel method of treating bed bug infested vehicles (Howerton 2020).

The use of whole home heat systems has become a widespread alternative to spray formulation applications. Bed bugs are known to harbor in hard-to-reach locations within a home and heat treatments do not require that every bed bug harborage be located prior to treatment. Therefore, to the average consumer, whole home heat systems sound like a safe and ideal way of

eliminating bed bugs throughout the house. However, although many types of heat systems have been marketed for bed bug control, not much known about the efficacy of different types of heat systems or their actual ability to eliminate bed bugs from homes of different sizes.

It is essential that the heat system being used is able to produce the required amount of heat needed to raise all hard to heat cracks and crevices to bed bug lethal temperature. In 2011, Kells and Goblirsch determined that the bed bug lethal temperature was 47.7°C for nymphs and adults, but that bed bug eggs required a temperature of 50.0°C to ensure mortality. Thus, every hard to heat crack and crevice inside a home must reach 50.0°C for all bed bugs to be killed. Needless to say, whole home heat treatments must be monitored rigorously to determine that all of the cubic footage, and all cracks and crevices achieve lethal temperature.

One of the common questions that our laboratory receives from bed bug infested consumers is "which is better, heat or chemical?" Although this question may sound simple, it is not. There are currently a large variety of heat systems on the market, but up until this time there have been no efficacy studies to evaluate their performance under different household conditions. Yet, it is very important to know which of these heat systems has the potential to eliminate bed bug infestations of different sizes in housing units of different cubic footage and different levels of household clutter. Therefore, it can be very difficult to say conclusively that a particular heat system will be able to eliminate bed bugs under all conditions. Studies are needed to compare the different attributes of individual heat systems (that can range in prices from $10K to $150K) that use different energy sources, pieces of equipment, and temperature monitoring devices for bed bug elimination in different housing units. Based on this current need, the purpose of this study was to compare and evaluate different whole home heat systems for their ability to eliminate bed bugs located in hard to heat locations in infested apartment units. The

heat systems evaluated were the GreenTech Titan® propane system, the Temp-Air Thermal Remediation® electric system, and the Heat Assault® forced convection glycol system.

Materials and Methods

Heat Systems Evaluated

In this study, three professional bed bug heat systems were evaluated for their ability to eliminate infestations in low-income housing units. Each heating system required a different energy source (glycol, propane, or electricity) and specific installment procedures. The three systems were evaluated to compare their treatment time (set-up to takedown), required amount of equipment, temperature monitoring method, treatment efficacy (bed bug mortality and number of sensors reaching bed bug lethal temperature), and the price per system. After observing several individual heat treatments, it became obvious that the attentiveness of the heat treatment technician was also critical for the treatment success (efficacy). Therefore, technician activities were also monitored as part of this study. Activity included entering the treatment zone to get temperature readings, move furniture, and/or move equipment to circulate heat.

The three bed bug heating systems that were compared in this study included the GreenTech Titan 800 (GreenTech Heat Solutions, Anaheim, California) propane heater, the Temp-Air Thermal Remediation EBB-40KW system (Temp-Air Inc, Burnsville, Minnesota), and Heat Assault 600X (Tamarack Industries, Winnipeg, Manitoba) heat system (Table 3.1).

Table 3.1. Properties of each whole-home heat system.

Properties	GreenTech	Temp-Air	Heat Assault
Heat Source	Propane	Electric	Glycol (heated)
Reported BTUs	≈990,000+	≈24,880 (per heater) ≈99,520 total	≈600,000

Equipment	1 heater 1 mylar tube, divided 2 propane tanks 2 fans	4 portable heaters 5 power cables 1 power distribution box 12 fans	5-10 radiators, 10-14 hoses, 2-4 supply/return manifolds 14-17 fans
Cost of System	~ $11,000.00	~ $150,000	~ $87,000

GreenTech Heat Solutions designs, manufactures, and sells both electric and propane-based heat systems. The GreenTech Titan is a portable "direct-fired" propane-based heater marketed to eliminate bed bugs and other indoor insect pests. The Titan heater is set at the front door, and the heat is directed from the Titan to the specific rooms of the home using mylar tubing. There are two types of GreenTech Titans; the Titan 450 and the Titan 800 which produce 550,000+ and 990,000+ BTUs, respectively (GreenTech 2021). The GreenTech 800 system was evaluated for this study.

The second system that was evaluated in this study was the Temp-Air Thermal Remediation EBB-40KW trailer package. Temp-Air Inc. manufactures Thermal Remediation® bed bug heat treatment packages that are marketed to pest management professionals, hotels, universities, and property management companies. Temp-Air Heat packages can be purchased that contain different numbers of electric heaters, fans, power distribution boxes, cables, sprinkler head covers, and temperature monitoring equipment. Temp-Air also manufactures trucks and trailers that can be used to transport diesel generators as well as to store all heating equipment (Temp Air 2021).

The EBB-40KW electric heat system consisted of four 99.8 kg heaters (each one putting out 25,000 BTUs), and 12 fans that could be moved around the home to aid in the heating process. The electric power for this system originated from a diesel generator within the trailer that was then transferred through a power cable connected to a distribution box. The distribution

box then distributed the electricity to the individual heaters through additional power cables in order to power them.

The Temp-Air Company is the only manufacturer in this study that offered a temperature monitoring system for purchase. The temperature monitoring system consists of 12 digital sensors that can be placed in hard to heat locations. The digital sensors display the local temperatures on a computer monitor outside of the heated apartment. This allows the technician to constantly monitor the temperatures within different locations of the home. The constant temperature display allows the technician to be aware of locations that may fail to reach bed bug lethal temperature. This information allows him/her to move the heat equipment into more focused locations if those locations are not heating adequately.

The third heating system evaluated in this study was the Heat Assault system. Heat Assault is a Canadian company that sells diesel-powered heat systems (Heat Assault 300X and Heat Assault 600X). The Heat Assault website states that the 300X and 600X are able to produce 300,000 and 600,000 BTUs per hour, respectively. Both the Heat Assault 300X and 600X packages include a trailer that contains the following equipment: heat exchangers/radiators, supply and return distribution manifolds, fans, and heat transfer fluid (glycol) (Heat Assault 2021). For this study, the Heat Assault 600X system was evaluated.

The Heat Assault glycol system is unique in the fact that it uses what is called “forced convection technology”. This refers to how the atmospheric air is put in contact with radiators heated by glycol and the heat expands to fill the treated home. This forced convection begins with heating the glycol prior to contact with the atmospheric air. The Heat Assault System basically consists of two closed loop systems: the “Heat Loop” and the “Field Loop.” Within the Heat Loop, glycol from the glycol reservoir enters a heat exchanger. This heat exchanger is

powered by burners fueled by a diesel generator. Within the heat exchanger, the glycol is heated to a temperature of 96°C. After reaching the desired temperature, the heated glycol is pumped into a reservoir. From the reservoir, the heated glycol can then be either returned to the exchanger for continued heating or pumped into the "Field Loop." Within the Field Loop, the heated glycol is pumped from the reservoir to the supply manifold within the treatment zone (apartment) using industrial-grade hoses. The supply manifold splits the heated glycol into different hoses that are each connected to individual radiators. These radiators are placed in multiple locations throughout the treated home. The glycol then heats the atmospheric air around each of the radiators. Fans are attached to the radiators in order to push the heated air into the treatment zone (apartment), thus increasing the temperature within the treated space. After heating the air, the spent glycol (from the different radiators) then returns to a return manifold. From the manifold, the cooled glycol completes the Field Loop by being returned to the reservoir to be reheated and reused throughout the multi-hour heating process.

Study Sites

All heat treatments were conducted in bed bug infested United States Housing and Urban Development (US HUD) housing facilities. All GreenTech System evaluations were conducted in the Richmond Rehabilitation and Housing Authority (RRHA) located in Richmond, Virginia. Heat Assault heat system evaluations were conducted in the Rocky Mount Housing Authority (RMHA) in Rocky Mount and Charlotte, North Carolina. The Temp-Air heat system evaluations were conducted in both Richmond, Virginia, and in Rocky Mount, North Carolina.

All heat system evaluations were conducted in single or two-story apartment units with one to three bed rooms. All units treated were slab-on-ground construction with cinderblock

walls. The infested units within each treatment group varied in their cubic footage, clutter, and bed bug infestation levels.

Sentinel Bed Bugs

The bed bugs used for this study were from the Richmond strain. The Richmond field strain was collected from a group home in Richmond, VA in 2008. The Richmond strain is currently maintained inside plastic rearing containers in the Dodson Urban Pest Management Laboratory (DUPML; Virginia Tech, Blacksburg, VA). All bed bugs are fed defibrinated rabbit blood once a week using an artificial feeding system. Bed bug colonies are kept in environmental chambers held at ca. 28°C, ca. 55% RH, and a photoperiod of 12:12 h L:D cycle. In 2017, the Richmond strain was one of ten bed bug field strains evaluated at Purdue University (West Lafayette, Indiana USA) for resistance to insecticide products containing chlorfenapyr or bifenthrin. The Richmond strain was found to be the most resistant of all field strains evaluated in that study (Ashbrook et al. 2017).

Sentinel Bed Bug Preparation

Five sentinel replicates of each bed bug life stage (eggs, nymphs, and adults) were prepared at the DUPML prior to each heat treatment. Each life stage replicate consisted of 10 bed bugs each, so that each heat treatment had a total of 150 sentinel bed bugs. The bed bugs were placed on filter papers (35mm) inside of nylon stockings that were tied at the end. To prepare the eggs, adult female bed bugs were fed one week prior the scheduled heat treatment. The females were then placed in Petri dishes lined at the bottom with filter paper. Adult females were left to lay

eggs on the filter paper *ad libitum*, so that there were at least 10 bed bug eggs laid on each filter paper in time for each heat treatment.

Resident Preparation Instructions

Heat treatment preparation instructions were provided to each resident by either the housing authority or one of the heat treatment professionals. These instructions were typically provided a couple of days prior to treatment initiation. While the preparation instructions for each heat system were somewhat different, all of them focused on the removal of items that had the potential to be damaged by high temperatures. All houseplants, pets, candles, and combustibles (aerosol cans and cigarette lighters) were typically required to be removed. Preparation instructions also recommended that all electronics be disconnected from outlets and that televisions be removed or wrapped in blankets prior to treatment. In addition, all non-refrigerated foods, medications, stringed instruments, family heirlooms, and photographs were suggested to be removed to avoid any potential heat damage. It was also typically recommended that blankets, linens and towels be put through a dryer cycle prior to treatment, and that all clothes closets and drawers be opened prior to treatment to allow for heat access.

Clutter Ratio Determination

Each heat-treated apartment unit was measured to determine the cubic footage of each room. Measurements were made using measuring tapes ("15 ft" and "25 ft" maximum reach) and a Bosch® (Robert Bosch Tool Corporation, Prospect, IL) Professional GLM 40 measuring laser. The Bosch® measuring laser was used to measure overall room size (length, width, and height) as well as clutter where appropriate. The measuring tapes were used exclusively for measuring

clutter. The combined cubic footage of the entire apartment unit and the cubic footage of all personal items, appliances, cabinets etc. were used to determine the "clutter ratio" for each unit. This was so that we could determine the percentage of the apartment space that was filled (e.g. 10%; 30%, 50% etc.) with clutter or other items. If needed, we could later determine if this ratio influenced the time required for treatment, or the treatment efficacy. In addition, the space and clutter measurements were used to create a diagram of each treated apartment unit illustrating the amount of open space, the amount and location of clutter, and the locations of all heat the sensors and sentinel bed bugs (see below).

Sentinel Bed Bug Placement

Sentinel bed bugs of each life stage were placed throughout each infested unit in hard-to-heat locations that would be challenging to get up to bed bug lethal temperature. Sentinel bed bug replicates were also placed in potential bed bug harborage locations. These included holes in the walls, between furniture cushions, inside closets, within clothes drawers, behind electrical outlet covers, and in gaps between the walls and baseboards. After the conclusion of a heat treatment, the replicates were collected and evaluated for mortality at the DUPML. Bed bug eggs were monitored for 14 days after treatment to determine egg mortality. Eggs that failed to hatch within this period were determined to have been killed by the heat treatment.

Temperature Monitoring During Heat Treatment

The researchers conducting this study used the Temp-Air Thermal Remediation wireless logger system to monitor each treated unit. The Thermal Remediation system includes 12 temperature sensors, a signal repeater, and a laptop computer monitoring system contained

within a case. The 12 temperature sensors were individually placed in hard to heat locations such as the corners of rooms, within hallway closets, along floor-wall junctions, on shelves, and within furniture. The repeater, which maintains the signal between the sensors and the monitoring device, was placed in the dining room or kitchen. The laptop computer monitoring system was placed outdoors in front of the apartment unit on a fold-out table. All sensor temperatures were recorded at 30-minute intervals throughout the heat treatment process.

GreenTech Heat Solutions Treatment Process

The GreenTech system evaluated in Richmond, VA, was the Titan 800. This heat system was owned and operated by Richmond Redevelopment & Housing Authority. To power the Titan 800, the technicians connected it to two 45 kg AmeriGas® (AmeriGas, Richmond, VA) propane cylinders that were confined to the bed of their work vehicle. The technicians placed two temperature resistant fans inside each apartment unit to circulate air. The technicians also had a Lasergrip® (Etekcity, Anaheim, CA) handheld infrared thermometer to monitor surface temperatures during the treatment.

During the setup phase of the treatment, the technicians placed the Titan 800 inside the doorway of the apartment unit's front door. They then connected mylar tubing to the front of the Titan 800 and extended the tubing to the uppermost floor (2nd floor if it was a 2-story unit). The two circulating fans were placed in the locations near the mylar tubing outlet. The fans dispersed the heated air from the mylar tubing outlet into the treated room. During setup, the technicians moved any observed heat sensitive items to outside of the unit.

After the mylar tube and the fans were in place, the technicians used clamps to secure a tarp over the unit's open doorway. After covering the doorway, the heat treatment was initiated. During the GreenTech heat treatment process, technicians entered the unit approximately every 1-3 hours to check surface temperatures. When a particular room reached lethal temperature (48.8°C), as determined by the technician (after testing multiple surfaces with the handheld thermometer), the mylar tubing outlet was either moved to another room or floor depending on the size and structure of the unit. Once all the rooms located away from the front door had achieved lethal temperature, the mylar tubing was disconnected from the propane heater so that the ground floor living room could be heated directly from the Titan heater itself.

The technicians determined treatment completion. The criteria used by the technicians was either the time of day, or when all surface temperature measurements had reached ≈48.9°C (120°F), in each of the heated rooms. The RRHA heat treatment technicians were restricted by time, which required them to be finished treating every unit by 3:00 PM. When the treatment was concluded, all heat equipment was removed from the premises. Once the equipment was cleared, and sentinel bed bugs were collected, the RRHA technicians performed crack, crevice, and spot treatments using the spray formulation insecticide product CrossFire® (Clothianidin 4.0%, Metofluthrin 0.1%, Piperonyl Butoxide 10.0%; MGK, Minneapolis, Minnesota).

Temp-Air Thermal Remediation Treatment Process

Temp-Air heat treatments were conducted in both Richmond, VA and Rocky Mount, NC. The Temp-Air system was housed in a trailer that contained a generator, four ≈91 kg electric heaters, 12 circulation fans, and 5 power cables (1 large, 4 small). Virginia Tech Facilities personnel conducted the first two heat treatments. Dodson Urban Pest Management Laboratory personnel

conducted the last three treatments. The Virginia Tech Facilities personnel had their own temperature monitoring system, but it was non-functional at the time of these treatments. Therefore, they resorted to using a handheld temperature measuring device (model and manufacturer unknown) and the researchers' temperature monitoring system.

During the setup phase of the Temp-Air treatment, the technicians had to conduct a number of preparatory tasks. They installed covers over the overhead sprinklers used for fire prevention in each unit. They then inspected the home for items that could be damaged by the heat treatment. Finally, they sealed all exterior windows with aluminum tape to reduce potential heat loss.

After the apartment preparation tasks were completed, the technicians began moving heat treatment equipment into the apartment unit. Each of the four heaters was brought in by two technicians (heater weight- 99.8 kg). Individual heaters were then placed in specific rooms, with upstairs bedrooms (highest priority) typically being treated first. At least one heater, if available, was also located downstairs at the beginning of the treatment. This heater was usually placed in either the dining area or living room. Once the heaters were in place, the power distribution box and the fans were brought into the unit. At least two fans were stationed with each heater. Additional fans were stationed where needed, such as the living room or the kitchen. The distribution box was stationed in the living room. A large cable ran from the outdoor generator to the distribution box, and four cables ran from the distribution box to each of the heaters. Finally, a tarp was hung from the ceiling in the stairwell between the upstairs and downstairs corridor. This was to limit the movement of heated air from the downstairs to the upper level.

After all of the Temp-Air system elements were set up, the exterior generator was started. Each heater was manually set to the "blower" setting. This allowed the individual heaters to

warm up. After approximately ten minutes, the heaters were put on the "heat" setting at 145º F. The fans remained turned off until the heater temperatures reached 37.8°C. After the heater temperatures reached 37.8°C, the fans were turned on to disperse and circulate the heat throughout the infested space. Once the fans were turned on, all doors to bedrooms that contained heat equipment were closed to confine the heat inside of that space.

Temperatures in the treated spaces were checked regularly throughout the Temp-Air heat treatment process. Temperatures were checked at least once every hour using a handheld laser thermometer. During the temperature monitoring process, the technicians moved furniture, clothing, and other personal items around to increase their heat exposure. They also repositioned fans to refocus the air flow when needed. When a bedroom was determined to have reached bed bug lethal temperature, the technician opened the door and moved the heater into the hallway to help heat the rest of the space upstairs. As the heat treatment came to completion (based on lethal temperatures being achieved) technicians moved all heaters into the living room. Once the living room was determined to have reached lethal temperature, the treatment was ended.

There was no specific time constraint on when the Temp-Air technicians needed to be finished. Once the technicians determined the treatment complete, they began the breakdown process. To initiate this process, the technicians put all heaters on the "blower" setting again to cool them down safely. Then, the fans were unplugged and removed from the premises to be stored in the trailer. After the fans were removed, the heaters were shut down completely. The generator was shut down, and the power cables were disconnected from the heaters and power distribution box. Once the cables had been stored in the trailer, then the heaters and distribution box were removed from the home and placed into the trailer as well.

Heat Assault Treatment Process

Heat treatments were performed using a Heat Assault 600x system that was owned and operated by Action Pest Control, of North Carolina. All Assault heat treatments were performed in the Rocky Mount Housing Authority in North Carolina.

The Heat Assault system was contained in a single trailer. This trailer held the glycol reservoir (≈378.54 liters), and a pair of heat exchangers to heat the glycol. A diesel-powered generator activated the burners of the heat exchangers, as well as the pumps. A circulating pump drew the glycol from the reservoir and ran it through the heat exchangers. The heated glycol was returned to the reservoir via the pump and was then drawn out by a field pump through an industrial hose (2.54 cm width, 22.86 m length) to a supply manifold located in the apartment unit that was to be heat treated. The supply manifold split the glycol into five smaller supply hoses (1.91 cm width, 7.62 m length) that then sent the glycol to the five radiators. The radiators put the ambient air in contact with the radiator heated by glycol and fans attached to the radiators pulled the heated air from the radiators out into the treatment zone (apartment unit).

After the heated air was released from the individual radiators the cooler glycol exited the radiators through return hoses (1.91 cm width, 7.62 m length). The return hoses then brought all of the used glycol together into the return manifold. From the manifold the glycol was sent through a larger return hose (2.54 cm width, 22.86 m length) back to the trailer reservoir where it was reheated and reused throughout the heat treatment process. At the end of the heat treatment, the pumps were shut down but a compressor in the trailer was left on to return the glycol back to the reservoir.

To initiate the setup phase of the Assault heat treatment, the technician positioned the necessary equipment outside of the apartment unit that was to be treated. The technician then

went indoors and checked the unit for infestations. He also removed any sensitive items (such as aerosols) from the apartment unit. In addition, he detached all socket plates from the electrical outlets. After the inspection, the technician began moving the heat equipment indoors. Each bedroom had at least one radiator (total of 5-10 radiators) that had a fan (total of 14-17) attached to it. The living room and dining room each had two radiators and fans. Five additional fans were placed throughout upper and lower floor hallways, living room, and kitchen to circulate the heated air that was being produced by the radiators. The ground floor and upper floor of each apartment had a supply manifold (2) and a return manifold (2) located near the stairway. The technician connected the supply and return hoses (2.54 cm) to the trailer's glycol reservoir, and to respective supply and return manifolds. The supply and return hoses (1.91 cm) were also connected between the manifolds and the appropriate radiators.

Once all of the equipment connections were completed and secure, the technician turned on the generator and began the heat treatment. Turning on the generator initiated the cycle of glycol moving through the hoses into the radiators and then back to the glycol reservoir. The fans were turned on to draw the heat out from the radiators and circulate it into the surrounding air. The technician then began to check surface temperatures every twenty minutes for the duration of the heat treatment using a FLIR handheld thermometer (FLIR systems Inc, Portland, Oregon, US). Halfway through the heat treatment, the average temperature reached ≥50°C. At that time, the technician began to move the furniture and other belongings to suddenly expose hiding bed bugs to the high heat. This exposure process included flipping mattresses and leaning them on their sides against the wall, displacing and flipping couch cushions, standing couches up on their end, and pulling drawers out from the dressers and other cabinets. Clothes on the floor were

shifted around and clothes in bags were pulled from the bag and draped across furniture to expose the bed bugs to high heat.

Each glycol heat treatment had a duration of approximately 6 h. The technician determined the heat treatment was completed once the lethal temperature was held for a sufficient amount of time (approximately 3 h). When this determination was made, the technician went outside to shut off the trailer pump. The technician then turned on the compressor to send air through the glycol supply lines to return the remaining glycol back into the reservoir. Once it was determined that the lines were clear, the technician shut off the compressor and went back inside the treated apartment unit to turn off the fans. The hoses were then disconnected from the radiators and manifolds and moved out of the apartment. After removing all of the heat equipment, the technician returned all furniture items back (more or less) to their original positions.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to determine which heat system caused the most overall bed bug mortality. The percent mortality for each bed bug life stage (egg, nymph, and adult) produced by each heat system was also compared using the one-way ANOVA. Differences between mean overall mortality and mean life stage mortality caused by each heat system was considered significant if P-value was ≤ 0.05. Mean overall and mean life stage mortality for heat systems were separated using Tukey's HSD. All one-way ANOVA statistical analysis was performed in JMP Pro 16 (SAS Institute Inc., Cary, NC).

The probability of a treatment resulting in 100% mortality was modeled for each life stage using mixed-effect logistic regression. Separate logistic regression models were fit for the egg, nymph, and adult life stage outcomes. Each model included the heat system, clutter ratio, treatment duration, the proportion of temperature sensors that reached the desired temperature, and the number of technician entries per treatment hour, as fixed effects and the replicate as a random effect. The treatment duration times included in the data set ranged from ≈4.4 to ≈10.6 h. The quantitative predictor variables were standardized to allow for direct comparison of effect sizes of predictor variables using the formula:

$$\frac{observed\ value\ -\ minimum\ observed\ value}{maximum\ observed\ value\ -\ minimum\ observed\ value}$$

The models were fitted using the 'glmmTMB' package in R (Brooks et al. 2017). Overall model fit and distributional appropriateness was evaluated using tools in the 'DHARMa' package in R (Hartig 2021). Stepwise regression using the Bayesian Information Criterion (BIC) as the determining criterion was performed on the full models using the 'stepAIC' function in R to select the most important predictors of bed bug mortality in each life stage (Venables and Ripley 2002). Predictor addition and elimination was allowed at each step. Predictors that were retained were considered significant by stepwise regression.

A logistic regression model was also used to model the proportion of temperature sensors that reached the lethal temperature of 50°C for each treatment. The system, clutter ratio, treatment duration, and the number of technician entries per treatment hour were included as fixed effects in this model and replicate was retained as a random effect. This logistic regression model allowed us to understand which predictor variable(s) had the greatest impact in allowing temperature sensors to reach lethal temperature.

Results

Bed Bug Mortality

Total bed bug mortality for each heat system is provided in Figure 3.1. Total mortality ranged from 85.2% to 98.3%, with no system reaching 100% total bed bug mortality. Statistical differences were detected in total bed bug mortality across the three systems ($F = 4.46$, $df = 2$, $P = 0.0127$). Total bed bug mortality was significantly greater for Heat Assault heat treatments than for GreenTech heat treatments. The Temp-Air total bed bug mortality was not significantly different from either the Heat Assault or GreenTech total bed bug mortality.

Bed bug treated and control mortality for egg, nymph, and adult life stages for each whole-home heat system is presented in Figure 3.2. Overall, each heat system produced ~80% to 100% mortality for each bed bug life stage. No statistical differences were detected between nymph ($F = 1.76$, $df = 2$, $P = 0.1796$) and adult ($F = 0.3347$, $df = 2$, $P = 0.7167$) bed bug mortality across the three systems. However, statistical differences were detected in egg mortality between the heat systems ($F = 3.88$, $df = 2$, $P = 0.0255$), with the Heat Assault system producing significantly greater egg mortality than the GreenTech system. Bed bug egg mortality caused by the Temp-Air system was not statistically different from either the Heat Assault or the GreenTech system.

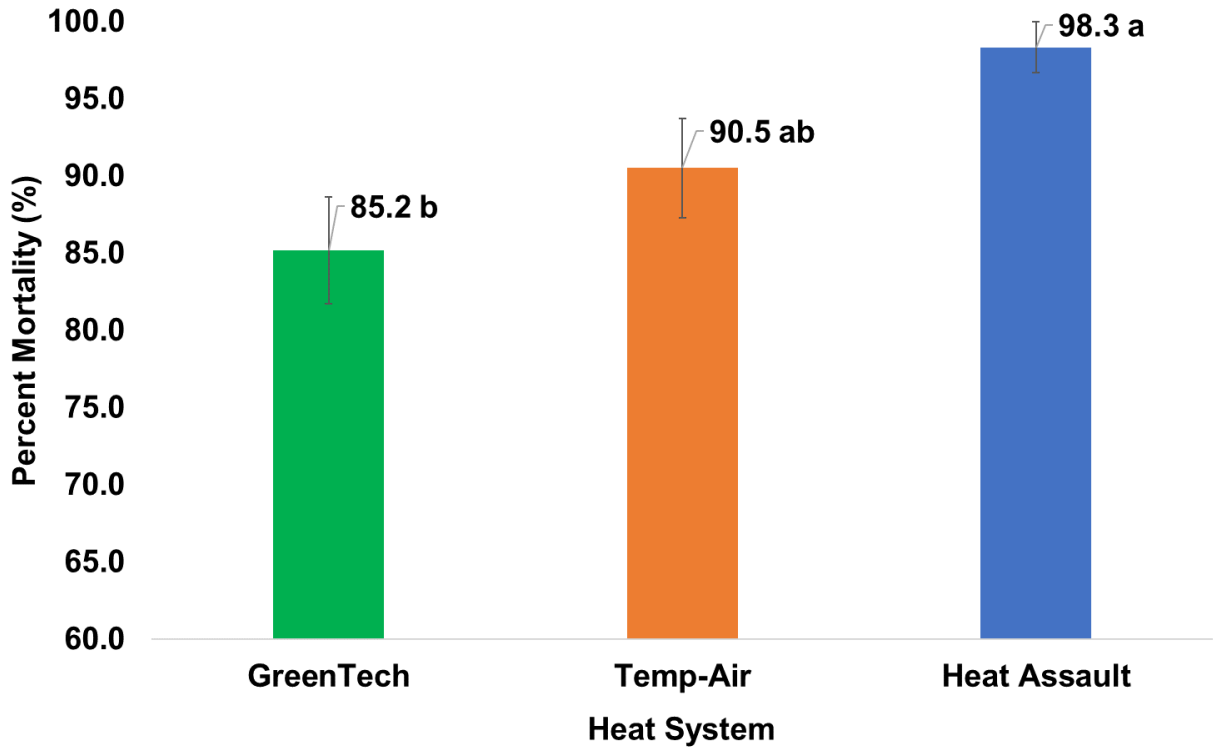


Figure 3.1. Average percent mortality (± SE) for all bed bugs (150) separated by whole-home heat system for each life stage. Overall mortality is given (% mean ± SE), and letters denote significance determined by Tukey's HSD comparisons.

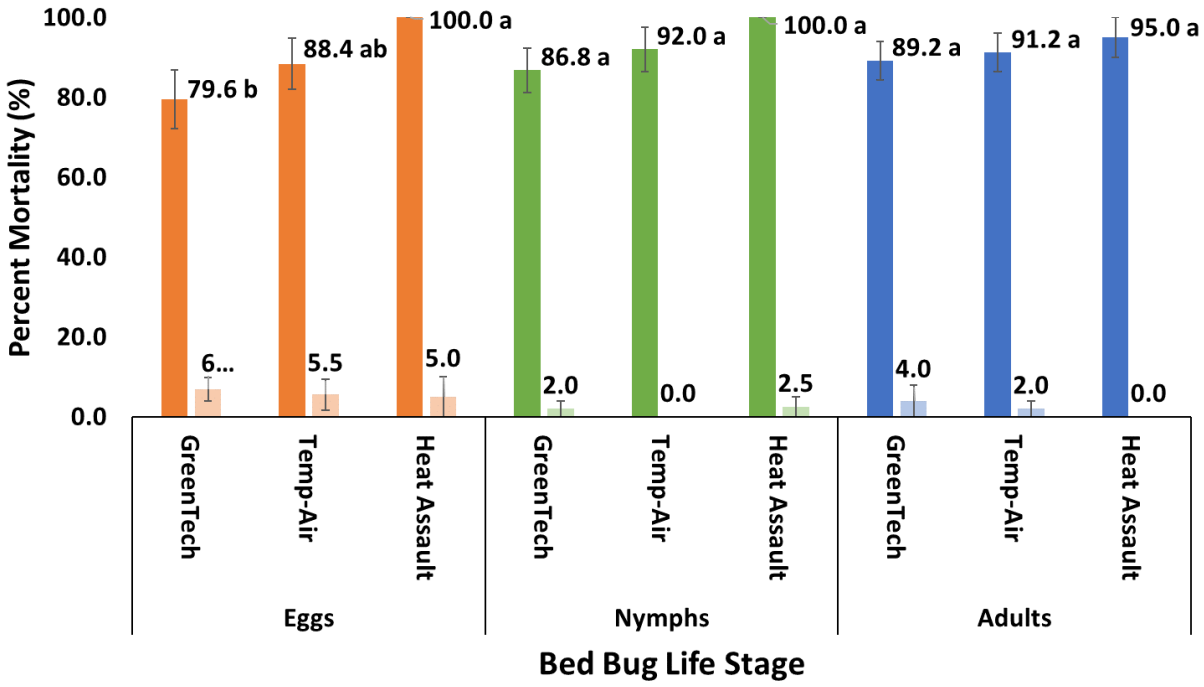


Figure 3.2. Bed bug average percent mortality (± SE) separated by life stage for each whole-home heat system. Darker colored bars represent treated bed bug mortality. Lighter shaded bars represent control bed bug mortality. Tukey's HSD comparisons was used to compare mortality of each life-stage when exposed to individual heat systems. Letters denote significance determined by Tukey's HSD comparisons.

Logistic Regression of Whole-Home Heat System Factors that Predict Bed Bug Mortality

Data ranges for the whole-home heat system factors can be found in Table 3.3. The logistic regression evaluates which factors best predict bed bug mortality for each life stage. After a stepwise regression was conducted, the proportion of temperature sensors reaching the bed bug lethal temperature was retained as a predictor of mortality for all life stages. The proportion of temperature sensors reaching 50°C was a significant predictor for adult mortality. Although the *P*-values for the nymph and egg stage mortality were somewhat higher ($P = 0.054$ and $P = 0.14$, respectively), the proportion of temperature sensors reaching lethal temperature no doubt had an effect on early life stage mortality (Table 3.4). In addition, treatment duration was found to be an

influential predictor in the nymph and egg life stage mortality models, though it was not a significant predictor of adult bed bug mortality (Table 3.4).

Table 3.3. Variable data for each whole-home heat system.

Variables[a]	GreenTech	Temp-Air	Heat Assault
Sensors (12) Reaching Lethal Temperature	4.60 (4 - 5)	5.00 (0 - 8)	11.25 (9 – 12)
Average Treatment Duration (min)	Setup: 24.8 Treatment: 249.6 (4.2 h) Breakdown: 11.8	Setup: 45.6 Treatment: 441.4 (7.4 h) Breakdown: 20.2	Setup: 39.0 Treatment: 337.5 (5.6 h) Breakdown: 39.0
Apartment Size Range (m^3)	131.08 - 233.78	84.23 - 247.87	131.39 - 232.01
Clutter Amount (m^3)	4.63 – 49.19	13.68 – 68.36	1.61 – 53.00
Clutter Ratio (Clutter/Apartment Size) (%)	2.93% - 37.53%	9.51% - 34.25%	1.02% - 22.84%
Technician Entries	2.00 – 6.00	8.00 – 16.00	14.00 – 21.00

a. Apartment size and raw clutter were not used in analysis. These variables were used to generate the clutter ratio (%) that was used in the analysis.

Table 3.4. Predictors of mortality for each bed bug life stage after stepwise regression.

Egg Life Stage	Estimate[b]	Standard Error	*P*-value
Intercept[a]	-4.40	2.84	0.12
Treatment Duration	7.27	5.01	0.15
Proportion of Sensors Reaching 122°F (50°C)	13.14	6.75	0.052
Nymphal Life Stage	Estimate	Standard Error	*P*-value
Intercept	-1.86	1.82	0.31
Treatment Duration	5.70	3.14	0.07
Proportion of Sensors Reaching 122°F	6.26	4.24	0.14
Adult Life Stage	Estimate	Standard Error	*P*-value
Intercept	0.18	0.70	0.79

Proportion of Sensors Reaching 122°F	3.48	1.54	0.02

a. Mean level of response variable (i.e. egg mortality) when other variables (i.e. treatment duration) equal zero.

b. Linear parameter estimates in terms of log(odds). Estimates can be compared as effect sizes.

Heat System	**Predicted Proportion of Temperature Sensors (12) Reaching 50°C.**	**Explanation**
GreenTech	0.81 **a**	This indicates that if technician entries between the GreenTech and Heat Assault systems were the same, then the number of temperature sensors reaching 50°C would be similar.
Temp-Air	0.39 **b**	
Heat Assault	0.78 **a**	

Table 3.5. Predicted influence of technician entries on temperature sensors reaching lethal temperature (50°C). Tukey's HSD comparisons were used to determine differences in proportion of sensors reaching lethal temperature. Letters denote significance determined by Tukey's HSD.

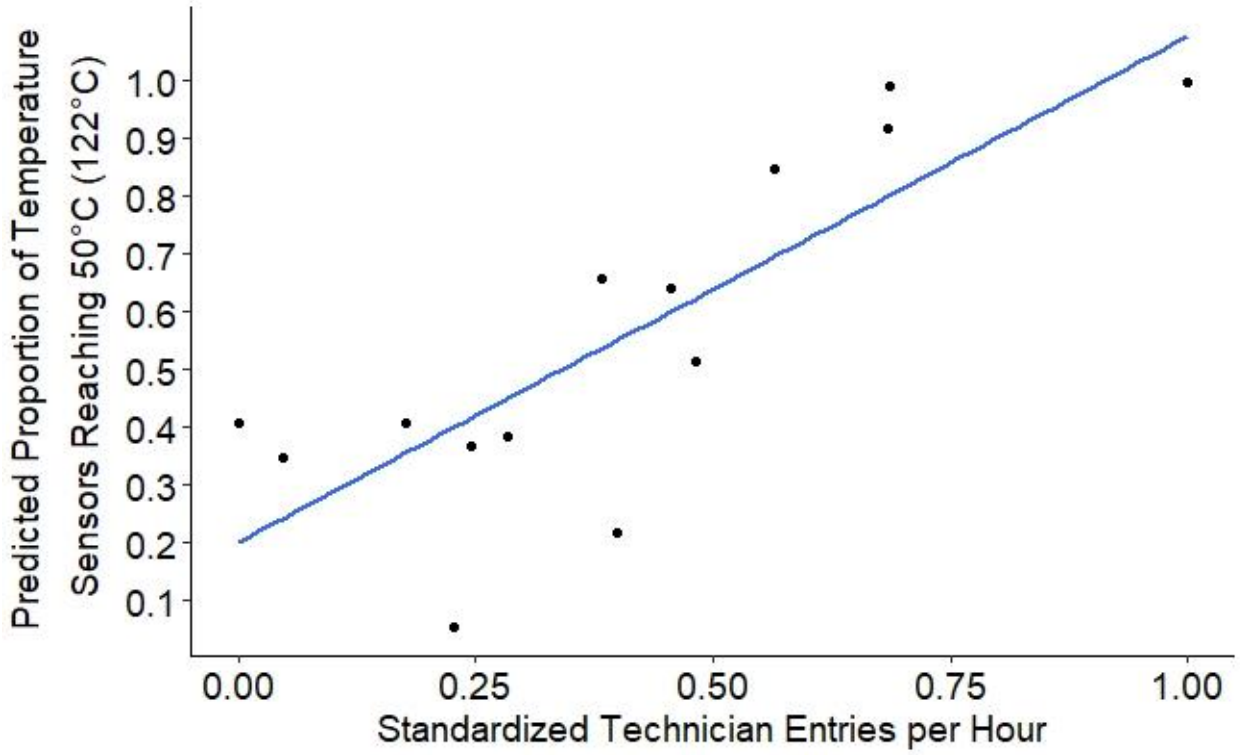


Figure 3.3. Predicted effect of technician entries per hour on the proportion of temperature sensors reaching lethal temperature when other variables are held constant. The range of technician entries per hour was 0.45 to 2.98 entries per hour.

Discussion

During this study we found that the Temp-Air system treated units of the smallest size but with the highest overall clutter ratios (Table 3.3). The Temp-Air system also had the longest average heat treatment (>8 hrs). The Heat-Assault system treated units that had the overall lowest clutter ratios. During the Heat-Assault heat treatments, all temperature sensors reached lethal temperature with the exception of one treatment where two sensors in the closets and one sensor within a living room table drawer did not reach 50°C. During the observations, technician entries were recorded. Technicians of the GreenTech system consistently conducted the least entries, while the Heat Assault technicians consistently conducted the most entries (Table 3.3).

Technicians of the Temp-Air systems had varied amounts of entries, which ranged from eight to sixteen entries throughout the treatment. Even with relatively low technician entries with the GreenTech system, total bed bug mortality for that system was 85.2% (Figure 3.1). The Heat Assault system produced the highest total bed bug mortality (98.3%), where only one adult bed bug replicate had survived inside a closet which was overlooked by the technician. The total bed bug mortality indicates that all systems were able to kill a high amount of the sentinel bed bugs within the units' hard-to-heat locations, however, no system was able to eliminate every bed bug in the treatment zone.

The question of whether or not bed bugs might develop resistance to heat treatments has been investigated due to the widespread observation of heat treatment failures. However, Ashbrooke (2019) found that bed bugs only have a limited ability to develop significant levels of heat resistance. Ashbrooke (2019) determined that susceptible-strains and insecticide-resistant field-strain bed bugs were equally susceptible to heat stress. Benoit (2009) also found that bed bugs are not known to display effective "heat hardening". This is when heat shock proteins are produced to protect other proteins from destruction due to heat exposure. Concerns of resistance development to heat treatments is reasonably low, and survival to heat treatments would be rather a product of heat treatment failure.

It has been documented that bed bugs can detect heat increases at close range (10-30 mm; Devries 2016: 25.4 mm; Berry III 2021). Therefore, temperature increases may induce an escape response during a heat treatment. Bed bugs have been observed moving to cooler locations (including outside of windows, personal observation) during heat treatments. If it were to take a long amount of time to heat a home or if the home is insufficiently heated over time, bed bugs will seek cooler environments to escape and shield themselves from heat within hard-to-heat

locations (Loudon 2017). Under more extreme circumstances, bed bugs may also attempt to escape the treated apartment entirely and enter a different apartment unit (Raab 2016). Given that there were sentinel bed bugs in hard-to-heat locations that did survive the observed heat treatments, reinfestations may occur in the future after a heat treatment is conducted if bed bugs were to escape to cooler locations such as where the sentinel bed bugs were placed.

While bed bugs may survive an inefficient heat treatment, there can be biological consequences of survival. Bed bug survivors of a heat treatment can suffer effects from sublethal temperatures, which can include reduced offspring proliferation, molting, egg production, and egg hatching (Rukke 2018). Ashbrooke (2019) also found that sublethal heat exposure caused reduced feeding and inhibited development in bed bugs. In spite of these sublethal consequences, heat treatment survivors are still alive and can feed post-treatment, which in consumer evaluations, can greatly reduce the value of heat treatments for bed bug control. Given that bed bug survivors can be negatively impacted by sublethal temperatures, it would be beneficial to perform supplementary treatments such as dusting and spray applications post-heat treatment.

Logistic regression analysis was unable to determine if the Heat Assault system would perform differently from the GreenTech system (Table 3.5), given that Heat Assault had consistently high technician entries while GreenTech entries were consistently low. Table 3.5 suggests that if technician entries were similar for the two systems, it is possible they would have similar efficacy with killing bed bugs. However, hard-to-heat locations struggled to reach lethal temperatures even as technician entries increased when using Temp-Air. It is possible that the GreenTech technicians may have seen greater control of bed bug populations if they had entered the treatment site and conducted more interventions. The GreenTech technicians were constrained by time and had the shortest treatments on average (Table 3.3), which may be a

contributing factor to bed bug heat treatment survival (Table 3.4). It would be beneficial for future studies to investigate the impact of different types of technician intervention (checking temperature versus checking temperature and moving furniture) and to compare heat systems while controlling for technician entries (equivalent number of entries) as much as possible.

The most important predictor, as suggested by the magnitude of the associated linear estimate in all of the life stage models, was the proportion of temperature sensors that reached 50°C (122°F). Because of this, the analysis of what factors impact how many temperature sensors reach lethal temperature was very important. A logistic regression analysis revealed that the number of technician entries per treatment hour was the most impactful factor for treatment success. However, it was also found that the Temp-Air system underperformed as compared to the other two systems even when the number of technician entries was taken into account.

Having lethal temperatures reached by sensors in hard to heat locations is positively correlated with the number of times a technician enters the treatment site to check temperatures, adjust equipment, and move furniture (Figure 3.3). Sensors in hard-to-heat locations reaching lethal temperatures is important given that stepwise regression indicated that the factor was influential in the mortality of all bed bug life stages. Consequentially, the importance of technician diligence during the heat treatment process cannot be understated and we hope that this result will help pest management professionals in learning how to conduct effective heat treatments.

Based on this study, the reason for bed bug survivors (heat treatment failure) appears to be a factor of failure to completely heat cracks and crevices to bed bug lethal temperatures. In spite of the deficiencies in heating all cracks and crevices, overall mortality was surprisingly high (Figure 3.1). Given that not all bed bugs were eliminated in this study, reinfestations after

heat treatment should not be blamed on residents. The results of this study indicate that it would be unfair to accuse residents of bed bug reintroductions when bed bug survivors occur with even the most conscientious technicians and high-tech systems. This study aids in dispelling any myths that heat treatments are a silver bullet, and we suggest that supplementary forms of control should be used after a heat treatment to produce the best possible level bed bug infestation control.

Chapter Four:

Efficacy of an oil-based fungal spore (*Beauveria bassiana*) formulation for control of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) under varying conditions of temperature and humidity

Introduction

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), was known to be a widespread pest in human environments since the start of early agricultural communities. Human and bed bug cohabitation continued with very little relief until the early 20th century when the use of dichlorodiphenyltrichloroethane (DDT) and other broad-spectrum insecticides eradicated most of their populations in developed nations (Potter et al. 2010, Potter 2011). While large populations of these 20th century bed bugs were eradicated by these insecticides, evidence of insecticide resistance to DDT was already documented by the 1950s (Johnson and Hill 1948, Busvine 1958). Between 1958 and 1968, organophosphate and carbamate insecticides replaced DDT due to the development of DDT-resistance (Rafatja 1971, Potter 2018). Although bed bugs are known to have developed resistance to these chemistries as well, by the 1970s, world-wide populations had been mostly eliminated, and bed bugs were no longer considered a human pest except in the most isolated developing nations.

However, in the late 20th century, bed bug populations began to re-emerge around the world. In the United States, the bed bug resurgence occurred at a particularly difficult time, due to the United States Environmental Protection Agency's (EPA's) implementation of the Food

Quality Protection Act (FQPA) of 1996 that eliminated multiple pesticide chemistries from being used indoors against bed bugs. The elimination of multiple broad-spectrum insecticides left the chemical manufacturing industry and pest management professionals with pyrethroids as the predominant chemistry available for indoor application when the bed bug resurgence began. Therefore, the success and continued proliferation of the common (and tropical; *Cimex hemipterus* F.) bed bug around the globe has been largely attributed to three major factors: (a) bed bugs developing resistance to the most commonly used pyrethroid insecticides; (b) regulatory changes that have limited the types of active ingredients that were allowed to be used indoors (EPA 1996); and (c) the increase in human population size around the globe combined with the increased ability of humans to travel internationally (Romero et al. 2007, Davies et al. 2012). With regard to insecticide resistance, modern bed bugs are known to have developed a variety of genetic mechanisms that limit their susceptibility to chemical formulations. These include target-site insensitivity, increased metabolic detoxification enzyme activity, and reduced cuticular penetration (Lilly et al. 2016a, b; Romero and Anderson 2016; Dang et al. 2017). Bed bug populations can have one or more of these resistance mechanisms, which make these bed bugs very challenging to control with current available chemistries (Zhu et al. 2013).

With the increasing levels of resistance to spray formulation insecticides, alternative treatments for bed bugs have been sought out by the pest control industry (as well as ambitious entrepreneurs) to provide pest management professionals with more novel control methods. Today, alternatives to spray pyrethroids include the use of whole home heat systems, spot treatments using steamers or frozen carbon dioxide applicators, vapor-type fumigants, pyrethroid impregnated mattress covers, sulfuryl fluoride fumigation, and even attractant traps (Kells and Goblirsch 2011, Olson et al. 2013, Phillips et al. 2014, Kells 2018, Wang et al. 2018). Some of

these methods have proven to be much more effective than others, but are sometimes accompanied by disadvantages (labor, cost, inconvenience, etc.).

One of the more novel approaches for eliminating insecticide-resistant bed bugs has been the indoor application of a fungal biological control agent, *Beauveria bassiana* (Barbarin et al. 2012, 2017, Shikano et al. 2019). *Beauveria bassiana* is an entomopathogenic fungus that is known to infect a wide range of insect pests such as mosquitoes, house flies, Colorado potato beetles, triatomine bugs, and German cockroaches (Lecuona 2001, Blanford et al. 2005, Wraight et al. 2009, Acharya et al. 2015, Davari et al. 2015, Ramírez et al. 2020). In 2012, GHA-strain *B. bassiana* was initially evaluated for its potential to control bed bug populations (Barbarin et al., 2012). In their study, *B. bassiana* was found to be highly infectious to bed bugs after short-term exposure. A 2017 study conducted by the same researchers (Barbarin et al. 2017) found that pyrethroid resistant bed bug strains were also very susceptible to *B. bassiana* infections.

Reduced cuticular penetration type resistance has been a particular concern when treating modern bed bugs with spray formulation insecticides. This is because the thicker cuticle protects the bed bug's interior physiology from insecticide exposure by slowing absorption of insecticide residues into the insect's body. Thus, reduced cuticular penetration often reduces any residual efficacy that spray formulations might have (Lilly et al. 2016a). However, a study by Pedini et al. (2009) found that pyrethroid-resistant kissing bugs (*Triatoma infestans* K.*)* that had both thickened cuticles and an increased number of cuticular lipids, were still susceptible to *B. bassiana* infections (Pedrini et al. 2009). Therefore, it is reasonable to assume that bed bugs with reduced cuticular penetration mediated resistance might also be susceptible to *B. bassiana* infections.

After multiple resistant bed bugs strains were evaluated for susceptibility to *B. bassiana* in multiple laboratory evaluations, the Aprehend® (ConidioTec LLC, Centre Hall, PA) product was successfully launched for bed bug control (Barbarin et al. 2017). Aprehend® (ConidioTec LLC, Centre Hall, PA) uses GHA-strain *B. bassiana* as a biological control agent to infect and kill bed bugs. The product is an oil-based formulation of *B. bassiana* fungal spores that is applied as a low volume spray. Aprehend® is applied using a specialized spray applicator provided by the company, which delivers the product at a rate of ≈14.8 ml. per 15.2 linear meters of surface. Following the manufacturer's instructions, the applicator creates a ≈5.1 cm wide treatment band that can be applied to furniture (box springs, behind dressers, the bottom and backs of chairs) and structural features inside the home (floor/wall and ceiling/wall junctions) where no direct human contact is expected. The infesting bed bugs walk across the treatment band and pick up the fungal spores on their bodies, the spores then germinate on the bed bug cuticle and infect the insect. Bed bug mortality has been shown to occur within a period of 4 to 10 days after the insects walk across the treatment zone (Barbarin et al. 2012, 2017; US EPA 2018, Pesticide Product Label, Aprehend). The Aprehend® label also claims the product has 3 months of residual activity after the initial application.

The biopesticide product is the subject of ongoing efficacy studies to evaluate its ability to eliminate bed bugs under the diverse conditions that exist in individual human homes. In homes, conditions such as heat and humidity can vary depending on the quality of the structure, human preferences, geography, and the seasonality of the local environment. The focus of this research project was to determine if these atmospheric conditions would influence the efficacy of Aprehend. Therefore, a laboratory study was conducted in which the environmental conditions could be controlled, and their influence on the infection and pathogenesis of the *B. bassiana*

could be observed. The specific purpose of this study was to evaluate the efficacy of the Aprehend oil-based product under varying combinations of temperature (15°C, 21°C, and 32°C) and humidity (30%, 50%, and 70%) that would be found inside human homes.

Materials and Methods

Insects

The bed bugs (*C. lectularius*) evaluated in this study were from the Richmond field-derived strain. This pyrethroid resistant bed bug strain was collected from a group home in Richmond, VA in 2008. The Richmond strain is frequently used in sponsored research trials at the Dodson Urban Pest Management Laboratory (DUPML; Virginia Tech University; Blacksburg, Virginia) and is therefore evaluated annually for insecticide resistance (and compared with the Harlan susceptible laboratory strain). In 2017, the Richmond strain was one of ten bed bug field strains evaluated for resistance to insecticide products containing chlorfenapyr or bifenthrin at Purdue University (Indiana, USA), and found to be the most resistant of all field strains evaluated in that study (Ashbrook et al. 2017).

The Richmond strain is currently maintained at the DUPML inside plastic rearing jars. All bed bugs are fed once a week with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using an artificial feeding system, maintained at 35.5°C by circulating hot water. All bed bug colonies at the DUPML are kept within environmental chambers at ca. 28°C, ca. 55% RH, and a photoperiod of 12:12 h L:D cycle.

Adult male bed bugs of the Richmond strain (henceforth referred to as adults) and mixed-instar nymphs were prepared for evalution at DUPML. At least 5 days before testing, 24 replicates of 4 adults and 6 mixed-instar nymphs (total of 10 bed bugs per replicate) were placed

inside Petri dishes (60 mm x 15 mm, CellTreat®, Pepperell, MA) lined at the bottom with filter paper. Bed bugs in this study were fed 5 to 7 days prior to experimentation.

Fungal Biopesticide

Aprehend was supplied by the company along with their specialized spray applicator. The spores of the *B. bassiana* are suspended in a proprietary oil carrier. Therefore, the formulation does not need any dilution before application. Currently, Aprehend is the only entomopathogenic fungal product that is registered for bed bug control.

Experimental Design

All laboratory bioassays were conducted at the DUPML in Blacksburg, Virginia. Three MyTemp-Mini incubation chambers (two 2200-HC, one 2200-H; Benchmark Scientific, Sayreville, New Jersey) were used for holding Aprehend exposed bed bugs at specific temperature/humidity combinations. Prior to each fungal sporulation evaluation, all three chambers were set up to be maintained at one humidity level (either 30%, 50%, or 70%), while each individual chamber was set at one of three different temperatures (15.6°C, 21.2°C, or 32.2°C). Temperature and humidity levels were monitored using both the chamber internal thermometer as well as a calibrated Fisher Scientific Traceable® thermo-hygrometer (Control Company, Webster, TX, USA).

To obtain the specific humidity level necessary for each sporulation evaluation, three different salts were obtained and formulated into solutions. These salts were magnesium chloride (hexahydrate), magnesium nitrate (hexahydrate), and potassium iodide. A Corning hot plate stirrer (PC-351) was used to create the saturated salt solutions used in each temperature and humidity level evaluation.

Magnesium chloride solutions were used to maintain a humidity level of 30% in all three chambers during the first temperature combination evaluation (Greenspan 1977). Each individual chamber containing the magnesium chloride solution was set at a temperature of either 15°C, 21°C or 32°C. Magnesium nitrate solutions were used to maintain a humidity level of 50% in all three chambers during the second combination evaluation. Potassium iodide solutions were placed in the three chambers to maintain a humidity level of 70% during the third combination evaluations.

Bed Bug Exposure Design

To mimic potential bed bug exposure to Aprehend in the field, impermeable surfaces were prepared for Aprehend application. Wooden boards (61 cm x 14 cm x 0.5 cm) were coated with three layers of fresh latex paint. After the paint was dry, the boards were cut into squares (14 cm x 14 cm x 0.5 cm). After the squares were cut, 2 cm x 2 cm pieces of masking tape were placed in the center of each square to create an unsprayed area for initial placement of bedbugs. Aprehend was then applied at a distance of ~12.7 cm to each square at the label rate (≥30.48 centimeters per second), using the product spray device. In total, we had one treated panel for each temperature and humidity combination for a total of 9 panels.

Three to 4 h after application, the masking tape was removed from the center of each of the treated squares. Bed bugs were then released from their Petri dish onto the untreated center of the square. The bed bugs were allowed to cross over the treated surface one time to achieve fungal spore exposure. After exposure, the ten bed bugs were placed inside plastic rearing containers. Inside of each jar container was a single folded filter paper to provide harborage for the bed bugs. After each of the four adult and six nymphal bed bugs were returned to their

rearing jar, the jar was placed in one of the incubation chambers that had been set at a particular temperature and housed with a specific salt solution to maintain the desired humidity level.

Each incubation chamber contained two shelves and a tray at the bottom of the chamber. The top shelf held the five Aprehend treated bed bug rearing containers and three untreated control jars. The bottom shelf held a specific salt solution in a glass bowl. Hygrometers were placed within the chambers to continuously monitor humidity levels.

Evaluation of Bed Bug Mortality

Bed bug mortality for each temperature/ humidity combination was monitored at 12-hour intervals for 15 days. Bed bug mortality (for each individual) was determined by lack of movement when abruptly disturbing the container as well as a lack of reaction to prodding the insect's body. To determine if bed bug mortality was the result of fungal exposure, dead bed bugs were removed from their container and surface sterilized by washing cadavers in a 10% aqueous solution of NaOCl (Clorox Performance Bleach 2, Clorox Professional Products Company, Oakland, CA, USA), water, and a small drop of dish soap. After surface sterilization, the bed bugs were rinsed in water, then excess removed by placing them on a sheet of paper towel to dry. After drying, the cadavers were placed into clean containers with a moist piece of paper towel provided to maintain high humidity. The surface sterilization process killed any organisms residing on the cuticle of the insect. Therefore, individual bed bugs that had been sterilized, were placed inside individual Petri dishes with a piece of moist paper towel. They were then monitored for a period of 7 days to observe any *Beauveria bassiana* fungal growth. The appearance of white fungal growth was used to indicate that bed bug death was due to *Beauveria bassiana* infection.

Statistical Analysis

The data used for Kaplan Meier survivorship analysis included survival times for 50 individual bed bugs (5 replicates) for each temperature and humidity combination (15°C, 21°C, and 32°C; 30%, 50%, and 70% humidity). The bed bugs held at each temperature/humidity combination were observed for 15 days, and bed bugs that survived to the end of the observation period were set as right-censored. In our survival analysis, right-censorship refers to individuals who have survived to the end (the "right side") of the observation period. Log-rank tests were used to determine if there were differences in bed bug mortality between each environmental combination. Median survival time (MST) in days was used to determine how long a homeowner might expect to see surviving bed bugs after treatment with the Aprehend product under specific environmental conditions.

Cox proportional hazards regression analysis was used to evaluate and compare the effect of different environmental conditions on bed bug mortality after exposure to *B. bassiana*. The 21°C and 50% humidity level condition was set as the baseline given that 21°C is closest to the optimal germination temperature (≈25°C; Fargues et al. 1997) and that 50% was the middle humidity level. Pairwise comparisons of estimated hazard ratios at each environmental combination were conducted to determine if conditions resulted in statistically significant effects.

Bed bug MST and hazard ratios after fungal exposure were calculated using the Rstudio Statistical software (R Core Team 2021). For estimates of final cumulative mortality under each condition Abbott's correction (Abbott 1925) was used to correct for control mortality in each of the temperature and humidity combinations except for those at 32°C. Exclusion of data from this last temperature was due to the high control mortality across all humidity levels.

Results

Bed Bug Mortality

All treated bed bug cadavers collected during the observation period were mycotic. Control bed bug cadavers did not exhibit signs of mycosis. Corrected final cumulative mortality for all temperature and humidity combinations is presented in Table 4.1. Table 4.2 lists bed bug median survival times for each temperature and humidity combination. Overall, the relatively low temperature of 15°C and 30% humidity combination produced the greatest percent bed bug mortality (71%) after 14 days, due to fungal infection. The mortality levels for the 15°C temperature were significantly less when the humidity was increased to 50% (55.1% mortality) and 70% (54% mortality). When the temperature was 21°C, the percent bed bug mortality was also the greatest at a humidity level of 30% (77.8%). However, this level of mortality due to fungal infection was not significantly greater than that recorded for infected bed bugs subjected to 21°C and 50% humidity (69%). The percent bed bug mortality at 21°C was significantly reduced (48.3%) for those replicates subjected to the 70% humidity level. At both the 15°C and 21°C temperature levels, a low percentage of mortality was observed in some of the control replicates (0 – 16.7%). However, this control mortality did not appear to be influenced by any these temperature/humidity combinations (Figure 4.1).

Table 4.1. Abbott's corrected final mean percent (%) mortality for Aprehend-treated bed bugs and untreated control bed bugs. High level of control mortality at 32°C; Compare mortality for humidity level within each temp. The trend is that the 30% humidity delivers the most mortality.

Temperature (°C)[a]	Humidity (%)	Treated (mean percent mortality ± SE)[b]	Control (mean percent mortality ± SE)[c]
15	30	71.0 ± 8.6 **bc**	3.3 ± 3.3
15	50	55.1 ± 6.5 **c**	2.5 ± 2.5
15	70	54.0 ± 6.8 **c**	0.0 ± 0.0

21	30	77.8 ± 7.1 **a**	10.0 ± 5.8
21	50	69.0 ± 8.4 **ab**	16.7 ± 3.3
21	70	48.3 ± 10.0 **c**	3.3 ± 3.3
32	30	98.0 ± 2.0	83.3 ± 3.3
32	50	74.0 ± 8.7	56.7 ± 8.8
32	70	66.0 ± 8.1	70.0 ± 5.8

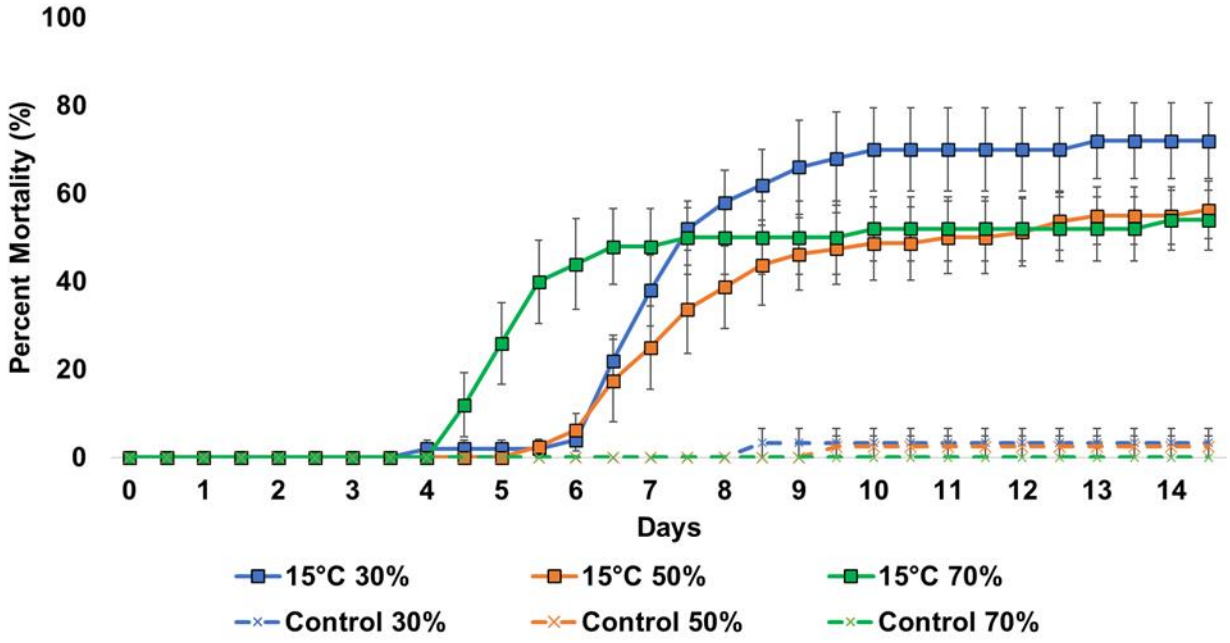
a. Treated mortality was corrected (Abbott's correction) for temperature 15°C and 21°C. Treated mortality at 32°C could not be corrected due to the high control mortality.
b. Letters denote significance of bed bug mortality from pairwise comparisons of Cox proportional hazards regression hazard ratios at 15°C and 21°C.
c. High control mortality at 32°C likely due to constant exposure to high temperature conditions. Letters denote significance of bed bug mortality from pairwise comparisons of Cox proportional hazards regression hazard ratios at 32°C.

Table 4.2. Median survival times and 95% confidence intervals of bed bugs under different combinations of temperature and humidity.

Temperature (°C)	Humidity (%)	n	Events (Mortality)	Median (in days)	95% LCL (in days)	95% UCL (in days)
15	30	50	36	8.0	7.5	10.0
15	50	80	45	12.0	9.0	>15
15	70	50	27	9.3	6.0	>15
21	30	50	40	4.5	4.5	6.5
21	50	50	35	4.5	4.5	7.0
21	70	50	25	14.5	8.0	>15
32	30	50	49	5.5	5.0	6.5
32	50	50	37	9.0	7.0	14.5
32	70	50	33	12.3	9.5	>15

At 15°C, bed bug mortality following exposure to the *Beauveria* began between days 4 and 6 (Figure 4.1). The MST values for exposed bed bugs at this temperature 8.0 days (CI = 7.50 – 10.00) at 30% humidity; 12.0 days (CI = 9.00 - >15.00) at 50% humidity, and 9.3 days (CI =

6.00 - >15.00) at 70% humidity (Table 4.2). Bed bug mortality at 21°C began earlier than those held at 15°C with mortality, beginning between days 3 and 4 (Figure 4.1). Infected bed bug median survival times were 4.5 days at 21°C and (CI = 4.50 – 6.50) at 30% humidity; 4.5 days (CI = 4.50 – 7.00) at 50% humidity, and 14.5 days (CI = 8.00 - >15.00) at 70% humidity (Table 4.2).



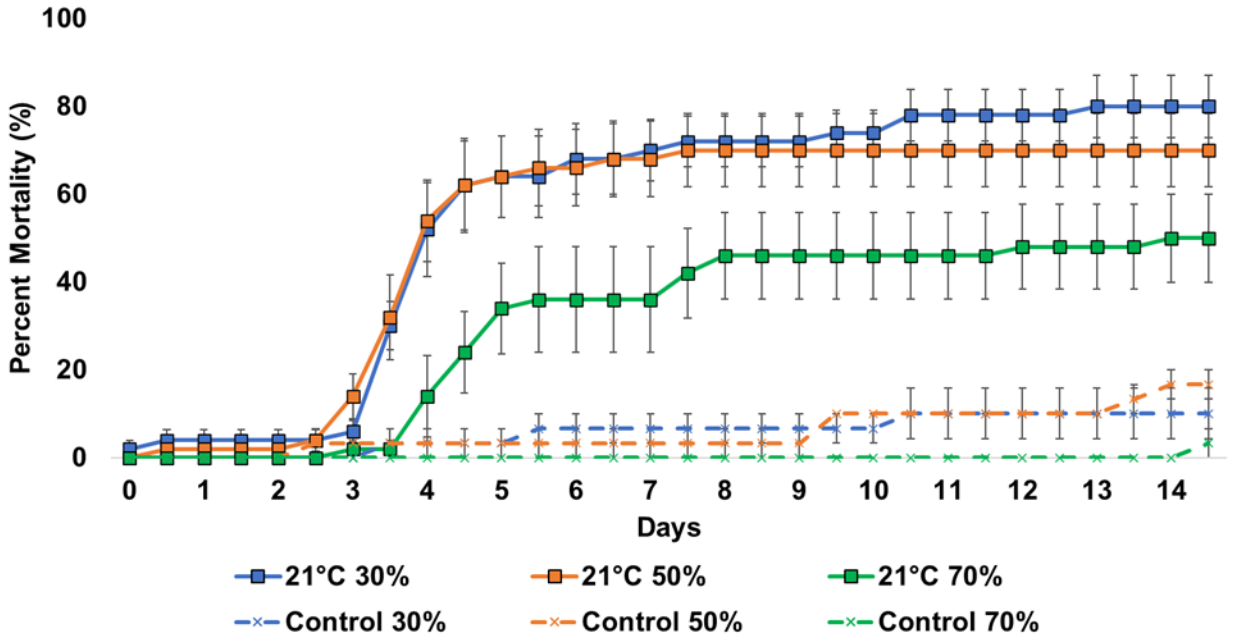


Figure 4.1. Aprehend-treated and control bed bug mean percent mortality (± SE) over time for temperatures 15°C and 21°C and humidity levels 30%, 50%, and 70%.

In the 32°C temperature replications, it was obvious that the high temperature was the factor responsible for the bed bug mortality, rather than fungal infection at any specific temperature/humidity combination. This temperature effect on mortality was made evident by the fact that the control replicates at 32°C experienced an average of 56.7% - 83.3% percent mortality at each of the humidity levels. The MST for fungus-exposed bed bugs recorded at 32°C were 5.5 days (CI = 5.00 – 6.50) at 30% humidity; 9.0 days (CI = 7.00 – 14.50) at 50% humidity, and 12.2 days (CI = 9.50 - >15.00) at 70% humidity (Table 4.2). However, because of the level of control mortality at 32°C, these median survival times can be attributed to the heat exposure, not to *Beauveria bassiana* infection time.

Environmental Influence on Post-Infection Bed Bug Mortality

A log-rank test was used to determine if there were differences between bed bug survival at 15°C, 21°C, and their various humidity levels. The test revealed that there were significant differences in bed bug survival across those conditions ($\chi^2 = 40.9$, $df = 5$, $P < 0.0001$). Cox proportional hazards regression analysis further supported that there were significant differences in bed bug mortality at different environmental conditions at 15°C and 21°C (Table 4.3). To conduct the Cox proportional hazards regression analyses the 21°C / 50% humidity was given a reference value of '1' (note that the 32°C temperature data was not included in this model). Values over 1 indicate that the bed bugs are more at risk of experiencing death from fungal infection, while values less than 1 indicated that the bed bugs are less at risk of dying from fungal infection. The hazard ratio values show that at 21°C and at 30% and 50% humidity, *B. bassiana* had the greatest impact on bed bug mortality (HR = 1.17, *P* = 0.496; HR = 1.00, respectively). *B. bassiana* had a lesser effect on bed bug mortality at 70% humidity at 21°C (HR = 0.42, P = <0.001). *B. bassiana* also had a reduced effect at 15°C at each humidity combination (HR = 0.57, *P* = 0.017 at 15°C / 30%; HR = 0.40, *P* = <0.001 at 15°C / 50%; HR = 0.46, *P* = 0.003 at 15°C / 70%). These hazard ratios suggest that bed bugs have a greater probability of death after exposure to *B. bassiana* at 21°C and lower humidity levels.

Table 4.3. Hazard ratio and *P*-values generated from the Cox proportional hazards model comparing bed bug survival environmental conditions.

Temperature (°C)	Humidity (%)	Hazard Ratio[a]	95% Confidence Interval	*P*-value[c]
15	30	0.57	0.35 – 0.90	0.017 **bc**
15	50	0.40	0.26 – 0.62	<0.001 **c**
15	70	0.46	0.28 – 0.77	0.003 **c**
21	30	1.17	0.74 – 1.84	0.496 **a**
21	50	1.00[b]	Reference	Reference **ab**
21	70	0.42	0.25 – 0.69	<0.001 **c**

a. Hazard ratios over "1" indicate that bed bugs are more at risk of death post-fungal exposure than at the reference condition. Hazard ratios under "1" indicate that bed bugs are less at risk of death post-fungal exposure than the reference condition.
b. 21°C and 50% was chosen as the reference environmental condition. 21°C is closest to the optimal growth rate of *Beauveria bassiana*. 50% is the midpoint humidity.
c. P-values indicate significance of environmental condition influence on bed bug mortality. Letters denote significance of bed bug mortality from pairwise comparisons of Cox proportional hazard regression hazard ratios.

Discussion

GHA-strain *Beauveria bassiana* is known to be highly lethal to bed bugs, regardless of the feeding status, sex, susceptibility to insecticides, or the life stage of the insect (Barbarin et al. 2012, 2017). Our concern about the biological control agent component of Aprehend was whether or not environmental conditions might impact the efficacy of the product. In the field, Aprehend is applied along the back, bottom and seams of furniture where human contact is either brief or non-existent. The product is also applied in bands along floor-wall junctions, ceiling-wall junctions, and wall-wall junctions. While the structure and furniture may be fairly stable in a home, the environmental conditions in homes can vary depending on seasonality of the location and the structural quality of the home.

In this study, bed bug mortality did not reach 100% in any of our bioassays. This is possibly due to the fact that the bed bugs crossed over the *Beauveria*-treated surfaces only once (across a treated band of ≈ 5.08 cm) and thus had limited exposure to the product, less than in operational conditions where bed bugs would presumably cross the treated zone twice, once to and again from their food source. While bed bug mortality did not reach 100%, the product was still able to produce an average of 48.3% to 77.8% mortality when held under the 15°C and 21°C temperature/humidity combinations, even though the bed bug exposure to the product was very

brief (Figure 4.1, Table 4.1). The greatest average bed bug mortality for both the 15°C and 21°C temperatures occurred at the 30% humidity level (71.0% and 77.8%, respectively). The fungus produced the most rapid kill when bed bugs were exposed to the 21°C under the 30% and 50% humidity combinations; 50% of bed bugs were dead within 4.5 days (Table 4.2).

Under the 32°C temperature condition, treated bed bug mortality was between 66.0% and 98.0% (Figure 4.2, Table 4.1). While mortality was high under these conditions, the mortality of the control bed bugs was also very high (70.0% - 83.3%). Under these high temperature conditions, GHA-strain *B. bassiana* is known to have a low germination rate (Fargues et al. 1997). The temperature tolerance curve shows a steep decline in *B. bassiana* germination rates when passing 28°C. Using the Fargues et al. 1997 *Beauveria* temperature tolerance curve as a guide, fungus vegetative growth rates at 32°C average ≈40% of optimal growth (at 25-28°C). This aspect would indicate that the chronic high temperature, may have played a significant role in bed bug mortality during the 32°C assays rather than fungal infection.

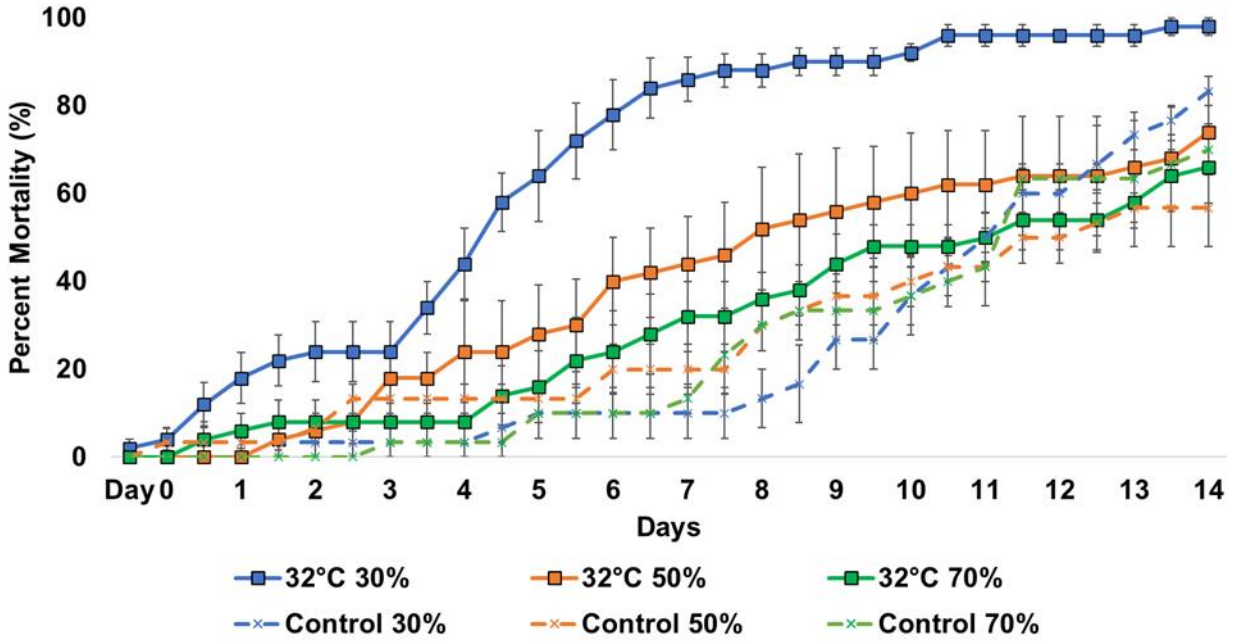


Figure 4.2. Aprehend-treated and untreated bed bug mean percent mortality (± SE) over time for temperature 32°C and humidity levels 30%, 50%, and 70%.

The Cox proportional hazards also indicated that the bed bugs were significantly more at risk of death from the fungus at the 21°C temperature and 30%-50% humidity levels than at other environmental conditions (Table 4.3). In conducting the analysis, the 21°C temperature and 50% humidity condition was considered the reference condition. This condition was chosen because 21°C is closest to the most optimal temperature for fungal spore germination and 50% humidity was the midpoint of the three levels. The Cox proportional hazard model suggested that bed bugs at 21°C and 30% were 17% more likely to experience death, however this was not significantly different from the reference condition (P = 0.496). At 15°C and the three humidity levels (30%, 50%, 70%), bed bugs were, relatively, 43%, 60%, and 54% less likely to die from the fungus than bed bugs under the reference environmental condition. Bed bugs in the 21°C

temperature and 70% humidity regime were 58% less likely to succumb to fungal infection than when held under the reference condition (21°C and 50% humidity).

At any of the three temperature regimes bed bug mortality was the lowest at the 70% humidity level. At 15°C and 70%, bed bug final mortality was 54.0%. At 21°C and 70%, bed bug final mortality was 48.3%. At 32°C and 70%, bed bug final mortality was 66.0%. We hypothesize that the insects may possibly be less stressed, and that they may be capable of better fending off *B. bassiana* infection under this humidity level or the fungus is less infective/ pathogenic. It may be possible that bed bugs with reduced cuticular penetration type resistance may have moisture collect on the dense wax layer of their exoskeleton and inhibit the penetration of the fungal spores along the more hydrophobic portions of their cuticle (Mishra et al. 2015). It would be beneficial to conduct further research to understand if there is a connection between this humidity level and bed bug survival when bed bugs are exposed to *B. bassiana*.

Our study indicates that *B. bassiana* may be most efficacious in environments that are somewhat cool and dry. Rardin (2022), a comfort expert with HVAC, states that the most ideal indoor humidity is between 30% and 50% during the winter and summer months, respectively. A residential indoor temperature study by Booten et al. (2017) found that mean indoor temperatures across the USA ranged from ≈70°F to ≈75°F (21.1°C to 23.9°C) during winter and summer, respectively. Given this information, many homes may already have conditions that allow *B. bassiana* to eliminate bed bugs most quickly and most effectively. These coexisting interior conditions would be likely to yield the fastest, and the highest bed bug mortality when using the Aprehend product.

Chapter Five:

Field evaluations of sulfuryl fluoride fumigation for control of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), using a 1.9X dosage factor in motor vehicles and filled cargo trailers

Introduction

The use of fumigation for control of the common bed bug (*Cimex lectularius* L.) has had a long but intermittent history in the United States (U.S.). In the first half of the 20th century, sulfur dioxide, and later hydrogen cyanide were widely used to control bed bugs in homes and personal belongings. Fumigation chambers were used as an efficient means of eliminating bed bugs from household items prior to moving them into apartments and other human living spaces (Potter 2011). Fumigation with hydrogen cyanide was also a method used for bed bug elimination in transportation vehicles such as trucks, ships, vans, and railway cars. However, hydrogen cyanide was also very dangerous, and the fumigation process required special care to avoid non-target mortality (Mallis 1945). With the advent of dichloro-diphenyl-trichloroethane (DDT) at the start of World War II, fumigations for bed bugs rapidly declined because DDT provided extremely effective bed bug control (Stenburg 1947). DDT was less expensive than fumigation and had long residual activity (Madden et al. 1944; 1945). In addition, DDT presented relatively little human health risk (Potter 2011) and was very easy to apply. The widespread use of DDT began to diminish after the 1950s when bed bug populations began to develop resistance to organochloride chemistry (Johnson and Hill 1948; Busvine 1958).

Organophosphate and carbamate insecticides replaced DDT between 1958-1968 (Potter 2018; Rafatja 1971), but by this time, bed bug infestations had been eliminated from developed nations (Potter et al. 2010; Potter 2011). During the last decade of the 20th century, the U.S. Environmental Protection Agency (EPA) began to terminate the registrations of organophosphate and carbamate insecticides from indoor and agricultural uses due to the requirements of the Food Quality Protection Act (EPA 1996). This left only the pyrethroid chemistries available for indoor use when bed bug populations began their world-wide resurgence in the late 1990s.

Interestingly, the bed bug resurgence has been largely attributed to their populations developing increased resistance to insecticides (Romero et al. 2007; Zhu et al. 2010; Davies et al. 2012). The modern bed bug is known to be highly resistant to pyrethroids and to some neonicotinoids, due to a variety of resistance mechanisms. These mechanisms include reduced cuticular penetration, increased metabolic detoxification (increased cytochrome P450 and esterase activity), and target site insensitivity (mutation of the alpha subunit; *kdr*-type) (Lilly et al. 2016a, Lilly et al. 2016b, Romero and Anderson 2016, Dang et al. 2017).

Because these modern populations of resistant bed bugs have continued to increase for the last 20 years, people are now experiencing bed bugs in more challenging locations in much the same way that they did in the early 20th century. Recently, bed bugs have become well publicized for infesting public transportation, such as city buses, taxis, Lyft, and Uber transport vehicles (Howerton 2020). At this time, no insecticidal (sprays or dusts) or heat treatment methods can be reliably recommended for treating vehicles due to their limited ability to access bed bug harborage locations and potential to damage electronic equipment, which is now standard in most vehicles.

Another major challenge with regard to managing modern bed bug infestations is that insecticide resistance, particularly reduced cuticular penetration type resistance, leaves bed bugs only marginally susceptible to dried pesticide residues (Romero et al. 2007; Zhu et al. 2010, 2013). Therefore, pyrethroid insecticides have little to no residual activity, and generally must be sprayed directly on the bed bugs to have any lethal effect (Davies et al. 2012; Lee et al. 2018b). Bed bugs, and particularly eggs, located in cracks and crevices or other hard to reach locations may not be killed during these bed bug "treatments". Clutter within the home gives bed bugs access to additional harborage locations, including many personal belongings such as electronic devices, books, shoes, toys, and bags of clothing, which cannot be treated with spray formulation insecticides.

Due to these challenges, much effort has been spent identifying novel treatments for managing bed bug infestations. Steam, cold, heat, and fungi have been (re-) introduced into the pest management market as methods for bed bug control (Kells and Goblirsch 2011, Kells 2018, Olson et al. 2013, Wang et al. 2018, Shikano et al. 2019). While heat, steam, and freezing can kill bed bugs, these methods also have limited ability to penetrate into and throughout all bed bug refugia. Because all bed bug refugia locations are not known at the time of treatment, determining that all bed bug refuge locations actually reached lethal temperature can be difficult. At this time, many infested homes have been known to require multiple treatments due to bed bug survivorship (Miller personal communication). Fungal spore application is another novel method recently marketed for bed bug control, but the fungal product is still new and under investigation to determine the number of treatments needed to control infestations of different sizes. The limitations of these more novel control methods, and the persistence of resistant bed bug

populations, have prompted renewed interest in using fumigation as a method for bed bug elimination.

Currently, Vikane® gas fumigant (99.8% sulfuryl fluoride, Douglas Products, Liberty, MO) is the most widely marketed gas fumigant for residential insect control in the United States. Vikane was developed in the 1950s by Dow Chemical for the control of drywood termites (Derrick et al. 1990). Sulfuryl fluoride is known for its excellent penetration through cracks, crevices, wood (Scheffrahn et al. 1992), and other materials, as well as its low water solubility. It is non-reactive as a gas to household materials and does not leave toxic residues on surfaces (Kenaga 1957, Derrick et al. 1990, Nead-Nylander 2013). In addition to drywood termites, sulfuryl fluoride has been used to control a variety of structure-infesting pest species including wood-destroying beetles (multiple species), carpenter ants (*Camponotus* spp.), carpet beetles (Dermestidae), cockroaches (*Blattella germanica, Periplaneta americana, and Supella longipalpa*), rodents, and the common bed bug (*Cimex lectularius* L.; Kenaga 1957, Derrick et al. 1990, Thoms and Scheffrahn 1994, Douglas Products 2016). Within the last decade, there has been new research investigating the dosage at which sulfuryl fluoride should be used for control of bed bugs and their eggs (Phillips et al. 2014, Gillenwaters and Scheffrahn 2018).

Sulfuryl fluoride products labeled for residential fumigation use the dosage required for control of drywood termites to calculate the dosage for other pests. The 3X dosage factor (3 times the dosage required for drywood termites) was the former Vikane label rate for bed bug control (Dow AgroSciences 2011). In 2014, there was a study to determine a more efficient dosage of sulfuryl fluoride that was capable of producing 100% mortality in common bed bug eggs, which is the most difficult life stage to kill for any pest insect. Laboratory studies conducted by Phillips et al. (2014) on a susceptible laboratory strain of the common bed bug determined that a 1.9X

dosage factor would control all life stages of bed bugs: eggs, nymphs, and adults. The 1.9X dosage factor subsequently replaced the 3X dosage factor for bed bugs in the Vikane labeling.

After sulfuryl fluoride enters the insect body through the spiracles (or through aeropyles and micropyles in the bed bug egg shell), it is broken down into sulfate and fluoride. The fluoride acts as the primary toxin and disrupts the glycolysis cycle within the insect's cells. With glycolysis inhibited, insects can only metabolize proteins and amino acids for energy, which is insufficient for maintaining the target insect's metabolic rate, thus the insect dies (Meikle et al. 1963). Insect eggs require a higher dosage of fumigant relative to post-embryonic life stages to achieve mortality. This is due to the decreased permeability of the egg shell, and the loss of the active ingredient due to binding to the egg chorion and embryonic membrane (Stanbridge 2011, Outram 1967). Exposure to sulfuryl fluoride also interferes with the metabolism of the developing insect embryo, indicated by a reduction in the egg's oxygen uptake (Outram 1970). At this time, there is no known resistance to the Fluorides (Group 8C) class of insecticides.

Since the resurgence of bed bugs twenty years ago, whole-structure fumigation using sulfuryl fluoride to eliminate bed bug infestations have been conducted in multiple regions of the U.S. in many types of buildings, including single family homes, multi-unit dwellings, and hotels (Miller and Fisher 2008, Thoms 2010). In a 2018 survey of all U.S. fumigation companies that use Vikane, fumigators reported that 9.5 ± 2.1% of Vikane fumigations were conducted for control of bed bugs (Kaplan et al. 2018). However, pest management professionals who are not familiar with fumigation and do not know physical properties and use patterns of sulfuryl fluoride have questioned the ability of this fumigant to penetrate certain bed bug refugia (i.e. plastic containers, bags of clothing, wall voids, books etc.; Miller personal communication), and to kill bed bug eggs. The purpose of this study was to investigate the efficacy of sulfuryl fluoride applied at the 1.9X

dosage factor to control insecticide-resistant bed bug eggs, nymphs, and adults that were hidden in vehicles and cargo trailers filled to 85% capacity with household items and challenging bed bug "refugia".

Material and Methods

Fumigation Study Site

All fumigation trials were conducted in the service vehicle parking lot (gravel and asphalt) at the Dodson Brothers' Pest Control home office in Lynchburg, VA. Fumigations were conducted under supervision of the company's Manager of Technical Services who is a licensed fumigator in the state of Virginia.

Regulatory Compliance

All label-required procedures, equipment, and safety precautions were followed for fumigant dosage calculations, introduction, initiation of aeration, and clearance testing. Fumigant introduction, aeration, and clearance testing were conducted by a licensed fumigator trained in the use of Vikane, using the required safety equipment and clearance devices.

Bed Bug Preparation

The bed bugs used in this study were from the Richmond field strain. This pyrethroid resistant bed bug strain was collected from a group home in Richmond, VA in 2008. The Richmond strain is currently maintained at the Dodson Urban Pest Management Laboratory (DUPML; Virginia Tech University; Blacksburg, Virginia) inside plastic rearing jars. All bed bugs are fed once a week with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using an artificial feeding system,

maintained at 35.5°C by circulating hot water. All colonies of bed bugs are kept within environmental chambers at ca. 28°C, ca. 55% RH, and a photoperiod of 12:12 h L:D cycle.

The Richmond strain is frequently used in sponsored research trials at the DUPML and is therefore evaluated annually for resistance (compared with the Harlan susceptible laboratory strain). In 2017, the Richmond strain was one of ten field bed bug strains evaluated for resistance to insecticide products containing chlorfenapyr or bifenthrin at Purdue University. The Richmond strain was found to be the most resistant of all field strains evaluated in that study (Ashbrook et al. 2017).

Richmond strain sentinel bed bug eggs, nymphs, and adult males (hence forth referred to as "adults") were prepared at the DUPML for fumigation trials to evaluate fumigation efficacy. Prior to testing, multiple groups of 10 fed adult females and two adult males were placed inside Petri dishes lined at the bottom with filter paper. These adults were left to lay eggs on the filter papers *ad libitum*, so that there were 9-11 eggs on each filter paper available for the fumigation study each day. Bed bug eggs ranged in age from two to seven days at the time of fumigation. On each test day, 19 sample units for each of three bed bug life stages; adults (n = 10), mixed-instar nymphs (n = 10), and eggs (n = 9-11), were placed on individual filter papers and inserted into individual knee-high stockings that were tied at the open end. The filter papers in stockings method for containing bed bugs has been used frequently in bed bug product efficacy evaluations by Virginia Tech researchers (Figure 5.1; Catron et al. 2017; Miller unpublished data). This bed bug containment method has proven to be very useful for maximizing bed bug exposure to specific treatments (fumigant, heat) while placing sentinel bed bugs in narrow, difficult to reach locations where bed bugs are known to harbor. Three groups of each life stage (adults, nymphs, and eggs) were placed within each fumigation and control replicate (90-93 individuals per treatment

replicate; a replicate consisted of one van or trailer). On each test day, bed bug sample units were transported from the laboratory in Blacksburg, VA to Lynchburg, VA in an ice chest (40 qt Wheeled Cooler, 6238-6341, The Coleman Company, Inc., Wichita, KS). One sample unit of each life stage (eggs, nymphs, and adults) was left behind at the laboratory each day to determine if handling influenced mortality.

Figure 5.1. All bed bug sample units (adults, nymphs, or eggs) were confined on filter papers inside of nylon stockings (as shown here) prior to placement in fumigation replicates.

Replicate Vans and Cargo Trailers

Dodge Grand Caravans ("vans"; model year 2012 (5.2 x 2.0 x 1.7 m), Chrysler Group LLC, Detroit, MI; model years 2015 (5.2 x 2.0 x 1.8 m) and 2019 (5.2 x 2.0 x 1.7 m), FCA US LLC, Detroit, MI) were used for van replicates. Two vans (2012 and 2015) belonged to the Dodson

Brothers' Pest Control company and were used for the fumigant trials. A third van (2019) was rented and served as the untreated control. These vans were selected for testing due to their complex interiors (multiple seats and storage locations including glove compartments, beverage holders, and door side-pockets) and stow-n-go seating (seats that fold into the floor), which limit their ability to be successfully treated for bed bugs using heat or spray formulation insecticides.

Cargo trailers ("trailer," 1.5 x 2.4 x 1.5 m U-Haul, Phoenix, AZ) of the same size and design were filled to 85% capacity with household belongings for all fumigation and control replications. One trailer was rented from a U-Haul dealer in Christiansburg, Virginia, and the other two trailers were rented from a U-Haul dealer in Lynchburg, Virginia.

For this study, there were six fumigation replicates and three control replicates for both vans and trailers. Control replicates were used to determine that bed bug mortality was not due to interior conditions of the individual replicates (such as heat or mechanical damage).

Interior Preparation of Fumigation Replicates

The vans were inspected to locate obscure, hard to reach locations that would serve as sentinel bed bug harborages. These locations included: the space in the floor where the stow-n-go seating is stored, air vents in the side of the vehicle and the dashboard, the intersection of the backrest and seat cushion of the driver's seat, beverage holders, between pages of the service manuals in the glove box, under floor mats, and other difficult to access locations. Bed bug sample units were placed in these locations in both the fumigated and control vehicles. Diagrams illustrating the placement of sentinel bed bugs were created. The positions of the bed bug sample units were rotated by life stage in each of the fumigation replications so that the bed bug eggs (in particular), nymphs, and adults were all exposed to the fumigant from multiple different locations.

To verify that the fumigant would not damage computer equipment in vehicles, one liquid crystal display (LCD) computer monitor (Sceptre, E205W-1600SR, City of Industry, CA) was placed inside of each van on one of the car seats. The LCD monitors were taken out of the packaging and turned on immediately before placement into a van to verify proper functionality before fumigant introduction. A different LCD monitor was used for each van replicate (each LCD monitor was fumigated once).

Each cargo trailer was loaded each test day to 85% capacity with furniture and other household items (e.g. couches, dressers, mattresses, bags of clothing, books, framed pictures, lamps, shoes, and bedding). To simulate challenging locations where bed bugs are known to harbor, three different types of "refugia" were assembled where sentinel bed bug eggs (and other life stages) were placed prior to fumigation. These egg refugia included: 1. an artificial wall void (126.0 cm (w) x 10.5 cm (d) x 92.0 cm (h)); 2. a sealed bag of clothes filled to 70% capacity (49-liter); and 3. a closed plastic storage container (56.8 cm x 40.3 cm x 33.3 cm) filled to 95% capacity with books where the bed bugs could be placed between book pages (Figure 5.2). These three different refugia were randomly placed among furniture items within each treated and control cargo trailer. After refugia placement, other items such as mattresses, pillows, and electronics were loaded into the trailer to reach the 85% fill capacity. The 85% capacity (volume) of the trailers had been previously determined by measuring the length, width, and height of the trailers, and marking the space to be left empty (15%) on the upper perimeter of the trailers. Adult and nymph bed bugs were then placed in dresser drawers, between couch cushions, and other locations where bed bugs might typically be found. Diagrams of each trailer configuration were made prior to fumigation to ensure that no two configurations were identical.

Figure 5.2. Placement of bed bug eggs in specific difficult to access refugia prior to fumigation in packed cargo trailers: A. Wall void behind an outlet; B. Inside of a book (stored with other books) inside of a closed plastic storage container; C. Inside a sealed bag of clothing.

All vans and trailers to be fumigated were supplied with a high-density polyethylene (HDPE) introduction hose (0.16 cm inside diameter (ID) and 6.1 m long; Hudson Extrusions Inc., Hudson, OH) which was attached to the front of a rotary fan (Honeywell HT-900, Helen of Troy Company, El Paso, TX) using a zip tie. In the vans, the fan was placed in the center cabin where the stow-n-go seating had been folded into the floor. In the trailers, the fan was placed in the headspace of each trailer where 15% of the cargo space was empty.

Two HDPE monitoring hoses (0.32 cm ID, Hudson Extrusions Inc., Hudson, OH) were placed in each van and trailer. In the vans, an upper monitoring hose terminated between the headrest and top of the front passenger seat. A lower hose terminated on the floor in the back of the van. In the trailers, the ends of both hoses were placed midway into the filled space, with one hose terminating in the upper quarter of the trailer and a second hose terminating in the bottom quarter. The hose ends outside of the fumigated space were labeled with their positions for later connection to a high concentration monitoring device.

Exterior Preparation of Vans and Trailers

Prior to fumigation, the vans and trailers (treated and controls) were rolled onto individual sheets of polyethylene (6 mm; HDX, The Home Depot, Atlanta, GA) that had been placed over the parking surface. After placement on the sheeting, the interiors of all vans and trailers were prepared and staged (see previous section). Another sheet of polyethylene was used to cover each van and trailer (treated and control) after they had been loaded and supplied with sentinel bed bugs. The perimeter borders of the two polyethylene sheets (top and bottom) were sealed to the ground using double or triple rows of overlapping tarpaulin tubes filled with sand ("sand-snakes"; ca. 10 cm dia. x 1.8 m length) that were sealed at the ends with plastic ties (Figure 5.3).


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Figure 5.3. Researcher using the SF-ExplorIR to check for fumigant leakage around tarped vans and cargo trailers. Fumigant leaks were remediated by adjusting the placement of sand snakes (around the perimeter of the sheeting) and by taping over holes in the plastic sheeting.

Weather conditions were highly variable during this 3-day study. To protect fumigation research equipment and researchers from precipitation (Days 1 and 2) and extreme temperature variation (Day 3), white pop-up canopies (3.0 x 3.0 m) were used to cover all work areas surrounding and adjacent to the fumigated enclosures (Figure 5.3). The walls of the canopies were installed for vans on Day 2 and on vans and trailers on Day 3.

Sulfuryl Fluoride Dosage and Dose Calculations

The dosage of sulfuryl fluoride required to kill a specific pest follows Haber's Rule and is a product of the fumigant concentration and the duration of exposure (concentration (C) x time (T) = CT dosage) (Abrams et al. 2020). Three factors determine the sulfuryl fluoride dosage: the pest organism, its life stage, and temperature at the site harboring the pest (Thoms and Scheffrahn 1994). Uptake of fumigant by the target pest determines toxicity. Temperature affects the dosage by influencing insect respiration rates. As temperatures increase, insects respire more rapidly, and thereby intake the fumigant at a faster rate and receive greater exposure. Thus, as temperature increases, the required dosage for insect control decreases.

The Vikane Fumiguide® calculator ("Fumiguide"; Douglas Products, Liberty, MO) is required by the product label to calculate the target dosage (g-h/m^3) of Vikane. The labeling for Vikane requires the temperature at the site of the target pest be used to calculate the dosage. The target dosage was determined by entering the 1.9X dosage factor for bed bugs, and the temperature

inside each van and trailer into the Fumiguide. The interior temperature was measured using a thermo-hygrometer pen (Supro THP1, Allenwood, NJ).

The Fumiguide also calculates the amount of fumigant, or dose (kg), required for introduction into the fumigated space (volume in m^3). The dose is based on the required dosage for the pest, the volume of the fumigated space, the desired fumigant exposure period (6 h planned), the monitoring status of the fumigation (e.g., a lower dose is required for fumigations where the fumigant concentrations are regularly measured in the fumigated space to confirm dosage accumulation), and fumigant confinement time, measured as half-loss time (HLT; Thoms and Scheffrahn 1994). The HLT is defined as "the amount of time for gas concentration to decrease by half" (Scheffrahn et al. 2005).

To estimate the HLT, the Fumiguide uses multiple factors: the quality of the seal (tarp condition, seal condition, and underseal), the windspeed, and the volume of the fumigated space. In this field evaluation, the seal conditions for the polyethylene sheeting were rated as excellent, and the "underseal" was rated as "slab" based on industry experience (the polyethylene sheeting placed underneath the vans and trailers is not a variable that can be entered into the Fumiguide). Wind speed was determined each day using weather.com (The Weather Channel, Atlanta, GA) to predict the average wind speed in Lynchburg, VA during the fumigation exposure period. The volume of fumigated space was determined by measuring the length, width, and height of each tarped van and trailer. Small variations in the volume of each van and trailer were recorded on each test day due to slight changes in the reapplication of the polyethylene sheeting and replacement of the sand snakes for each replicate. The Fumiguide predicted HLTs ranging from 7.9 – 8.8 h for vans, and 7.4 – 8.3 h for trailers. A preliminary trial indicated that increasing volumes of each fumigated space by 100-fold would more accurately estimate the HLT. Using this

method, the subsequent doses calculated by the Fumiguide were then reduced by 100-fold to obtain the doses applied.

Sulfuryl Fluoride Introduction

Outside of each tarped van and trailer, the proximal end of the introduction hose was connected to a needle valve (N400B, Park Hannifin, Elyria, OH) to control the fumigant flow rate. The needle valve was attached to a commercial cylinder of Vikane that was placed on top of a digital scale (Yellow Jacket Flatbed Scale; Ritchie Engineering Co., Bloomington, MN). The sulfuryl fluoride dose was then measured gravimetrically as the fumigant was released from the cylinder. Fumigant introduction was conducted sequentially, not concurrently, for each tarped van and trailer.

After sulfuryl fluoride was introduced, a SF-ExplorIR (Spectros Instruments, Hopedale, MA; Limit of Detection [LOD] ± 1 ppm) was used to check for fumigant leakage around each tarped van and trailer (Figure 5.3). Any detected leaks were remediated by adjusting the placement of sand snakes or by using tape (10.2 cm wide Intertape PM2 Brown Flatback Tape, Intertape Polymer Group, Sarasota, FL) to seal any observed punctures or tears in the plastic sheeting.

Monitoring Sulfuryl Fluoride Concentrations

A SF-ReportIR (Spectros Instruments, Hopedale, MA; LOD: 24 ppm) was used to measure the fumigant concentrations inside each van and trailer replicate throughout the exposure period by withdrawing air samples through the previously placed monitoring hoses. A gas standard of 4,380 ppm sulfuryl fluoride in a compressed gas cylinder (Scott Marin, Riverside, CA) was used before and after the study to verify the proper functional status of the SF-ReportIR.

Fumigant concentrations were measured in each replicate (from both upper and lower hoses) at ca.10-15 min intervals post fumigant introduction until the readings stabilized. Once

readings were stabilized, the introduction fans were turned off and the initial sulfuryl fluoride concentration readings were entered into the Fumiguide to begin dosage accumulation calculations. Sulfuryl fluoride concentration readings were taken every hour to track the accumulated dosage for each replicate. As the accumulated dosage approached the label-required dosage, readings were taken more frequently. Aeration was initiated once the label-required dosage was achieved.

Aeration

To begin aeration, a trained fumigator in a self-contained breathing apparatus (SCBA; Dräger PAS® Lite, Draeger, Inc., Telford, PA) removed all sand-snakes, lifted the tarps, and opened the doors of the fumigated vans and trailers. The fans previously used for fumigant introduction were turned on again and repositioned to ventilate the interior of the vans and trailers. To further aid in the aeration of the trailers, 6 mm polyethylene sheeting was rolled into a tube (ca. 0.3 m ID and 4.6 m long). The terminal end of the tube was inserted into the headspace of the trailer, and the proximal end was attached to a fan located outside (Lasko 1365634 Super Fan, Lasko Products, LLC, West Chester, PA) forcing fresh air into the trailer.

A SF-ExplorIR was used to measure fumigant concentrations to ensure researchers were working in areas where their exposure would not exceed 1 ppm, which is the label-required concentration for re-entry. After completing aeration, the SF-ExplorIR was used to test the headspace of each trailer and the breathing zones (e.g., where individuals would typically stand, sit, or lie down) in each van. This was done to confirm the fumigant concentrations were 1 ppm or less before re-entry by researchers to remove the sentinel bed bugs from fumigated and control replicates.

Post-Treatment Evaluation of Sentinel Bed Bug Mortality

Sentinel bed bugs were returned to the DUPML to be scored for mortality the day after aeration was completed. Bed bug nymphs and adults were considered dead if they did not react to any stimulation (i.e. shaking their container or prodding). Gillenwaters and Scheffrahn (2018) observed latent mortality up to 22 days post fumigation in bed bug eggs fumigated at a 0.67X dosage factor (ca. 1/3 the label rate of the 1.9X dosage factor used in this study.) Therefore, treated and control bed bug egg hatch was recorded every day for 23 days post fumigation. Eggs that failed to hatch after 23 days were considered to be dead. Mortality of control nymphs and adults were recorded on the day of their return to the laboratory, and the following day (controls only).

Post-Treatment Evaluation of LCD Monitor Functionality

After each fumigation, the LCD monitors that had been placed in treated and control vans were returned to the DUPML to verify their functionality. A monitor was determined to be functional if the screens could be turned on and there was no discolorations or evidence of dead pixels. Screen functionality was evaluated every day for 15 days post fumigation.

Statistical Analysis

The Fisher's Exact Test (Hoffman 2015) was used to evaluate a two by two contingency table that had the fumigation replicates versus control replicates on one axis, and "success" (100% mortality) versus "failure" (< 100% mortality) on the other axis. In this study, the null hypothesis tested was that the control and the fumigated chambers were equally likely to result in 100% bed bug mortality. The alternative hypothesis was that the fumigated chambers were more likely to result in 100% mortality than the controls. For both the van and the cargo trailer comparisons, values of $P \leq 0.05$ indicated significance. The statistics were calculated using the SAS Institute Inc.; Proc.

Freq. for 2 x 2 contingency tables (SAS Institute 2004). The average percent difference between target dosages and accumulated dosages (± SE), and standard errors for bed bug control mortality (for each life stage) were calculated using Microsoft 365 for Business Excel Version 2005 (Microsoft, Redmond, WA).

Results

Weather Conditions During Fumigation

Weather conditions and ambient temperatures in Lynchburg, VA varied each day during this fumigation study. Table 5.1 lists the weather conditions for each of the three fumigation dates. Recorded temperatures were 16.7°C inside each vehicle and cargo trailer on Day 1 of the fumigation study. All temperatures were recorded in the late morning to early afternoon before fumigant introduction on each fumigation day. Precipitation occurred throughout the day with a total rainfall of 10 mm. On Day 2, the temperatures and total rainfall were very similar to Day 1. However, wind gusts and maximum hourly rainfall were two to three times greater than they had been the previous day (24 km/h and 7.6 mm/h). On Day 3, temperatures increased to 25°C and there was neither wind nor rainfall during the fumigation period.

Table 5.1. Fumigation weather conditions: Temperatures (recorded inside replicates); mean (min – max) wind speed, wind gusts, and hourly and total rainfall for Lynchburg, VA[a].

June 2020[b]	Temp (C)[c]	Wind Speed (km/h)	Wind Gusts (km/h)	Hourly Rainfall (mm/h)	Total Rainfall (mm)
16th	16.7	18.5 (14.5-24.1)	12.9 (0.0-35.4)	1.3 (0.0-2.5)	10.2

17th	18.3	20.9 (14.5-29.0)	24.0 (0.0-40.2)	1.5 (0.0-7.6)	12.7
18th	25.0	12.7 (9.7-19.3)	0.0 (0.0-0.0)	0 (0.0-0.0)	0.0

[a] Recorded wind and rainfall conditions for Lynchburg VA Regional Airport weather station obtained from the Weather Underground (wunderground.com, San Francisco, CA).

[b] The fumigation dates 2020: Day 1 (June 16th); Day 2 (June 17th); Day 3 (June 18th).

[c] Temperatures recorded inside the vans and trailers using a thermo-hygrometer pen.

Fumiguide Input, Target Dosage, and Temperature

For all fumigations, the accumulated dosages were within 0.107 ± 0.002% for trailers, and 0.296 ± 0.006% for vans of the target dosages. As a result of varying daily temperatures, target dosages differed each day, ranging from 149.0 to 251.9 g-h/m^3 (Tables 5.2 and 5.3). As previously discussed, the lower the temperature at the site of the target insect pest, the higher the dosage of sulfuryl fluoride required to control the pest. For example, on Day 1, the temperature (16.7°C) input resulted in a Fumiguide target dosage of 251.9 (g-h/m^3) in both the vans and cargo trailers. On Day 2, the temperature was slightly higher at 18.3°C so the target dosage calculated by the Fumiguide was slightly lower at 223.1 (g-h/m^3). However, on Day 3 when the temperature was considerably higher (25.0°C), the target dosage was reduced to 149.0 (g-h/m^3) in both the vans and cargo trailers (Tables 5.2 and 5.3). Note that the dose (kg; output) on all test days was slightly greater in the vans than in the cargo trailers. This was no doubt due to the vans having a greater volume (m^3; input).

Table 5.2. Dodge Grand Caravan input, output, and recorded data from the Vikane Fumiguide® calculator.

		Input[c]			Output				Actual Recorded Data			
June 2020[a]	Replicate (Van ID)[b]	Temp (°C)[d]	Wind (km/h)[e]	Replicate Volume (m^3)[f]	HLT (h)[g]	Dose (kg)[h]	Target Dosage (g-h/m^3)	Target Initial Concentration (g/m^3)	HLT (h)	Accumulated Dosage (g-h/m^3)	Initial Concentration (g/m^3)	Exposure Time (h)
16th	1 (A)	16.7	16.1	12.7	32.8	0.57	251.9	44.7	12.4	251.9	57.9	5.9
16th	2 (B)	16.7	16.1	13.0	33.0	0.58	251.9	44.7	26.9	251.7	50.8	5.7
16th	Control (C)											
17th	3 (C)	18.3	17.7	14.4	32.9	0.57	223.1	39.6	19.0	222.9	60.3	4.5
17th	4 (B)	18.3	17.7	13.9	32.4	0.55	223.1	39.6	10.8	223.4	51.9	5.5
17th	Control (A)											
18th	5 (A)	25.0	12.9	14.7	37.0	0.39	149.0	26.3	11.5	151.3	43.2	4.5
18th	6 (C)	25.0	12.9	13.6	36.0	0.36	149.0	26.3	15.8	149.4	43.6	4.1
18th	Control (B)											

[a] The fumigation date. Day 1, 2, and 3 are June 16, 17, and 18, respectively.

[b] All Dodge Grand Caravans used in this study were manufactured in either 2012 (A), 2015 (B), or 2019 (C).

[c] Parameters entered into the Fumiguide before the fumigation. For all replicates, tarp condition and seal condition were rated as "excellent," and underseal was rated as a "slab."

[d] Temperatures recorded inside the vans using a thermo-hygrometer pen.

[e] Wind speed information for Lynchburg, VA retrieved from weather.com.

[f] Volumes were increased by 100-fold for input into Fumiguide to more accurately estimate HLT based on HLTs measured during a preliminary trial.

[g] HLT = half loss time.

[h] Dose reduced by 100-fold to correct for previously increased volume.

Table 5.3. Cargo trailer input, output, and recorded data from the Vikane Fumiguide® calculator.

	Input[b]	Output	Actual Recorded Data

June 2020[a]	Replicate (Trailer ID)	Temp (°C)[c]	Wind (km/h)[d]	Replicate Volume (m^3)[e]	HLT (h)[f]	Dose (kg)[g]	Target Dosage (g-h/m^3)	Target Initial Concentration (g/m^3)	HLT (h)	Accumulated Dosage (g-h/m^3)	Initial Concentration (g/m^3)	Exposure Time (h)
16th	1 (A)	16.7	16.1	9.9	30.2	0.45	251.9	44.9	20.5	252.7	68.6	4.3
16th	2 (B)	16.7	16.1	9.6	29.9	0.43	251.9	45.0	16.9	252.1	54.9	5.7
16th	Control (C)											
17th	3 (C)	18.3	17.7	9.3	28.5	0.37	223.1	40.0	37.1	223.2	47.8	5.2
17th	4 (B)	18.3	17.7	8.8	28.0	0.35	223.1	40.0	12.9	223.4	48.4	5.9
17th	Control (A)											
18th	5 (A)	25.0	12.9	8.8	31.4	0.23	149.0	26.5	10.8	148.7	33.8	6.2[h]
18th	6 (C)	25.0	12.9	9.9	32.5	0.26	149.0	26.5	35.4	149.4	30.4	5.9
18th	Control (B)											

[a] The fumigation date: Day 1, 2, and 3 were June 16, 17, and 18, respectively.

[b] Parameters entered into the Fumiguide before the fumigation. For all replicates, tarp condition and seal condition were rated as “excellent,” and underseal was rated as a “slab.”

[c] Temperatures recorded inside the cargo trailers using a thermo-hygrometer pen.

[d] Wind speed information for Lynchburg, VA retrieved from weather.com.

[e] Volumes were increased by 100-fold for input into Fumiguide to more accurately estimate HLT based on HLTs measured during a preliminary trial.

[f] HLT = half loss time.

[g] Dose reduced by 100-fold to correct for previously increased volume.

[h] Trailer replicate 5 had a 6.2 h exposure time because of a delay in recording the initial concentration caused by a mislabeled monitoring hose.

Exposure Time and Fumigant Concentration

In the van and trailer replicates, the exposure times required to achieve the target dosages were less than the planned 6 hours, with the exception of one trailer replicate (#5). This was due to initial measured sulfuryl fluoride concentrations being 1.1 to 1.7-fold greater than planned initial concentrations (Tables 5.2 and 5.3). The increased initial fumigant concentrations resulted in decreased exposure times because dosage is a product of concentration and time. Trailer replicate #5 had a 6.2 h exposure time due to the seal being opened during the exposure period to verify the location of a monitoring hose. Opening the seal lowered the fumigant concentration resulting in the need for a slightly longer than planned exposure period.

The observed increase in initial fumigant concentrations was caused by the contents and even certain void spaces that occupied the air space within the replicates, reducing the space available for fumigant diffusion. Trailers were filled with materials, such as books, wood, drywall, and sealed LCD screens which occupied space after sulfuryl fluoride diffused throughout these trailer contents. The vans had sealed, impermeable voids, such as tires, the engine block, radiator, and gas tank, that excluded sulfuryl fluoride and increased the interior concentration of the fumigant.

Sulfuryl Fluoride Half-Loss Times

Actual HLTs ranged from 10.8 - 26.9 h for vans (Table 5.2) and 10.8 - 37.1 h for trailers (Table 5.3). These actual HLTs were generally less than those estimated by the Fumiguide using the 100-fold increase in volume. However, the HLTs were greater (i.e., better confinement) than those estimated using the actual volume. Procedures conducted in this study to improve fumigant confinement included using new polyethylene sheeting, placing sheeting underneath each van and

trailer, and sealing sheeting at ground level using double and triple rows of sand snakes. Additionally, each replicate was repeatedly checked for leaks using the SF-ExplorIR during the fumigation, and any leaks discovered were immediately sealed. Each of these procedures are used by fumigators for specific situations, but they are not mandated by fumigant labeling and were not used in field trials to develop the Fumiguide algorithms for estimating HLT.

The variability in the measured HLTs between similar replicates treated the same day, was due in part to the small volumes of the vans and trailers. These small volume replicates (compared to the size of a home) have a large surface area relative to total volume to lose fumigant. Because of this, any changes in confinement conditions (e.g., intermittent wind gusts, abrasion or small punctures in the polyethylene tarps, and changes in the seal after re-tarping) had a greater effect on HLTs for the vans and trailers than they would on the HLTs of larger structures.

Bed Bug Mortality

Upon return to the laboratory, any physical damage to adult and nymph control sample units was recorded. No physical damage for the fumigated adult and nymph units could be determined (due to mortality). However, one sample unit of control nymphs from a trailer replicate on Day 3 did have damage recorded. Eggs in both fumigated and control replicates were inspected daily for 23 days. One sample unit of eggs taken from the van control on Day 3 appeared to have two damaged eggs. No damage was recorded for fumigated eggs.

Mortality for all fumigated bed bug nymphs and adults, in both vans and trailers, was 100% on the day of their return to the laboratory, and the following day (Table 5.4; 720 bed bugs totals; 360 each for vans and trailers). There was no mortality observed in the nymph and adult controls on the day of their return to the laboratory, except for one trailer sample unit of nymphs on Day 3

of the trial. This sample unit had been placed into a bag of trash. At the time of collection following aeration, it was discovered that a metal sprinkler-head had fallen onto the bag, killing five of the 10 nymphs in the sample unit. Because all fumigated bed bug nymphs and adults were dead after aeration and none recovered the following day, the assessment of mortality of fumigated and control bed bug nymphs and adults ended at this time.

Table 5.4. Average percent (%) mortality for all eggs, nymphs, and adult bed bugs from fumigated and control replications (vans, and cargo trailers filled to 85% capacity).

	Vans (Mean percent mortality ± S.E.)			Cargo Trailers (Mean percent mortality ± S.E.)		
June 2020	Eggs[a]	Nymphs[b]	Adults[b]	Eggs[a]	Nymphs[b]	Adults[b]
Treated[c]	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Controls[d]	9.9 ± 6.7	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 0.2	5.6 ± 5.6	0.0 ± 0.0

[a] Fumigated and control egg mortality was recorded for 23 days post fumigation.

[b] Fumigated and control mortality for nymphs and adults was recorded the day after aeration was completed, and the following day.

[c] There were 10 bed bugs per sample unit; nine sample units (3 per life stage) per fumigation replicate; and six fumigation replicates for vans and trailers. This resulted in 1,080 fumigated bed bugs total (540 total bed bugs for vans, and 540 for trailers).

[d] There were 10-11 bed bugs per sample unit; nine sample units (3 per life stage) per untreated field control; and 3 control replicates for vans and trailers. This resulted in 546 control bed bugs (272 total bed bugs for vans, and 274 for trailers).

Note: Laboratory controls (9-11 per life stage on each test day) were not included in the analysis due to no nymph or adult mortality being observed post-fumigation, and only a single egg failing to hatch by day 23.

For the Fisher's Exact Test, bed bug mortality between the treated and control sample units was compared using the data taken the day after fumigation. The average mortality for the 90 adult bed bugs in control vans was 0%. The average mortality for the 90 nymphs in control vans was 0.0%. The average mortality for the 90 adult bed bugs in control cargo containers was 0.0%. Mortality for bed bug nymphs in control cargo containers was 5.6 ± 5.6% (5 bed bugs).

In all fumigation replicates, 100% of the bed bug eggs failed to survive. In almost every replicate, egg deterioration could be viewed under the microscope during the 23-day observation period. At the end of ~10 days, the eggs were noticeably shriveled and contracted as they began to dehydrate. Interestingly, there was one fumigated egg that displayed latent mortality. This egg was from a sample unit in a fumigated cargo trailer on Day 1. The embryo in this egg developed eye spots that could be seen from outside of the egg. Fourteen days post-fumigation, the cap of the egg shell came off and the embryo began to emerge (Figure 5.4). The first instar managed to push its head out of the shell, and the body could be seen undulating as it attempted to complete the eclosion process. However, only the head successfully emerged from the egg shell prior to nymphal mortality.

Figure 5.4. Latent mortality of a single fumigated bed bug egg was observed in one replicate. Eclosion was incomplete with the nymph only surviving long enough for the head to emerge. Although two additional eggs developed eye spots, no additional eggs hatched in this group and all eggs began to shrink within the following five days.

In the field control replicates, the mean egg mortality was 9.9 ± 6.7% in vans and 6.4 ± 0.2% in trailers after 23 days (Table 5.4). In the control van replicate on Day 3, a total of seven eggs did not hatch. Five of those bed bug eggs were contained in a single sample unit, the other two sample units each had one failed egg. It is not known exactly why the egg mortality for the control van on Day 3 was so high, but upon return to the laboratory two of the eggs had appeared to be crushed when examined under the microscope. The sample unit had been placed in the stow-and go seating storage area in the floor of the van. Thus, five of the eggs may have been damaged

when the floor panel covering the seating storage space was closed. However, the remaining 5 eggs in the sample unit survived, and all had hatched by Day 12.

In the three laboratory controls (one for each life stage on each test day), bed bug mortality was exceptionally low. In the adult and nymph laboratory controls, there was no mortality recorded the day after fumigation. Laboratory control eggs were observed for 23 days, and only one of the eggs failed to hatch during the observation period.

The Fisher's Exact Test was used to determine the success of the 1.9X fumigation dosage factor to kill all bed bugs in both vans and cargo trailers. In both the vans and cargo trailers, the fumigated replications (6 vans and 6 trailers) achieved 100% bed bug mortality, while the controls (3 vans and 3 trailers) did not. Therefore, in fumigated replicates (vans and cargo trailers), the "success" (100% mortality) was significantly greater than in the controls (< 100% mortality; *P*-values 0.0119 for both vans and cargo trailers; and a 95% C.I. value of 2.22 Infinity (Inf.)). The confidence interval indicated that the odds ratio (probability of success, divided by probability of failure) for fumigation was within the 2.22 Inf. confidence interval. Laboratory controls were not included in the analysis due to the fact that only one egg out of all life stage sample units failed to survive.

All fumigated LCD monitors were checked each day post-fumigation for 15 days, and as expected, were found to be completely functional (Bell et al. 2003; Mueller 2012). This study confirms that the fumigation process has no negative impacts on LCD monitors in vehicles.

Discussion

The current study is the first field evaluation to document the ability of Vikane (at the 1.9X dosage rate) to eliminate bed bugs from vehicles, and chambers filled with household items that included

difficult to access bed bug refugia. The results obtained in this study confirmed the laboratory results published by Phillips et al. (2014) which determined the 1.9X dosage factor was sufficient to kill all bed bug life stages. It should be noted that Phillips et al. 2014, study used pesticide-susceptible bed bug strains. This field evaluation was the first to use pyrethroid-resistant bed bugs to document the efficacy of sulfuryl fluoride for killing resistant bed bugs. In the current study, all fumigated replicates resulted in 100% mortality, and the Fisher's Exact Test determined that the process of fumigation with Vikane was successful in eliminating all resistant bed bugs in every replicate ($P = 0.01$).

The 1.9X dosage factor determined by Phillips et al. (2014) decreased the required amount of fumigant by one-third, compared to the 3.0X dosage factor that was formerly used for bed bug control (Dow AgroSciences 2011). The authors suggested that this reduction in dosage might represent a significant fumigant cost reduction by requiring less fumigant (Phillips et al. 2014). The efficacy of the 1.9X dosage factor, and the potential for reduced fumigant costs, suggests that Vikane fumigation has the potential to become one of the more widely used methodologies for eliminating resistant bed bugs in vehicles (e.g. cars, city buses, or tractor-trailers) and other difficult to access locations which may be filled with infested materials, including storage facilities, trailer homes, and campers.

The original motivation for conducting the fumigation studies in vehicles was to address the recent issue of bed bug infestations in cars (Howerton, McFarland, Rittman 2020). Bed bugs in vehicles are a unique problem due to the potential for passengers to transport bed bugs into un-infested buildings. Vehicles cannot not be treated effectively with spray formulation insecticides due to inaccessible harborage locations. Using heat treatment is also challenging because heat has the potential to damage the electronic equipment which is often standard in modern vehicles.

Therefore, a more comprehensive solution for treating vehicles is currently needed. In the past, sulfuryl fluoride has been used to fumigate a variety of pests in vehicles, including buses (Dow AgroSciences 1998), passenger rail cars (Douglas Products 2015), and watercraft. The current study verified that sulfuryl fluoride fumigation at the 1.9X rate will eliminate bed bugs in vehicles, confirming that Vikane fumigation is a viable bed bug treatment option.

The fumigation trials in cargo trailers filled to 85% capacity were intended to address two specific objectives: 1). to evaluate the efficacy of chamber fumigations for eliminating bed bugs in household items and 2). to evaluate the ability of Vikane to kill bed bug eggs located in difficult to access "refugia". The authors wanted to identify an effective alternative for treating infested furniture, clothing, and other household items that are either difficult to treat with insecticides or cannot be treated at all (books, shoes, artwork, photographs, etc.). This was due to the fact that current pest management "preparation instructions" recommend that these infested items either be thrown away or be placed indefinitely into sealed bags. These instructions ensure that the items are out of the way when the technician comes to treat, but the preparation requirements do not address how to eliminate bed bugs from those items that the residents want to keep. This study determined that fumigation is the alternative to having the resident throw away furniture that might be irreplaceable, and to prevent residents from having to put their keepsakes and belongings (indefinitely) into bags. Container fumigation is a superior method that allows pest management professionals to eliminate bed bugs from belongings, and also prevents bed bug reintroductions due to resident handling of their personal items after treatment.

The cargo trailer fumigation also validated the ability of the fumigant to access and kill bed bug eggs in refugia such as wall voids behind electrical outlets, sealed bags full of clothing, and in books stored in plastic containers. For this objective, the chambers filled to 85% capacity were

intended to mimic highly cluttered indoor environments that might challenge the fumigant's ability to contact and kill bed bug eggs in these difficult to access refugia. As stated previously, researchers did observe the head of a single bed bug nymph emerging from an egg 14 days post fumigation. However, the nymph failed to eclose any further prior to mortality (Figure 5.4). This was the first replicated research trial to verify that sulfuryl fluoride would kill bed bug eggs in hidden refugia where the larger treatment space is so filled with clutter that it could not be treated effectively using residual insecticides or heat. This research confirmed clutter and lack of accessibility to bed bug refugia are not obstacles to sulfuryl fluoride fumigation.

Fumigation using sulfuryl fluoride is the only residential pest control method that provides single-treatment eradication of the target pest throughout a treated structure. The dosage calculation tool (e.g., Fumiguide) provides precision in applying the required dose to control the target pest based on specific site and fumigation parameters. The efficacy of the fumigation can be verified by measuring sulfuryl fluoride concentrations during the fumigation process and inputting these readings into the dosage calculation tool. The calculation tool will report if the required dosage for control is being accumulated and if not, provides directions (amount of additional fumigant and/or time to add to the fumigation) to obtain this dosage. The tools and methods used to confirm the required sulfuryl fluoride dosage to eliminate target pests have been validated over decades of field use and research, that now include this study.

Prior to the bed bug resurgence twenty years ago, the use of sulfuryl fluoride in central and northern states (e.g. Indiana, Washington, Connecticut, Delaware, Montana, Nebraska, New Hampshire, New Jersey, New York; Missouri, Oklahoma, Ohio, etc.) was limited due to a lack of drywood termites and primarily used for control of wood-destroying beetles in buildings. However, bed bugs have infested the major cities in these states at a rapid rate (Miller and Fisher

2008; Anonymous 2019; Anonymous 2020b). As a result, sulfuryl fluoride fumigation for bed bug control is expanding in these states as well as throughout the U.S. In a 2018 survey of pest control companies in these central and northern states, fumigators reported that 66.3% of all Vikane fumigations were specifically conducted for bed bug control (Kaplan et al. 2018).

While some pest management companies might be reluctant to offer fumigation as part of their service due to their lack of familiarity with the process, or fear of the potential hazard associated with misapplication (Miller personal communication), the fumigation process using sulfuryl fluoride has been extensively researched and has been an integral part of the pest management industry for 60 years. Those that may not want to fumigate buildings, might consider the utility of offering containerized fumigation to control bed bugs in vehicles or trailers filled with personal belongings. Vehicular and containerized fumigation is a proven and reliable method for bed bug control in states where there is currently no other method that can guarantee 100% elimination of all bed bugs and their eggs. The results of this study are intended to provide guidance to pest control professionals who are in the market for novel and practical options for eliminating insecticide-resistant bed bugs from homes, vehicles, and household items.

Chapter Six:

Summary

The most recent world-wide resurgence of the common bed bug, *Cimex lectularius* L., is largely due to their genetic variability, which has allowed modern populations to become highly resistant to a variety of the insecticide chemistries designed by humans. Just prior to the bed bug resurgence, the number of chemistries labelled for indoor usage in the United States had been greatly reduced due to the implementation of the 1996 Food Quality Protection Act (FQPA).

The FQPA eliminated almost most types of insecticide chemistries from indoor use, except for pyrethroids, pyrroles, IGRs, and neonicotinoids. This was done in order to reduce the potential of childhood pesticide exposure. While the FQPA has certainly reduced insecticide exposure in human children, the bed bug resurgence had not been anticipated, and the unforeseen consequences of the FQPA has left the pest management industry with very few chemical options with which to address modern bed bug infestations. Needless to say, repeated indoor applications of pyrethroids led to widespread pyrethroid resistance among all common household pests such as German cockroaches, cat fleas, house flies, and of course, the common bed bug (Rust 2016, Wu and Appel 2017, Freeman et al. 2019). Within common bed bug populations, repeated pyrethroid exposures led to their selection for three different types of resistance, which facilitated the world-wide bed bug population explosion.

The most extensively researched type of resistance that was documented in modern bed bug populations is *kdr* resistance (Yoon et al. 2008, Zhu et al. 2010, Romero and Anderson 2016). *Kdr* resistant populations develop when repeated pyrethroid exposure selects for those individuals with mutated receptors on their nerve cells. These mutations are known to reduce or

even eliminate the pyrethroid insecticides' ability to bond to the receptor sites and thus inhibit nerve function. Due to the active ingredient's inability to attach to the bed bug's mutated nerve receptor, pyrethroid exposure is no longer a neurological threat to the insect, and will not result in mortality.

Repeated pyrethroid exposure has also selected for bed bugs with a second type of resistance: production of detoxification enzymes in large quantities within their bodies. These exceptional enzyme producers are capable of detoxifying insecticides, thus increasing the bed bug's survival potential after exposure. While these enzyme-producing bed bugs may be "knocked down" after pesticide exposure, they will recover within a few hours due to their physiological ability to detoxify the pyrethroid (and other insecticide) molecules (Romero 2018).

The third, and very influential type of resistance that has been selected in the modern bed bug is increased cuticular thickness. Increased cuticular thickness reduces the cuticular penetration of insecticides and can intensify other forms of resistance (Lilly et al. 2016b). The advantage of being selected for this particular trait is that increased density of the exoskeleton significantly delays pesticide absorption into the bed bug's body (Balabanidou et al. 2018). In addition to bed bugs having physically thicker cuticles, bed bugs can also be selected to have increased P450 and ATP-binding cassette transporter expression in the epidermal layer to detoxify insecticides in the integument (Zhu et al. 2013). While reduced cuticular penetration type resistance may only protect some bed bugs from direct spray applications, it can completely protect them from exposure to dried pesticide residues, thus eliminating any insecticide residual activity.

This history of artificial selection due to insecticide overexposure has been largely responsible for the bed bug resurgence. While there have been other factors that have contributed

to the resurgence (the overall increase in the human population, proliferation of human travel, the extensive use of pyrethroid treated bed nets for malaria control, etc.), the decades of selection for pyrethroid resistant populations is the major factor contributing to the modern bed bug resurgence.

Many of the modern bed bug populations have now been evaluated for different types of resistance, and almost all have been found to have at least one type (Zhu et al. 2010; Lilly et al. 2016a, 2016b). Thus, the modern bed bug resurgence has forced pest management professionals to reduce their reliance on spray formulation insecticides, and to seek out more novel methods for addressing modern bed bug infestations.

Due to the resurgence of resistant bed bugs in the 2000s, most pest control companies in the United States were relatively inexperienced when it comes to effective bed bug control. However, many have already come to realize that bed bug elimination cannot be achieved by spraying or fogging with pyrethroid products alone. Therefore, many pest management companies have investigated a number of alternative methods in their attempts to eliminate bed bug infestations. Over the last decade many novel products have come into the bed bug market. Unfortunately, many of these products were fairly short lived when it became evident that they did not have the ability to eliminate infestations like they had originally claimed (e.g., neem oil products; cedar oil, bed bug sticky monitors; permethrin impregnated mattress covers, etc.). However, some of these non-traditional alternatives to insecticides have proven to be effective and are becoming more widely used in infested environments. These alternative control methods have included desiccant dusts, frozen CO_2 applications, chamber and whole-home heat treatments, fumigants, and a fungal spore formulation of *Beauveria bassiana*. While these novel methodologies have been found to be reasonably effective in the field, some of these less

conventional control methods have not yet been evaluated in controlled scientific studies. Given the success of the common bed bug resurgence and the need for reliable (alternative) control methods, it is essential that these alternative methods be thoroughly evaluated to determine how they can be used most effectively in the field.

The purpose of this study was to address this need for evaluation of these novel treatment methods. The specific intention of this research was to determine the conditions under which whole-home heat systems and the spray formulation fungal product *B. bassiana* (Aprehend®) is the most effective for controlling insecticide-resistant bed bugs. I also wanted to determine if the fumigant, sulfuryl fluoride, was effective for controlling insecticide-resistant bed bugs in challenging situations (heavily cluttered environments) while also not harming electronics that may be present in vehicles. In this thesis, each of these alternative products/methods for bed bug control were evaluated in a way that was intended to reveal how environmental factors and/or application practices would enhance their functionality, increase their bed bug elimination potential, and verify bed bug elimination in challenging situations.

Currently, there are a variety of whole home heat systems on the market, and although they are all believed to be a single solution for bed bug elimination, many of the commercial heat systems do not have the heating capacity to kill all bed bugs (and their eggs) in homes of different cubic footage. Therefore, one of the problems that bed bug afflicted customers unknowingly face when hiring a heat treatment company is determining whether the heat system that the company uses has the power and ability to bring all hard to heat cracks and crevices in the home up to bed bug lethal temperature. In addition, many inexperienced or negligent pest technicians believe that the heat system simply has to be put into place and left for a certain period of time to be effective. They have not been trained to monitor the temperatures regularly

in hard to heat places, or to move the heaters and fans during the process in order to cover the complete cubic footage of the home. A major factor contributing to the technicians' ignorance is the fact that prior to this study, no effort has been made to compare and evaluate the capabilities of different whole-home heat systems for their ability to eliminate bed bugs in homes under different conditions. While whole-home heat treatments are presumed to be a one-treatment solution for eliminating bed bugs in homes, this study found that none of the three heat systems tested were able to simply provide 100% elimination in every situation. This study found that several factors other than the system's attributes were the major contributors to any system's potential for bed bug elimination success. Factors that were initially anticipated to be influential contributors to heat treatment success (or failure) included the heat system itself (BTUs; pieces of equipment, and its energy source), and the clutter ratio of the treatment zone. However, these two factors were not found to be significant. Two factors that were found to be significant was a system's ability to get multiple crack and crevices (monitored by our temperature sensors) up to bed bug lethal temperature during the treatment process and, not surprisingly, the length of the treatment duration. When we sought to identify the specific factors that contributed to the hard-to-heat locations reaching bed bug lethal temperature, it was found that technician diligence was the single most influential factor. Therefore, we had to conclude that the technician's attention to the heat treatment process was just as important to bed bug elimination as the heat system itself. This information will be very important to report to the pest management industry and to heat treatment customers. That being said, we did have bed bug survivors occur under even the most diligent technician's watch. Therefore, it is advisable that post-treatment spray applications be used to achieve maximum bed bug kill. A spray application of the Aprehend® product

(discussed below) would be highly recommended as a supplement to be applied after a heat treatment is completed to maximize treatment efficacy.

One of the most interesting evaluations conducted in this study focused on the influence of environmental conditions (temperature and humidity) on the efficacy of the Aprehend product. Bed bugs were exposed to the Aprehend fungal agent *Beauveria bassiana,* and then held under different combinations of temperature and humidity to evaluate how these environmental factors might influence the ability of the fungal spores to infect and kill the bed bugs. The bed bugs were forced to cross a 5.08 cm Aprehend spray barrier only once prior to being placed into environmental chambers maintained at different temperature (15°C, 21°C, and 32°C) and humidity levels (30%, 50%, 70%). In spite of the brief single-exposure design of these assays, high levels of bed bug mortality still occurred. After the bed bugs were exposed to the fungal application, groups of bed bugs were monitored in environmental chambers held at the different temperature and humidity combinations. This study determined that both the temperature and humidity levels did indeed have an influence on bed bug fungal infection and mortality. Median survival times were also calculated for each temperature/humidity combination, and it was found that bed bugs were killed the fastest at room temperature (21°C) at the 30% and 50% humidity levels. It was found that bed bug mortality was the greatest for all three test temperatures at the 30% humidity level (71.0% mortality at 15°C; 77.8% mortality at 21°C; and 98.0% mortality at 32°C). Surprisingly, we found that bed bug mortality was at the lowest across all temperatures at the highest humidity level (70%) tested (54.0% mortality at 15°C; 48.3% mortality at 21°C; 66.0% mortality at 32°C). While 32°C temperature produced the most bed bug mortality, this high temperature also killed the control bed bugs that had no fungal exposure. Therefore, we are unable to consider fungal infection as the cause of mortality in the 32°C humidity combinations,

given that the growth rate of *B. bassiana* is known to be reduced by 40% at this high temperature (Fargues et al. 1997).

One beneficial discovery of *B. bassiana*'s thermal profile and the optimal humidity levels observed in this study, is that these temperature and humidity levels are typically found in homes where humans regulate their environmental conditions. Therefore, we can be reasonably confident that fungal sporulation will be optimal under the conditions found in most household environments (Booten et al. 2017; Rardin 2022). It is important to note that bed bugs in this study were provided with only a single brief exposure to *B. bassiana*, and yet, relatively high levels of mortality (+70%) were achieved. Therefore, we were able to conclude that application of the Aprehend product would be an effective bed bug control method in the field where bed bugs are likely to have a higher frequency of exposure. Given the results of the heat treatment and *B. bassiana* studies, I would recommend a future study a heat treatment is followed up with Aprehend treatments for the control of bed bugs.

The final, and most consistently effective method of bed bug elimination evaluated in this study, was fumigation using Vikane® gas fumigant (sulfuryl fluoride). Sulfuryl fluoride fumigation is commonly used to eliminate drywood termite infestations in homes throughout the southern and western states in the US. Vikane has also been used to control a variety of indoor pests, including the bed bug. While Vikane has existed for some time, bed bug fumigation with sulfuryl fluoride was not heavily investigated until recently. The original dosage factor required for bed bug control was 3.0X. However, in 2014, it was found that the 1.9X dosage factor was sufficient for elimination of bed bugs and their eggs (Phillips et al. 2014). While our study was specifically focused on the elimination of bed bugs in vehicles and containers filled (85%) with personal belongings, it should be noted that this study was also the first field evaluation to

determine sulfuryl fluoride's ability to eliminate bed bugs from hard-to-reach locations at the 1.9X dosage factor. We found that fumigation using sulfuryl fluoride eliminated all bed bug eggs, nymphs, and adults in both vehicles and tightly packed cargo trailers. The results of this study clearly indicate that fumigation is a single treatment solution for eliminating bed bugs from personal belongings, furniture, computer equipment, and many other items (such as toys) that cannot be treated with chemical insecticides. While fumigation can be a single treatment solution, it does not provide residual activity and preventative measures should be used to prevent reintroduction of bed bugs. Fumigation would also provide a unique solution for the growing problem of bed bugs in rideshare vehicles (e.g., Uber, Lyft, and taxis; Howerton 2020). Currently, fumigation is also the only solution for treating infested vehicles where 100% elimination can be guaranteed.

While some pest management companies may not want to get into the home fumigation market due to a lack of familiarity or for financial reasons, they could get into the fumigation market on a smaller scale as seen in this study. Pest management companies could perform both containerized fumigations and/or vehicular fumigations on their company property, thus reducing any liability potential that is typically associated with whole-home fumigation.

Overall, this evaluation study of alternative bed bug control methods indicated that these novel methods could be very effective for eliminating insecticide resistant populations. Heat treatments can be effective when the treatment is applied for a sufficient length of time, and when the technician makes numerous inspections and any necessary equipment modifications throughout the treatment. The Aprehend fungal product (*Beauveria bassiana*) was capable of killing over 50% of bed bugs that received a single exposure from crossing over a small, treated surface. *B. bassiana* was also found to kill bed bugs the most rapidly when held at 21°C (4.5 –

7.0 days) and lower humidity levels (30% and 50%), which are standard environmental conditions within a home. Sulfuryl fluoride fumigation at the 1.9X rate was able to eliminate 100% of the bed bugs hidden in motor vehicles as well as infested belongings and furniture placed inside of packed cargo trailers.

While fumigation was the only alternative method that provided 100% mortality in every replication, heat treatments and fungal spore applications were found to be very successful at producing significant reductions in all of the treated bed bug populations. While these methodologies certainly require more technician labor and focus, they do provide reasonable alternatives to pyrethroid applications, which are largely no longer effective for modern bed bug control. In addition, heat treatments and fungal spore applications can be supplemented with desiccant dusts to ensure maximum treatment efficacy. It is my hope that these novel product evaluations will aid pest management professionals in their continued search for alternative and supplemental methodologies for treating modern bed bug infestations.

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