ABSTRACT

SIERRAS, ANGELA JUVENTINA. Toward a Liquid Bait Against Bed Bugs: Evaluation of Ingestible Insecticides. (Under the direction of Dr. Coby Schal).

Bed bugs have become a challenging ubiquitous urban pest over the past two decades. As ectoparasites of humans, and obligatory blood feeders, bed bugs must ingest a blood meal to develop and molt to the next life stage and reproduce. Current bed bug control options have significant constraints and limitations. Using ingestible baits in pest management has proven to be an effective method of controlling several urban pest species. In the following studies, I investigated the concept and potential of an ingestible bait for bed bugs.

In the first study, four active ingredients were evaluated for their effectiveness at causing mortality in two different life stages of an insecticide-susceptible strain of bed bugs. Bed bugs were assayed using an artificial feeding system. Dose-response studies with abamectin, clothianidin, fipronil, indoxacarb and indoxacarb's bioactive metabolite decarbomethoxylated JW062 (DCJW) were evaluated. To demonstrate that active ingredients are more effective by ingestion than direct contact we conducted dose-response studies by topically applying fipronil and indoxacarb to bed bugs. Abamectin, clothianidin and fipronil were effective at causing bed bug mortality in both males and 1st instars, and their time-course of mortality was similar. Indoxacarb and DCJW were ineffective against both males and 1st instars by ingestion. Fipronil was also effective by topical application, but indoxacarb was ineffective. However, fipronil was much more effective by ingestion than topical application. We therefore concluded that abamectin, clothianidin and fipronil could be potential candidate active ingredients for incorporation into a bed bug bait and ingestion is a much more efficient route of insecticide delivery.

Boric acid has long been used as a residual application insecticide in the urban environment. It has successfully controlled many other urban pests. Boric acid is formulated as a dust and presumably abrades the cuticle, causing desiccation of the insect, as well as potentially being a stomach poison once ingested. The mode of action of boric acid is still not well understood. In our studies of bed bugs in infested homes, we observed that boric acid was used by residents in efforts to control bed bugs. In the second study, we evaluated boric acid for its efficacy at causing mortality by ingestion and residual contact. As in the first study, dose-response ingestion studies were conducted and boric acid was assayed for residual activity in petri dishes. Boric acid was effective by ingestion but not by residual application, even when its particle size was manipulated. Therefore, boric acid also has the potential to be incorporated into a liquid bait.

Insect growth disruptors (IGDs), also known as insect growth regulators (IGRs), are frequently used in combination with other insecticides in pest management programs. Juvenile hormone analogs (JHAs) are a subset of IGDs. JHAs disrupt the insect's ability to metamorphose and ultimately become a sexually mature adult. Two JHAs, hydroprene, which is currently labelled for bed bug control, and methoprene were evaluated for their ability to cause lethal and sublethal effects by ingestion. Both JHAs were effective at high concentrations at causing mortality and reducing oviposition. However, females were more susceptible than males and methoprene was more effective than hydroprene.

Toward a Liquid Bait Against Bed Bugs: Evaluation of Ingestible Insecticides

by
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DEDICATION

To my mother, Mary Pat Sierras, and to my wife, Jonas Shawn, for your endless encouragement, patience, support and love. To my grandparents, Mike and Barbara Orozco, I could not have done this without both of you. Grandpa, you were a true inspiration to me and I miss you every day, but I always carry you in my heart.

BIOGRAPHY

Angela Juventina Sierras was born and raised outside of Los Angeles, CA. She attended California State Polytechnic University-Pomona, before transferring to North Carolina State University where she earned her B.S in Zoology. Her passion for pest management inspired her desire to pursue her Ph.D. in Entomology.

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CHAPTER 1

Introduction

Bed bugs, *Cimex lectularius*, are one of the most challenging urban pests to eradicate. They were a common pest before WWII but were virtually eradicated with the development of the organochlorine insecticide DDT (Usinger 1966). In the late 1990s, bed bugs began to resurge around the world (Boase 2001, Gbakima et al. 2002, Doggett et al. 2004, Potter 2005, Hirao 2010), with infestation rates continually rising. In the past two decades bed bugs have become a ubiquitous pest, and by 2006, they were reported in all 50 states in the US (Gangloff-Kaufmann et al. 2006). The resurgence has been attributed to increased human travel, proliferation of thrift, used furniture, and mattress shops, the elimination of DDT and other organochlorine insecticide that were relatively effective in bed bug control, cross resistance between current and previously used insecticides, a general dearth of knowledge about bed bugs, and shortage of effective control options (Pinto et al. 2007).

Bed Bug General Biology

Bed bugs are reddish-brown in color and adults are about 4.5 mm, wingless, oval in shape and dorsoventrally flattened, allowing them to shelter in small and narrow wall voids, and cracks and crevices in and around furniture, which allows them to go undetected by humans (Usinger 1966, Evison et al. 2018). Often, they are misidentified as early instar cockroaches, carpet beetle larvae, fleas and ticks (Goddard 2009, Vaidyanathan and Feldlaufer 2013). Bed bugs are obligatory blood feeders and must coexist in relatively close proximity with their host. They are ectoparasites of humans, and although humans are the primary and preferred host, bed bugs successfully feed on other mammals, birds and rodents (Usinger 1966). Their bites are often misidentified as other blood feeding arthropods (Fallen and Gooderham 2011). After hatching, each successive instar must take a blood meal to grow, molt to the next life stage, metamorphose into an adult, and ultimately reproduce (Usinger 1966, Reinhardt and Siva-Jothy 2007, Evison et al. 2018). Reinhardt et al. (2009) showed that fed females attracted more male mating attempts than unfed females. To find a host, they depend on cues such as heat, carbon dioxide (CO_2) and host odors (Usinger 1966, Reinhardt and Siva-Jothy 2007, Suchy and Lewis 2011, DeVries et al. 2016, Evison et al. 2018). They are active at night, a behavior pattern that allows bed bugs to remain undetected by their diurnal host (Romero et al. 2010, Evison et al. 2018). Once they arrive at the host they use their piercing-sucking mouthparts to penetrate through the skin and begin to ingest blood. Unlike ticks, bed bugs do not remain attached to their host for an extended period. Feeding is relatively short, approximately 5 min, and after feeding they return to their harborage (Reis and Miller 2011, Evison et al. 2018).

Public Health Significance

In 2002, several US government agencies officially acknowledged bed bugs as a pest of significant health importance because bed bugs can cause an array of physical and mental health problems (Centers for Disease Control and Prevention 2010). While feeding bed bugs inject various salivary components to facilitate feeding. These components induce vasodilation and inhibit clotting (Francischetti et al. 2010) and can cause adverse skin reactions (Leverkus et al. 2006, Hwang et al. 2018). The severity of the reaction can range from a mild response to anaphylaxis (Thomas et al. 2004, Goddard 2009). Most of the reactions present as cutaneous reddish inflammations (urticaria) (Doggett et al. 2012, Hwang et al. 2018), but more systemic reactions can result in more extreme symptoms such as difficulty breathing (Minocha et al. 2017).

These bite sites can be extremely itchy and elicit frequent scratching which can lead to secondary infections (Thomas et al. 2004, Rossi and Jennings 2010, Hwang et al. 2018).

Physical manifestations of bed bug bites are not the only problem that sufferers may experience. Many individuals have reported suffering from psychological disorders that include, but not limited to, anxiety, depression, delusional parasitosis, paranoia and ostracism (Doggett et al. 2012, Goddard and de Shazo 2012, Susser et al. 2012, Ashcroft et al. 2015, Perron et al. 2018).

Whether bed bugs can vector disease has been debated for decades. Although their gut has been shown to be infected with over 45 pathogens, there has not been a single documented case of pathogen transmission from bed bugs to humans under natural conditions (Lai et al. 2016, Doggett 2018b). However, in recent laboratory studies bed bugs successfully transferred the pathogen causing Chagas disease, *Trypanosoma cruzi*, to mice (Salazar et al. 2015, Doggett 2018b) and the pathogen was able to persist in bed bugs after molting to the next life stage (Blakely et al. 2018, Doggett 2018b).

Economic Significance

Bed bugs establish infestations in any human inhabited environment, without regard to socioeconomic status (Kells 2006). All structures, including retail stores, hotels, movie theatres, public transportation, hospitals, schools and private residences are subject to bed bug establishment. Commercial businesses, especially retail stores and hotels, suffer large economic losses due to treatment costs, loss of sales, lawsuits and subsequent damage to their reputation (Potter et al. 2008, National Pest Management Association 2011, Penn et al. 2014, Liu and Pennington-Gray 2015, Doggett et al. 2018). In private residents can incur costs of anywhere from a few hundred to a few thousand dollars for treatments. Potter and Haynes (2014) found that the average cost of treating a single apartment was ~ $1,000. Overall, economic damages attributed to bed bugs have been extensive and continue to be a burden on society.

Interventions

Several treatment practices are currently deployed against bed bugs. These include non-chemical and chemical interventions. However, employing a combination of both in an integrated pest management (IPM) program is the most effective treatment strategy not only against bed bugs, but in pest management in general. Nonetheless, all options available for bed bug control have their limitations and constraints (Doggett and Feldlaufer 2018).

Mechanical practices include altering the environment to limit habitats and micro-habitats that are preferred by bed bugs. Since bed bugs hide and form aggregations behind baseboards, wall voids and within clutter (Usinger 1966), sealing these areas and eliminating clutter can reduce harborages. Using devices such as pitfall traps can help detect bed bugs and also to monitor for bed bugs after an intervention has been performed and assess its effectiveness (Wang et al. 2011b, Wilson 2018). Vacuuming can help remove bed bugs and their eggs (Kells 2018). Vacuuming has been shown to reduce populations of other indoor pests, including fleas (Rust and Dryden 1997) and cockroaches (Kaakeh and Bennett 1997). Most vacuum devices, however, cannot reach into areas where bed bugs aggregate (Potter 2006), so vacuuming can only dispose of eggs and bugs that are readily accessible (Kells 2018). Mattress and box-spring covers can be used to preserve the mattress and box-spring (Cooper 2007, Wilson 2018). Nevertheless, many people unnecessarily dispose of bed bug-infested furniture. While this can remove some live bugs and eggs from the residential environment, it can also pose significant problems by distributing bed bugs to new areas, including common hallways in apartment buildings and hotels, and even distant places, as people tend to pick up discarded furniture at dumpsters and road sides (Wang et al. 2011a, Kells 2018).

Using extreme temperatures has become a more common treatment method, despite its higher cost. Heat treatments have been found to be one of the most effective treatment options. Bed bugs and eggs are killed when exposed to temperatures above 48^oC for at least 2 hours (Pereira et al. 2009, Kells and Goblirsch 2011) and -16^oC (freezer temperature) kills 100% of adult females that are exposed for 1 hour (Benoit et al. 2009a). The cost of heat treating a home can be a burden for most people, especially in low-income communities (Eddy and Jones 2011). The use of high temperatures also requires extensive preparation and it can damage various household items, requiring careful removal of some items and insulation of others. Some bed bug hiding places, such as electronic equipment, need to be removed, treated separately, and then re-introduced after treatment. Heat treatments are often combined with applications of residual insecticides, meant to intercept bed bugs that are re-introduced after the heat treatment (Kells 2018).

There are few active ingredients labelled for the control of bed bugs. Pyrethroids are the primary class, but neonicotinoids have become more popular, as well as combination products containing both pyrethroids and neonicotinoids (Lee et al. 2018). Often, the insect growth regulator (IGR) hydroprene is combined with neuroactive insecticides to target nymphal stages of the bed bug. Juvenile hormone analogs (JHAs) can disrupt molting, reduce the frequency of reproductive adults, and disrupt oviposition and embryogenesis. However, studies have shown that at the current label rate (*S*)-hydroprene is ineffective when applied as a residual insecticide, and 10-fold the label rate is necessary to disrupt molting, fecundity and egg viability in bed bugs (Goodman et al. 2013). Studies with (*S*)-methoprene, which is not labelled for bed bug control, conveyed similar results (Naylor et al. 2008).

Dust formulations are also used for bed control. These can be either dusts containing organic insecticides, or inorganic dusts (Lee et al. 2018). Insecticide impregnated dusts are used but usually they must be combined with one or more other approaches to be effective (Benoit et al. 2009b, Aak et al. 2017). Also, bed bugs recently have been shown to spend less time and avoid areas where dusts have been applied (Agnew and Romero 2017). Inorganic dusts, such as diatomaceous earth and boric acid, have been highly effective at controlling other pests (Quarles 1992, Gore and Schal 2004, Durmus and Buyukguzel 2008, Naranjo et al. 2013). Factors such as humidity, dust particle size (Doggett et al. 2008), the absolute need to contact the dust and incorporate other components (Benoit et al. 2009b) can decrease the efficacy of diatomaceous earth against bed bugs and increase the amount of time it takes to cause mortality. However, the efficacy of boric acid on bed bugs has not been investigated.

Biological control agents have not been used to control bed bugs. Recently, however, a study by Barbarin et al. (2012) found that *Beauveria bassiana* has residual efficacy against bed bugs after 1 h of exposure, causing mortality and it can be horizontally transferred. *B. bassiana* is also efficacious against field collected bed bugs (Barbarin et al. 2017, Aak et al. 2018, Kells 2018). Under laboratory conditions, entomopathogenic bacteria can be effective against bed bugs, but higher temperature is required to increase its virulence (Pietri and Liang 2018). Nevertheless, no studies have evaluated these biological control agents in the field.

Insecticide Resistance and Environmental Health

Pyrethroid resistance is globally prevalent in populations of bed bugs (Romero et al. 2007, Lilly et al. 2009, Davies et al. 2012, Zhu et al. 2013, Romero 2018), and recently, neonicotinoid resistance has been documented (Romero and Anderson 2016). Several mechanisms confer resistance to insecticides. Metabolic resistance can involve elevated gene expression and activity of detoxifying enzymes, such as P450s, carboxylesterases and glutathione-*S*-transferases, target site mutations result in decreased sensitivity to insecticides, and cuticular thickening can reduce insecticide penetration through the cuticle (Zhu et al. 2010, Adelman et al. 2011, Booth et al. 2015, Benoit et al. 2016, Lilly et al. 2016, Romero 2018). Insecticide resistance often results in repeated applications of residual insecticides, which not only select for greater resistance, but also can cause adverse health and environmental effects (Jacobson et al. 2011). Multiple applications of combination products can expose people and pets to insecticide residues (Gangloff-Kaufmann et al. 2006, Doggett 2018a), and over-the-counter products that are available to consumers pose significant health risks, especially when users fail to follow the label and application instructions (Jones and Bryant 2012) . New management strategies for bed bugs must be explored to alleviate limitations and constraints of currently available treatment options.

Baits in Urban Pest Management

Baits have been effective against major urban pests such as cockroaches, ants, termites, and wasps, all with chewing mouthparts (Klotz et al. 1998, Su and Scheffrahn 1998, Nalyanya et al. 2001, Sackmann et al. 2001). Baits offer several major advantages over residual insecticides. Baits are formulated with attractants and phagostimulants which attract the insects to the bait and stimulate consumption (Müller et al. 2010). Bait formulations also provide protection against degradation in the environment, which increases their residual life. Baits usually provide secondary kill, which amplifies their effects as the active ingredient spreads among other members within the colony or aggregation (Silverman et al. 1991, Buczkowski et al. 2001). Also, like pharmaceuticals, the active ingredients in baits are more bioavailable, which significantly lowers the amount of insecticide needed to cause lethality. Baits can also be placed in desirable locations based on knowledge of pest behavior and locations of harborages, as well as lower non-target effects and risk of human exposure. Although baits often require that competing food sources be reduced, and bait applications can be time consuming, these interventions are much simpler than the preparation needed for residual sprays, heat treatments or fumigation.

Bed Bug Bait?

Arthropods with piercing-sucking mouthparts are managed in agricultural landscapes and veterinary medicine with a modification of a baiting concept. Plants are treated with a systemic insecticide that is transported and distributed in plant tissues, targeting pests that feed on various parts of the plant (Horowitz et al. 1998). Likewise, animals can be treated topically, injected or fed an active ingredient that enters their circulatory system. Fleas and ticks are frequently controlled using this technique (Shoop et al. 1995, Holmstrom et al. 2012). A recent review contends that this approach, sometimes referred to as “host intoxication”, or “xenointoxication”, may be viable for hematophagous vectors of human and animal pathogens in general, including mosquitoes and biting flies (Miglianico et al. 2018)

Recently, bed bugs were shown to be susceptible to humans and mice treated with ivermectin and moxidectin (Sheele et al. 2013, Sheele and Ridge 2016). Because bed bugs are obligate blood-feeders and they are known to feed on a variety of non-human hosts, such as chickens (Steelman et al. 2008) and bats (Booth et al. 2015), a live host bait is an appealing concept. Notably, in xenointoxication systems the plant or animal serves as the vehicle for the active ingredient. Therefore, it is not necessary to develop specific host attractants and phagostimulants, because the natural host effectively attracts the arthropod pest. However, whether a live host or artificial baiting system is used, it is imperative to investigate and identify active ingredient(s) that would be effective in controlling the target pest.

The overall goal of this dissertation is to explore a novel control strategy for bed bugs, using an artificial feeding system, and to identify active ingredients that would be effective at impacting bed bug populations through lethal and/or sublethal effects. The ultimate goal is to demonstrate a proof-of-concept that an ingestible bait can be highly effective against bed bugs and could be expanded to other hematophagous arthropods.

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Chapter 2

Comparison of ingestion and topical application of insecticides against the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae)

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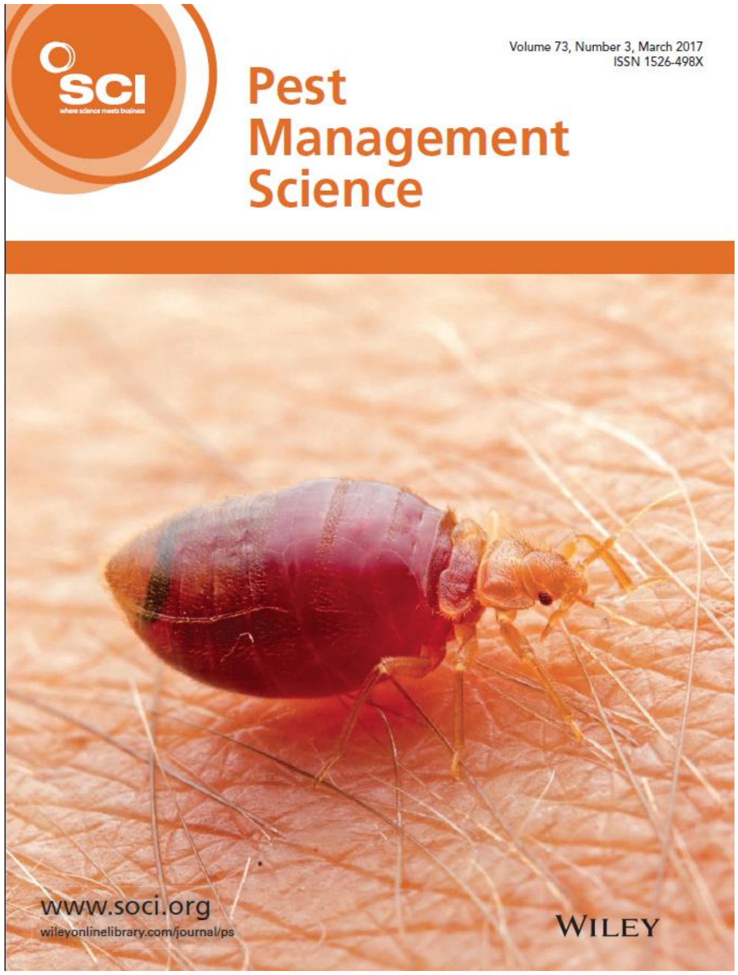

Cover of *Pest Management Science*, showing blood-fed bed bug nymph.

Photo credit: Matt Bertone

Abstract

BACKGROUND: The global prevalence of *Cimex lectularius* infestations has challenged current intervention efforts, as pyrethroid resistance has become ubiquitous, availability of labeled insecticides for bed bugs is limited, and non-chemical treatment options, such as heat, are often unaffordable. We evaluated representative insecticides toward the goal of developing a novel, ingestible liquid bait for hematophagous arthropods.

RESULTS: LC_{50} values were estimated for adult males and first instar nymphs of an insecticide-susceptible strain for abamectin, clothianidin, fipronil and indoxacarb, after ingestion from an *in vitro* feeder. LD_{50} values were calculated based on the ingested blood volume. Ingested abamectin, clothianidin and fipronil caused rapid mortality in both life stages. Fipronil was ~43-fold more effective by ingestion than by topical application. Indoxacarb and its bioactive metabolite decarbomethoxyllated JW062 (DCJW) were ineffective at causing bed bug mortality even at concentrations as high as 1000 ng mL^{-1} blood.

CONCLUSIONS: Fipronil, clothianidin and abamectin have potential for being incorporated into a liquid bait for bed bug control; indoxacarb and DCJW were not effective. Bed bugs are a good candidate for an ingestible liquid bait because systemic formulations generally require less active ingredient than residual sprays, they remain contained and more effectively target hematophagous arthropods.

Keywords: Bed bug; *Cimex lectularius*; mortality; systemic control; liquid bait

1. INTRODUCTION

The incidence and prevalence of bed bug infestations are on the rise worldwide. There does not appear to be a human environment that is unacceptable to bed bugs[1] and because *Cimex lectularius* accepts a variety of vertebrate hosts, bed bug reservoirs can be sustained in pet shops and confined animal production farms. Infestations can have significant economic impacts on households, the hospitality industry and poultry farms.[2] The public health impacts of bed bugs are arguably as important, not only because they are obligatory blood feeders with potential to vector pathogens,[3] but also because of the anxiety, sleeplessness and ostracism associated with infestations.[4] Bed bug saliva can cause skin irritation, ranging from mild annoyance to severe dermatitis, and immune responses can occur soon after feeding or days or even weeks later.[5] Itching may be so severe that chronic scratching often leads to secondary infection(s).[6]

Bed bug infestations are often controlled with insecticide sprays, heat treatments, fumigation and sometimes freezing. However, each of these approaches has significant constraints and short-comings. Pyrethroids are the most common class of insecticides labelled for bed bug control, but resistance to pyrethroids has become widespread globally.[7-10] Bioassays of field-collected populations confirm extensive resistance to pyrethroids,[11] but its pervasiveness is especially highlighted by molecular analyses showing that >80% and >95% of USA and European bed bug populations, respectively, carried one and/or both mutations in the voltage-gated sodium channel gene, conferring target-site based pyrethroid resistance.[11, 12] Multiple molecular mechanisms that underlie resistance to pyrethroids also include differential gene expression of carboxylesterases, P450s, glutathione-*S*-transferases and cuticular protein genes that contribute to a thicker cuticle.[9, 11, 13-16] Combinations of pyrethroids with other classes of insecticides (e.g., imidacloprid/β-cyfluthrin or acetamiprid/bifenthrin) also tend to be less effective due to pyrethroid resistance.[17] Overall, the pervasiveness of pyrethroid resistance, and lack of reliable alternative insecticides have resulted in repetitive applications by pest controllers, which ultimately results in large amounts of residual insecticides in the home environment.[18]

Baits have been successfully deployed against major groups of urban pests, including ants, cockroaches, termites and wasps.[19-21] Bait formulations have several noteworthy advantages. They are slower acting which allows time for foraging insects to return to their harborage and potentially deliver the active ingredient (AI) – often in a palatable excretion – to nestmates or aggregations,[22] providing secondary effects of the insecticide.[23, 24] Baits often include attractants and phagostimulants that facilitate intake, and the formulation tends to protect the AI from environmental factors (e.g., UV) giving it longer residual activity.[22] Baits can be formulated to be insect-specific and they can be placed in desired locations to more effectively target the pest. Finally, ingested insecticides (like pharmaceuticals) tend to be more bioavailable and hence more effective at a lower dose than other formulations. Because baits require greater concerted effort, including reduction of competing food sources, they are generally a preferred formulation in the heterogeneous built environment more than in vast agricultural fields where plants, often in monocultures, are the preferred food.

In plant protection, systemic insecticides are effectively used to control a variety of hemipteran species with piercing/sucking mouthparts.[25, 26] AIs are taken up by the plant, transported in phloem and are thus ingested in a liquid medium. An advantage of this system is that attractants and phagostimulants are not necessary because the host plant serves this function. A similar strategy has been exceptionally successful with vertebrate hosts in veterinary medicine, where systemic insecticides are used to protect pets and farm animals from ectoparasites. AIs such as afoxolaner,[27] members of the avermectin family,[28] and a combination of spinosad and milbemycin oxime[29] are ingested by dogs, cats and livestock to control blood feeding arthropods, such as fleas and ticks.

Bed bugs are known to feed on a variety of vertebrate hosts associated with humans and human-made structures.[3] For example, *C. lectularius* populations thrive in poultry farms[30, 31] and associate with bats in roosts within urban structures.[12] Their hemimetabolous development and the absolute requirement for a blood meal by both sexes and all mobile life stages[3] (unlike fleas, mosquitoes, and various flies including sand flies) make a baiting system targeting bed bugs particularly appealing. Whether artificial formulations or live hosts are considered as baits, it is essential to identify effective insecticides that would kill bed bugs at low concentrations and would be compatible with these prospective approaches.

We report the evaluation of four insecticides with different modes of action as potential AIs for incorporation into an artificial liquid baiting system or a live baiting system. None of the following insecticides are currently labeled for bed bug control. Abamectin is a member of the avermectin (macrocylic lactones) family which are naturally derived from the soil bacterium, *Streptomyces avermitilis.*[32] It has insecticidal, nematicidal and acaricidal properties, and it targets the γ-aminobutyric (GABA) receptors and glutamate gated chloride channels, resulting in paralysis.[33, 34] Clothianidin is a neonicotinoid and an agonist to the nicotinic acetylcholine receptors (nAChR).[35, 36] Fipronil is a phenylpyrazole that can act by contact or systemically and, like abamectin, affects the GABA receptors and chloride channels in the central and peripheral nervous system.[33] However, unlike abamectin, fipronil causes hyperexcitation of the nerves.[37] Indoxacarb is an oxadiazine and is unique in that it must be bioactivated within the insect and blocks sodium channels,[38] consequently pseudoparalysis occurs and neural activity ceases.[39]

All four of these insecticides are active on insects with piercing/sucking mouthparts. Fipronil and indoxacarb are used in topical treatments for dogs and cats. Fipronil has been shown to cause mortality in fleas that feed on treated mice[40] and indoxacarb not only causes mortality in adult fleas, but also interrupts the development of flea eggs and larvae upon contact with treated cats.[41] Clothianidin has systemic activity in crop protection against many species with piercing/sucking mouthparts.[42] Lastly, the avermectin class is widely known for its systemic activity. Ivermectin kills mosquitoes and causes sublethal effects such as lower fecundity of females that fed on treated cattle.[43] A recent study showed that ivermectin also caused mortality in bed bugs fed on treated mice and humans.[44]

2. MATERIALS AND METHODS

2.1. Bed bugs and rearing procedures

Cimex lectularius colonies of the Harold Harlan strain (HH; also known as Fort Dix, collected in 1973), which is known to be susceptible to pyrethroid insecticides, were maintained in an incubator at 27°C, ~50% RH, and LD 12:12, and all experimental insects were maintained in the same conditions. Colonies were fed defibrinated rabbit blood in an artificial feeding system, as used in Romero and Schal (Fig. 1A).[45] Briefly, custom-fabricated water-jacketed glass condensers (Prism Research Glass, Raleigh, NC) served as feeders. An internal reservoir (blood chamber) was surrounded by an outer reservoir (circulating water chamber) connected to a thermal circulator that circulated heated water through the condenser to maintain blood near human body temperature (38.4°C). The reservoir held up to ~4 mL of blood, but 2 mL were sufficient to eliminate air from the feeder. Nescofilm® (Alfresa Pharma Corporation, Osaka, Japan) was stretched across the bottom of the feeder and blood was introduced through an opening at the top. Feeders were connected in series so several colonies could be fed concurrently. Bed bugs were housed in containers or vials with paper inserts for harborage and plankton netting (BioQuip Products, Rancho Dominguez, CA) on the top through which all life stages could feed.

All bed bugs were starved for 7–10 days prior to feeding or topical application assays. We used adult males because they were more readily obtained and their physiological state is relatively independent of reproductive cycles. In comparison, we also tested first instar nymphs because they are much smaller and therefore take a much smaller blood meal.

2.2. Measurement of blood meal volume

Starved adult male bed bugs (5 males per replicate, 5 replicates, total *n*=25) and first instar nymphs (10 per replicate, 5 replicates, total *n*=50) were placed into 4 mL glass vials and weighed prior to feeding. They were allowed to feed for 10 min on defibrinated rabbit blood. Only fully fed individuals (determined visually) (Fig. 1D) were then reweighed and the mass gain per individual was adjusted by the number of individuals that fed. We converted mass to blood volume without making any corrections for the specific gravity of blood. However, assuming a specific gravity of blood of 1.0506 at 37ºC,[46] our volumetric estimates may be inflated by ~5%.

2.3. *In vitro* feeding assays

2.3.1. Dimethyl sulfoxide (DMSO) dose response

DMSO was used as the vehicle for all insecticides. Various amounts of DMSO were added to defibrinated rabbit blood to obtain final concentrations of DMSO of 0% (blood only), 0.25%, 0.5%, 0.75% and 1%. Only adult males were used in this assay. We used 3 replicates of 10 males for each concentration of DMSO. Each replicate was placed in a 4 mL glass vial with a strip of Manila folder (clinging substrate) that reached the top of the vial to facilitate feeding.

Each group was allowed to feed for 30 min. After the first 15 min a glass Pasteur pipette was inserted into the internal reservoir and the blood was agitated. After 30 min of feeding, only fully engorged bed bugs were separated and monitored daily for mortality for 7 days.

2.3.2. Insecticide in vitro feeding bioassays

Technical grade abamectin (98-99%, Whitmire [now BASF], Research Triangle Park, NC), fipronil (88.7%, Aventis [now Bayer], Research Triangle Park, NC), clothianidin (99.5%, Chem Service Inc., West Chester, PA), indoxacarb (98.5%, Chem Service Inc., West Chester, PA) and DCJW (active form of indoxacarb, 98%, DuPont, Wilmington, DE) were dissolved in DMSO to make stock solutions of 10 mg AI mL^{-1} DMSO and then serially diluted in DMSO to obtain the desired concentrations.

For each insecticide concentration we used 28–30 adult males and 24–30 first instar nymphs per treatment group, with two control groups (defibrinated rabbit blood and defibrinated rabbit blood plus 0.75% DMSO) and 6 treatment groups for each life stage tested. All feedings were made in the scotophase, 2–8 hrs after lights-off.

2.4. Topical application assays

Fipronil and indoxacarb were dissolved in acetone to make a stock solution of 20 mg AI mL^{-1} and then diluted in acetone. For each insecticide we used 30 adult males per dose (solvent control group and 5 treatment groups; n=180). Each replicate of 10 males was placed in a 4 mL vial, immobilized on ice ~10–15 min, bed bugs were placed ventral side up onto a filter paper (Whatman #1, 55 mm) in a petri dish (Falcon®, 60x15 mm), and 0.5 µL of acetone or one of the pesticide doses in acetone was applied onto the thorax, between the coxae, using a Hamilton syringe held in a manual micro-applicator (Hamilton Co., Reno, NV). The acetone was allowed to evaporate and the Petri dish lid was then taped in place to prevent the bugs from escaping. All applications were made in the scotophase, 2–8 hrs after lights-off. Individuals were monitored and mortality was recorded daily for 5 days.

2.5. Statistical Analysis

Probit analysis was used to determine the concentration required to kill 50% of test insects (LC_{50}) of each insecticide on days 1–7 using PoloPlus© (LeOra Software Company, Petaluma CA). This software was also used to estimate the dose required to kill 50% of test insects (LD_{50}) values from topical application assays.

3. RESULTS

3.1. Ingested blood volume by adult males and first instars

All adult bed bugs fed to repletion (Table 1), and each adult male consumed a mean of 3.92 ± 0.21 µL (SE, *n*=25) of defibrinated rabbit blood (without DMSO). The blood meal represented 147.4% of the unfed body mass of an average adult male and resulted in ~2.5-fold greater body mass of fed males. Fully engorged first instar nymphs (47 of 50) consumed 0.46 ± 0.04 µL (*n*=47) of blood, representing 219% of their unfed body mass, or a ~3.2-fold greater body mass after feeding. Thus, there was nearly a tenfold difference in the blood meal volume ingested by first instars and adult males, coincident with a tenfold difference in their unfed body mass (Table 1). Therefore, on a mass AI per body mass basis, the dose-response curves of insecticides are expected to be similar for these two life stages.

3.2. *In vitro* feeding assays

3.2.1. Effect of DMSO on bed bug mortality

A dose-response study was conducted to determine the concentration of DMSO in defibrinated rabbit blood that could be used in subsequent dose-response studies with insecticides. Most of the bed bugs fully engorged even on the highest concentration of DMSO (Fig. 2). However, there was a dramatic increase in mortality as DMSO concentration increased >0.75%. Thus, 0.75% DMSO in defibrinated rabbit blood was deemed suitable because it caused only 10% mortality 7 days post ingestion (n=30 bed bugs), which was the same as the blood-only control.

3.2.2. In vitro *feeding assays with insecticides*

The results of the *in vitro* feeding bioassays are shown in Table 2 and Fig. 3. Mortality was recorded for 7 days (supplemental information, Figs. S1-S4) but only results for day 3 are shown. Generally, the HH bed bugs readily accepted and ingested the insecticides offered in rabbit blood, even at their highest concentrations (Tables S1-S4). The order of toxicity of the four insecticides against fully engorged bed bugs was determined to be fipronil > clothianidin > abamectin >>> indoxacarb for both adult males and first instars based on results of probit analysis. We also estimated the LD_{50} ingested by adult males and first instars (LC_{50} x blood volume ingested); these LD_{50} values may be compared to the results of topical applications.

Fipronil, clothianidin and abamectin were highly effective at causing 100% mortality in both adult males and first instars by day 3 (Fig. 3, Table 2). HH strain bed bugs accepted fipronil, with 85–100% fully engorging (Table S3). Fipronil was most effective with an LC_{50} value of 13.4 ng mL^{-1} blood and 6.84 ng mL^{-1} blood for adult males and first instars, respectively, corresponding to LD_{50} values of only 52 pg $male^{-1}$ (3 pg $nymph^{-1}$) and 8 pg mg^{-1} fed male body mass (0.4 pg mg^{-1} fed nymph body mass); fipronil caused 100% mortality at 100 ng mL^{-1} blood in both adult males and first instars.

Both adult males and first instar nymphs readily accepted clothianidin, with 97-100% fully engorging at all concentrations (Table S2). A concentration of 1000 ng mL^{-1}

blood clothianidin caused 100% mortality in adult males and first instars by day 4 (Fig. S2). The LC_{50} values for adult males and first instars 3 days after ingestion of clothianidin were similar to fipronil (Table 2). There were no appreciable changes in mortality between days 3 and 7 for both males and first instars (Fig. S2).

Abamectin was readily ingested by males and nymphs, with 93-100% of bed bugs fully engorging even on the highest concentrations of abamectin (Table S1). Abamectin caused 100% mortality at 100 ng mL^{-1} blood in both adult males and first instars, with LC_{50} values ~2.5-fold higher than for fipronil and clothianidin for adult males. LC_{50} values for first instars were similar for clothianidin and ~3-fold higher compared to fipronil (Table 2). Like clothianidin, abamectin mortality increased only slightly between days 3 and 7 (Fig. S1).

Indoxacarb was variably ingested by the HH bed bugs. All first instar nymphs fully engorged even on 1000 ng indoxacarb mL^{-1} blood, but 67–93% of adult males fully engorged on 25–1000 ng indoxacarb mL^{-1} blood (Table S4). Surprisingly, ingested indoxacarb was ineffective, with only <20% mortality at the highest concentration (1000 ng mL^{-1} blood; ~4 ng $male^{-1}$ or ~0.5 ng $nymph^{-1}$) on day 3 (Fig. 3) and <90% mortality by day 7 (Fig. S4). Probit analysis could not be performed with indoxacarb, but adult males were more susceptible to it than first instars. The active metabolite of indoxacarb, DCJW, was slightly more effective, causing 20% and 30% mortality by day 3 at concentrations of 100 and 1000 ng mL^{-1} blood, respectively (Fig. 3).

For clothianidin, the LC_{50} values were not significantly different for adult males and first instar nymphs, based on overlap of the 95% confidence intervals of both life stages. Using the same criterion, first instars were significantly more sensitive to fipronil and abamectin than adult males (Table 2). However, on a per mg body mass basis the clothianidin LD_{50} values were similar for males and first instars, 19 and 15 pg mg^{-1}, respectively.

3.3. Topical Applications

Mortality of HH strain males was monitored for 5 days (Fig. S5), but only day 3 results are shown in Table 3 for each insecticide tested. Fipronil again was highly effective at causing bed bug mortality 3 days after topical application; the LD_{50} was 2.21 ng $male^{-1}$ (Table 3, Fig. 4) compared to 0.052 ng $male^{-1}$ by ingestion (Table 2).

Indoxacarb caused some bed bug mortality 3 or 7 days after ingestion (Table 2, Fig. S4) or 5 days after topical application (Fig. S5), but only at extremely high doses of 3.9 ng $insect^{-1}$ and 10,000 ng $insect^{-1}$, respectively. Doses as high as 10 µg indoxacarb killed only <60% of the HH bed bugs 5 days after topical application.

4. DISCUSSION AND CONCLUSIONS

4.1. Ingestible insecticides for bed bug control

Overall, three of the four insecticides we assayed by ingestion – abamectin, clothianidin and fipronil – proved to be highly effective at causing bed bug mortality. Patterns of mortality were similar for adult males and first instar nymphs of the Harold Harlan population, an insecticide-susceptible strain that has been maintained in laboratory culture since 1973. On the other hand, indoxacarb and DCJW were surprisingly ineffective at causing bed bug mortality by ingestion, and topical applications confirmed that this insecticide is ineffective against bed bugs. The same lot of indoxacarb was tested on the German cockroach, *Blattella germanica*, and it was found to be effective,[47] indicating that the differential efficacy of indoxacarb is related to its species-specific processing. It is unlikely that reduced cuticular penetration of indoxacarb was involved because it was ineffective by ingestion. It is also unlikely that indoxacarb was rapidly catabolized because it is a pro-insecticide and its major metabolite, DCJW, was also ineffective. Although indoxacarb is particularly effective on lepidopteran larvae, it is much less potent, especially on a per mg body mass basis, on piercing sucking insects, beetles and even cockroaches.[38] Notably, while indoxacarb was active on *Aedes aegypti* mosquito larvae, it failed to kill adult female mosquitoes.[48] Likewise, indoxacarb was ineffective by topical application on first instars of the kissing bug *Triatoma infestans*,[49] suggesting that it may be generally ineffective on hematophagous insects. Nevertheless, topical spot-on applications of indoxacarb were shown to be highly effective against cat fleas on cats.[50] We considered the possibility that bed bugs lack the enzymes – presumably esterases – necessary to cleave indoxacarb to its N-decarbomethoxyllated metabolite which is the active blocker of sodium-dependent action potentials. However, DCJW, the active metabolite, was only slightly more effective by ingestion than indoxacarb. Clearly, further research is needed to understand why indoxacarb was ineffective on bed bugs and whether its activity can be synergized in an ingestible formulation.

Oral administration of fipronil was much more effective than topical application, as expected. Based on calculations of LD_{50} values, *C. lectularius* males were ~43-fold more sensitive to fipronil by ingestion than by topical application, with remarkably high toxicity of 8 pg per mg fed body mass by ingestion. The LC_{50} value for fipronil on bed bugs was 10-fold lower than for afoxolaner on fleas; monthly treatments of dogs with oral dosages of 2.5 mg kg^{-1} afoxolaner maintains a plasma concentration >100 ng mL^{-1}, and results in 100% efficacy on fleas.[27] These results support the notion that effective AIs can be combined with phagostimulants[45] in a liquid bait that would use much less AI than is often used in residual applications of insecticides.

Overall, our results with an *in vitro* feeding system provide support for the concept that liquid baits should be developed as a novel and valuable insecticide delivery system against bed bugs and other hematophagous arthropods. Our experiments only evaluated the responses of adult males and first instar nymphs. Future studies should investigate adult females and sub-lethal effects on fecundity, egg viability, egg hatch and the ability of bed bugs to refeed after sublethal exposure to insecticides. Also, recently collected field populations should be examined to evaluate their susceptibility to these insecticides via ingestion and topical application. The HH strain bed bugs readily accepted and ingested even the highest concentrations of these insecticides, but the palatability of these and other AIs should be tested on recently collected bed bugs. Moreover, more insecticides representing these and other classes with different modes of action should be evaluated to assess their potential inclusion in a liquid baiting system. Finally, this research addresses only one component, the AI dissolved in blood, as a proof-of-principle for prospective fully synthetic liquid baiting formulations. Before this concept can be implemented, attractants (including heat), phagostimulants and other formulation ingredients will need to be explored.

5. SUMMARY

The goal of the study was to screen several common AIs with different modes of action for their potential inclusion in a liquid baiting system. Moreover, we sought to compare two modes of delivery of insecticides, ingestion and contact. A baiting system could serve as a safer alternative to residual insecticides because less AI would be used in containerized formulations placed in desired locations. Three AIs, abamectin, clothianidin and fipronil, were highly effective by ingestion. However, indoxacarb and DCJW were ineffective by either ingestion or topical application. The concept of an artificial liquid or live baiting system has high appeal, and these results highlight its potential as well.

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Table 1. Relationship between *in vitro* blood meal volume and body mass of unfed adult males and first instars.

Life Stage	**Number of bugs (*n*)**	**Number of bugs fully fed (%)**	**Unfed body mass (mg) mean ± SE**	**Blood meal size (µL) mean ± SE**	**Increase in body mass (%)**
Adult males	25	25 (100)	2.66 ± 0.33	3.92 ± 0.21	147%
First instars	50	47 (94)	0.21 ± 0.04	0.46 ± 0.04	219%

Table 2. *In vitro* feeding bioassay results 3 days after ingestion of four different insecticides by bed bugs of the Harold Harlan strain.

Active ingredient	Life stage	*n*	LC_{50} Slope (± SE)	LC_{50} (95% CI)[a] (ng mL^{-1} blood)	LD_{50} [b] (ng $insect^{-1}$)	LD_{50} [c] (ng mg^{-1} fed body mass)
Abamectin	Adult males	240	7.79 (1.66)	32.59 (27.14–37.88)[d]	0.127	0.019
	First instars	229	3.96 (0.61)	22.10 (17.87–26.87)[d]	0.010	0.015
Clothianidin	Adult males	238	2.64 (0.51)	14.19 (9.12–18.91)	0.056	0.008
	First instars	228	1.59 (0.24)	20.66 (4.40–49.75)	0.010	0.015
Fipronil	Adult males	240	2.45 (0.43)	13.37 (9.51–21.29)[d]	0.052	0.008
	First instars	234	7.16 (1.35)	6.84 (5.89–7.99)[d]	0.003	0.004
Indoxacarb	Adult males	216	-	-		
	First instars	232	-	-		

[a] LC_{50} values and 95% confidence intervals. Values are in ng AI mL^{-1} defibrinated rabbit blood.

[b] LD_{50} values are in ng AI $insect^{-1}$ and were calculated based on the observation that each adult male bed bug consumed 3.9 µL blood and each first instar ingested 0.46 µL blood (LC_{50} X blood volume consumed).

[c] LD_{50} values are in ng AI per mg fed body mass and were determined based on a fed body mass value of 6.58 mg for each adult male bed bug and 0.67 mg for each first instar (LD_{50} X fed body mass).

[d] Significant differences between the LC_{50} values of males and nymphs due to non-overlap of 95% CI for the two life stages tested.

Table 3. Topical application bioassay results, 3 days after application, with two different insecticides tested against adult males of the Harold Harlan population.

Technical Insecticide	***n***	**Slope (±SE)**	**LD_{50}[a] (95% CI)**
Fipronil	180	3.89 (0.93)	2.21 (1.54–2.87)
Indoxacarb	180	-	-

[a] LD_{50} values (ng technical insecticide per male) were derived from Probit analysis on day 3.

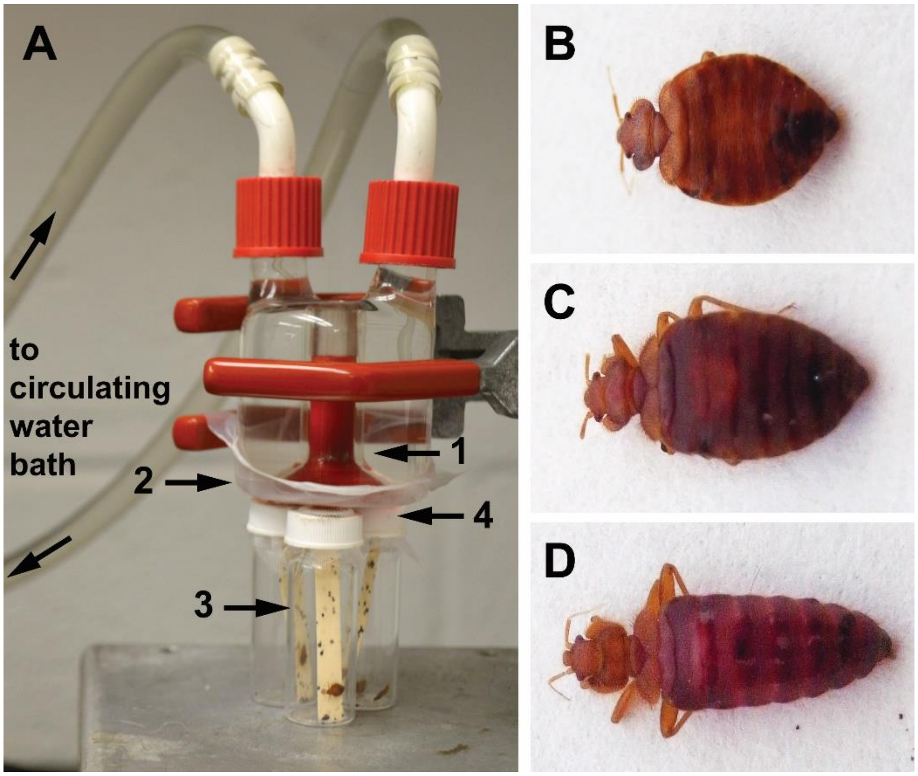

Figure 1. (A) Apparatus used to feed bed bugs insecticide-treated defibrinated rabbit blood. Blood was placed into the internal reservoir of a custom-fabricated glass condenser (1) heated with a circulating water bath and held in place by a ductile, malleable, thermoplastic paraffin film (2). Bed bugs were placed in glass vials containing Manila paper (3) and capped with plankton screen (4) through which they could insert their mouthparts. (B–D) Unfed (B), partially fed (C) and fully fed (D) adult male bed bugs. Only fully fed individuals were included in our assays.

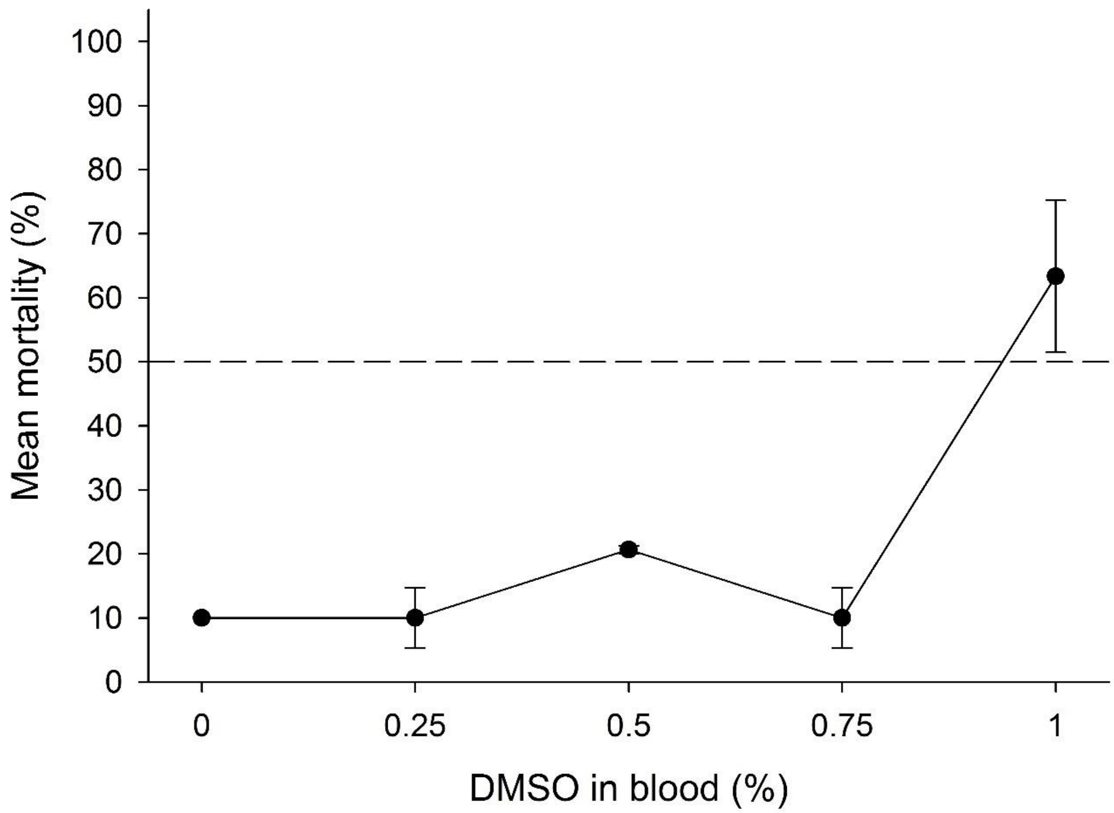

Figure 2. Dose-response for DMSO in defibrinated rabbit blood, showing adult male bed bug mortality 7 days after ingestion. Each mean (± SE) represents 3 replicates of 10 bed bugs each.

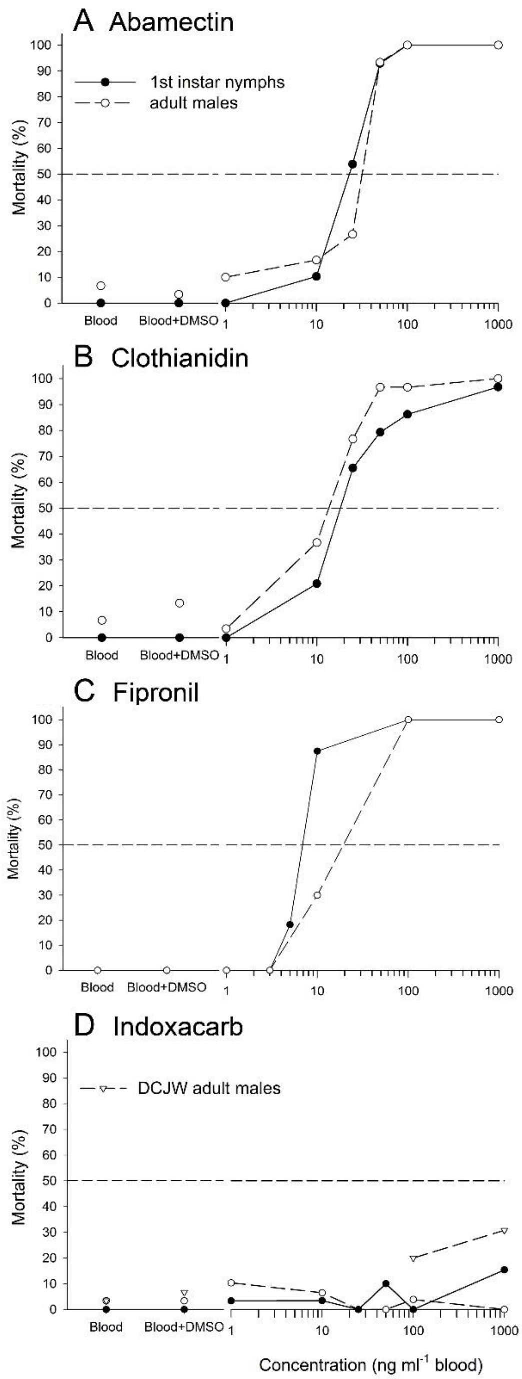

Figure 3. Mortality dose-response curves for abamectin, clothianidin, fipronil and indoxacarb, three days post ingestion, for both first instar nymphs (solid line) and adult males (dashed line). Each insecticide was dissolved in DMSO and mixed with defibrinated rabbit blood to a final concentration of 0.75% DMSO.

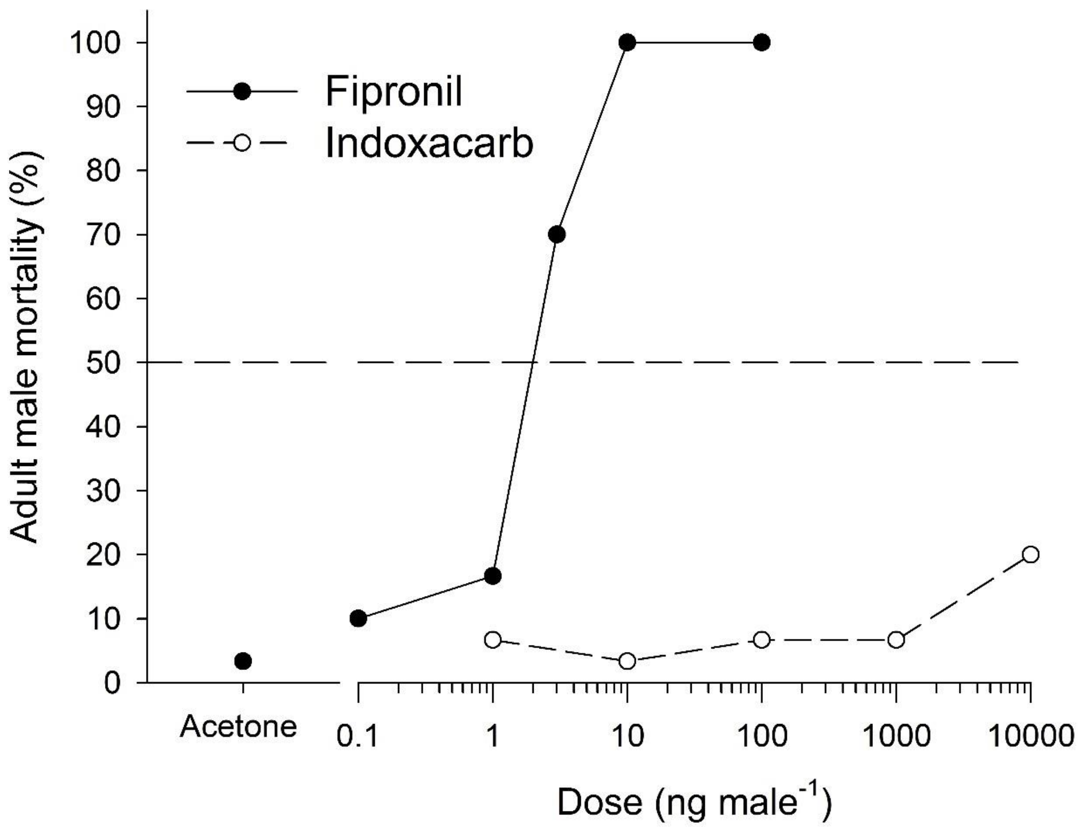

Figure 4. Dose-response mortality curves for topical applications of fipronil and indoxacarb to adult male bed bugs, 3 days post application.

Chapter 3

Effectiveness of boric acid by ingestion, but not by contact, against the common bed bug (Hemiptera: Cimicidae)

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Abstract

Boric acid has been used as an insecticide in the successful control of agricultural, public health and urban pests long before the advent of synthetic organic pesticides. Boric acid products, formulated as dusts, sprays, granular baits, pastes, gels and liquids, are widely available to consumers and pest management professionals, especially to control pest infestations within homes. Boric acid dust is commonly used against bed bugs (*Cimex lectularius* L.), but its efficacy has not been demonstrated. We evaluated the efficacy of boric acid as an ingestible and residual contact insecticide on bed bugs, and compared its efficacy on the German cockroach (*Blattella germanica* L.) which is known to be susceptible to boric acid by both routes. Dose-response studies of 0–5% boric acid in blood demonstrated that ingested boric acid caused rapid mortality at concentrations ≥2%, and even 0.5% and 1% boric acid caused 100% mortality, albeit at a slower time-course. In contrast, bed bugs survived contact with high concentrations of boric acid dust. Smaller boric acid particles did not increase mortality of either unfed or recently fed bed bugs. The same boric acid products were effective at causing mortality of German cockroaches by both contact and ingestion. We thus conclude that while boric acid is an excellent candidate active ingredient for an ingestible bait formulation, residual applications of dust or spray would be ineffective in bed bug interventions.

Keywords: chemical control, IPM-Urban, Public Health Entomology, Urban and Structural Entomology, bed bug

Introduction

Bed bug infestations continue to increase worldwide, and in the United States all 50 states are plagued with their presence. Bed bugs exert a range of health risks in infested homes, hotels or other indoor settings. Although they have not been shown to vector any diseases to humans, in a laboratory setting bed bugs are able to successfully transfer the pathogen in Chagas disease, *Trypanosoma cruzi,* to mice (Salazar et al. 2015) and *T. cruzi* persists across bed bug molts (Blakely et al. 2018). Bed bug bites can also cause severe dermatologic reactions in some people, and excessive scratching can lead to secondary infections (Rossi and Jennings 2010). A recent study showed that bed bug feces contains large amounts of histamine, which accumulates in household dust and has the potential to elicit adverse respiratory and dermatological responses (DeVries et al. 2018). Other adverse effects include increased anxiety, depression (Susser et al. 2012) and avoiding public places for fear of bringing bed bugs home (Goddard and de Shazo 2012). Residents in infested homes often over-use and misuse “do-it-yourself” insecticides in efforts to eradicate bed bug infestations (Jacobson et al. 2011).

Current interventions to eliminate bed bug infestations include the application of residual insecticides as sprays or dusts, spatial heat treatments, and targeted steam or freezing treatments to areas such as the seams of mattresses. All these approaches have significant constraints and shortcomings. Residual insecticides have become an increasing concern due to the rapid evolution of resistance to pyrethroids (Davies et al. 2012) and neonicotinoids (Romero and Anderson 2016), the major classes of insecticides labeled for use against bed bugs. Bed bugs have evolved diverse mechanisms of resistance, including target site mutations that reduce sensitivity to insecticides (Zhu et al. 2010, Booth et al. 2015), expansions of detoxifying enzyme families high expression of which supports resistance (Adelman et al. 2011, Benoit et al. 2016), and cuticular thickening, which is a general resistance mechanism to a wide range of residual, contact insecticides (Koganemaru et al. 2013, Benoit et al. 2016, Lilly et al. 2016)}. Resistance can trigger recurrent applications of marginally effective insecticides, ultimately resulting in greater human exposure to insecticide residues (Gangloff-Kaufmann et al. 2006). Although heat treatments are often successful (Pereira et al. 2009), the treatment protocol requires complex, specialized and expensive equipment, and there is potential of damage to belongings due to the high temperatures required to eradicate bed bugs (Kells and Goblirsch 2011).

Desiccant dust formulations, such as diatomaceous earth and silica gel, have been incorporated into bed bug interventions. However, application of these products has practical constraints, as does their efficacy. Bed bugs also exhibit behavioral avoidance of repellent dusts, reducing direct contact which is required for efficacy of diatomaceous earth, dinotefuran and silica gel (Agnew and Romero 2017). In most cases, the dust alone is not sufficient to control infestations. Studies have shown that in combination with CO_2 (Aak et al. 2017), alarm pheromone (Benoit et al. 2009), or when formulated as an aerosol spray (Akhtar and Isman 2016), the efficacy of silica and/or diatomaceous earth dusts can be substantially improved.

Boric acid has been deployed successfully for controlling a wide range of pests in agricultural, structural, and public health settings (Durmus and Buyukguzel 2008, Bicho et al. 2015, Lachance et al. 2017). In combination with a phagostimulant, often in a simple water-based formulation, boric acid was shown to be effective at reducing populations of ants, cockroaches and mosquitoes (Klotz et al. 1998, Xue and Barnard 2003, Gore and Schal 2004, Gore et al. 2004, Naranjo et al. 2013, Wang et al. 2017). Boric acid is also commonly used in more complex gel formulations against cockroaches, and even as a feeding deterrent when used at high concentrations to protect wood against termites (Tsunoda 2001).

The mode of action of boric acid is still equivocal, but presumably includes destruction of the lining of the foregut (Cochran 1995) and neurotoxicity (Habes et al. 2006). Early studies also demonstrated that boric acid caused mortality in German cockroaches (*Blattella germanica*), even when their mouthparts were sealed, suggesting that it penetrated the cuticle (Ebeling et al. 1975). Major advantages of boric acid as component for a yet to be developed ingestible bed bug bait would be its low toxicity to humans and pets compared to other insecticides (Weir and Fisher 1972, Murray 1995, 1998), low cost, and high solubility in water. Moreover, despite a century of use, there is no evidence of insect resistance to boric acid (Cox 2004), possibly related to its inorganic properties.

Previously, we performed a proof of concept study showing that several neuroactive insecticides were highly effective when ingested by bed bugs in blood using an artificial feeding system (Sierras and Schal 2017). The lethal dose by ingestion was about 45-fold lower than the dose required in topical applications. Similar studies with ivermectin and moxidectin ingestion by humans and mice also showed mortality of bed bugs that ingested the treated blood (Sheele et al. 2013, Sheele and Ridge 2016).

Boric acid is commonly used in do-it-yourself control of household pests, including in bed bug infested homes (Moore and Miller 2009). We are not aware, however, of any formal assessment to determine if boric acid is effective for controlling bed bugs. Our objective was to evaluate the efficacy of boric acid against bed bugs by ingestion and residual application, for its possible incorporation into a bed bug bait and integrated pest management program. We demonstrated that whereas boric acid was highly efficacious on bed bugs by ingestion, it had marginal efficacy as a dust in residual applications. We used the German cockroach in a comparative study to show that the same boric acid was effective on *B. germanica* by both routes of delivery.

Materials and Methods

Insects

Colonies of *Cimex lectularius* (Harold Harlan [HH] = Fort Dix strain, collected in 1973; insecticide susceptible) and *Blattella germanica* (Orlando Normal = American Cyanamid strain, collected >70 years ago in Florida; insecticide susceptible) were maintained in separate incubators at 27^{o}C, ~50% RH and on a 12:12 L:D regime. Only adult males were used in all experiments because their physiological state is less variable and less dependent on reproduction status than females. Moreover, our previous investigation with ingestible insecticides showed no major differences in their toxicity on first instar nymphs and adult males (Sierras and Schal 2017). All experimental insects were held in the same conditions. Bed bug colonies were fed defibrinated rabbit blood using the artificial feeding system described in Sierras and Schal (2017). German cockroaches were fed rodent chow (Purina No. 5001 Rodent Diet, PMI Nutrition International, Brentwood, MO) and given water as described in Wada-Katsumata et al. (2013).

Experiment 1: Feeding assays with bed bugs

Technical grade boric acid (≥ 99% purity, Fisher Scientific, Fairlawn, NJ) was dissolved in rabbit blood to 0, 0.5, 1, 2, 3, 4 and 5% (wt/vol). A 1% boric acid solution is 0.16 M. Ten adult male bed bugs were collected randomly from the colony and placed in a 4-mL glass vial with a plankton screen top (BioQuip Products, Rancho Dominguez, CA) through which they could feed. A paper substrate (strip of manila folder) in the vial reached the screen, so bed bugs could reach the feeding membrane. Each group of 10 bugs was given 30 min to feed, but after 15 min a glass Pasteur pipette was used to remix the blood-boric acid solution, as in Sierras and Schal (2017). Only fully fed (engorged) males, determined visually as detailed in Sierras and Schal (2017) (see **Fig. 1A–C**), were kept and mortality was monitored daily for 7 d. Three replicate vials (N = 30 males) were used per treatment.

Experiment 2: Residual contact assays with bed bugs

Boric acid (BORiD, Waterbury Companies, Waterbury, CT) was weighed (Sartorius 1712 MP8 Silver Edition, Sartorius Lab Instruments, Goettingen, Germany) and distributed into 60 x 15 mm Petri dishes (Falcon, Corning, NY, USA). To determine whether (a) higher concentrations of boric acid are more effective, and (b) fed bed bugs are more susceptible to boric acid dust because their intersegmental membranes are more exposed after they feed, we exposed bed bugs to 13.8 mg of boric acid per dish, representing the label rate (0.48 mg/cm^2) or 138 mg per dish representing 10 times the label rate; controls (no boric acid) were also included. Bed bugs placed in the assays were either fully fed or starved 7–10 d. Each replicate consisted of 9–10 adult males per dish and a total of 118 starved and 120 fully fed adult male bed bugs were used. Bed bugs remained in their treatment dishes for 24 hrs and then bed bugs from each replicate dish were placed into a clean Petri dish. Mortality was monitored daily for 14 d.

We also hypothesized that boric acid particle size might affect mortality by influencing the interaction with intersegmental membranes and spiracles. To reduce the particle size, ~0.35 g of boric acid (BORiD) was placed into 2 ml tubes (FastPrep, MP Biomedicals, Santa Ana, CA) with 1.44 mm ceramic beads (MP Biomedicals) and ground in a reciprocating homogenizer (FastPrep24 5G, MP Biomedicals) with four 30 sec cycles (total of 2 min). Bed bugs were assayed on the ground boric acid as described above, but only at the label rate (0.48 mg/cm^2), and mortality was monitored daily.

We quantified the size of boric acid particles to confirm that grinding reduced their size distribution. Boric acid particles were scattered on conductive carbon tape on a scanning electron microscopy (SEM) stub, coated with gold/palladium (Hummer 6.2 Sputtering System, Anatech, Hayward, CA), and observed by SEM in high vacuum (JSM-5900LV, JEOL, Peabody, MA). We also observed the cuticular surface of adult male bed bugs exposed to boric acid dust and control males that were not exposed to boric acid. Three treatment groups included three males each: (a) control males; (b) males exposed to non-ground boric acid; and (c) males exposed to ground boric acid. Bed bugs were exposed to 13.8 mg boric acid in Petri dishes (60 x 15 mm; 0.48

mg/cm^2) for 24 hrs. Each bed bug was then killed by decapitation and prepared for SEM. We arbitrarily defined a 100 µm radius around the right spiracle on the ventral side of the seventh abdominal sternite and quantified the size of all boric acid particles in this region (0.03 mm^2). ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify the size of boric acid particles to which bed bugs were exposed, as well as the boric acid particles that adhered to the cuticular surface of treated males.

Experiment 3: Residual contact assays with cockroaches

Adult male German cockroaches (9–11 d old) were used. To control for their ingestion of boric acid, two treatment groups were used, one with glued mouthparts and a control treatment. Both groups were immobilized briefly with CO_2 and placed on ice. The mouthparts of some males were then glued (Loctite Super Glue ULTRA Gel Control, Henkel Corporation, Rocky Hill, CT) to prevent ingestion of boric acid after grooming. The control group received the same treatment but mouthparts were not glued. Cockroaches were placed into Petri dishes that were 100 x 20 mm (Falcon, Corning, NY) in replicates of 3–6 males per dish (n_{total} = 26–41 males per control or treatment group). Males were then exposed to boric acid dust at the label rate (0.48 mg/cm^2, 38.4 mg per dish) and mortality was monitored daily.

Experiment 4: Injection assays with bed bugs

To assess the toxicity of boric acid to bed bugs, we injected it directly into the hemocoel. Boric acid was solubilized in phosphate buffered saline (PBS, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride and 0.01 M potassium chloride, Fisher Scientific, Fairlawn, NJ) to obtain a 5% stock solution, and diluted in PBS to the desired concentrations for 5 treatment groups, including PBS-only controls. All adult males (20 per treatment group; *n*= 100) were starved 4 days prior to injection. Bed bugs were immobilized briefly with CO_2 and placed ventral side up on ice, and 0.2 µl or 0.5 µl of the PBS solution was injected through the intersegmental membrane using a custom-made glass capillary attached to a 31-gauge syringe (Hamilton, Reno, NV), delivering 0, 1, 10 or 25 µg boric acid/bed bug. Bed bugs were placed individually into Petri dishes (60 x 15 mm) lined with filter paper (Whatman #1, 55 mm, Fisher Scientific, Fairlawn, NJ). Treatment groups were then placed into plastic containers with a vented lid and a moist paper towel lined the bottom of the container. Mortality was recorded daily for 7 d.

Statistical Analysis

Survivorship analysis was used for residual, ingestion and injection assays using a Kaplan-Meier and log rank test performed in SAS (2012). A Sidak adjustment was used for log rank tests for multiple comparisons. Kolmogorov-Smirnoff test was used to analyze differences in the particle size distributions of ground and non-ground boric acid in Petri dishes and on adult male bed bugs after 24 hrs of exposure.

Results

Mortality of bed bugs after ingesting boric acid

A dose-response study was conducted with different concentrations of boric acid in defibrinated rabbit blood, using an artificial feeding system. Bed bugs readily accepted low concentrations of boric acid, as 97% fed to repletion on up to 2% boric acid (**Table 1**). However, at boric acid concentrations ≥ 3%, the number of bed bugs that fully engorged (**Fig. 1B**) declined to 80% at 5% boric acid. We also calculated the amount of boric acid each adult male ingested based on previous findings that each adult male consumes 3.9 µl when fully engorged (Sierras and Schal 2017), and at the lowest (0.5%) and highest (5%) concentration each bed bug consumed 19.5 µg and 195 µg boric acid, respectively (**Table 1**).

None of the control bed bugs fed defibrinated blood died in the 7-d assay, but all bed bugs that ingested 1 to 5% boric acid died within 4 d (**Fig. 1D**). There were significant differences among all groups (log rank, $\chi^2 = 264.76$, df = 6, $P < 0.0001$) and between the control group (0% boric acid) and all other treatment groups (0.5, 1, 2, 3, 4, 5%) ($P < 0.0001$ for all comparisons). Pairwise comparisons revealed significant differences between the 0.5% treatment group and the 2, 3, 4, and 5% groups ($P < 0.0001$ for all, Sidak adjustment). The lowest concentration of boric acid, 0.5%, killed 50% of the males by day 5 and only 20% survived to 7 d. The highest concentration of boric acid that was readily ingested, 2%, killed 70% of the bed bugs within 1 h and 100% within 24 hrs after ingestion.

Mortality of bed bugs after contact with boric acid particles

We found low mortality in assays with unfed adult male bed bugs exposed to the label rate (0.48 mg/cm^2) of boric acid dust distributed in a Petri dish. Only 33% of the bed bugs that were exposed to boric acid particles for 24 hrs died within 14 d (**Fig. 2**). We then examined the effect of larger amounts of boric acid and feeding status to assess if fed bed bugs with exposed intersegmental membranes were more susceptible than unfed bed bugs (**Fig. 1A–C**). We predicted that (a) higher concentrations of boric acid would be are more effective, (b) fed bed bugs would be more susceptible to boric acid dust because their intersegmental membranes are more exposed after they feed , and (c) smaller particles of boric acid dust would be more effective.

Although there were significant differences among the treatment groups (χ^2 = 22.51, df = 8, P = 0.004; log rank test), a 10-fold higher concentration of boric acid dust (4.8 mg/cm^2) increased mortality of unfed males by only 10% (**Fig. 2**). Likewise, we found no significant differences between the fed treatment groups and the unfed bed bugs. Fed bed bugs exposed to the highest concentration (10x label rate) exhibited only 15% mortality, and unfed bed bugs exposed to the same concentration experienced a similar mortality of only 33% after 14 d (**Fig. 2**). Therefore, both increasing the boric acid concentration and exposing the intersegmental membranes did not increase the susceptibility of bed bugs to boric acid.

Finally, to determine if smaller particles of boric acid would be more effective at causing mortality in bed bugs, we ground boric acid to smaller particles to which bed bugs were exposed for 24 hrs. First, we confirmed by SEM that the grinding procedure reduced the particle size distribution of boric acid dust. The size of the unground particles (n = 338, $\bar{x}$ = 56.3 µm, median = 20.7 µm) was significantly reduced after grinding (n = 340, $\bar{x}$ = 14.2 µm, median = 9.1 µm) ($P < 0.0001$; Kolmogorov-Smirnov; **Fig. 3A**). Approximately 20% of the unground boric acid particles were >100 µm, whereas the largest particles after grinding were 35 µm or smaller (**Fig. 3A**).

SEM observations revealed that boric acid particles on bed bugs represented only a fraction of the distribution of boric acid particles to which bed bugs were exposed. Thus, when bed bugs were exposed to non-ground boric acid, small particles were disproportionately represented on their cuticle (n = 150, $\bar{x}$ = 7.3 µm, median = 5.9 µm), compared to the overall distribution of dust particles with $\bar{x}$ = 56.3 µm, showing that large boric acid particles were excluded from the cuticle (**Fig. 4B, C**). Bed bugs that were placed in Petri dishes with ground boric acid dust also picked up smaller particles (n = 150, $\bar{x}$ = 6.1 µm, median = 4.7 µm) than the overall distribution of ground boric acid with $\bar{x}$ = 14.2 µm (**Fig. 4A**). Although the size distributions of the non-ground and ground particles on the cuticle converged onto much smaller sizes of $\bar{x}$ = 7.3 µm and $\bar{x}$ = 6.1 µm respectively, they remained significantly different ($P = 0.04$; Kolmogorov-Smirnov).

However, reducing the size of boric acid particles did not improve their efficacy at causing bed bug mortality (**Fig. 2**). The label rate of boric acid that was applied to Petri dishes (0.48 mg/cm^2) was ineffective at causing mortality within two weeks after exposure for all combinations of ground and non-ground boric acid,and fed and unfed bed bugs. More than 85% of the fed bed bugs survived exposure to non-ground or ground boric acid at both the label rate and 10x the label rate. Survivorship also did not differ significantly between the non-ground and ground boric acid treatment groups for either fed or unfed males, even at 10x the label rate ($\chi^2 \leq 7.23$, $P \geq 0.22$; **Fig. 2**).

Mortality of cockroaches after contact with boric acid

The effectiveness of boric acid on *C. lectularius* by ingestion, and its marginal effectiveness as a dust prompted us to use *B. germanica* as an overall control because it is known to be highly susceptible to boric acid by both ingestion and contact. Because cockroaches ingest the dust that they groom off their body, we sealed their mouthparts to prevent ingestion of boric acid. Control cockroaches with sealed mouthparts that were not exposed to boric acid did not experience more mortality than control cockroaches with functional mouthparts ($\chi^2 = 6.25$, $P = 0.07$; **Fig. 5**). However, mortality was significantly higher in both treatment groups that were exposed to boric acid than in both control groups with glued or functional mouthparts ($\chi^2 \geq 29.68$, $P < 0.0001$ for all comparisons). There was no significant difference between the two treatment groups that were exposed to boric acid dust ($\chi^2 = 1.46$, $P = 0.77$), with 98–100% mortality within 24 hrs, indicating that contact with boric acid dust caused significant mortality in *B. germanica*.

Mortality of bed bugs after injection of boric acid

Boric acid was highly effective at causing mortality when injected into the hemocoel, a procedure that bypassed the digestive system (**Fig. 6**). We injected two different PBS controls (0.2 and 0.5 µl) and three doses of boric acid in PBS: two doses (1 and 10 µg boric acid per bed bug) that were less than the lowest effective concentration ingested by bed bugs (0.5%, corresponding to 19.5 µg boric acid per bed bug; **Table 1**) and one dose (25 µg boric acid per bed bug) that was greater than the 19.5 µg exposure through ingestion. There were significant differences among the treatment groups (log rank, χ^2 =

71.50, df = 4, $P < 0.0001$) (**Fig. 6**). The lowest dose, 1 µg per bed bug, did not differ significantly from the two control groups ($P > 0.28$), but the two higher doses resulted in greater mortality than the control groups ($P \leq 0.0014$). However, the 1 µg and 10 µg per bed bug injections did not differ significantly from each other ($P = 0.05$), exhibiting 40% and 64% mortality at day 7, respectively. Injections of 25 µg boric acid per bed bug, however, resulted in 95% mortality by day 7 (**Fig. 6**). These findings indicate that boric acid delivered to the hemocoel is toxic to bed bugs, that passage through the alimentary system is not necessary for mortality to be expressed, and that boric acid dust is somehow prevented from penetrating the bed bug cuticle.

Discussion

Overall, our results show that whereas boric acid is highly effective on bed bugs by ingestion, it has marginal efficacy by contact. In ingestion assays, we observed that relatively low concentrations of boric acid, up to 2% in rabbit blood, were readily ingested, as indicated by a high percentage of bed bugs fully engorging on the boric acid containing blood. Higher concentrations of boric acid deterred some bed bugs from feeding, yet 80% of the bed bugs accepted 5% boric acid, the highest concentration that we tested. Concentrations ≥ 2.25% boric acid in a 10% sucrose solution were shown to deter feeding in house flies (Hogsette and Koehler 1994) and ≥ 6.25% boric acid in dry-mixed and wet-mixed baits deterred German cockroaches from feeding (Strong et al. 1993). Deterrence of boric acid at high concentrations has prompted the use of borates to protect and preserve wood against termites (Kartal 2010, Han et al. 2012, Lopez-Naranjo et al. 2016) and wood-boring beetles (Robinson 1967, French 1969, Palanti et al. 2012).

Ingested boric acid was highly effective at causing bed bug mortality. Even a single blood meal of 0.5% boric acid, the lowest concentration we tested, killed 80% of the bed bugs in 7 d, and concentrations ≥ 1% killed 100% of the bed bugs within 4 d. Studies that have evaluated boric acid as a component of ingestible baits have shown it to be highly effective. German cockroaches ingesting an aqueous solution containing 0.5–2% boric acid with common sugars experienced rapid population declines when tested in the laboratory (Gore and Schal 2004), and using similar concentrations in the field in a 0.5 M sucrose solution significantly decreased cockroach infestations in swine farms (Gore et al. 2004). Likewise, argentine ants are susceptible to 0.5% boric acid in a 25% sucrose in water solution (Klotz et al. 1998, Hooper-Bui and Rust 2000). Toxic sugar-bait solutions that contain 1% boric acid are effective at reducing blood-fed, gravid female mosquitoes by 98% within 48 hrs in laboratory tests (Xue and Barnard 2003) and in the field spraying 1% boric acid in a melon derived solution, which was naturally attractive to both male and female mosquitoes, reduced *Anopheles gambiae* populations by 90% (Muller et al. 2010).

Surprisingly, bed bugs were relatively unaffected by contact with boric acid dust. Even 24 hrs of continuous exposure to 10x the label rate of boric acid dust resulted in only 15% and 32.5% mortality 14 d post exposure in fed and unfed bed bugs, respectively. Because the bed bug cuticle is covered with many setae that may prevent larger particles from contacting the cuticular surface, we suspected that smaller particles might adhere to and interact more with the cuticle and spiracles, thus increasing efficacy. Smaller particle size, however, did not improve the efficacy of boric acid dust. In fact, we found that regardless of the particle size distribution of boric acid dust applied to the dish, only small particles adhered to the bed bug cuticle. We further hypothesized that feeding status influences survivorship because the dust adheres to the abdominal intersegmental membranes, which are more exposed in engorged bed bugs. However, both groups experienced low mortality, with more fed than unfed bed bugs surviving the 10x label rate of boric acid.

Our results showed that the route of boric acid administration greatly affected its efficacy. Ingested boric acid was highly effective against bed bugs, whereas boric acid administered by contact was ineffective. This surprising finding prompted us to use the same boric acid materials on the German cockroach, *B. germanica*, which is known to be susceptible to boric acid by both ingestion and contact (Ebeling 1978). The same boric acid dust that was ineffective on bed bugs was highly effective on the German cockroach. Thus, it appears that this otherwise insecticide-susceptible strain of bed bugs possesses mechanisms that prevent boric acid from compromising or penetrating the cuticular barrier.

The mode of action of boric acid remains unclear. Its effectiveness on bed bugs by ingestion, but not by contact, suggests that it might specifically disrupt the digestive system, as suggested by Cochran (1995) for cockroaches. To experimentally bypass both the alimentary canal and cuticle, we injected boric acid into the hemocoel. Ebeling (1995) and Zurek et al. (2002) showed that boric acid caused high and faster mortality when injected into German cockroaches than when ingested. Boric acid was effective by injection on bed bugs as well. A minimal ingested dose of 19.5 µg boric acid killed ~80% of fully engorged bed bugs, and similarly, an injected dose of 25 µg boric acid killed ~95% of the bed bugs in 7 d. These results confirm that the mode of action of boric acid in bed bugs involves neither the cuticle (e.g., desiccation) nor the digestive system (e.g., starvation or tissue degradation). Rather, the adverse effects of boric acid on the insect are expressed after it enters the hemocoel and may potentially have neurotoxic effects on bed bugs, such as elevated glutathione *S*-transferases and reduced acetylcholinesterase activity observed in German cockroaches exposed to boric acid (Habes et al. 2006).

Ebeling et al. (1975) suggested an explanation for the differential mortality in cockroaches after exposure to boric acid dust, its ingestion in liquid, and injection. Boric acid dust that adheres to the cuticle is groomed off the body and ingested, but it remains in the foregut and takes a long time to penetrate the thick cuticle that lines the foregut. When dissolved in water, boric acid readily passes to the midgut and hindgut, from where it more quickly penetrates the hemocoel. Injection bypasses these barriers and exerts the fastest effects on the insects. Our observations with bed bugs are consistent with these suggestions, but with the added constraint that boric acid dust cannot be ingested by bed bugs. Interestingly, the rapid mortality we observed in bed bugs that ingested boric acid in a blood meal may be related to the adaptation of hematophagous insects to rapidly transfer water from the blood meal to the hindgut for excretion. This process may also facilitate the rapid transfer of dissolved boric acid out of the foregut.

Boric acid is commonly used to control a wide variety of urban pests, including bed bugs. A 2011 study found that boric acid was available as a “do-it-yourself” (DIY) product in 70% of 120 stores surveyed in New York City, and it was the most common non-spray product found (Horton et al. 2011). Indeed, dust formulations of boric acid are effective against other crawling insects, including cockroaches, silverfish, and fleas (Hinkle et al. 1995, Rust and Dryden 1997, Wang and Bennett 2009). We have observed that many residents use boric acid dust against bed bugs as well (AS, personal observations). But the low morality we observed in bed bugs exposed for 24 hrs of continuous contact with boric acid dust indicates that interventions using boric acid dust would not be effective against bed bugs. Drivers for such misguided use of boric acid dust for bed bug control likely include lack of efficacy data documenting its inefficacy, high cost of alternative methods of bed bug control, and the wide availability of boric acid dust to consumers.

Our research considered only adult male bed bugs of the Harold Harlan population, which is susceptible to all insecticides and maintained in the laboratory for ~45 years. Future studies should evaluate females, nymphs, as well as recently field-collected populations. Also, only fully engorged individuals were monitored for lethal effects following the ingestion of boric acid. Further research should examine the sublethal effects of ingested boric acid and its effects on partially fed individuals. Furthermore, future research should investigate why boric acid is ineffective against bed bugs as a residual insecticide, and strategies that might correct this deficiency. For example, spatial heat can synergize the effects of boric acid in cockroaches and beetles (Ebeling 1995). Because spatial heat is a common though expensive bed bug intervention, its interaction with boric acid dust should be further investigated.

A major challenge in developing effective bed bug interventions is to develop, validate and implement a bed bug bait. Their piercing-sucking mouthparts make this task particularly challenging for hematophagous arthropods. In previous research, we showed that several neuroactive insecticides were much more effective by ingestion in an artificial feeding system than by topical application (Sierras and Schal 2017). Ivermectin and moxidectin administered by ingestion to humans and mice also showed systemic effects and mortality of bed bugs that ingested the treated blood (Sheele et al. 2013, Sheele and Ridge 2016). Boric acid appears to be an excellent candidate for a bed bug baiting system, and its solubility and stability in water, low mammalian toxicity, low cost, and no evidence of resistance to it in any insect species make it a particularly appealing candidate. The research we report here was conducted with boric acid dissolved in rabbit blood. Phagostimulants in blood likely maximized blood intake and possibly obscured some deterrence at higher concentrations of boric acid. However, Romero and Schal (2014) showed that the addition of ATP to water could simulate bed bugs to ingest large amounts of water. Furthermore, heat itself strongly stimulates close-range attraction and feeding in bed bugs (DeVries et al. 2016), so the combination of ATP, heat and boric acid could be important components of a bed bug baiting system.

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Table 1. Percentage of fully engorged adult male bed bugs that ingested different concentrations of boric acid in defibrinated rabbit blood and amount of boric acid they ingested.

Concentration of boric acid in blood (%)	Number in treatment group (*n*)	Number fully engorged [a]	% fully engorged	Ingested boric acid (µg/adult male) [b]
0	30	30	100	0
0.5	30	30	100	19.5
1	30	30	100	39
2	30	29	97	78
3	30	27	90	117
4	30	25	83	156
5	30	24	80	195

[a] Adult male bed bugs were given 30 min to feed

[b] Calculated based on the observation that each adult male bed bug consumes 3.9 µl blood (Sierras and Schal 2017)

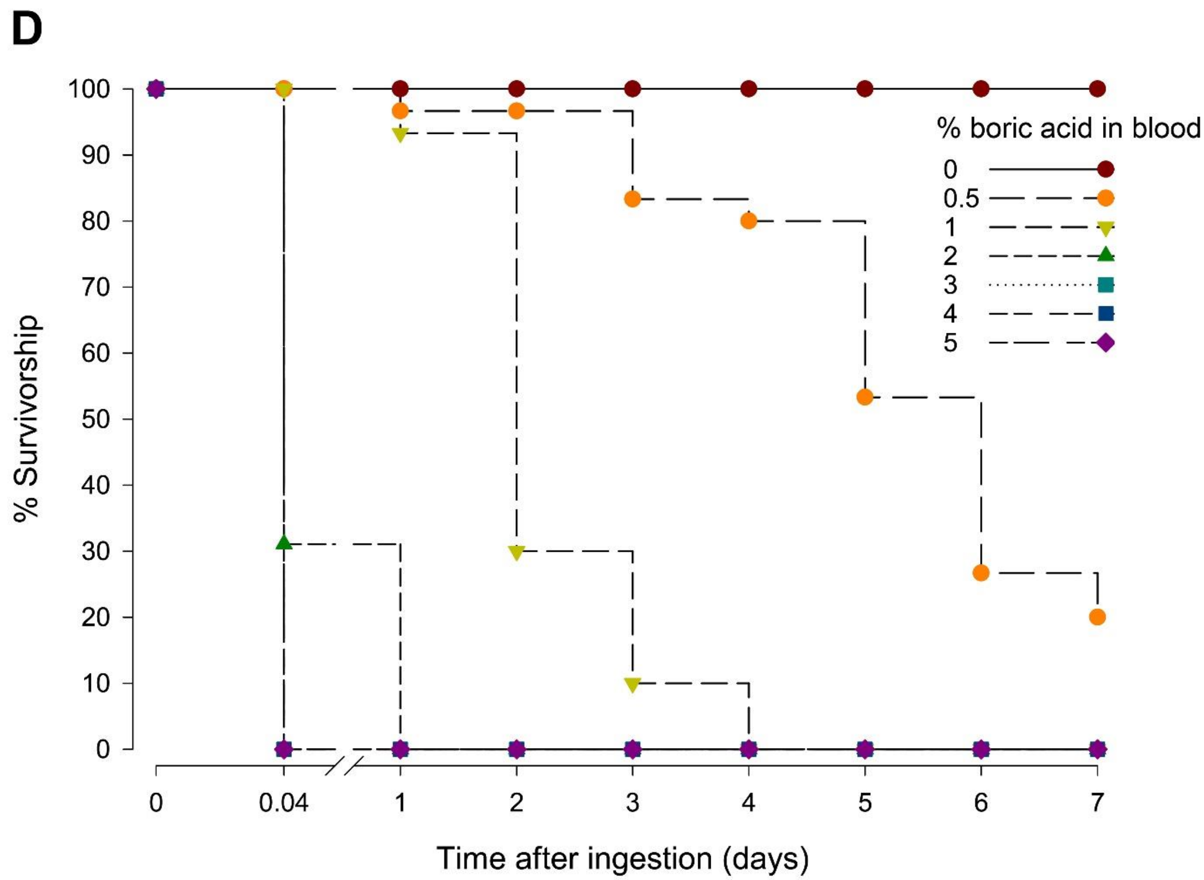

Figure 1. Unfed (A) and fully engorged (B) adult male *Cimex lectularius*, showing the exposed abdominal intersegmental membranes in fed bed bugs (C). (D) Kaplan-Meier survivorship of adult male *Cimex lectularius* fed once to repletion on various concentrations of boric acid in defibrinated rabbit blood. Each treatment group consisted of three replicates (*n* = 24–30 per group, see Table 1). Mortality was recorded for 7 d post ingestion.

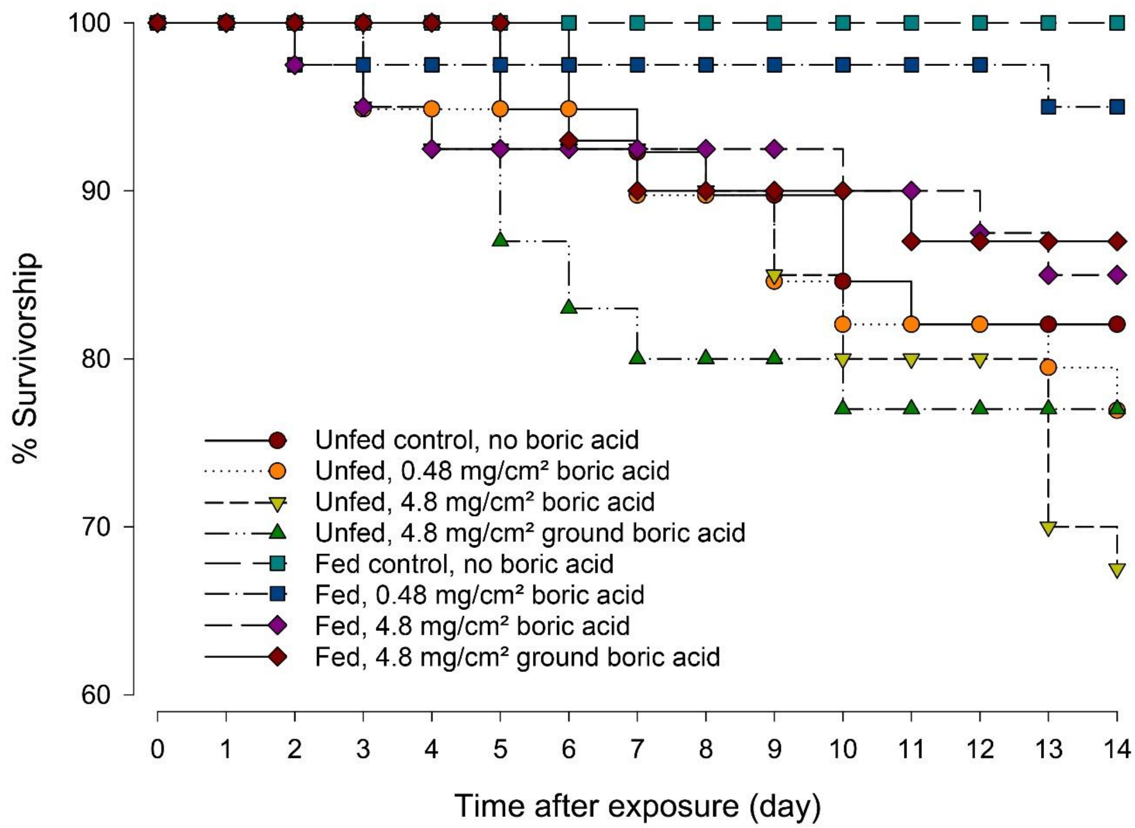

Figure 2. Kaplan-Meier survivorship of adult male *Cimex lectularius* that were exposed to different concentrations of boric acid dust for 24 hrs and monitored for mortality for 14 d post exposure. The label rate of boric acid dust is 0.48 mg/cm^2. Each treatment group consisted of 3–4 replicates (*n* = 30–40 bed bugs per treatment).

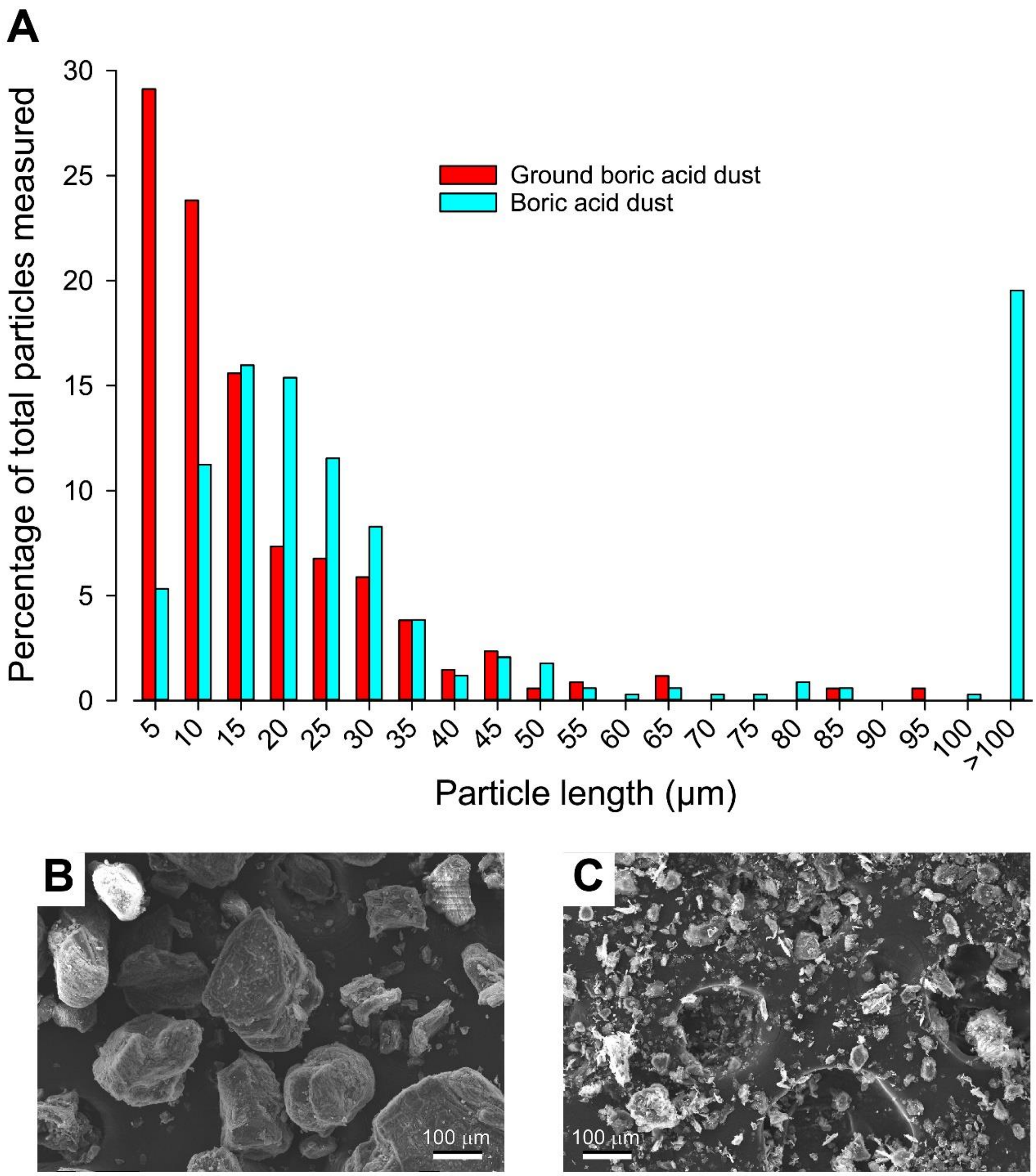

Figure 3. Particle size distributions of non-ground (*n* = 338 particles) and ground (*n* = 340 particles) boric acid dust (A). Boric acid particles were measured from SEM images (magnified 130x) as the longest axis of each particle. These images represent similar images used for measurements. Particle sizes were binned in 5 µm increments and each bin was represented as a percentage of the total number of particles measured for non-ground (B) and ground (C) dust.

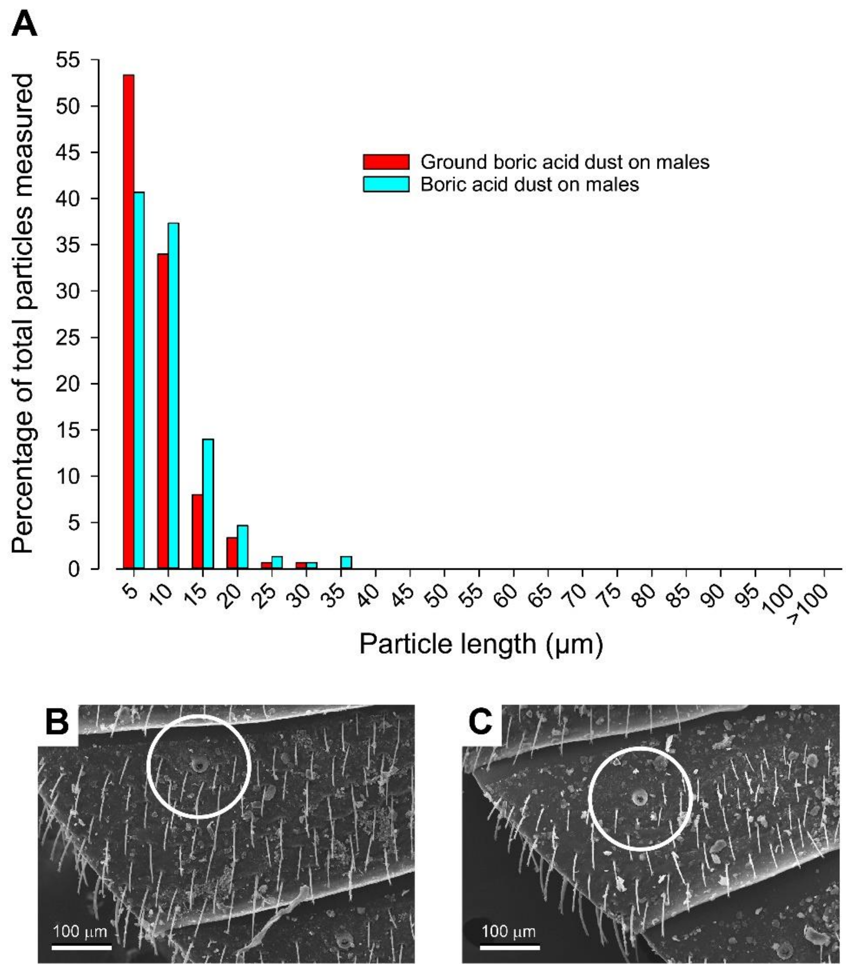

Figure 4. Particle size distributions of non-ground (3 replicates; n_{total} = 150) and ground (3 replicates; n_{total} = 150) boric acid dust, to which bed bugs were exposed in Petri dish assays, quantified from the cuticular surfaces of male bed bugs after 24 hrs of exposure (A). Boric acid particles were measures from SEM images (magnified 200x) as the longest axis of each particle. These images represent similar images used for measurements. Particle sizes were binned in 5 µm increments and each bin was represented as a percentage of the total number of particles measured for non-ground (B) and ground (C) dust on the cuticular surface. Measurements of particles were conducted in an area of 100 µm radius around the spiracle on the right ventral side of the 7th abdominal segment (as shown in white).

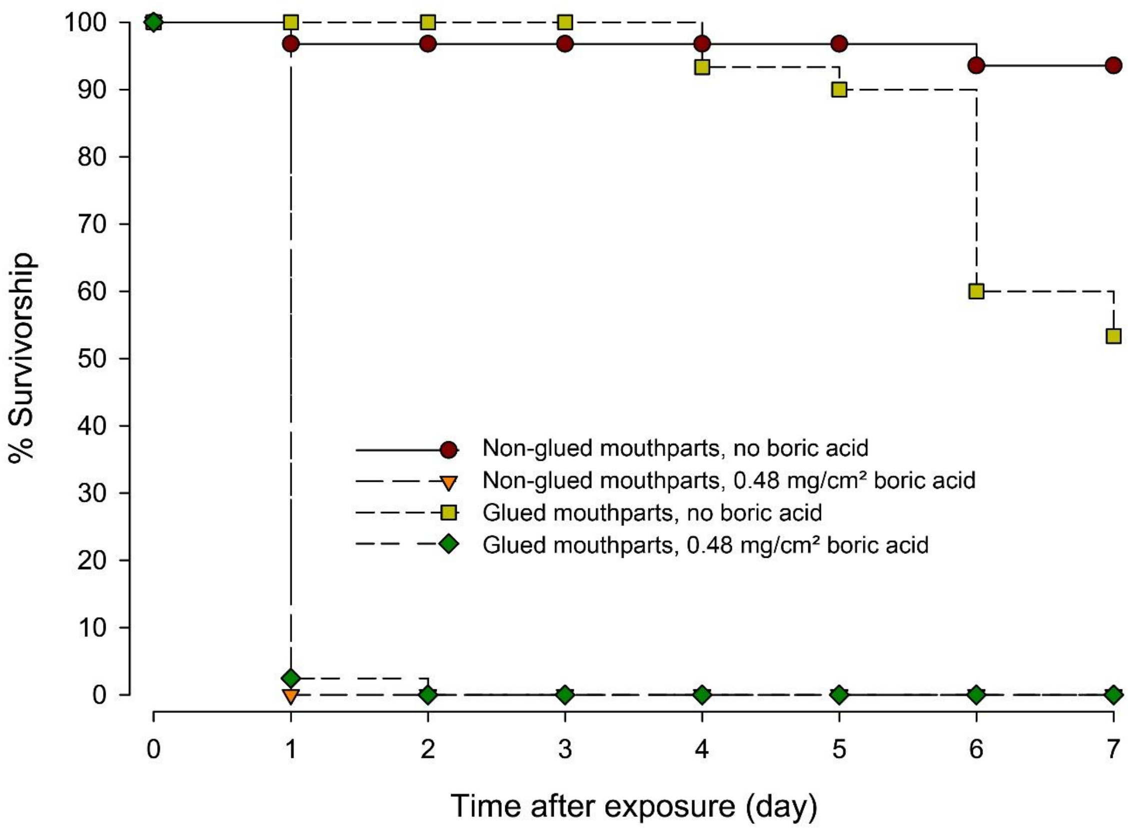

Figure 5. Kaplan-Meier survivorship of adult male *Blattella germanica* that were exposed in Petri dishes to 0.48 mg/cm^2 (label rate) of boric acid (BORiD) for 24 hrs. Treatments included glued mouthparts to prevent cockroaches from ingesting boric acid. Mortality was monitored for 7 d post exposure. *n* = 26–41 per treatment group.

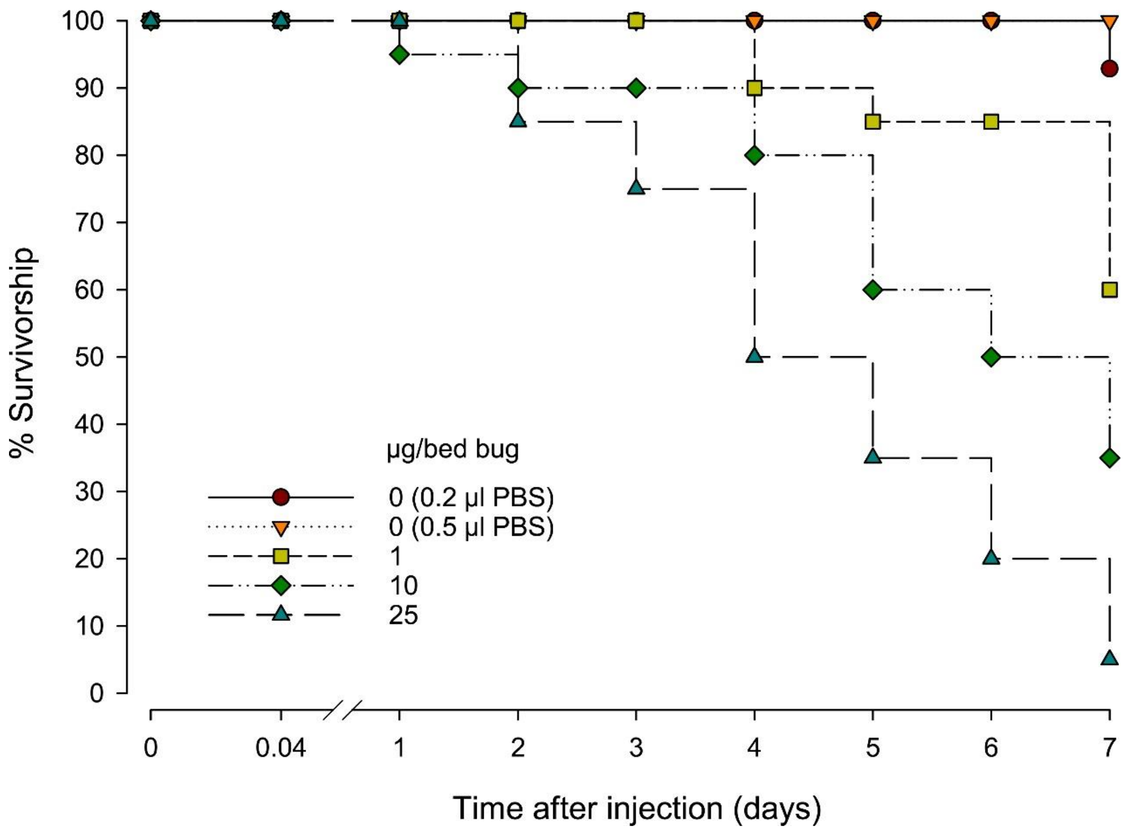

Figure 6. Kaplan-Meier survivorship of adult male *Cimex lectularius* injected with various amounts of boric acid in PBS. Each treatment group consisted of 20 individuals. Mortality was recorded for 7 d post injection.

Chapter 4

Lethal and Sublethal Effects of Ingested Hydroprene and Methoprene on Development and Fecundity of the Common Bed Bug (Hemiptera: Cimicidae)

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Abstract

In the last two decades, bed bugs (*Cimex lectularius* L. [Hemiptera: Cimicidae]) have become a perennial and difficult to control indoor pest. Current pest control options are severely constrained by high prevalence of insecticide resistance and relatively high costs of alternative interventions. Measures to counter the drawbacks of insecticide resistance include efforts to diversify the modes of action with residual applications of combinations of insecticides which frequently include a juvenile hormone analog (JHA). JHAs, such as hydroprene and methoprene, have a desirable safety profile and are effective against many indoor pests. We evaluated the potential of hydroprene, which is currently labeled for bed bug control, and methoprene, to be incorporated into an ingestible bait, with dose-response studies on fifth instar male and female bed bugs. Females were more susceptible than males to both JHAs, and methoprene was more effective by ingestion than hydroprene at inducing both lethal and sublethal effects. Ingestion of high concentrations (≥10 μg/ml blood) of either JHA by last instar nymphs reduced oviposition in adult females; untreated females that mated with males exposed to high concentrations of either JHA also exhibited lower oviposition. We conclude that methoprene could be incorporated into integrated pest management programs in two ways: high concentrations of methoprene alone could be used in a liquid bait, and both high and possibly lower concentrations of methoprene could be used in residual sprays in combination with other insecticides.

Key words: Urban IPM, Bed Bugs, *Cimex*, Juvenile Hormone Analog, Insect Growth Regulator

Introduction

Bed bugs are ubiquitous, obligatory blood feeding insects. Their preferred host are humans, but they readily feed on other vertebrates (Usinger 1966). Like lice, but unlike other blood feeding urban pests, such as fleas, mosquitoes, sand flies, and other hematophagous flies, every mobile life stage is dependent on a blood meal for growth, development and reproduction. When a host is available, bed bugs feed every 3–7 d (Reinhardt and Siva-Jothy 2007). Their obligate ectoparasitic association with humans make bed bugs a particularly loathed pest. During feeding, they introduce salivary secretions that can elicit local cutaneous or systemic immune responses in the host; these responses can be serious in some individuals (Goddard and deShazo 2009, Hwang et al. 2018). Subsequent scratching of bite sites can lead to secondary infections such as impetigo and lymphangitis (Thomas et al. 2004).

As bed bugs have become more prevalent, so has their impact on people and their public health importance. Bed bugs have been shown to adversely affect quality of life and psychological health, causing depression, anxiety, and paranoia (Rossi and Jennings 2010, Perron et al. 2018). Bed bug feces contain histamine, and significant amounts levels of histamine accumulate and persist in household dust even after successful intervention with heat treatments (DeVries et al. 2018). Although the health impacts of environmental histamine remain unknown, adverse respiratory and dermatological reactions are likely because of the close proximity of bed bug feces to our breathing space. As with histamine, the importance of bed bugs as pathogen vectors is largely unknown. Although more than 40 pathogens have been found in different body parts or excretions of the bed bug (Lai et al. 2016), transmission of these harmful pathogens has not been documented under natural conditions (Doggett 2018). In the laboratory, bed bugs can successfully transmit *Trypanosoma cruzi* to other bed bugs (Blakely et al. 2018) as well as to mice (Salazar et al. 2015, Blakely et al. 2018) and they vertically transmit *Bartonella quintana* (Leulmi et al. 2015).

Indirect health and environmental concerns include the large amounts of insecticides used to control bed bug infestations. Residual insecticide sprays, often consisting of combinations of pyrethroids and neonicotinoids, are the primary approach to bed bug control because this approach is less expensive than fumigation or heat treatment, and residual sprays are readily available for do-it-yourself pest control (Doggett et al. 2012, Lee et al. 2018). High levels of resistance to these insecticides (Zhu et al. 2010, Adelman et al. 2011, Romero and Anderson 2016, Agnew and Romero 2017, Romero 2018), however, necessitate repeat applications, leading to greater insecticide use, and strong selection for higher resistance and multiple resistance mechanisms.

The practice of combining insecticides with different modes of action to combat resistance includes insect growth disruptors (IGDs), formerly termed insect growth regulators (IGRs) (Pener and Dhadialla 2012). Although IGDs are slower acting than neurotoxic insecticides, they can be highly effective as they disrupt the insect life cycle, particularly at juvenile stages (Pener and Dhadialla 2012). The most common IGDs used within the urban environment are chitin synthesis inhibitors, such as lufenuron and novaluron (Su et al. 2014, Seccacini et al. 2018), which interfere with the formation of chitin, and juvenile hormone mimics (e.g., pyriproxyfen, hydroprene and methoprene), which disrupt metamorphosis (Invest and Lucas 2008, El-Sheikh et al. 2016). The juvenile hormone analogs (JHAs), namely hydroprene and methoprene, are structurally similar to naturally occurring juvenile hormones (JHs), and have a desirable safety profile, including low mammalian toxicity and relatively rapid degradation in the environment (Mohandass et al. 2006).

Both hydroprene and methoprene have been used in the successful control of various urban pests. Both have been shown to cause larval mortality and affect egg production and egg hatch in fleas (Moser et al. 1992, Graf 1993), and genitalia and ootheca deformities and sterility in German cockroaches (King and Bennett 1989, 1991, Atkinson et al. 1992) and Oriental cockroaches (Short and Edwards 1992). Hydroprene has also been used to successfully control several stored product pests (Mohandass et al. 2006). Likewise, methoprene, the first commercially registered JHA, has been used to control mosquito larvae, various flies, fleas, as a feed-through insecticide mainly against flies, and in baits to control pharaoh ant (Lim and Lee 2005) and fire ant (Williams et al. 2001) colonies.

Juvenile hormone analogs have been assessed for bed bug control. Goodman et al. (2013) found that hydroprene and methoprene were ineffective against bed bugs when they were applied directly to various life stages at the label rates. Likewise, Naylor et al. (2008) found similar results with residual methoprene applications. Earlier studies have yielded similar results (Takahashi and Ohtaki 1975, Shaarawi et al. 1981, 1982).

Previously, we reported a proof of concept study, showing that insecticides delivered through ingestion could be more effective at causing bed bug mortality than by topical application (Sierras and Schal 2017). In this study we evaluated two JHAs, hydroprene and methoprene, and demonstrated their lethal and sublethal effects against 5th instar male and female nymphs and adults that emerged from the treated nymphs.

Materials and Methods

Insects

Bed bugs (*Cimex lectularius* L. [Hemiptera: Cimicidae]) from the HH (Harold Harlan; also known as Fort Dix) population were used for all experiments. This strain was collected in Fort Dix, NJ, in 1973, and maintained in our laboratory on defibrinated rabbit blood since 2008 as a standard insecticide-susceptible strain. Colonies were maintained in an incubator at ~27^{o}C and ~50% RH on a 12:12 (L:D)-h regime, and experimental insects were held in the same conditions. Bed bugs were fed defibrinated rabbit blood using an artificial feeding system, as described in Sierras and Schal (2017), and all experimental chemicals were fed to bed bugs using this system.

Rearing Procedure for Experimental Insects

To generate staged 5th instars, we first separated a large cohort of eggs. Groups of blood-fed adult females (~75) and males (~25) were placed into 118-ml (4 oz) plastic wide-mouth jars (57-mm diameter x 70-mm; with 58-mm screw cap) (Consolidated Plastics Company, Stow, OH) containing pleated paper inserts that served as shelter and substrate for oviposition. The adults were removed from the jars after 10 d, and 10 d later 1st instars were fed and left for another 10 d to molt to 2nd instars. Nymphs were similarly fed every 10 d to generate synchronous same-stage cohorts, until 5th instars were present. Nymphs were then separated by sex into 20-ml vials capped with modified plastic caps with a center hole that was sealed with plankton netting (BioQuip Products, Rancho Dominguez, CA) to enable feeding.

Feeding Assays

To determine the concentration of dimethyl sulfoxide (DMSO) suitable for use as the solvent for JHAs, different concentrations of DMSO (0, 0.1, 0.25, 0.5, 0.75, and 1% [vol/vol]) were dissolved in defibrinated rabbit blood. Groups of 24–31 adult females per plastic container (59-ml, 2 oz; 54-mm diameter x 47.5-mm; with 53-mm screw cap; Consolidated Plastics) were starved 7–10 d prior to the feeding assay. Concurrently, adult males were starved 7–10 d and fed untreated defibrinated rabbit blood. A paper substrate was enclosed in the container and plankton netting allowed blood-feeding. Females and males in separate containers were given 30 min to feed, but after 15 min a Pasteur pipette was used to mix the blood-DMSO solution. Only fully engorged females were placed 4–6 females per 4-ml glass vial, along with 3–4 males, with a strip of paper for shelter and oviposition substrate and capped with plankton netting. Females oviposited for 5 d and then males and females were moved as a cohort to a new vial with a new paper insert for another 5 d. Eggs were thus counted in each vial every 5 d, after adults were removed. The number of 1st instars were counted 10 d later and the percentage eggs that hatched was determined.

Feeding Assays with Hydroprene and Methoprene: Effects on Mortality and Molting

Technical grade (*S*)-hydroprene (98%, Zoëcon, Schaumburg, IL, USA) and (*S*)-methoprene (98%, Chem Service, West Chester, PA) were dissolved in DMSO to make a stock solution of 100 mg/ml DMSO and then diluted further in DMSO to obtain the desired concentrations. Fifth instar male and female nymphs were used in separate vials (35–77 each) for each treatment group (0.1, 1, 10, and 100 µg JHA/ml blood) and for two control groups (defibrinated rabbit blood and 0.1% DMSO in blood). The experimental feeding procedure was the same as described above. Only fully engorged nymphs were placed individually into 2-ml microcentrifuge tubes with a small hole in the cap and a paper insert as substrate. Mortality was recorded daily for 10 d for both JHAs, but molting was recorded daily over the 10 d only for methoprene.

Feeding Assays with Hydroprene and Methoprene: Sublethal Effects

All bed bugs of the same treatment group and sex that survived to adults 10 d after ingesting JHA as 5^{th} instars were placed into 20-ml vials and fed untreated defibrinated rabbit blood. Fully fed bugs were then placed individually into new 2-ml microcentrifuge tubes with a new paper insert and paired with a single untreated adult of the opposite sex of the same stage and feeding status. Pairs could mate and oviposit freely for 5 d, after which they were transferred to a new 2-ml microcentrifuge tube with a new paper insert and given another 5 d to oviposit. Eggs were counted on d-5 in the first vial, and again on d-10 in the second vial. First instars were counted 10 d after counting eggs and percentage egg hatch was calculated.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD to compare the effect of various ingested concentrations of hydroprene and methoprene on egg production over a 10-d period among treatments using SAS Institute (2016). Kaplan-Meier analysis was performed using SAS Institute (2012) to compare time until molting among methoprene treatment groups and Sidak correction was performed for all group comparisons.

Results

Effect of Ingested DMSO on Oviposition and Egg Hatch

To determine the amount of DMSO that would be suitable to use as a solvent for JHAs, without adversely affecting females, we conducted a dose-response study of DMSO in defibrinated rabbit blood. Overall, DMSO concentration in blood significantly affected egg production (ANOVA; $F_{5,29} = 4.24$, $P = 0.0051$; **Fig. 1A**). The control females (0% DMSO) oviposited significantly more eggs per day (1.8 ± 0.27) than females that ingested 0.75% (1.3 ± 0.14; $P = 0.035$) and 1% (1.2 ± 0.40; $P = 0.009$) DMSO in blood. Likewise, females that ingested 0.1% DMSO oviposited more eggs (1.7 ± 0.12) than females that ingested 1% DMSO ($P = 0.043$). All other group comparisons did not significantly differ from one another ($P \geq 0.130$).

Eggs were given 10 d to hatch, 1st instars were counted, and the percentage hatch was calculated for each treatment group (**Fig. 1B**). The control, 0.1%, and 0.25% DMSO groups resulted in 87%, 85%, and 80% successful egg hatch, respectively. Higher concentrations of DMSO – 0.5, 0.75, and 1% – caused a sharp decline in egg hatch, with only 60%, 40%, and 15% of the eggs successfully hatching, respectively. Based on these results we considered 0.1% DMSO in blood suitable for dose-response assays with hydroprene and methoprene.

Mortality of Fifth Instars After Ingesting Hydroprene or Methoprene

Newly ecdysed 5th instar male and female bed bugs were evaluated in an ingestion assay in which nymphs were fed hydroprene or methoprene in 0.1% DMSO using the artificial feeding system, and mortality was recorded daily for 10 d post ingestion only for fully engorged nymphs. After ingestion of JHAs, bed bugs that successfully molted exhibited a less melanized cuticle than nymphs fed untreated blood (**Fig. 2A,B**). Mortality generally resulted from disruption of ecdysis (**Fig. 2C,D**). The control females fed only blood, or blood supplemented with 0.1% DMSO, experienced <6% mortality and male nymphs experienced <18% mortality (**Fig. 3A**). Low concentrations of both JHAs, 0.1 and 1 µg/ml blood, had negligible effects on both males and females, with <16% mortality (**Fig. 3B, C**). However, the two JHAs differentiated at higher concentrations (10 and 100 µg JHA/ml blood), with females experiencing higher mortality on methoprene (38% and 84%, respectively) than on hydroprene (0% and 40%). The highest dose (100 µg/ml blood) was not used on males, but results with the 10 µg methoprene/ml blood dose suggest that females might be more susceptible to the JHA than males (**Fig. 3C**).

Effect of Methoprene on the Imaginal Molt

We recorded daily for 7 d the number of insects that successfully molted to adults after ingesting methoprene as newly emerged 5th instar males or females. Control females and males (no methoprene) synchronously ecdysed to adults between day 4 (0% adults) and d-5 (nearly 100% adults) (**Fig. 4A,B**). The lowest concentration of methoprene (0.1 µg/ml blood) did not affect the temporal pattern of ecdysis, with ≥96% of both sexes successfully molting to adults. Higher concentrations of methoprene (≥1 µg/mL blood) caused earlier molting, with 70–93% of the females and 58–96% of males molting to adults by d-4. Only females received the 100 µg methoprene/ml blood treatment and they experienced high mortality (**Fig. 3C**). Nevertheless, methoprene accelerated their imaginal molt, with 88% becoming adults by d-4. For both females (**Fig. 4A**) and males (**Fig. 4B**) there were significant differences among the treatment groups (log rank test; females: $\chi^2 = 101.28$, df = 4, $P < 0.0001$; males: $\chi^2 = 77.3$, df = 3, $P < 0.0001$). The time to molt did not differ for the females in the control (0) and 0.1 µg methoprene/mL blood, as well as for females in the 1 and 10 µg methoprene/ml blood groups ($P \geq 0.961$). However, all other female groups significantly differed from one another ($P \leq 0.021$). The same was true for males in the control and 0.1 µg/ml blood, as well as the males in the 1 and 10 µg methoprene/ml blood ($P \geq 0.13$). In all control and treatment groups for both males and females 100% of the nymphs molted by d-7.

Sublethal Effects of Hydroprene and Methoprene on Oviposition

After the imaginal molt, individuals that successfully molted were fed untreated rabbit blood and paired with an untreated adult of the opposite sex. The mean number of eggs per female per day was recorded over a 10-d period. Control bed bugs oviposited 1.3–1.7 eggs/d (**Fig. 5A**). Both males and females that survived JHA treatment as nymphs and allowed to mate with untreated bed bugs experienced sublethal effects (ANOVA; hydroprene: $F_{10,476} = 31.11$, $P < 0.0001$, **Fig. 5B**; methoprene: $F_{10,459} = 54.14$, $P < 0.0001$, **Fig. 5C**). Females that ingested the two lowest concentrations of hydroprene (0.1 and 1 µg/ml blood) were unaffected and produced as many eggs as control females. The two highest concentrations of hydroprene (10 and 100 µg/ml blood) significantly reduced egg production relative to control females, to 1.01 ± 0.73 and 0.00 ± 0.0 eggs/d, respectively ($P \leq 0.003$). Males were less affected by hydroprene than females at all concentrations (0.1, 1, and 10 µg hydroprene/ml blood). When these males mated with untreated females, these females' egg production was not significantly different from control females that mated with untreated males ($P \geq 0.148$).

Methoprene had more significant sublethal effects than hydroprene on both females and males. Only female nymphs that survived the lowest concentration of methoprene (0.1 µg methoprene/ml blood) and emerged as adults produced the same number of eggs as control females. All other females (fed 1, 10, or 100 µg methoprene/ml blood) oviposited significantly fewer eggs (0.11, 0.00, and 0.00 eggs/d, respectively; P <0.0001) than females fed on 0.1 µg methoprene/ml blood (**Fig. 5C**). Females fed 1 and 10 µg methoprene/ml blood also oviposited significantly fewer eggs/d than females fed the respective concentrations of hydroprene (P <0.001) (**Fig.**

5B,C). Males also were more affected by methoprene than hydroprene. Untreated females that mated to males fed 0.1 µg methoprene/ml of blood as nymphs produced the most eggs (1.94 ± 0.51 eggs/d) of any treatment, including controls; egg production was significantly higher than in females mated to males that ingested 1 µg methoprene/ml of blood as nymphs (1.49 ± 0.72; p = 0.022). Ingestion of 10 µg methoprene/ml blood by males significantly reduced egg production in their untreated mates relative to blood-only controls (1.34 ± 0.49; P = 0.002) to only 0.60 ± 1.22 eggs/d. Overall, methoprene appeared to be more effective at reducing female oviposition at concentrations ≥1 µg/ml blood, whereas the sublethal effects of hydroprene on oviposition were evident at concentrations ≥10 µg/ml blood. The sublethal effects of methoprene were also more significant when female nymphs ingested the JHA and mated as adults with untreated males than on untreated females that mated with males that ingested the JHA as nymphs.

Discussion

Our results showed that relatively high concentrations of hydroprene and methoprene were needed to obtain significant lethal and sublethal effects. Female bed bugs were more susceptible to both JHAs than males, and methoprene was more effective than hydroprene at causing mortality and reducing oviposition when either female nymphs were treated, or male nymphs were treated and mated with untreated females. Overall, we conclude that both JHAs are more effective by ingestion than by contact and although methoprene was substantially more effective, both could serve as active ingredients in bait formulations to reduce bed bug populations.

The lethal effects of the two JHAs were evident in 5th instar nymphs that ingested JHA-supplemented blood early in the instar and died during the imaginal molt. Mortality caused by hydroprene was low and not different from control mortality on untreated blood for both males and females. Even the highest dose of hydroprene (100 µg/ml blood; 0.01%) that we tested, caused only 40% mortality in females. In contrast, methoprene was more effective at causing mortality in females, with 10 µg/ml blood causing 40% mortality in females and 100 µg/ml blood (0.1%) causing nearly 90% mortality. We observed that most of the mortality occurred during ecdysis from the 5th instar to either adult, adultoid, or supernumerary nymph.

JHAs often delay development and increase melanization of the cuticle (Pener and Dhadialla 2012). In bed bugs, however, both hydroprene and methoprene accelerated development and reduced cuticular melanization. Some individuals that were treated with ≥1 µg JHA/ml blood molted to adults with lighter cuticle and patchiness of discoloration, as observed by Naylor et al. (2008). Almost all untreated 5th instar females and males molted to normal adults 5 d after ingesting blood. We observed a hitherto unreported dose-dependent acceleration of the imaginal molt. More than 80% of the females that survived 10 or 100 µg methoprene/ml blood molted on d-4, and >95% of males fed 10 µg methoprene/ml blood molted by d-4. We are not aware of similar findings with other insects.

Oviposition in females that survived exposure to JHAs in the last nymphal stadium was concentration dependent. The sublethal effects of hydroprene on reproduction were evident only in female bed bugs treated with ≥10 µg hydroprene/ml blood, with ingestion of 100 µg hydroprene/ml blood by nymphs completely suppressing reproduction in adult females over the 10-d observation period. Methoprene's sublethal effects were significantly greater than with hydroprene: treatment of female nymphs with 1 µg methoprene/ml blood greatly reduced reproduction of adults, and reproduction in females treated as nymphs with ≥10 µg methoprene/ml blood was completely suppressed. The indirect effects on female reproduction of treating male nymphs with JHAs were less severe. Male nymphs that survived ingesting up to 10 µg hydroprene/ml blood did not affect reproduction of untreated females, suggesting that relatively high doses of hydroprene did not affect sperm viability. However, males that ingested methoprene as nymphs and survived, significantly affected reproduction of their female mate in a dose-dependent manner. Thus, males fed 10 µg methoprene/ml blood as nymphs and mated with an untreated female, caused an 80% reduction in oviposition, indicating either that their genitalia morphology or sperm viability were adversely affected by methoprene. We did not dissect the females, but it is possible that lower oviposition was related to egg retention, as observed by Goodman et al. (2013). Certainly, delivery of inviable sperm would fail to induce females to switch from a virgin (low oviposition of unfertilized eggs) to mated state (elevated oviposition of fertilized eggs).

Three important observations are worthy of further discussion: (a) the relative activity of different JHAs, (b) their relative effectiveness on males and females, and (c) their relative efficacy by contact and ingestion. In the bed bug, methoprene was more effective than hydroprene, yet in other hemimetabolous insects, including the German cockroach (King and Bennett 1989, Kramer et al. 1989), Oriental cockroach (Bao and Robinson 1990, Short and Edwards 1992, Edwards and Short 1993) and mole crickets

(Parkman and Frank 1998), hydroprene is more effective than methoprene. Both are isoprenoids, and structural analogs of natural JHs, whose mode of action is to provide excess JHA at the time in development when endogenous JH is absent, thus interfering with metamorphosis and embryogenesis. Methoprene has a broad spectrum of activity against a variety of insects, including many dipterans (mosquitoes, flies, sand flies) and stored products pests (moths, beetles), whereas hydroprene has a more limited activity spectrum on insects (Mohandass et al. 2006). The natural JH of hemipteran insects remains unknown, and it is possible that methoprene has greater affinity to the JH receptor in bed bugs than hydroprene. Regardless, two recommendations emerge from these observations: First, given its superior performance, it appears that methoprene should be labeled for bed bug control. Second, in this context, it would be important to also consider other JHAs, such as kinoprene, which is particularly effective against homopterans (Henrick 2007), and heterocyclic analogs of JH, such as pyriproxyfen, which is the most potent JHA available with perhaps the broadest spectrum of activity that likely would include bed bugs. Pyriproxyfen has been shown to be effective on various hemipterans (Pener and Dhadialla 2012), and it is already labeled for indoor use against cockroaches, fleas, ants and beetles.

While both sexes of bed bugs are vulnerable to the effects of JHAs, *C. lectularius* females were more susceptible than males, whereas in other insects, males are especially affected. In the German cockroach, for example, hydroprene can cause melanization, morphological deformities of external appendages (e.g., genitalia, antennae, wings) and internal tissues (e.g., ovaries, testes), and ultimately sterilization (Bennett and Reid 1995). As little as 40 µg hydroprene/g body mass (ca. 2 µg/male), applied topically, can sterilize male nymphs, but double this dose is needed to sterilize female nymphs (King and Bennett 1989). In our investigation, females experienced greater lethal and sublethal effects than males. However, we caution that last instar females likely ingested more blood than males and therefore were exposed to higher doses at equivalent concentrations of the JHAs.

The bed bug is obviously much less sensitive to the effects of JHAs in topical application and residual assays; 3-fold to 8-fold the label rate of hydroprene and methoprene were required for any effects on bed bugs (Naylor et al. 2008, Goodman et al. 2013). Ingestion assays, however, suggest otherwise. Based on the estimation that adult male bed bugs ingest approximately 3.9 µl of blood in a single blood meal (Sierras and Schal 2017), we estimate that ingestion of methoprene, the more effective of the two JHAs, at a concentration of 10 µg/ml blood, would expose the bed bug to only 39 ng of methoprene (390 ng at 100 µg methoprene/ml blood). Thus, the efficacy of methoprene by ingestion is much greater than by topical treatment. The highly apolar nature of most JHAs likely affects their cuticular penetration and presentation to receptors. Pener et al. (1997) reported that pyriproxyfen was over 1,000-fold more effective on locusts by injection in oil than by topical application. They attributed this enormous difference to both the delivery by injection and the use of oil as a slow-release solvent. Greater efficacy of neuro-active insecticides, inorganic insecticides and JHAs by ingestion (Sierras and Schal 2017, Sierras et al. 2018) should stimulate greater interest in bait development for bed bugs.

Notably, a wide range of insecticides can be used against bed bugs in either a liquid bait or in a systemically treated host. We used blood in our present assays, but bed bugs readily accept a saline solution supplemented with various phagostimulants, notably adenosine triphosphate (ATP) (Romero and Schal 2014). Even DMSO has potential as an insecticide in an artificial liquid bait. Our present assays showed that 1% DMSO fed to last instar females significantly reduced their fecundity and fertility as adults. The same concentration of DMSO fed to adult males resulted in >60% mortality (Sierras and Schal 2017, Sierras et al. 2018), and 5% caused 100% mortality (not shown).

In conclusion, formulating insecticides as ingestible baits would enable a dramatic reduction in the amount of insecticide applied for bed bug control in the indoor environment, thus reducing potential exposure of humans and pets. Our results suggest that while methoprene may be effective at suppressing bed bug populations, a host of other insecticides kill bed bugs much faster and at much lower concentrations. Nevertheless, JHAs, and more generally IGDs, should continue to be investigated against bed bugs because other IGDs may be more effective than methoprene, the JHAs might synergize the activity of other insecticides, and the unique mode of action of JHAs, such as activity on embryos (Takahashi and Ohtaki 1975), may serve to forestall resistance to other active ingredients. An important step would be to screen kinoprene, pyriproxyfen and various chitin synthesis inhibitors not only on standard susceptible strains but also on recently field-collected bed bug populations.

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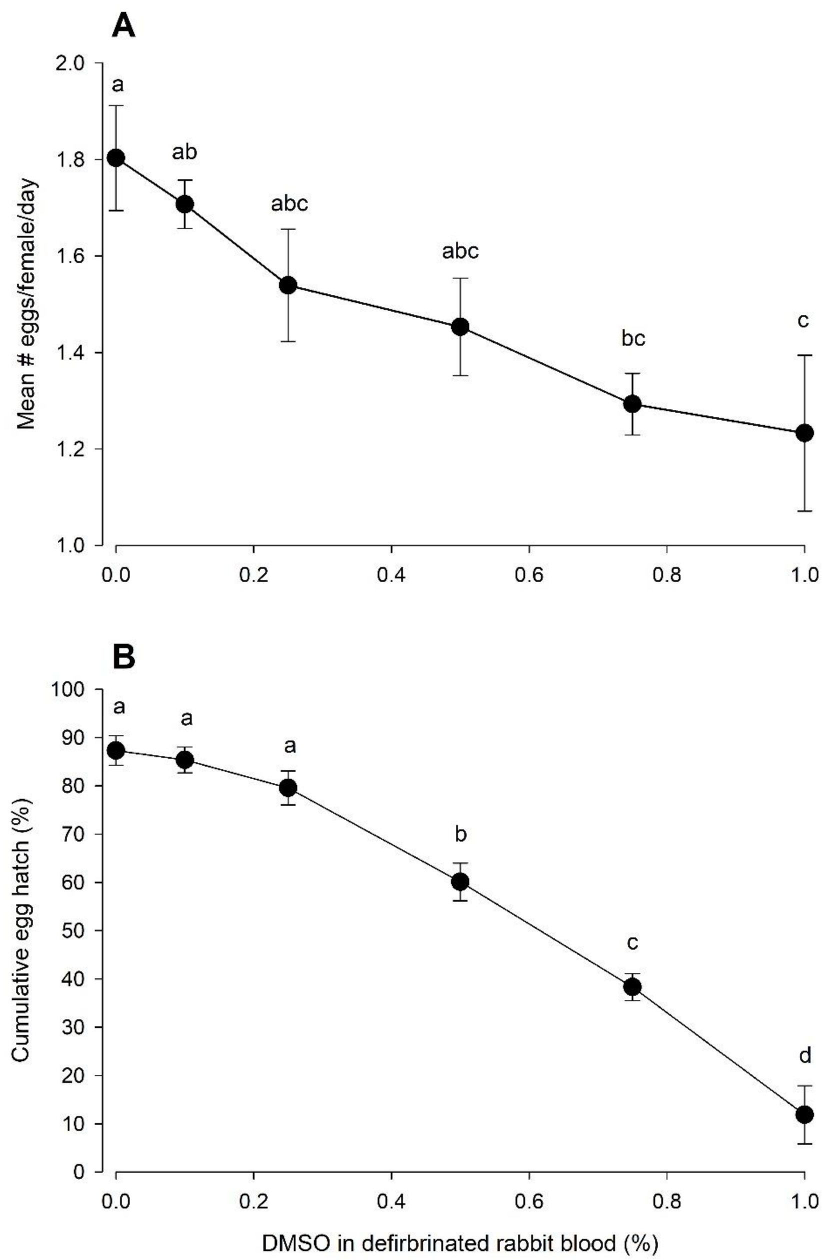

Figure 1. Dose-response of DMSO in defibrinated rabbit blood, showing (A) mean ± SEM number of eggs deposited by females per day during a 10-d period (*n* = 24–31 females per treatment group, 4–6 females per replicate), and (B) the percentage ± SEM of eggs that successfully hatched. Letters not shared by means indicate significant differences between the treatments (ANOVA, Tukey's HSD, $P < 0.05$).

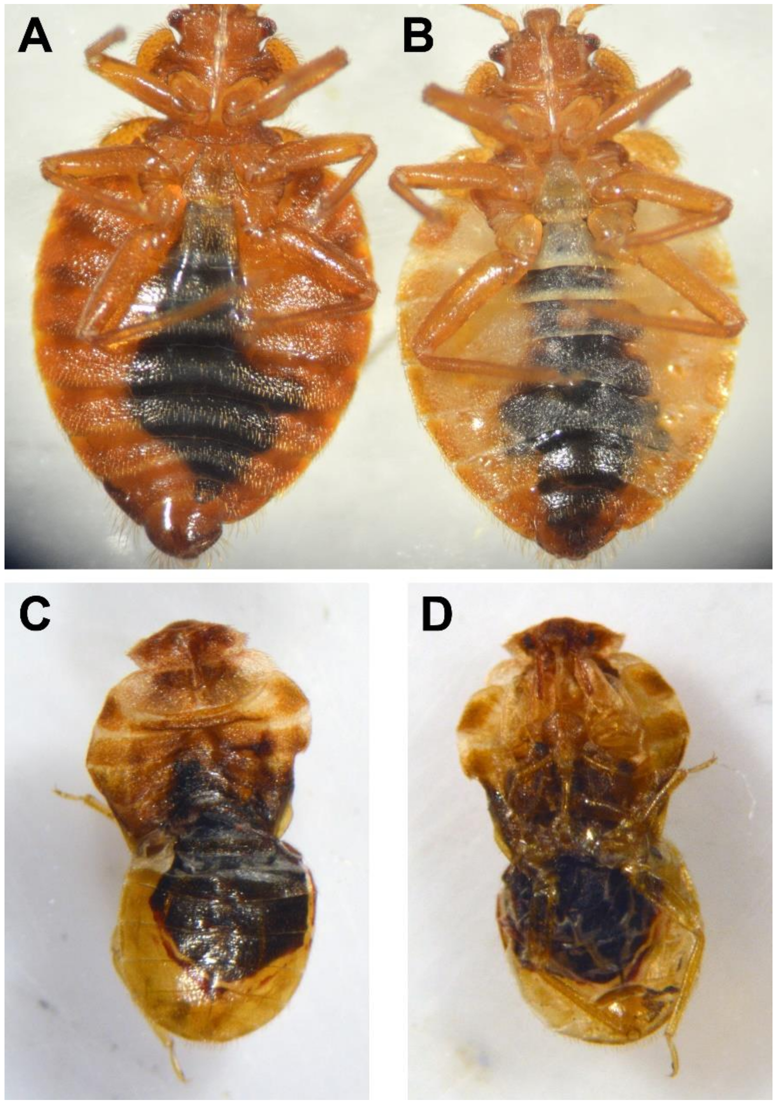

Figure 2. Post-ingestion effects of hydroprene on last instar male bed bugs. Compared to a control male fed untreated blood (A), hydroprene-bed bugs exhibited less melanization of the cuticle (B). Mortality generally occurred during the molt, as shown in dorsal (C) and ventral (D) views.

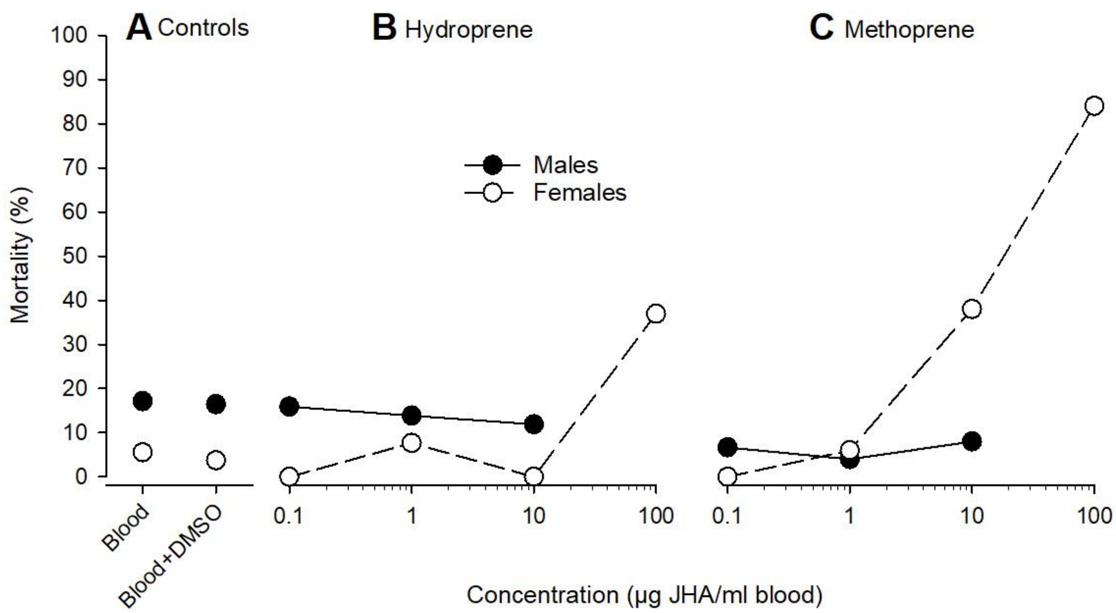

Figure 3. Cumulative mortality (%) of 5th instars 10 days after ingesting a JHA. Only fully engorged 5th instars were assessed after feeding on untreated blood and blood supplemented with DMSO (A), or various concentrations of hydroprene (B) and methoprene (C). Each assay consisted of 29–66 males and 29–77 females. Males were not fed 100 µg/ml blood for either JHA.

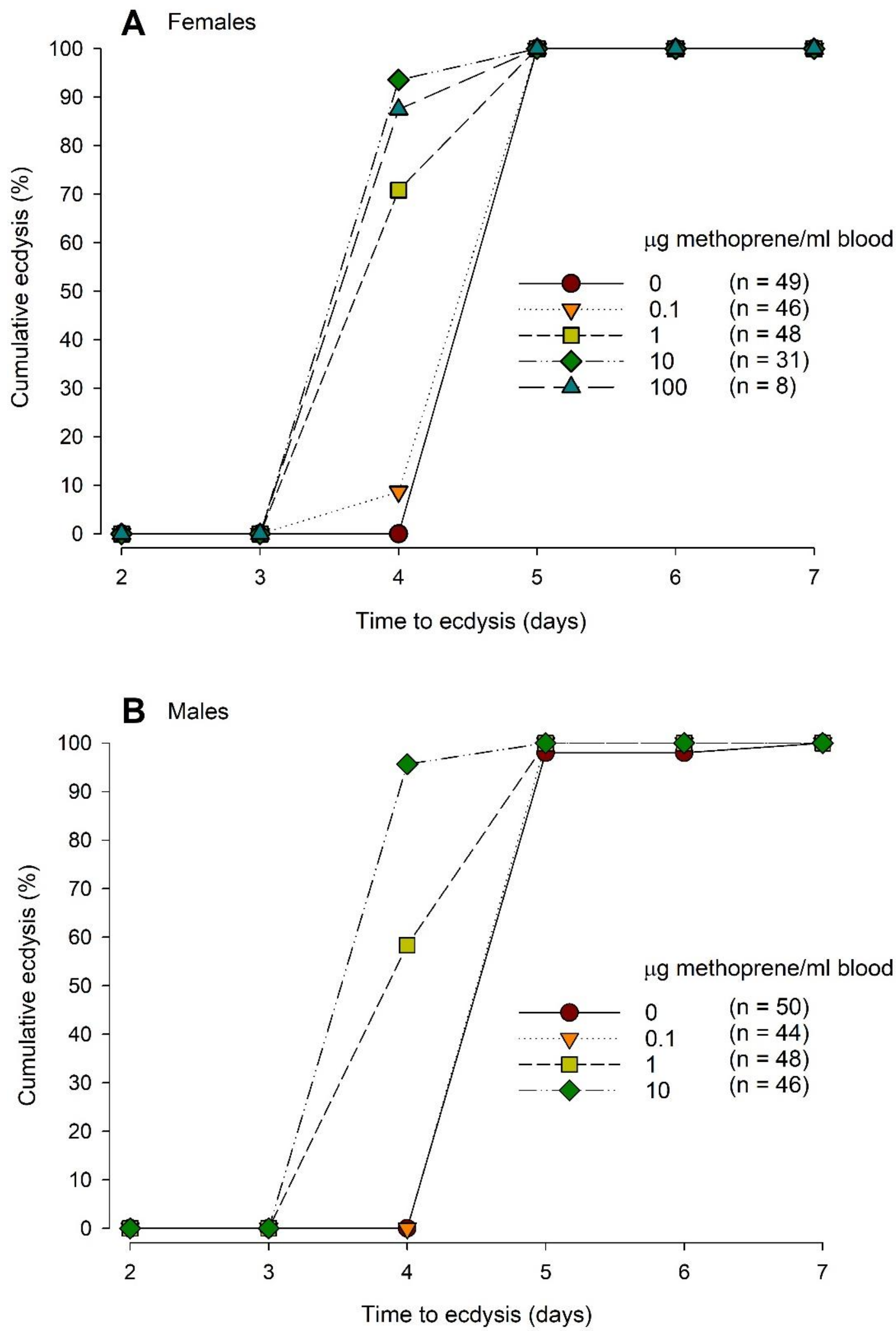

Figure 4. Cumulative daily molting (%) of 5th instars after ingesting methoprene. Only fully engorged 5th instar females (A) and males (B) were assessed after feeding on blood supplemented with 0.1% DMSO (0% methoprene), or various concentrations of methoprene in DMSO. Males were not fed 100 µg methoprene/ml blood.

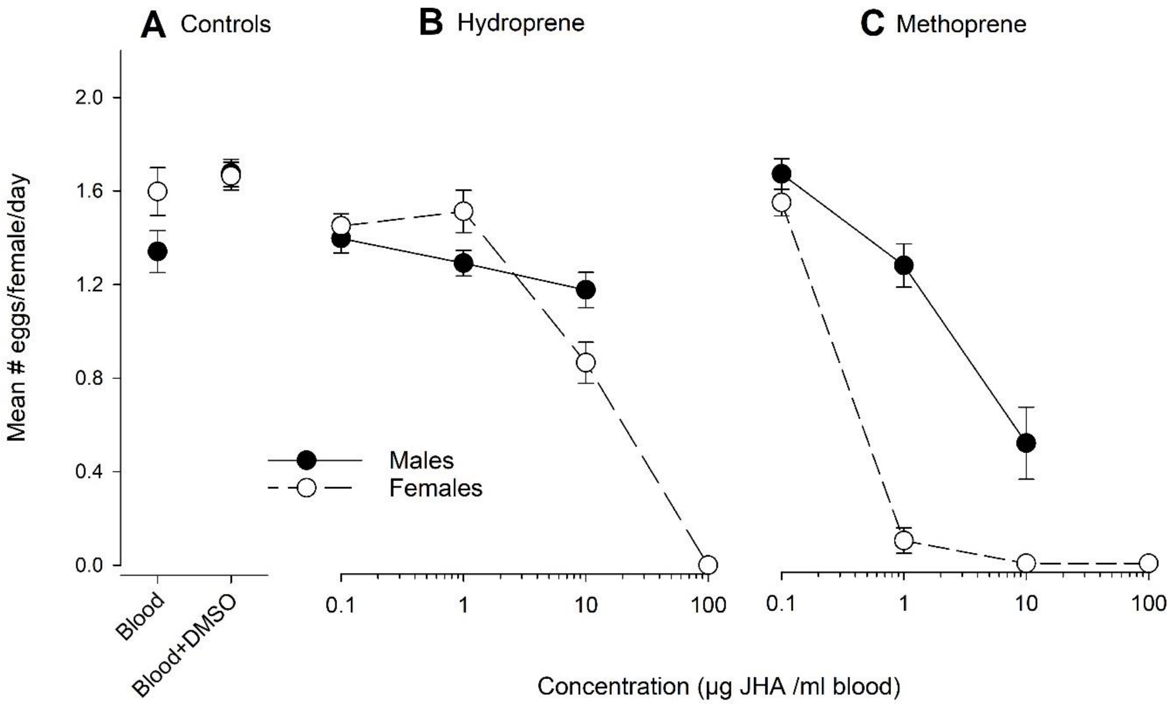

Figure 5. Dose-response including two controls (A), hydroprene (B) and methoprene (C) in defibrinated rabbit blood fed to 5th instar nymphs, given 10 d to molt and then fed untreated defibrinated rabbit blood. Mean ± SEM number of eggs deposited per d by females are shown. Males were not fed 100 µg/ml blood of either JHA.

Appendix

Table S1. Percent of Harold Harlan adult males and first instar nymphs that fully engorged on different concentrations of abamectin in defibrinated rabbit blood.

Concentration of Abamectin in blood (ng mL^{-1})	Life stage	Number in treatment group (*n*)	Number fully engorged	% fully engorged
Blood only	Adult males	30	30	100
	First instars	30	29	97
Blood + 0.75% DMSO	Adult males	30	30	100
	First instars	30	30	100
1	Adult males	30	30	100
	First instars	30	28	93
10	Adult males	30	30	100
	First instars	30	29	97
25	Adult males	30	30	100
	First instars	26	26	100
50	Adult males	30	30	100
	First instars	28	28	100
100	Adult males	30	30	100
	First instars	30	30	100
1000	Adult males	30	30	100
	First instars	30	29	97

Table S2. Percent of Harold Harlan adult males and first instar nymphs that fully engorged on different concentrations of clothianidin in defibrinated rabbit blood.

Concentration of Clothianidin in blood (ng mL^{-1})	**Life stage**	**Number in treatment group (*n*)**	**Number fully engorged**	**% fully engorged**
Blood only	Adult males	30	30	100
	First instars	29	29	100
Blood + 0.75% DMSO	Adult males	30	30	100
	First instars	30	30	100
1	Adult males	29	29	100
	First instars	28	28	100
10	Adult males	30	30	100
	First instars	24	24	100
25	Adult males	30	30	100
	First instars	29	29	100
50	Adult males	30	30	100
	First instars	29	29	100
100	Adult males	30	30	100
	First instars	29	29	100
1000	Adult males	30	29	97
	First instars	31	31	100

Table S3. Percent of Harold Harlan adult males and first instar nymphs that fully engorged on different concentrations of fipronil in defibrinated rabbit blood.

Concentration of Fipronil in blood (ng mL^{-1})	Life stage	Number in treatment group (*n*)	Number fully engorged	% fully engorged
Blood only	Adult males	30	30	100
	First instars	28	25	89
Blood + 0.75% DMSO	Adult males	30	30	100
	First instars	24	22	92
0.3	Adult males	30	30	100
	First instars	26	23	88
1	Adult males	30	29	97
	First instars	29	27	93
3	Adult males	30	28	93
	First instars	25	24	96
5	Adult males	-	-	-
	First instars	25	22	88
10	Adult males	30	29	97
	First instars	25	24	96
100	Adult males	30	30	100
	First instars	27	23	85

Table S4. Percent of Harold Harlan adult males and first instar nymphs that fully engorged on different concentrations of indoxacarb in defibrinated rabbit blood.

Concentration of Indoxacarb in blood (ng mL^{-1})	Life stage	Number in treatment group (*n*)	Number fully engorged	% fully engorged
Blood only	Adult males	30	30	100
	First instars	29	29	100
Blood + 0.75% DMSO	Adult males	30	30	100
	First instars	29	29	100
1	Adult males	30	30	100
	First instars	29	29	100
10	Adult males	29	29	100
	First instars	31	31	100
25	Adult males	29	25	86
	First instars	28	28	100
50	Adult males	29	20	67
	First instars	31	31	100
100	Adult males	28	26	93
	First instars	26	26	100
1000	Adult males	30	26	87
	First instars	29	29	100

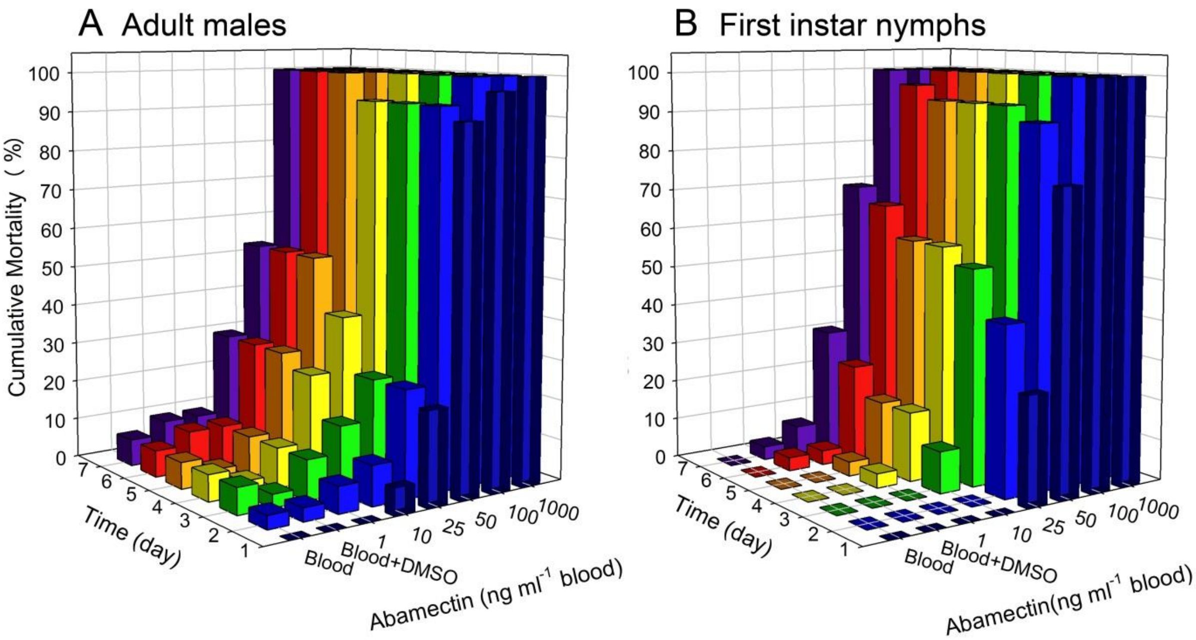

Figure S1. Daily mortality of bed bugs (Harold Harlan strain) over 7 days after ingesting various concentrations of abamectin. (A) Adult males, (B) First instar nymphs.

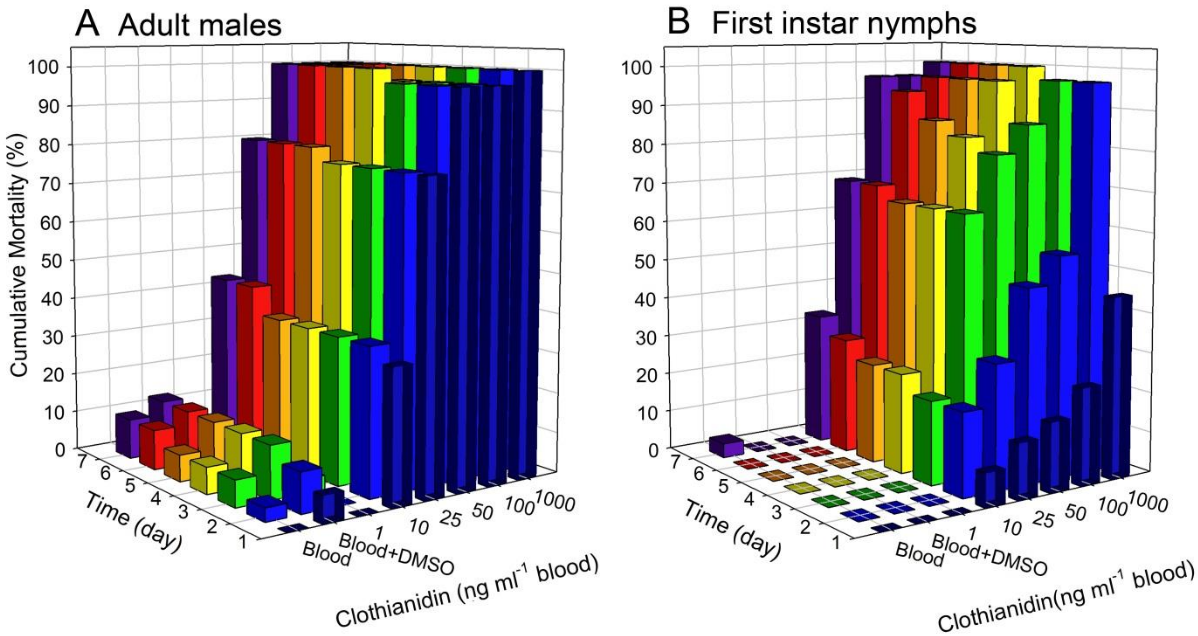

Figure S2. Daily mortality of bed bugs (Harold Harlan strain) over 7 days after ingesting various concentrations of clothianidin. (A) Adult males, (B) First instar nymphs.

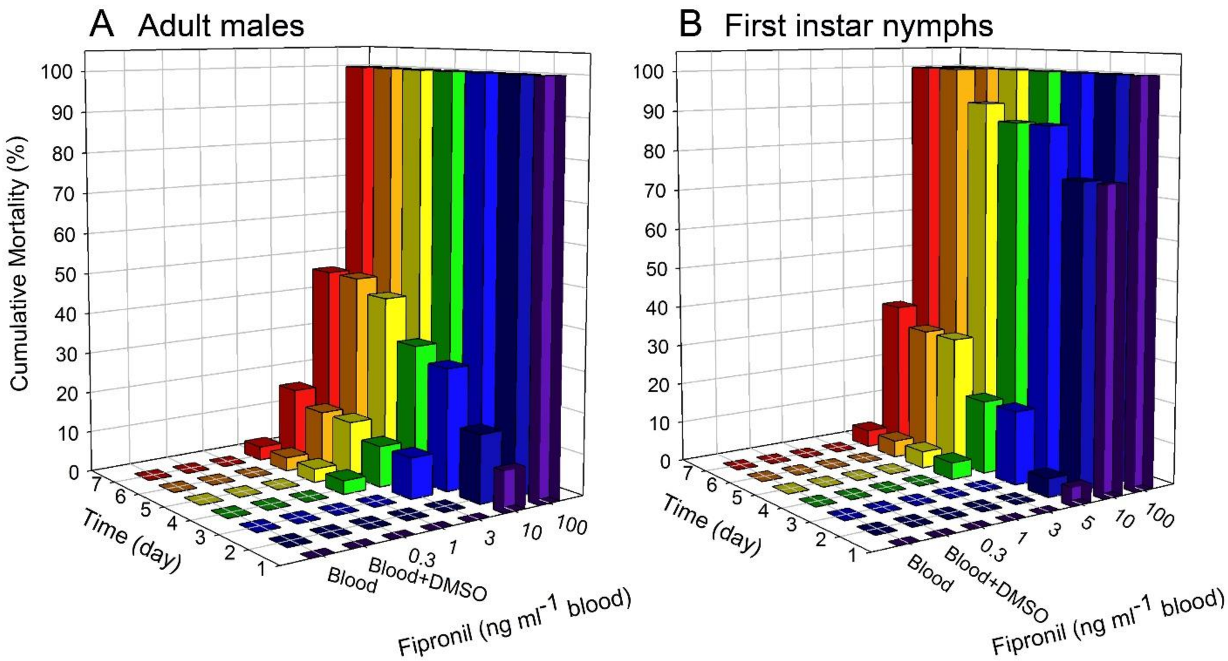

Figure S3. Daily mortality of bed bugs (Harold Harlan strain) over 7 days after ingesting various concentrations of fipronil. (A) Adult males, (B) First instar nymphs.

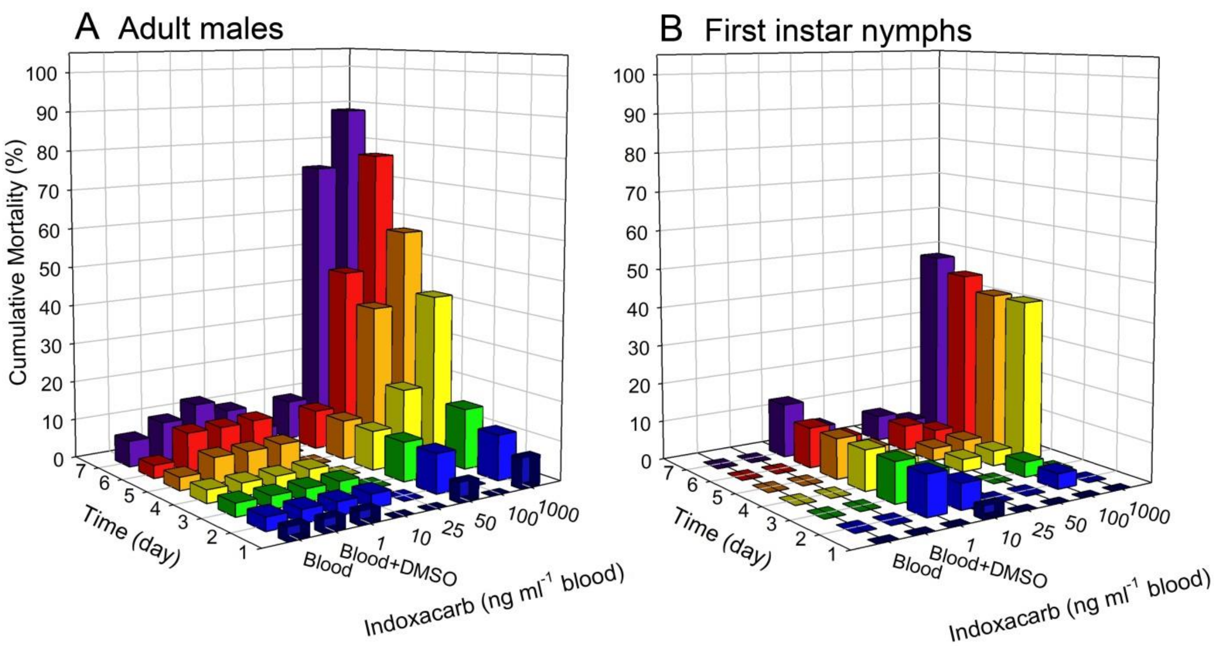

Figure S4. Daily mortality of bed bugs (Harold Harlan strain) over 7 days after ingesting various concentrations of indoxacarb. (A) Adult males, (B) First instar nymphs.

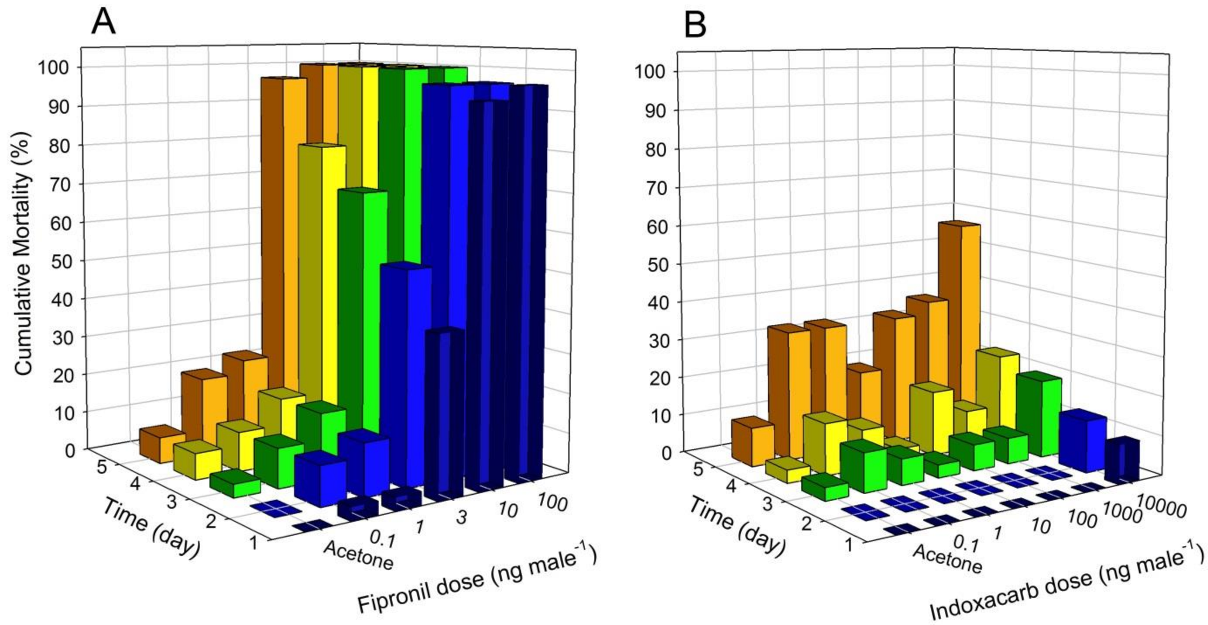

Figure S5. Daily mortality of adult male bed bugs (Harold Harlan strain) over 5 days after topical applications of various concentrations of (A) Fipronil and (B) Indoxacarb.