ABSTRACT

GONZALEZ-MORALES, MARIA ANGELICA. Insecticide Resistance in The German Cockroach and Common Bed Bug and Evaluation of Ectoparasitic Drugs to Control Bed Bugs in Poultry Farms (Under the direction of Dr. Coby Schal).

Bed bugs and cockroaches are medically and economically significant pests with detrimental effects on human health. The goal of eradicating these pests in residential settings is practically hindered by operational constraints and insecticide resistance. We aimed to understand temporal trends and mechanisms of insecticide resistance and evaluate potential solutions for challenging residential and farm environments.

Bed bugs collected from infestations on a global scale have been found to be resistant to major active ingredients. Therefore, it is critical to develop new active ingredients with different modes of action against bed bugs. In the first study, we evaluated the efficacy of fipronil, a broad-spectrum active ingredient not labeled for bed bug use, in recently field-collected populations from North America and Europe. We also evaluated the potential for insecticide resistance and mechanisms involved. Highly variable and high levels of resistance were found in bed bug (*Cimex lectularius*) populations from both continents, ranging from 1.4- to >985-fold. We evaluated metabolic resistance mechanisms involving specific classes of detoxifying enzymes using synergists, and target-site insensitivity was assessed by sequencing a fragment of the *Rdl* gene to detect a mutation that results in the A302S amino acid substitution. Enzymes play an important role in fipronil resistance in *C. lectularius*. However, we did not detect the *Rdl* mutation in any of the strains. We thus report substantial fipronil resistance for the first time in bed bugs. We discuss the importance of understanding routes of exposure of bed bugs to insecticides not labeled for their control and how this information can be used to improve integrated pest management.

Similarly, insecticide resistance has been reported worldwide in the German cockroach (*Blattella germanica*) to a variety of active ingredients with multiple modes of action. Fipronil, has been available in the market for >20 years for indoor use and although it is extensively used in cockroach bait formulations, the highest resistance level reported is 38-fold. Therefore, we evaluated the current status of fipronil resistance in the German cockroach in five field-collected populations. Resistance ratios ranged from 22.4 to 37.4, indicating only marginal changes in resistance over two decades. We assessed the roles of detoxification enzymes in fipronil resistance and sequenced a fragment of the *Rdl* gene to assess target site insensitivity as a resistance mechanism. Detoxifying enzymes appear to play a minimal role in fipronil resistance, and all field-collected strains were found to be homozygous for a mutation in the *Rdl* gene (A302S), that confers resistance to fipronil. Insights on the mechanisms by which cockroaches can rapidly evolve high levels of resistance to some insecticides and not others, despite intensive selection pressure, will provide to more efficacious pest management techniques.

Indoor pests are notorious for the harm and nuisance they can cause in residential settings. However, the same pests can invade animal production settings, where their control is much more challenging. Bed bugs have become an important ectoparasite in poultry farms. We evaluated two commonly used ectoparasitic veterinary drugs on bed bugs. Using an artificial feeding system, we conducted dose-response experiments with ivermectin and fluralaner. Both drugs caused high mortality in bed bugs from an insecticide-susceptible reference strain. Fluralaner was also tested and found to be highly effective on in five field-collected bed bug strains. Fluralaner was also highly effective at killing bed bugs that fed on medicated chickens. This is the first post-DDT study to report quantitative data in support of an alternative approach using systemic veterinary drugs to control bed bug infestations in poultry. Findings from this

research also validate the efficacy of a new group of active ingredients, the isoxazolines, on bed bugs.

Insecticide Resistance in The German Cockroach and Common Bed Bug and Evaluation of Ectoparasitic Drugs to Control Bed Bugs in Poultry Farms

by
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DEDICATION

To my husband, Kevin Sullivan, for all the love, support, and patience. Thank you for always listening to me talk about insects for infinite hours, for believing in me unconditionally and for encouraging me to give my best and never give up.

Special appreciation to my mom, family and friends, my beloved island, and its people. To my pets, Phillip and Atlas, for the unconditional love. In the memory of, Milagros Gonzalez and Hector Ortiz, I hope this makes you proud in heaven.

BIOGRAPHY

María A. González Morales was born in Humacao, Puerto Rico and grew up in Maunabo, Puerto Rico, a small rural town. She attended University of Puerto Rico at Mayaguez, PR where she earned her B.S in Agronomy in Crop Protection. After graduating her B.S., her interest in insects led her to pursue a M.S. in Agricultural Biology in Entomology at New Mexico State University, in Las Cruces, NM. She evaluated the effect of synergists on deltamethrin resistance in the common bed bug. Afterwards, her interest in insecticide resistance and urban entomology drove her to pursue a Ph.D. in Entomology, with a focus on insecticide resistance and novel control techniques to control challenging urban pests.

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CHAPTER 1

Introduction

Urban pests

Modern human civilization has witnessed important changes in climate, ecology, urbanization, and residential construction that have favored a closer coexistence with arthropods (Kotze et al. 2011). Rapid urbanization, changes in construction practices, and extensive global travel have contributed to the closer association and pervasiveness of pests adapted for indoor life (Gratz 1999). In the indoors environment, insects are considered pests when they become a nuisance, affect the health or residents and pets, or inflict aesthetic or economic damage to the human environment. Thus, insects in human environments can be the causal agents of structural damages, vector-borne diseases such as malaria and dengue, mechanical transmission of pathogens such as *Salmonella*, consumption and contamination of food and many other physical and mental health impacts (Gratz 1999).

In the US, the German cockroach (*Blattella germanica*, Blattodea) and the common bed bug (*Cimex lectularius*, Hemiptera) are the two most challenging and expensive indoor insect pests to control. Although bed bug and cockroach infestations can occur in all human environments, independent of social status, ethnicity, or sanitation, both pests are more prevalent in low-income communities, largely because of limited resources and ineffective pest control (Wang et al. 2008, Wang et al. 2016). Higher prevalence of these pests in disadvantaged communities contributes to new and preexisting health risks, exacerbates economic disparities, and can perpetuate social discrimination and stigmas associated with poverty.

The common bed bug

Cimex lectularius is an obligate hematophagous flightless insect that belongs to the order Hemiptera, Family Cimicidae. Bed bugs are small (about 5 mm long), oval and flattened

ectoparasitic insects with cryptic and photophobic behaviors that entirely depend on their hosts for survival, development, and reproduction at all mobile life stages (Usinger 1966). Hosts are exclusively warm-blooded animals, mainly humans, poultry, and bats. Although bed bug re-emergence in Europe was reported earlier, resurgence in the US began in the late 1990s and it has been attributed to increased global commerce and travel, changes in pesticide regulation, misuse of pesticides, and development of insecticide resistance (Doggett et al. 2018). Since the early resurgence, bed bug infestations have become widespread, reported in health care facilities, laundromats, theaters, public transportation, housing, college dormitories, and various other environments that humans frequent throughout all 50 US states (Potter et al. 2008). More recently, heavy bed bug infestations have been reported in poultry farms, reminiscent of infestations reported in the 1940s, before the extensive use of organochlorine, organophosphate, and carbamate insecticides (Axtell 1985).

Although bed bugs are not known to transmit any diseases, their feeding habits can inflict significant health impacts. Bed bug bites can cause skin reactions, itchiness, and skin lesions in humans. In fact, about 70% of people that experience bed bug bites have reported at least some skin reaction (Reinhardt et al. 2009, Potter et al. 2010). In more severe cases, infestations can result in anemia and secondary infections caused by skin damage caused by the bites. The presence of bed bugs can inflict mental stress in people that suffer from infestations in their dwellings, potentially causing insomnia, anxiety, depression and delusory parasitosis (Goddard and deShazo 2009). The impacts of bed bugs in other hosts such as poultry or companion animals have not been clearly documented, although even greater impacts can be expected because bed bug populations can reach very high levels. Moreover, bed bugs void large amounts of histamine in their feces (DeVries et al. 2018, Kakumanu et al. 2020), and their role in

respiratory disease and asthma in humans is yet to be determined.

The German cockroach

Blattella germanica is exclusively associated with human-built structures and human food or waste (Schal 2011). German cockroaches are omnivorous at all mobile life stages and generally nocturnal and cryptic. *B. germanica* is a perennial pest considered to be the most abundant, prevalent, and globally widespread indoor insect pest (Lee and Wang 2021). Its worldwide spread is largely attributed to early global trade and more recent global travel (Tang et al. 2019). Infestations of German cockroach are important from a public health and medical perspective. They are causal agents of numerous health problems including allergies, vectors of pathogens like *Salmonella* and antibiotic resistant microbes, and are known to sensitize and trigger asthma in children. Moreover, infestations are also associated with psychological distress and social stigma, especially in lower socioeconomic households (Schal and DeVries 2021).

Challenges in pest eradication

Both bed bugs and the German cockroach are considered the hardest pests to eradicate in the US. Behavioral traits such as cryptic and photophobic behaviors and high reproduction rates contribute to failure to control infestations. Over several decades, indoor pest control has shifted from broadcast applications of residual insecticides to more targeted baseboard sprays, and ultimately to applications of residual insecticides directly into cracks and crevices where these insects aggregate. In the past two decades, baits have been shown to be far more effective in controlling German cockroach infestations (Appel and Rust 2021). To date, there is no bait or systemic drug designed to manage bed bug populations. Control programs for both pest species

typically involve a combination of practices including intense monitoring, sanitation, effective insecticide formulations, inorganic dusts, and in bed bugs the use of heat and mattress encasements. However, some of these approaches may be unaffordable (e.g., heat), or ineffective due to insecticide resistance. Moreover, their establishment and proliferation in complex and sensitive environments such as hospitals and animal production facilities (e.g., poultry and swine houses) can pose completely new challenges to efforts to eradicate infestations. The proper implementation of comprehensive Integrated Pest Management programs has been effective in residential settings. However, the philosophy and practice of pest eradication have not been broadly adopted in the pest management industry.

Factors contributing to failure to eradicate pests in the indoor environment include the overuse of insecticide formulations that can rapidly select for insecticide resistance in genetically closed populations (Mallet 1989, Rust et al. 1995), including adaptive behavioral traits such as glucose aversion in cockroaches (Wada-Katsumata et al. 2013) and the limited availability of active ingredients with different modes of action. Thus, populations of bed bugs and the German cockroach on a global scale have evolved resistance to various classes of insecticides (Romero 2018, Scharf and Gondhalekar 2021). In recent years, for example, the overuse of pyrethroids and neonicotinoids has led to high levels of resistance reported worldwide in both pest species (Romero 2018, Scharf and Gondhalekar 2021). Moreover, exposure over 70 years to successive classes of neurotoxic insecticides – organochlorines, carbamates, and organophosphates – has selected for resistance to most of these compounds in both species (Romero 2018, Scharf and Gondhalekar 2021).

Insecticide resistance mechanisms

Multiple mechanisms are responsible of insecticide resistance in arthropods. Altered behaviors, reduced penetration, increased sequestration, metabolic detoxifying enzymes, and target site insensitivity are among the most common mechanisms. However, metabolic and target site alterations are the most widely reported mechanisms in bed bugs and cockroaches (Romero 2018, Scharf and Gondhalekar 2021).

Metabolic resistance involves changes in the catalytic activities of detoxifying enzymes or upregulation of their expression levels (Panini et al. 2016). Cytochrome P450 monooxygenases (P450s), estereases, and glutathione S-transferases (GSTs) have been shown to underlie resistance to various insecticides in bed bugs (Romero et al. 2009, Lilly et al. 2016, Gonzalez-Morales and Romero 2019) and the German cockroach (Scharf and Gondhalekar 2021). The involvement of these enzymes can be explored *in vivo* with specific enzyme inhibitors such as piperonyl butoxide (PBO) that inhibits P450s and ESTs (Bergé et al. 1998), triphenyl phosphate (TPP) and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF), which inhibit the activity of ESTs (Plapp Jr et al. 1963) and diethyl maleate (DEM), an inhibitor of GSTs (Motoyama and Dauterman 1974).

Target site insensitivity results from modifications in the active binding site of insecticides, reducing the binding efficacy of the insecticide, thus reducing toxicity to the targeted insect (Ffrench-Constant 1999). For example, nucleotide substitutions in specific genes, such as *kdr*, result in amino acid substitutions that reduce the affinity of the insecticide to the voltage-gated sodium channel; such single nucleotide polymorphisms (SNPs) have been shown to confer pyrethroid resistance in a wide range of insects, including the common bed bug (Dang et al. 2015, Romero 2018) and German cockroach (Dong 1997, Scharf and Gondhalekar 2021).

Mutations in the target site are often evaluated by PCR-based sequencing target DNA fragments to identify SNPs and determine their homozygosity or heterozygosity.

Focus of the Dissertation

Despite extensive knowledge of the biology and management of bed bugs and cockroaches, emergence of novel formulations and technologies to control them, and demonstrations of outstanding efficacy in the field, a substantial gap remains between commercial efforts to eradicate infestations and the efficacy of such efforts. This gap can largely be attributed to (a) the low economic margins of pest control, especially in multi-family low-income buildings; (b) extensive use of residual spray formulations despite demonstrated resistance to them; (c) lack of formulations that uniquely target bed bugs, as baits do with cockroaches; and (d) poor translation of timely research findings to the pest management industry.

Each chapter of this dissertation is designed to contribute to closing the knowledge gap between insecticide resistance and practical pest control, as well as to identify, validate, and demonstrate unique tools to control cockroaches and bed bugs in challenging environments. The fundamental research questions addressed in this dissertation are translated to applicable solutions. Overall, in this dissertation I investigate the efficacy of fipronil, a common and highly effective active ingredient, quantify fipronil and pyrethroid resistance, and identify resistance mechanisms in the common bed bug and German cockroach. Fipronil is a broad-spectrum neurotoxic insecticide that belongs to the phenylpyrazole class that is commonly used in and around structures and has proven to be effective against cockroaches (Kaakeh et al. 1997), ants (Hooper-Bui and Rust 2000, Wiltz et al. 2010), and termites (Vargo and Parman 2012). Fipronil is also a commonly used veterinary product to protect dogs and cats from ectoparasites (Dryden

et al. 2000). Resistance to fipronil in many arthropods involves both metabolic mechanisms and target-site mutations. Fipronil acts as an antagonist for the gamma-aminobutyric acid (GABA) receptor that mediates synaptic inhibition in the insect central nervous system (Caboni et al. 2003). It also blocks the glutamate-activated chloride channels, which are unique invertebrate channels involved in locomotion, feeding, and sensory input (Zhao et al. 2004, Narahashi et al. 2010). The GABA-gated chloride channel, encoded by the *Rdl* (Resistant to dieldrin) gene, is also the target of cyclodiene insecticides (Ghiasuddin and SM 1982, Ffrench-Constant 1999). Substitutions at the A302S/G site confer high resistance to dieldrin in various insect species, and generally limited resistance to fipronil (Zhao et al. 2004, Nakao 2017). Fipronil resistance has been detected in the German cockroach in the late 1990s (Holbrook et al. 2003) and both metabolic mechanisms and the *Rdl* mutation A302S apparently contribute to fipronil and dieldrin resistance in this species (Hansen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013). Sierras and Schal (Sierras and Schal 2017), showed that fipronil was highly effective on a reference insecticide-susceptible bed bug strain by both ingestion and topical application. However, the efficacy of fipronil in field-collected populations remains unknown.

Another goal of this research was to investigate the efficacy of systemic veterinary drugs on bed bugs, and to quantify the time-course of their efficacy in chickens. A major constraint in bed bug control in poultry houses is the limited availability of insecticides, and resistance to most of the commonly used insecticides. Moreover, the use of residual insecticides is restricted by the production cycle and chicken health. The systemic treatment of animal hosts is widely used in veterinary and animal production settings to kill parasites and ectoparasites, such as ticks and fleas. Studies that supplemented blood in artificial feeders with insecticides, have demonstrated considerable mortality in the common bed bug; effective active ingredients

include the conventional insecticides abamectin and fipronil (Sierras and Schal 2017), ivermectin and moxidectin (Sheele et al. 2020) and fluralaner (Sheele 2020).

Therefore, we evaluated two commonly used ectoparasitic drugs, ivermectin and fluralaner. Ivermectin is commonly known as the “wonder drug”, it is remarkably safe and commonly used in humans to control parasitic infections transmitted by mosquitoes (e.g., lymphatic filariasis), reduce malaria transmission, and to treat scabies, onchocerciasis and myasis (Ashour 2019). Currently, ivermectin-based formulations are available to control some ectoparasites and endoparasites in chickens (Whitehead and Roberts 2014). Fluralaner is a relatively new drug, introduced to the market in 2014 as a flea treatment for dogs and in 2019 for cats. Several studies have evaluated the efficacy of fluralaner administered to hens to control the poultry red mite (Brauneis et al. 2017, Thomas et al. 2017) and the northern fowl mite (Hinkle et al. 2018). Currently, there are no formulations of fluralaner registered for use in poultry in North America. However, in Europe, fluralaner is approved for the use in the poultry industry to control mites. The ultimate goal of this project is to implement the use of systemic ectoparasitic drugs in chickens to target bed bug infestations with new insecticid

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CHAPTER 2

Resistance to Fipronil in the Common Bed Bug, *Cimex lectularius* L. (Hemiptera: Cimicidae)

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Abstract

Cimex lectularius L. populations have been documented worldwide to be resistant to pyrethroids and neonicotinoids, insecticides that have been widely used to control bed bugs. There is an urgent need to discover new active ingredients with different modes of action to control bed bug populations. Fipronil, a phenylpyrazole that targets the GABA receptor, has been shown to be highly effective on bed bugs. However, because fipronil shares the same target site with dieldrin, we investigated the potential of fipronil resistance in bed bugs. Resistance ratios in eight North American populations and one European population ranged from 1.4 to >985-fold, with highly resistant populations on both continents. We evaluated metabolic resistance mechanisms mediated by cytochrome P450s, esterases, carboxylesterases and glutathione *S*-transferases using synergists and a combination of synergists. All four detoxification enzyme classes play significant but variable roles in bed bug resistance to fipronil. Suppression of P450s and esterases with synergists eliminated resistance to fipronil in highly resistant bed bugs. Target site insensitivity was evaluated by sequencing a fragment of the *rdl* gene to detect the A302S mutation, known to confer resistance to dieldrin and fipronil in other species. All nine populations were homozygous for the wild-type genotype (susceptible phenotype). Highly resistant populations were also highly resistant to deltamethrin, suggesting that metabolic enzymes that are responsible for pyrethroid detoxification might also metabolize fipronil. It is imperative to understand the origins of fipronil resistance in the development or adoption of new active ingredients and implementation of integrated pest management programs.

Key words: Bed bugs, *Cimex*, fipronil, insecticide resistance

Introduction

Over the past two decades, the common bed bug, Cimex lectularius L. (Hemiptera: Cimicidae), has re-established as a perennial indoor pest, causing global public health concerns (Boase 2001, Doggett et al. 2004, Potter 2006, Masetti and Bruschi 2007, Levy Bencheton et al. 2011, Doggett et al. 2018). Although bed bugs are not known to transmit any diseases to humans, their presence in homes is a nuisance, their bites can lead to secondary infections, and significant psychological stress can be associated with bed bug infestations (Doggett et al. 2018). Bed bugs also pollute the indoor environment with biocontaminants and they can adversely alter the indoor microbiota (DeVries 2018, Kakumanu et al. 2020). Eradication of bed bug infestations is particularly difficult and costly because they are cryptic, shelter on surfaces that often cannot be treated with insecticides, and multiple insecticide treatments may be required to eradicate bed bug populations (Lee et al. 2018). Moreover, a limited list of labelled insecticides and pervasive resistance to them has hampered effective management of bed bug infestations (Romero 2018).

Comprehensive approaches to controlling bed bugs include mattress encasements, trapping, vacuuming, spot and spatial heat treatments, inorganic dusts, and chemical treatments with pyrethroids, neonicotinoids and pyrroles (Lee et al. 2018). Resistance to pyrethroids has been reported worldwide, whereas resistance to neonicotinoids appears to be increasing, mainly in the U.S. (Romero 2018). Multiple resistance mechanisms to pyrethroid insecticides have been reported in *C. lectularius*, including various metabolic mechanisms involving cytochrome P450 monooxygenases (P450s), glutathione *S*-tranferases (GSTs), carboxylesterases (CESTs) and esterases (ESTs) (Adelman et al. 2011, Bai et al. 2011), target site insensitivity (e.g., knockdown resistance *kdr*) (Yoon et al. 2008) and reduced cuticular penetration (Koganemaru et al. 2013).

Metabolic resistance often involves the upregulation of detoxification enzymes, which can be functionally detected *in vivo* with enzyme inhibitors that synergize the activity of the insecticide. For example, piperonyl butoxide (PBO) inhibits P450s and ESTs (Bergé et al. 1998). Likewise, triphenyl phosphate (TPP) inhibits CESTs and *S,S,S*-tributyl phosphorotrithioate (DEF) inhibits the activity of ESTs (Plapp Jr et al. 1963), and diethyl maleate (DEM) is an inhibitor of GSTs (Motoyama and Dauterman 1974). Studies with bed bugs have shown that PBO (Romero et al. 2009, Lilly et al. 2016, Gonzalez-Morales and Romero 2019), as well as DEM, DEF, and TPP (Gonzalez-Morales and Romero 2019) can partially overcome pyrethroid resistance. Target site insensitivity results from alterations in the active binding site of insecticides, reducing the binding efficiency of the insecticide and thus reducing mortality (Ffrench-Constant 1999).

Fipronil is a broad-spectrum phenylpyrazole insecticide that is commonly used in and around structures to control cockroaches (Kaakeh et al. 1997), ants (Hooper-Bui and Rust 2000, Wiltz et al. 2010) and termites (Vargo and Parman 2012). Fipronil is also used in veterinary products to protect dogs and cats from ectoparasites (Dryden et al. 2000). Resistance to fipronil may involve both metabolic mechanisms and target site mutations. Fipronil acts as an non-competitive antagonist on the gamma-amino butyric acid (GABA) receptor that mediates synaptic inhibition in the insect central nervous system (Caboni et al. 2003) and it blocks glutamate-activated chloride channels that are involved in locomotion, feeding and sensory input (Zhao et al. 2004, Narahashi et al. 2010). The GABA-gated chloride channel, encoded by the *Rdl* (Resistant to dieldrin) gene, is also the target of cyclodiene insecticides (Ghiasuddin and Matsumura 1982, Ffrench-Constant et al. 1991). Substitutions of a conserved alanine residue with serine or glycine (A302S/G) confer high resistance to dieldrin in various insect species, and

generally limited resistance to fipronil (Zhao et al. 2003, Nakao 2017). However, the magnitude of the cross-resistance to phenylpyrazoles varies across species and even across populations of the same species, possibly related to mutations at other sites in the *Rdl* gene, as documented for planthoppers (Garrood et al. 2017). Fipronil resistance has been detected in the German cockroach (*Blattella germanica* L. (Blattodea: Ectobiidae)) (Holbrook et al. 2003) and both metabolic mechanisms and the *Rdl* mutation A302S apparently contribute to fipronil and dieldrin resistance in this species (Hansen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013).

The need for new active ingredients with different modes of action to eradicate bed bug infestations prompted us to investigate the efficacy of fipronil. Sierras and Schal (2017) showed that fipronil was highly effective on an insecticide-susceptible laboratory-reared bed bug population by both ingestion and topical application. However, because dieldrin was historically used to control bed bugs (*C. lectularius* and *Cimex hemipterus* (Hemiptera: Cimicidae)) and resistance to dieldrin had been documented (Armstrong et al. 1962, Gaaboub 1971, Lilly 2017), it is important to screen fipronil against recently collected populations of bed bugs. In this report, we screened nine bed bug populations for resistance to fipronil, evaluated the effects of inhibitors of detoxifying enzymes as potential fipronil synergists, determined the importance of detoxifying enzymes in fipronil resistance and screened bed bugs for target site mutations that might confer insensitivity to fipronil.

Materials and Methods

Experimental Insects

We screened nine field-collected *C. lectularius* populations and one standard insecticide-susceptible population (**Table 1**). The susceptible population (Harlan Harold = Harlan) was collected at Ft. Dix, NJ in 1973, and maintained in the laboratory thereafter. Since its collection,

the Harlan population has not been challenged with insecticides, and therefore it was used in this study as an insecticide susceptible reference strain. Since December 2008, this strain (Harlan-NCSU) has been fed defibrinated rabbit blood (below). To test for potential inadvertent exposure of this colony to fipronil in rabbit blood, we also tested the same Harlan strain that was maintained solely on human blood by Regine and Gerhard Gries at Simon Fraser University (Harlan-SFU).

Bed bug colonies were reared in 118 cm^3 plastic jars with cardstock paper substrate at 25°C, 50 ± 5% relative humidity (RH), and a photoperiod of 12:12 (L:D) h. Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) delivered through an artificial feeding system modified after Montes et al. (2002), as described in Sierras and Schal (2017). It consisted of a heated water bath (blood heated to 35°C) circulating through a series of water-jacketed custom-fabricated glass feeders. We used stretched plant grafting tape (A.M. Leonard Horticultural Tool and Supply Co., Piqua, OH) to hold the blood within each feeder. Healthy adult males were separated from the colony after feeding and tested 4 d post-feeding.

Fipronil and Deltamethrin Resistance

Fipronil ((RS)-5-Amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)pyrazole-3-carbonitrile; CAS 120068-37-3), 88.7% purity, was obtained from Sigma-Aldrich Co. (St. Louis, MO). The lethal dose of fipronil that killed 50% of each population (LD_{50}) was determined by topical application. Healthy adult male bed bugs of unknown ages, 4 d post-feeding, were placed in plastic Petri dishes (d = 60 mm, Thermo Fisher Scientific, Waltham, MA) lined with filter paper (Whatman No. 1, Sigma-Aldrich) and briefly anesthetized with CO_2. Topical applications of fipronil in acetone were made with a

microapplicator (Hamilton Co., Reno, NV) equipped with a 25 µl glass syringe (Hamilton Co.) that delivered 0.5 µl of solution on the ventral thorax of each bed bug. Fipronil concentrations ranged from 0 (acetone control) to 20 µg in 0.5 µl acetone and varied by population tested. Mortality was assessed every 24 h for 96 h by gently touching individual bed bugs with entomological forceps, categorizing them as alive (coordinated avoidance movement) or dead (no response or unable to right themselves after touching with forceps). Three replicates of 10–15 adult male bed bugs were performed per dose.

Deltamethrin ([(*S*)-cyano-(3-phenoxyphenyl)methyl] (1*R*,3*R*)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate; CAS 52918-63-5), 98.9% purity, was obtained from Chem Services (West Chester, PA). We conducted a dose-response study with the Harlan-NCSU strain, as above, to estimate the LD_{99} dose. We used seven doses between 0.25 and 10 ng, 20 male bed bugs per dose, for a total of 160 bed bugs (including 20 in the acetone control treatment). Mortality was assessed 2 d post treatment. The LD_{99} was used as a diagnostic dose on eight of the nine field-collected populations.

Effect of Synergists on Fipronil Toxicity

The synergists we tested were: *S*,*S*,*S*-triphenyl phosphorotrithioate (DEF, 97.7%) (Chem Services), triphenyl phosphate (TPP, 99%), piperonyl butoxide (PBO, 99%), and diethyl maleate (DEM, 97%) (Sigma-Aldrich). We evaluated the effects of these detoxification enzyme inhibitors on fipronil resistance in four populations: The susceptible Harlan-NCSU population, two moderately resistant populations (Cincinnati and Winston Salem), and a highly resistant population (Shanda). Bed bugs were topically treated with 50 µg of PBO, DEF, DEM or TPP in 0.5 µl acetone, based on previous reports of pyrethroid synergism (Romero et al. 2009, Lilly et al. 2016). After the bed bugs recovered at room temperature for 2 h, they were briefly

anesthetized again with CO_2 and topically treated with either acetone alone (control) or the population-specific LD_{50} for each bed bug population. Three replicates of 10 adult male bed bugs were performed for each population-synergist combination. Mortality was assessed every 24 h for 96 h, as described above.

Relative Importance of Detoxifying Enzymes in Fipronil Resistance

We compared the most resistant population, Shanda, to the Harlan-NCSU insecticide-susceptible population to understand the relative importance of metabolic detoxification of fipronil. Because limited numbers of test insects were available, we did not conduct dose-response studies with the synergists. Instead, we topically treated adult males with 50 µg PBO and 2 h later they received an application of the Harlan-specific fipronil LD_{50} dose (20.3 ng/male). Two to five replicates of 10–25 adult male bed bugs per replicate (30–55 total per treatment) were performed and compared to responses of the susceptible population (Harlan-NCSU) to the same treatments. Mortality was assessed every 24 h for 96 h.

Bed bugs from the Shanda population were also treated with a mix of the two most effective inhibitors, PBO and DEF, to determine whether inhibiting the detoxifying enzymes could eliminate resistance in these highly resistant bed bugs. First, an application of 50 µg of PBO was made and then, 5 min later, it was followed by a second application of 50 µg of DEF. After the bed bugs recovered (2 h), we delivered by topical application either acetone alone (control) or the Harlan-NCSU population-specific fipronil LD_{50} (20.3 ng/male). Three replicates of 10 adult male bed bugs were performed for each treatment group. Mortality was assessed every 24 h for 96 h, as described above.

Rdl Mutation Detection

Ten bed bugs from each population were screened for the *Rdl* mutation A302S. Genomic DNA was extracted with the DNeasy Blood & Tissue extraction kit (Cat. 69506, Qiagen, Germantown, MD). The head and thorax of each bed bug were homogenized for 30 s with glass beads in a FastPrep 24 5G homogenizer (MP Biomedicals, Solon, OH), to which we added 180 µl of ATL solution and 20 µl of proteinase-K and incubated it for 4 h at 56°C. The rest of the protocol followed the manufacturer's instructions. DNA was eluted in 50 µl sterile nuclease-free H_2O and stored at -20°C until further use.

A 245-bp genomic fragment of the GABA receptor gene that includes the A302S mutation site was amplified with a primer pair designed for this study. The primers were CL-Rdl-F (5′-GTGCGATCCATGGGCTACTA -3′) and CL-Rdl-R (5′-AGAGATGCGAAGACCATGAC -3′). The PCR reactions were conducted in a 20 µl reaction mix comprising 10 µl of AmpliTaq Gold 360 2X Master mix (Cat. 4398881, Applied Biosystems, Thermo Fisher), 1 µl of 10 µM of each primer, 0.2 µl BSA (20 mg/ml) and 2 µl of bed bug genomic DNA as template for the PCR reaction. A negative control with no template DNA was included in every PCR run. The following thermal cycle program was used for amplification: Initial activation at 95°C for 10 min followed by 35 cycles of 94°C for 30 s, 58.4°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. Each PCR product was verified by running 2 µl on 1.2% agarose gel. The remaining PCR product was ExoSAP-IT-purified (Applied Biosystems, Foster City, CA) and direct sequenced at the Genomic Sequencing Laboratory (North Carolina State University, Raleigh, NC) with CL-*Rdl*-R as sequencing primer. Each sequence was determined by manually checking for the GCC to TCC mutation that results in the A302S substitution.

Statistical Analysis

The LD_{50} for each bed bug population was determined using log-dose probit-mortality analysis in PoloPlus (LeOra Software Company, Petaluma, CA). The toxicity of deltamethrin for each population was estimated by applying to bed bugs the Harlan-NCSU LD_{99} as a diagnostic dose. The toxicity of fipronil to each population was compared relative to the susceptible Harlan population using a Resistance Ratio (RR_{50}), calculated as (LD_{50} resistant population)/(LD_{50} Harlan-NCSU population). We used the lethal dose ratio significance test: The 95% confidence limits (CL) of the RR_{50} were calculated, and if this confidence interval did not include the value of 1.0, then the RR_{50} was considered significant (Robertson et al. 2017). Abbott's correction (Abbott 1925) was used to correct for control mortality, as needed. The effects of various treatments, including synergists, on fipronil toxicity were determined using ANOVA and Tukey's HSD test (JMP 2020).

Results

Fipronil and Deltamethrin Resistance

We conducted topical application dose-response assays with technical fipronil applied to 10 populations. The LD_{50} values ranged over >3-orders of magnitude from 20.3 ng/male for the insecticide-susceptible Harlan-NCSU population, to >20 µg/male for the Beroun (Czech Republic) population (**Table 1, Fig. 1**). The resistance ratios (RR_{50}s) for the recently field-collected populations, relative to the Harlan-NCSU population, ranged from 1.44- to >985-fold. However, because the highest doses (10 µg and 20 µg per male) killed only 23% of the Shanda bed bugs and 42% of the Beroun bed bugs, respectively, their actual RR_{50} values were substantially higher than 985-fold, indicating extremely high resistance to fipronil in both

populations. We found moderate resistance to fipronil in the Fuller Miller (RR_{50} = 44.4-fold) and Winston Salem (RR_{50} = 65.7-fold) populations, with relatively shallow slopes of their dose-response curves, suggesting heterogeneous populations with individuals responding to fipronil over a broad range of concentrations. Lower resistance levels to fipronil were found in five populations, with RR_{50} values ranging from 1.4 to 10.7-fold. With the exception of the Lafayette population (RR_{50} = 1.4-fold), all field-collected populations were significantly resistant to fipronil compared to the susceptible Harlan population (**Table 1**).

The deltamethrin log dose-probit response study indicated that the LD_{50} was 1.431 ng (95% CI: 1.00, 2.14; total n = 140; slope = 2.54 ± 0.353 (SE); χ^2 = 5.80, df = 5; t-ratio = 7.193 (P < 0.05)). The estimated LD_{99} diagnostic dose of 11.79 ng (95% CI: 5.91, 51.46) was applied to six populations, and the percentage mortality (and n) are shown in **Table 1**. Interestingly, populations with relatively low resistance to fipronil experienced high mortality with deltamethrin, whereas there was no mortality with the LD_{99} diagnostic dose of deltamethrin in the two populations we tested that had the highest resistance to fipronil (Winston Salem and Shanda).

Metabolic Enzyme Inhibitors Synergize Fipronil Toxicity

We selected four bed bug populations for further studies with four detoxification enzyme inhibitors as potential fipronil synergists. For each population, topical applications of an inhibitor (or acetone-only control) were followed 2 h later by topical application of the population-specific LD_{50} dose of fipronil. Each of the enzyme inhibitors, alone, caused <10% mortality and the overall ANOVA for each population was significant (**Table 2**). Piperonyl butoxide (PBO), an inhibitor of P450s and esterases, significantly enhanced fipronil toxicity in all four populations that we screened, as the addition of PBO to the population-specific LD_{50} dose of fipronil

significantly increased mortality to 100% in the all four populations – Harlan-NCSU, Cincinnati, Winston Salem and Shanda (**Fig. 2**). *S,S,S*-trybutyl phosphorotrithioate (DEF), an esterase inhibitor, significantly increased mortality caused by the LD_{50} dose of fipronil in the two most resistant populations, Winston Salem and Shanda (**Fig. 2**). DEF was less effective on the less fipronil-resistant population, Cincinnati, and on the Harlan insecticide-susceptible population.

Triphenyl phosphate (TPP), a carboxylesterase inhibitor, also significantly synergized the mortality caused by the population-specific LD_{50} dose of fipronil in three of the four bed bug populations (Harlan-NCSU, Winston Salem, Shanda) (**Fig. 2**). Diethyl maleate (DEM), a GST inhibitor, was the least effective inhibitor of detoxification enzymes. It significantly synergized fipronil only in the Harlan-NCSU population, but DEM failed to significantly increase mortality in Cincinnati and Shanda bed bugs (**Fig. 2**). Curiously, in the Winston Salem population mortality significantly decreased when bed bugs were treated with DEM and then fipronil, suggesting an antagonistic interaction.

Fipronil and Deltamethrin Resistance

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Relative Importance of Metabolic Resistance

To assess the relative contribution of metabolic mechanisms to fipronil resistance in bed bugs, we treated bed bugs from the most resistant population, Shanda, with the Harlan-NCSU-specific fipronil LD_{50} dose (20.3 ng/male) in combination with either PBO or DEF, or a mix of PBO and DEF. The overall ANOVA for each population was significant (**Table 3**). The Harlan-NCSU-specific fipronil LD_{50} dose (20.3 ng/male) killed 63.0% ± 7.0 (SEM, n = 35) of the Harlan bed bugs and only 12.4% ± 9.0 (n = 34) of the Shanda bed bugs. Pre-treatment of bed bugs with PBO elevated mortality significantly in both populations: 100% mortality in Harlan bed bugs and

94.8% mortality in Shanda bed bugs (**Fig. 3**). DEF was effective on Harlan bed bugs, but less effective on Shanda bed bugs.

PBO was a highly effective synergist of fipronil, but pre-treatments with a combination of PBO and DEF further increased mortality in the Shanda population to 100%. Thus, suppression of major metabolic detoxification enzymes eliminated resistance to fipronil in highly resistant bed bugs.

Rdl Mutation

A 245 bp fragment of the GABA receptor gene targeting the *Rdl* mutation A302S was amplified and sequenced from the Harlan-NCSU population (n = 10) and the nine field-collected populations (n = 10 per population). Based on previous studies correlating fipronil resistance to this mutation, and the high level of resistance we detected in some populations, we expected to detect genotypes corresponding to homozygous susceptible wild-type (Ala302/Ala302; S/S), homozygous putatively resistant (Ser302/Ser302; R/R) and the heterozygous genotype (Ala302/Ser302; S/R). However, we did not find the A302S mutation in any of the 10 bed bug populations (**Table 4**). The sequences of the amplified GABA receptor gene fragment in all bed bugs exactly matched GenBank Accession number XM_014385500.2 (predicted *Cimex lectularius* gamma-aminobutyric acid receptor subunit beta (LOC106661780), transcript variant X18, mRNA). All 90-individual field-collected bed bugs were homozygous for the wild-type (susceptible) sequence (Ala302/Ala302; GCC/GCC), as were the 10 fipronil-susceptible Harlan-NCSU bed bugs (**Fig. 4**).

Synergism in Two Susceptible Bed Bug Sub-Populations

Two observations led us to evaluate the idea that the Harlan-NCSU might have been previously exposed to fipronil, possibly in rabbit blood, because fipronil is used as a veterinary ectoparasitic treatment (Dryden et al. 2000). First, fipronil was significantly synergized by all four enzyme inhibitors in the Harlan-NCSU population (**Figs. 2, 3**), suggesting detoxification enzyme activity in the fipronil-susceptible population. Second, the fipronil LD_{50} values in this study (20.3 ng/male) were nearly 10-fold higher than in a previous study (2.21 ng per adult male) (Sierras and Schal 2017). Therefore, we compared the Harlan-NCSU population, which was fed rabbit blood, to another lineage of the same original population, Harlan-SFU, which was fed exclusively on human blood. We found no difference in mortality between the two Harlan populations using the Harlan-NCSU LD_{50} dose of fipronil (**Fig. 5**). Moreover, the addition of PBO to the LD_{50} dose of fipronil (20.3 ng/male) increased mortality to 100% in both populations.

Discussion

Bed bug populations collected in the last 12 years from various parts of the U.S. and Europe had a wide range of resistance levels to fipronil. The LD_{50} level of the Lafayette population (IN) was not significantly different from that of the standard insecticide-susceptible Harlan population. At the other extreme, two recently collected populations, Shanda (NC) and Beroun (Czech Republic), were highly resistant to fipronil, beyond our ability to quantify their LD_{50}, suggesting that their RR_{50} was >985-fold relative to the Harlan population. To our knowledge, this is the first documentation of fipronil resistance in bed bugs. This finding is especially interesting and important because fipronil (a) is not labeled for any bed bug control products, (b) is unlikely to be used extensively in areas where bed bugs would commonly be found, and (c) its indoor use in Europe is even more restricted than in the U.S.

Causes of Resistance to Fipronil in Bed bugs

A fascinating conundrum emerges from our findings: How did such geographically diverse populations of *C. lectularius* presumably independently evolve high resistance to fipronil? We propose three non-mutually exclusive hypotheses. First, that resistance to fipronil could represent cross-resistance to previously used cyclodienes. Second, that contemporary use of fipronil or related compounds is selecting for fipronil resistance. A third hypothesis is that metabolic enzymes that are upregulated in response to other insecticides, such as pyrethroids, have broad substrate specificity and also detoxify fipronil.

Cross-Resistance to Cyclodienes. The first hypothesis, that resistance to fipronil represents a relic of cross-resistance to cyclodienes, was suggested to explain fipronil resistance in German cockroach populations before fipronil-containing products were introduced in the U.S. (Holbrook et al. 2003). Cyclodienes, such as dieldrin, are a class of organochlorine insecticides that were used extensively indoors in the 1940s through early 1980s. Bed bugs were selected by these treatments and some populations were shown to have evolved resistance to dieldrin by 1954 in Italy, 1958 in various countries in Asia (Busvine 1958, World Health Organization 1963) and by 1976 around the globe (World Health Organization 1976). The closely related tropical bed bug *Cimex hemipterus* (F.) also evolved resistance to dieldrin (Armstrong et al. 1962). Cyclodienes and fipronil share not only the same CNS target site, GABA-gated chloride channels, but also common detoxifying enzymes (Kristensen et al. 2004). Thus, it is plausible that modern bed bugs have retained the alleles that conferred resistance to dieldrin.

However, two lines of evidence might argue against this hypothesis. The dieldrin cross-resistance mechanism could be most effective if resistance to fipronil in *C. lectularius* bore little

fitness costs and was retained for decades after dieldrin use was discontinued, as discussed in other systems (Bass 2017). However, in the planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), for example, relaxing fipronil selection for only 15 generations reduced the LD_{50} from 166 to 9 µg/g body mass (Yang et al. 2014), suggesting that relatively high fitness costs are associated with fipronil resistance. Likewise, resistance to fipronil in most indoor and peridomestic arthropods that are directly exposed to fipronil appears to be constrained, possibly due to fitness costs. Despite heavy selection with fipronil, resistance ratios in field-collected cat fleas (*Ctenocephalides felis* (Bouché) (Siphonaptera: Pulicidae)) ranged from 0.5 to 2.2-fold (Rust et al. 2015), in the brown dog tick (*Rhipicephalus sanguineus* Latreille (Ixodida: Ixodidae) from 2.6 to 13.8-fold (Becker et al. 2019) and in the German cockroach resistance ratios ranged from an average of 17-fold (Holbrook et al. 2003) to 36.4-fold in a more recent study (Gondhalekar and Scharf 2012). These values suggest that substantial fitness costs constrain the evolution of high resistance to fipronil. Yet, most of our bed bug populations had much higher resistance to fipronil, suggesting that fipronil resistance does not inflict strong fitness costs in *C. lectularius*.

To our knowledge, this is the first investigation of *Rdl* mutations in the common bed bug. The A302S/G mutation in the *Rdl* locus is a relic of dieldrin selection in some insects (Ffrench-Constant et al. 1993). Multiple cockroach populations in the U.S. and Europe have been shown to have the A302S mutation in the *Rdl* locus (Hansen et al. 2005, Ang et al. 2013), consistent with previous selection with dieldrin and with a significant correlation between dieldrin resistance and low levels of fipronil resistance before fipronil was introduced for cockroach control (Holbrook et al. 2003). However, as noted above, cockroach resistance to fipronil is relatively low, ~30-fold, and the A302S mutation also appears to confer low fipronil resistance in

planthoppers (Nakao 2017), fleas (Rust et al. 2015) and flies (Gao et al. 2007). It thus appears that the substitution at the A302S substitution, by itself, confers low resistance or cross-resistance to fipronil (Garrood et al. 2017). However, all the bed bug populations we investigated did not have the *Rdl* mutation, suggesting little relationship to the dieldrin selection several decades ago. Moreover, the resistance levels of some U.S. and a European population were extremely high. This pattern suggests contemporary selection rather than a vestige of dieldrin selection. We hasten to note, however, that bed bugs might have evolved other mutations in the *Rdl* gene in response to dieldrin selection, and these might cause the high levels of fipronil resistance that we found. However, in other insects (e.g., planthoppers and flies), additional *Rdl* mutations appear to occur in tandem with the mutation in the 302 position.

Exposure to Fipronil in Ectoparasitic Products. The second hypothesis is that modern use of fipronil or related compounds is selecting for fipronil resistance. Fipronil-containing products are widely used to control cockroaches, termites, and ants. In all these cases, however, the areas where fipronil is applied are ecologically different from where bed bugs are usually found, as cockroach baits are rarely deployed in bedrooms and living rooms and little fipronil is translocated from bait applications (DeVries et al. 2019). Ants and termites are targeted with baits and soil treatments, respectively. However, fipronil is also used in ectoparasite control in veterinary products, targeting mainly fleas, lice, mosquitoes and ticks to protect dogs and cats. Although *C. lectularius* is most often associated with humans, they often associate with non-human hosts (e.g., bats, birds, chickens), they accept the blood of various vertebrates, including domesticated animals (e.g., chickens and dogs) (Usinger 1966, Axtell 1999, Beugnet et al. 2021), and a recent preliminary report detected cat DNA in a pool of two bed bug nymphs collected in a New Jersey apartment (Potts et al. 2021). Thus, bed bugs might be exposed to fipronil through

contact or feeding on dogs, cats or chickens treated with fipronil. This proposition can readily be tested with blood meal analysis of bed bugs in homes with pets.

Cross-Resistance to Other Insecticides, Such as Pyrethroids. Our third hypothesis is that selection with other insecticides resulted in upregulation of metabolic enzymes with broad substrate specificity that can also detoxify fipronil. In the four populations of bed bugs that we tested, PBO significantly synergized the activity of fipronil, indicating an overall importance of cytochrome P450s in metabolic resistance to fipronil in bed bugs. Likewise, esterases appear to be significant in fipronil detoxification in all four populations, as indicated by synergism of fipronil activity by both DEF and TPP. In our most resistant population, Shanda, the combination of a low fipronil dose (LD_{50} of the Harlan population, 20.3 ng/male) and the two most potent synergists PBO and DEF was able to eliminate metabolic resistance. These results suggest that fipronil resistance is mostly dependent on enhanced P450 and esterase enzymes. On the other hand, GSTs appear to not be prominently involved in fipronil resistance, because DEM synergized the activity of fipronil only in the Harlan population but not in any of the field-collected populations.

High pyrethroid resistance in bed bug populations has been reported to be significantly reduced by synergists, such as PBO (Romero et al. 2009, Lilly et al. 2016, Gonzalez-Morales and Romero 2019), DEF, DEM, and to a lesser extent TPP (Gonzalez-Morales and Romero 2019). Cross-resistance of pyrethroids and neonicotinoids, conferred by metabolic detoxifying enzymes, has been suggested for U.S. bed bug populations, where highly pyrethroid resistant populations were shown to be resistant to neonicotinoids before the latter were introduced in the U.S. market (Romero and Anderson 2016). Similarly, Liang et al. (2017) showed that exposure to fipronil increased cross-resistance of cockroaches to indoxacarb, an oxadiazine insecticide that blocks the

sodium channel. In the house fly *Musca domestica* L. (Diptera: Muscidae), permethrin selection in the laboratory dramatically elevated multi-insecticide resistance to various pyrethroids, an organophosphate, carbamate, neonicotinoid and fipronil (Liu and Yue 2000). Thus, exposure to various insecticides and possibly other household xenobiotics with different modes of action from fipronil, might select for metabolic enzymes that also detoxify fipronil, even in the absence of direct exposure to fipronil.

Screening six of the nine bed bug populations with deltamethrin provided support for this idea. The LD_{99} dose of deltamethrin, estimated from the Harlan-NCSU population, killed 88% of the least fipronil-resistant population (Lafayette), but none of the two most fipronil-resistant populations (Winston Salem and Shanda). It is important to note, however unlikely, that the apparent association between deltamethrin resistance and pyrethroid resistance could be the result of concurrent selection by fipronil and pyrethroids on the same populations that could independently select for resistance to both classes of insecticides. Transcriptome analysis should be able to identify specific detoxification enzymes (mainly P450s and esterases) that are upregulated in the most fipronil-resistant populations, and these enzymes should then be assessed for their substrate specificity with pyrethroids and fipronil.

Enzyme Inhibitors Synergize Fipronil in the Susceptible Population

We observed that fipronil was significantly synergized in the Harlan-NCSU insecticide-susceptible population by all four enzyme inhibitors, PBO, DEF, TPP and DEM. Moreover, the fipronil LD_{50} in *C. lectularius* males (20.3 ng/male or ~7.6 µg/g) was substantially higher than for insecticide-susceptible populations of other insect species (0.04 µg/g for *B. germanica* (Ko et al. 2016); 0.4 µg/g for cat flea assuming a body mass of 0.5 mg (Rust et al. 2014); 0.475 µg/g for *M. domestica*, assuming body mass of 12 mg (Liu and Yue 2000); 0.07 µg/g for *N. lugens* (Yang

et al. 2014)). It is possible that bed bugs are inherently less susceptible to fipronil than other insect species, perhaps related to less penetration or selectivity of the species-specific RDL receptor site. Alternatively, we considered that the Harlan-NCSU population might have been chronically exposed to fipronil in rabbit blood, because fipronil is a common ectoparasitic veterinary active ingredient. However, we rejected this idea by showing that the Harlan-NCSU population exhibited a similar fipronil dose-mortality relationship as the Harlan-SFU population that has been fed on human volunteers since it was collected in 1973. Nevertheless, it is possible that the Harlan population was exposed before it was collected in 1973 not only to cyclodienes, but also to carbamate and organophosphate insecticides, and low levels of fipronil cross-resistance might be related to exposure to a broader range of legacy insecticides.

Perspective

Recently, fipronil has been approved for use in residual sprays (0.65%, Fipronil-Plus-C, EPA Reg. No. 55431-15) for controlling a wide range of crawling insects indoors, including cockroaches. Direct exposure to fipronil is expected to select for higher fipronil resistance in bed bug populations. Moreover, pyrethroids continue to be used extensively to control bed bug populations, despite the high levels of resistance documented on a global scale. If pyrethroid resistance also confers fipronil cross-resistance, as suggested by our results, then resistance to fipronil is expected to increase and become more prevalent across populations, as documented for pyrethroids (Romero 2018). Nevertheless, combining the fipronil dose that killed 50% (LD_{50}) of the susceptible bed bugs with PBO and DEF eliminated fipronil resistance in the most resistant population. These results suggest that formulating fipronil with PBO and DEF should be explored to control pyrethroid and neonicotinoid resistant bed bug populations.

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Table 2.1. Fipronil dose-response assays, resistance ratios and deltamethrin LD_{99} percentage mortality of recently collected *C. lectularius* populations relative to an insecticide-susceptible (Harlan-NCSU) population

			Fipronil					Deltamethrin
Population, abbreviation (year collected)	Collection location	*n*	LD_{50} µg/male (95% CI)[b]	Slope ± SE	χ^2 (df)	*t*-ratio[c]	RR_{50}[d]	% mortality LD_{99} dose (*n*)[a]
Harlan-NCSU, HA (1973) (susceptible)	Fort Dix, NJ	124	0.0203 (0.0122–0.0310)	2.66 ± 0.62	0.5 (1)	4.40*	-	-
Lafayette, LAF (2009)	Lafayette, IN	184	0.0289 (0.0171–0.0451)	2.24 ± 0.30	3.3 (3)	7.39*	1.4	88 (40)
Campus Courtyard, CC (2009)	Raleigh, NC	137	0.0765 (0.0566–0.1009)	2.47 ± 0.38	0.0 (1)	6.33*	3.8*	0 (50)
Jersey City, JC (2008)	Jersey City, NJ	153	0.0880 (0.0252–0.1514)	1.28 ± 0.41	0.3 (2)	3.15*	4.4*	20 (50)
Cincinnati, CIN (2012)	Cincinnati, OH	119	0.1671 (0.1231–0.2013)	6.48 ± 2.15	0.7 (1)	4.64*	8.4*	13 (45)
Liberty, LIB (2017)	Liberty, NC	108	0.2147 (0.1017–0.3333)	2.15 ± 0.54	0.4 (1)	4.18*	10.7*	ND
Fuller Miller, FM (2017)	High Point, NC	224	0.8877 (0.4807–1.576)	1.00 ± 0.21	1.5 (3)	5.04*	44.4*	ND
Winston Salem, WS (2008)	Winston Salem, NC	161	1.314 (0.1703–2.648)	0.86 ± 0.26	0.6 (2)	3.33*	65.7*	0 (48)
Shanda, SHA (2017)	Raleigh, NC	195	>10 (23%)[e]	-	-		>492	0 (46)
Beroun, BER (2014)	Czech Republic	183	>20 (42%)[e]	-	-		>985	ND

[a] The LD_{99} dose of deltamethrin, determined with the Harlan-NCSU population, was applied in 0.5 µl acetone solution. Percentage mortality at 2 d and (*n*) are reported. ND = not determined.
[b] Adult male bed bugs were treated with 0.5 µl acetone solution containing fipronil. Lethal dose that killed 50% of the bed bugs (LD_{50}) was determined from probit analysis for each population.
[c] *t*-ratio of the slope. Values >1.96 denote a significant regression (* $P < 0.05$).
[d] Resistance Ratio (RR_{50}) was calculated as (LD_{50} resistant population)/(LD_{50} Harlan-NCSU population). RR values with (*) are considered significant when the 95% confidence interval does not include 1.0 (Robertson et al. 2017).
[e] Maximum mortality is indicated in parenthesis. LD_{50} could not be estimated and therefore a formal test of the lethal dose ratios (RR_{50}) could not be done

Table 2.2. Synergistic effects of four enzyme inhibitors, assayed with four *C. lectularius* populations[a]

Population	Source	DF	Sum of squares	Mean square	*F* ratio	Prob > *F*
Harlan-NCSU	Treatment	9	58336.7	6481.8	64.8185	**<0.0001**
	Error	20	2000.0	100.0		
	Total	29	60336.7			
Cincinnati	Treatment	9	43200.0	4800.0	16.3636	**<0.0001**
	Error	20	5866.7	293.3		
	Total	29	49066.7			
Winston Salem	Treatment	9	49950.0	5550.0	138.7500	**<0.0001**
	Error	20	800.0	40.0		
	Total	29	50750.0			
Shanda	Treatment	9	44830.0	4981.1	99.6222	**<0.0001**
	Error	20	1000.0	50.0		
	Total	29	45830.0			

[a] Four bed bug populations were assayed. Adult male bed bugs from each population were treated with 0.5 µl acetone solution in the following 10 treatments (see Fig. 2): acetone alone, DEM, TPP, DEF, PBO, fipronil at the population-specific dose that killed 50% of the bed bugs (LD_{50}), fipronil + DEM, fipronil + TPP, fipronil + DEF, and fipronil + PBO. Tukey's HSD tests for comparisons of treatments within each population are shown in Fig. 2.

Table 2.3. Synergistic effects of PBO, DEF, and a combination of both, assayed with two *C. lectularius* populations[a]

Population	Source	DF	Sum of squares	Mean square	*F* ratio	Prob > *F*
Harlan-NCSU	Treatment	7	47483.3	6783.3	172.4576	**<0.0001**
	Error	17	668.7	39.3		
	Total	24	48152.0			
Shanda	Treatment	7	38473.8	5496.3	92.5238	**<0.0001**
	Error	16	950.5	59.4		
	Total	23	39424.3			

[a] Two bed bug populations were assayed. Adult male bed bugs from each population were treated with 0.5 µl acetone solution in the following eight treatments (see Fig. 3): acetone alone, DEF, PBO, fipronil at the Harlan-specific LD_{50} dose (20.3 ng per male), fipronil + DEF, fipronil + PBO, and fipronil + PBO + DEF. Tukey's HSD tests for comparisons of treatments within each population are shown in Fig. 3.

Table 2.4. Frequency of *Rdl* mutations in fipronil-resistant and susceptible populations of *C. lectularius*

Population	RR_{50}[a]	*n*	No. of bed bugs		
			A302/A302 (S/S)	S302/S302 (R/R)	A302/S302 (S/R)
Harlan-NCSU	-	10	10	0	0
Lafayette	1.4	10	10	0	0
Courtyard	3.8	10	10	0	0
Jersey City	4.4	10	10	0	0
Cincinnati	8.4	10	10	0	0
Liberty	10.7	10	10	0	0
Fuller Miller	44.4	10	10	0	0
Winston Salem	65.7	10	10	0	0
Shanda	>492	10	10	0	0
Beroun	>985	10	10	0	0

[a] Values from Table 1

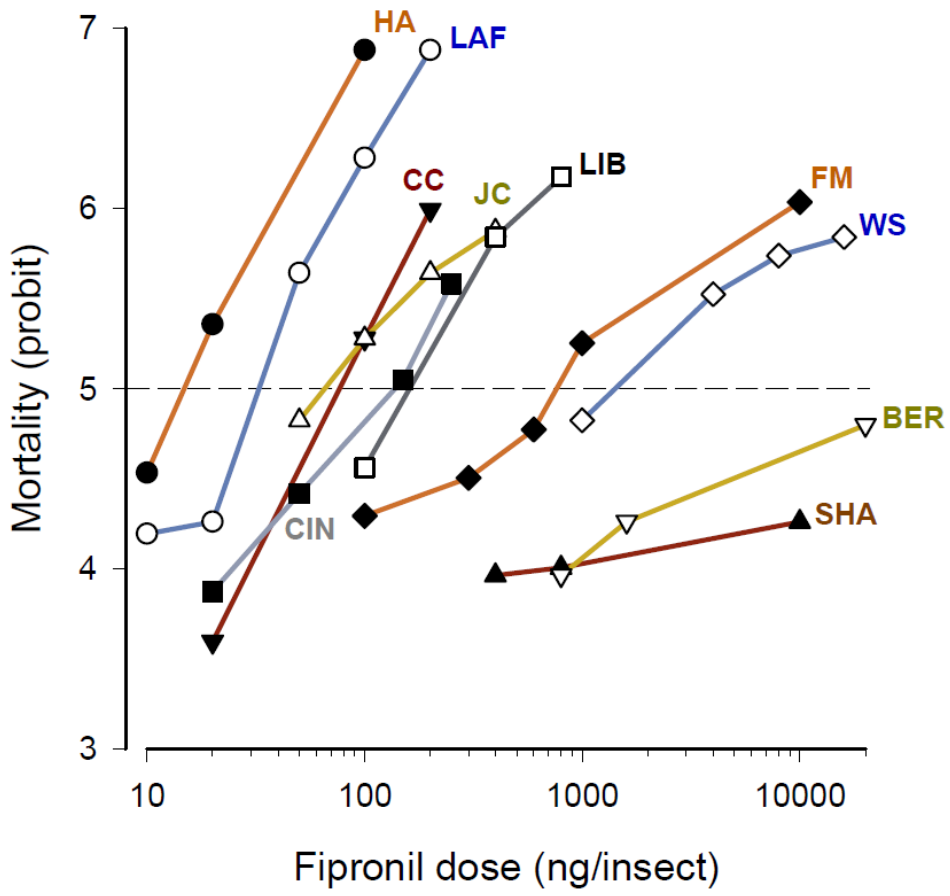

Fig. 2.1. Fipronil dose-response probit-transformed curves for *C. lectularius* adult males from 10 populations, including nine field-collected populations and our laboratory-reared population (Harlan-NCSU). Abbreviations are explained in **Table 1**. The lethal dose of fipronil that killed 50% of each population (LD_{50}) was determined by topical application. Fipronil concentrations ranged from 0 (acetone control) to 20 µg in 0.5 µl acetone and varied by population tested. Mortality was assessed every 24 h for 96 h, and mortality at 96 h is reported. Mortality in the control group, treated with acetone alone, was <5%. At least three replicates of 10 adult male bed bugs were performed per dose.

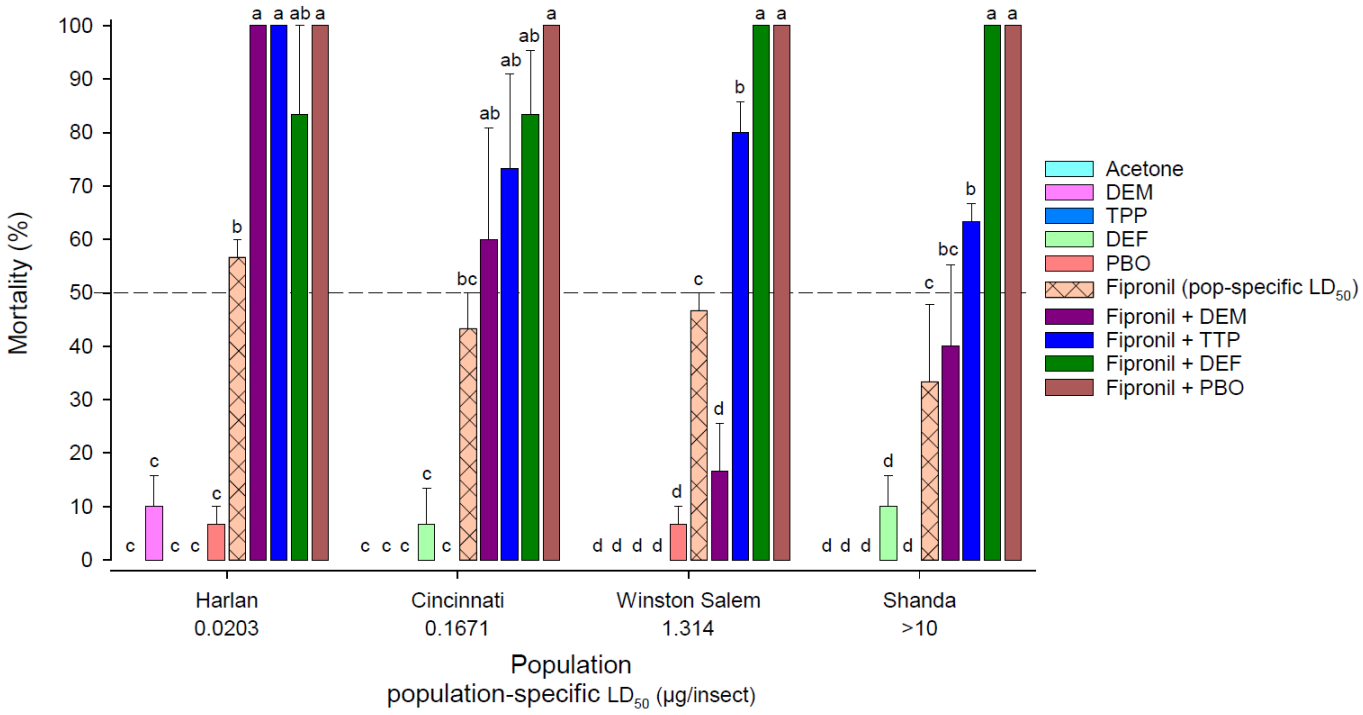

Fig. 2.2. Effects of four insecticide synergists on fipronil toxicity in *C. lectularius* adult males. Each synergist (piperonyl butoxide [PBO], *S*,*S*,*S*-tributyl phosphorothrithioate [DEF], diethyl maleate [DEM], and triphenyl phosphate [TPP]) was topically applied in 0.5 µl acetone 2 h prior to application of a population-specific LD_{50} dose, as shown on the X-axis. Percent mortality was determined 4 days after treatment and mortality was corrected for control mortality (synergist only). Means ± SEM (n = 30 bed bugs per treatment) are shown. For each population we used 300 bed bugs. Statistical differences among treatments within each population were determined using ANOVA (shown in **Table 2**) and Tukey's HSD test, with significant differences ($P < 0.05$) indicated by different letters.

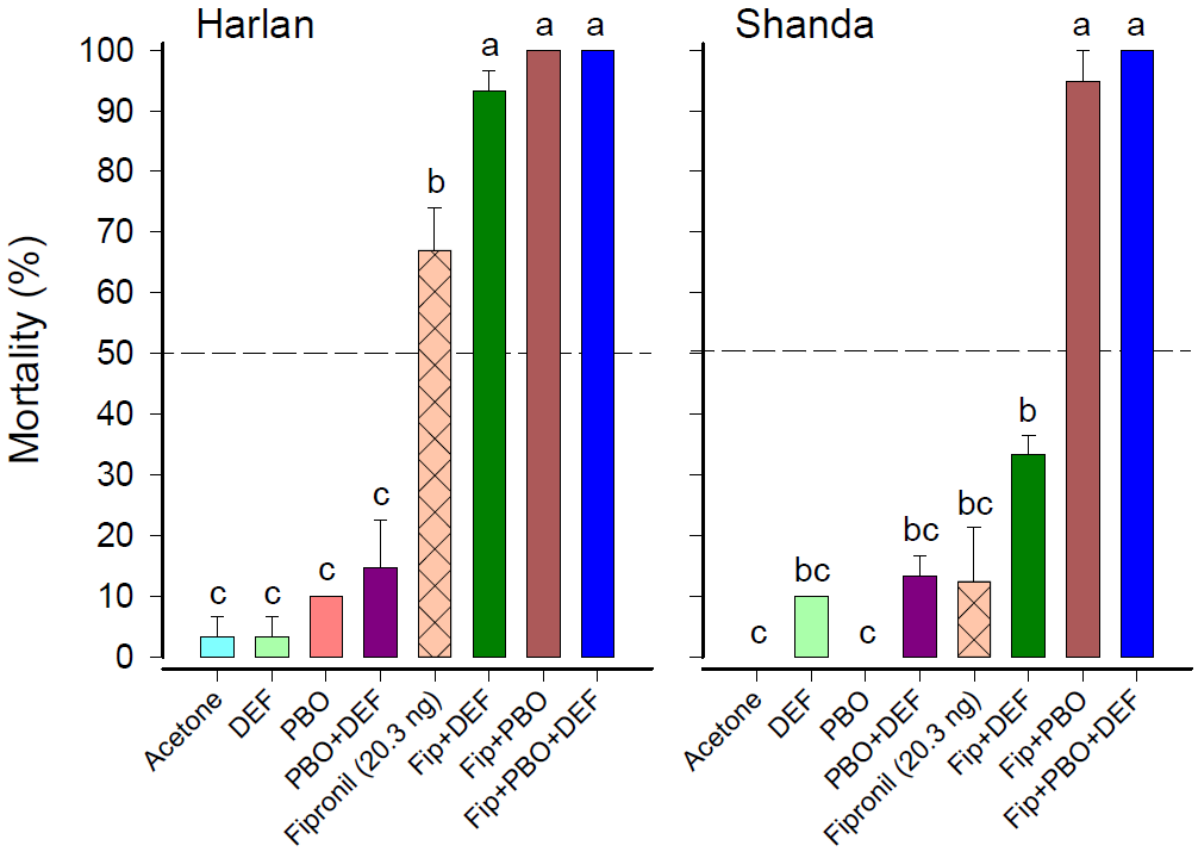

Fig. 2.3. A combination of piperonyl butoxide [PBO] and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) eliminates resistance in highly resistant adult male *C. lectularius*. PBO, DEF or a mix of both (50 µg each) were topically applied, and 2 h later the Harlan-NCSU-specific LD_{50} dose of fipronil (20.3 ng) was applied. Mortality was assessed 4 days after treatment. Means ± SEM (n = 30–55 bed bugs per treatment) are shown. We used 285 Harlan bed bugs and 283 Shanda bed bugs. Significant differences among treatments within population are indicated with different letters (ANOVA (shown in **Table 3**) and Tukey's HSD test, $P < 0.05$)

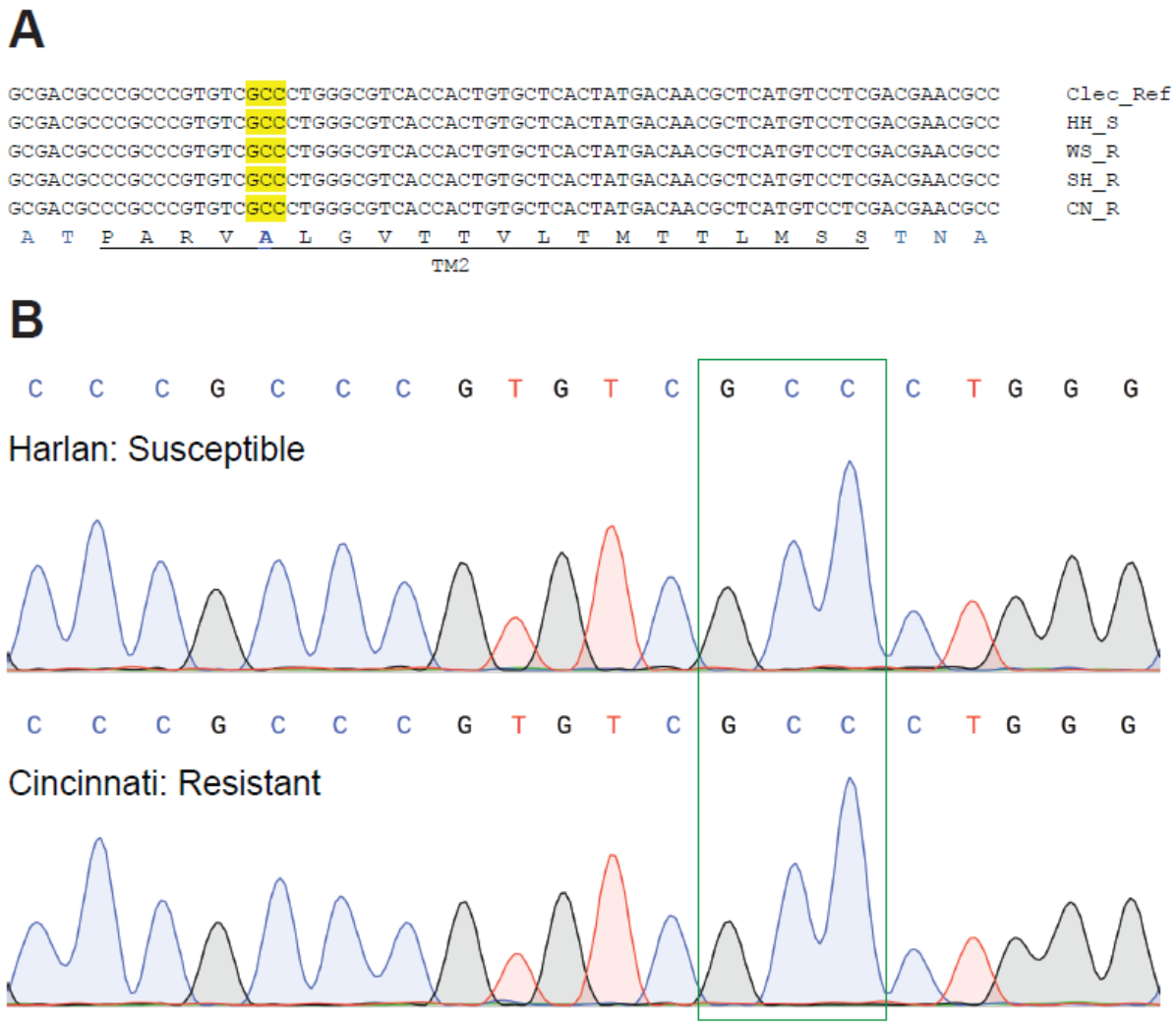

Fig. 2.4. Frequency of the *Rdl* mutation A302S in *C. lectularius* populations. Ten bed bug males from each of 10 populations were screened for the A302S mutation. Genomic DNA was extracted with the DNeasy Blood & Tissue extraction kit and a 245-bp genomic fragment of the GABA receptor gene that includes the A302S mutation site was amplified with a primer pair designed for this study. Each sequence was determined by manually checking for the GCC to TCC mutation that results in the A302S substitution.

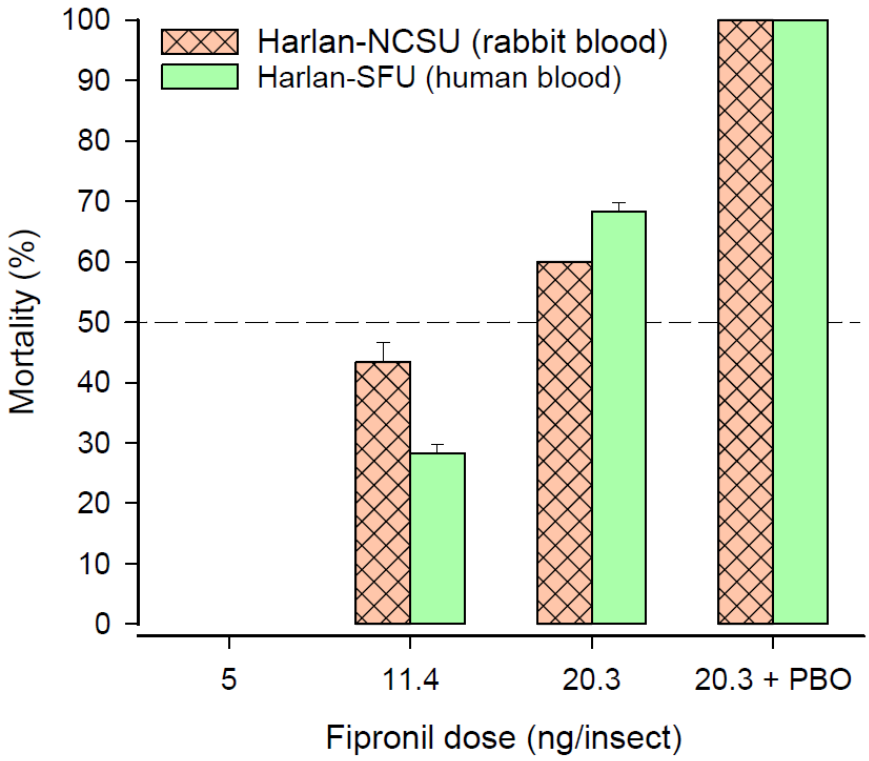

Fig. 2.5. Comparison of the responses of two related insecticide-susceptible populations of *C. lectularius* to treatment with fipronil. Both populations originated from a single collection in 1973. Harlan-NCSU has been fed defibrinated rabbit blood using an artificial feeding system since 2008. Harlan-SFU has fed on human volunteers since 1973. A dose-response study was conducted with topical applications of fipronil ranging from 5 to 20.3 ng (Harlan-NCSU LD_{50}). PBO was used in combination with the highest fipronil dose (20.3 ng) to assess the relative role of P450s in the response to fipronil. Each treatment consisted of two replicates of 7–15 adult males each. A total of 92 bed bugs of each population was

CHAPTER 3

Multiple Mechanisms Confer Fipronil Resistance in the German Cockroach: Enhance Detoxification and Rdl Mutation

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Abstract

Populations of *Blattella germanica* (L.) (German cockroach) have been documented worldwide to be resistant to a wide variety of insecticides with multiple modes of action. The phenylpyrazole insecticide fipronil has been used extensively to control German cockroach populations, exclusively in baits, yet the highest reported fipronil resistance is 38-fold in a single population. We evaluated five populations of German cockroaches, collected in 2018–2019 in apartments in North Carolina and assayed in 2019, to determine the status of fipronil resistance in the state. Resistance ratios in field-collected strains ranged from 22.4 to 37.4, indicating little change in fipronil resistance over the past 20 years. In contrast, resistance to pyrethroids continues to escalate. We also assessed the roles of detoxification enzymes in fipronil resistance with four synergists previously shown to diminish metabolic resistance to various insecticides in German cockroaches – piperonyl butoxide, *S*,*S*,*S*-tributyl phosphorotrithioate, diethyl maleate, and triphenyl phosphate. These enzymes appear to play some, but minimal role in fipronil resistance. We also sequenced a fragment of the *Rdl* (*resistant to dieldrin*) gene that encodes a subunit of the GABA receptor. Our findings showed that all field-collected strains are homozygous for a mutation that substitutes serine for an alanine (A302S) in RDL and confers low resistance to fipronil. Understanding why cockroaches rapidly evolve high levels of resistance to some insecticides and not others, despite intensive selection pressure, will contribute to more efficacious pest management.

Key words: Urban IPM, German cockroach, *Blattella germanica*, Fipronil, Insecticide Resistance, Rdl, Target site insensitivity

Introduction

The German cockroach (*Blattella germanica* L., Blattodea: Ectobiidae) is arguably the most prevalent and harmful indoor pest in the U.S., and often the most difficult to eradicate, especially from multi-family apartment complexes. Chronic cockroach infestations produce potent aeroallergens that trigger allergies and asthma in atopic children (Rosenstreich et al. 1997, Pomés and Schal 2020). Cockroaches also harbor a diverse microbial community in their digestive system and feces (Kakumanu et al. 2018) and have been implicated in pathogen transmission in hospitals and residential settings (review: Schal and DeVries 2021). Controlling and eradicating cockroach infestations is the most effective strategy to mitigate potential health risks (review: Schal and DeVries 2021). However, German cockroach eradication strategies are seriously constrained by the rapid evolution of resistance to numerous active ingredients across a wide array of modes of action (review: Scharf and Gondhalekar 2021).

Strategies to manage German cockroach populations in residential settings have transitioned from residual insecticides to various bait formulations (review: Appel and Rust 2021). Baits offer several noteworthy advantages over sprays. Namely, they target the pest more effectively, the active ingredient in baits is more bioavailable, and baits leave significantly less residues on household surfaces (DeVries et al. 2019a). Moreover, there is a much wider assortment of active ingredients available for use in bait formulations than in sprays, and the dose that insects receive from ingesting baits is typically higher than in sprays, representing a more effective “high dose” strategy.

Fipronil has been used in baits for cockroach control for nearly three decades (Kaakeh et al. 1997). It is a member of the broad-spectrum phenylpyrazole class of insecticides that act as antagonists of gamma-aminobutyric acid (GABA)-gated chloride channels (Gant et al. 1998).

Fipronil blocks both GABA-gated channels that mediate synaptic inhibition in the insect central nervous system (Gant et al. 1998) and glutamate-gated chloride (GluCl) channels involved in locomotion, feeding and sensory input (Zhao et al. 2004, Narahashi et al. 2010). It was first registered in the U.S. for cockroach control in 1996, but one year later, resistance was reported in the German cockroach (Scott et al. 1997, Valles et al. 1997). Both reports documented low levels of resistance ranging from a resistance ratio based on the LD_{50} value (RR) of 1.3 in three cockroach strains (Valles et al. 1997) to 7 in seven strains (Scott et al. 1997) (**Table 1**). Holbrook et al. (2003) collected cockroaches from 20 populations in central North Carolina before fipronil baits were introduced in commercial products and found moderate levels of resistance, ranging from 1.2 to >17-fold (**Table 1**). However, despite intensive selection pressure with baits over the past three decades, resistance levels have increased minimally to 17–38-fold (Wang et al. 2004, Gondhalekar et al. 2012). Similar patterns of relatively low fipronil resistance were also reported in Europe (Kristensen et al. 2005) and Malaysia (Ang et al. 2013).

Two major mechanisms have been proposed for fipronil resistance in the German cockroach – metabolic resistance and target site insensitivity. Metabolic mechanisms usually involve upregulation of detoxification of enzymes such as cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs), carboxylesterases (CESTs) and esterases (ESTs). Activity of these enzymes *in vivo* can be inferred with specific enzyme inhibitors such as piperonyl butoxide (PBO) that inhibits P450s and ESTs (Bergé et al. 1998), triphenyl phosphate (TPP) and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF), which inhibit the activity of ESTs (Plapp 1963), and diethyl maleate (DEM), an inhibitor of GSTs (Motoyama and Dauterman 1974). Application of PBO to cockroaches affects the efficacy of fipronil, but results have been inconsistent, with some researchers finding synergism, while others found antagonism (Scott et

al. 1997, Valles et al. 1997, Gondhalekar et al. 2012, Ang et al. 2013). The enzyme inhibitors TPP and DEM have not been evaluated on the effectiveness of fipronil in the German cockroach.

Single nucleotide polymorphisms (SNPs) in the binding sites of insecticides can result in target site insensitivity. The GABA-gated chloride channel is encoded by the *Rdl* (Resistant to dieldrin) gene. A mutation that results in substitution of serine or glycine in place of an alanine residue (A302S/G) in Rdl confers different levels of resistance in various insect species, and the response to various phenylpyrazole insecticides varies across species and populations of the same species (Zhao et al. 2003, Nakao 2017). In *B. germanica*, the *Rdl* mutation appears to contribute more to dieldrin resistance than to fipronil resistance (Scott et al. 1997, Hansen et al. 2005, Kristensen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013).

High levels of insecticide resistance can severely undermine interventions to eradicate German cockroach infestations. Resistance to most insecticides, such as pyrethroids, evolves rapidly, often within <5 years after their extensive use against *B. germanica* (Fardisi et al. 2019, Tang et al. 2019). Despite extensive and intensive selection pressure from fipronil-containing baits, the highest recorded resistance level is approximately 38-fold, in a single strain (Gondhalekar at al. 2012) (**Table 1**). In contrast, we recently reported 1,000-fold resistance to fipronil in the bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae), even though there are no fipronil-containing commercial products labeled for its use against bed bugs (González-Morales et al. 2021). These observations suggested that fipronil resistance in the German cockroach might be somehow constrained. They prompted us to re-examine fipronil resistance in more recently sampled cockroach populations, assess responses to synergists that inhibit specific classes of detoxifying enzymes, and determine the frequencies of *Rdl* target site mutations.

Materials and Methods

Experimental Insects

German cockroaches were sampled from five North Carolina apartments and one insecticide-susceptible reference strain and screened for fipronil resistance (**Table 2**). The insecticide-susceptible strain of *B. germanica* (Orlando Normal = American Cyanamid) was collected in 1947 in a Florida apartment. All other populations (VS101, DR2800, DR2820B, CC29 and PR515F) were collected recently (2018–2019) in homes in Raleigh, NC, under Institutional Review Board approval (NC State University #12188). All cockroach colonies were maintained in plastic bins (20 × 15 × 10 cm) and provided *ad libitum* with water and rodent chow pellets (Purina No. 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO). Temperature was kept at 27°C, relative humidity at 40–70%, and the photoperiod was 12L:12D. All assays with these colonies were conducted in 2019, 2–4 generations after the field collections.

Fipronil Toxicity

Fipronil ((RS)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile; CAS 120068-37-3), at 98.7% purity, was obtained from Sigma-Aldrich (St. Louis, MO). The lethal dose of fipronil that killed 50% of each population (LD_{50}) was determined by topical application. Groups of 10 adult male cockroaches were briefly anesthetized with CO_2 in a plastic Petri dish (90 × 15 mm). Fipronil was topically applied in acetone with a micro-applicator (Hamilton, Reno, NV) equipped with a 50 µl glass syringe (Hamilton Co.) that delivered 1 µl on the ventral thorax of each cockroach. We chose this area, between the coxae, because it is not groomed as frequently as other regions of the body. Fipronil dilutions ranged from 0 (acetone control) to 120 ng/µl acetone and varied by population tested. Mortality was assessed every 24 h for 96 h post application by gently touching individuals with

forceps, with morbid cockroaches (unable to right themselves after touching with forceps) considered dead.

Metabolic Enzyme Inhibitors in Fipronil Resistance

We used the following enzyme inhibitors: DEF (97.7% purity, Chem Services, West Chester, PA), TPP (99%), PBO (99%), and DEM (97%) (Sigma-Aldrich). We evaluated the effects of detoxification enzyme inhibitors on fipronil toxicity in six populations: The susceptible population (Orlando Normal), and the five strains collected in North Carolina. Each adult male was topically treated with a non-lethal dose of one of the inhibitors in 1 µl acetone: 100 µg PBO, 30 µg DEF, 100 µg DEM or 30 µg TPP. These doses were based on previously reported values. After the cockroaches recovered at room temperature for one h, they were briefly anesthetized again with CO_2 and treated topically with either acetone (control) or the strain-specific LD_{50}. Three replicates of 10 adult male German cockroaches were performed for each population-inhibitor combination. Mortality was recorded every 24 h for 96 h, as described above.

Pyrethroid Resistance

Cypermethrin (98% purity, Sigma-Aldrich) resistance was evaluated in the Orlando Normal strain and three apartment-collected strains DR2820B, CC29, and VS101. We topically treated 240 adult males of the Orlando Normal strain, as described for fipronil, to generate a dose-response curve and estimate the LD_{50} from Probit analysis. The field-collected cockroaches were then treated with the estimated LD_{50} of the Orlando Normal strain, as well as 10-fold and 100-fold the LD_{50}. Mortality was assessed 48 h post-treatment. We also weighed individual males of these strains, as well as the Orlando Normal strain.

Detection of *Rdl* Mutation

Ten adult males from each of the six cockroach populations were screened for the presence of the *Rdl* mutation that results in the A302S substitution. The head of individual cockroaches was homogenized for 30 s with glass beads in a FastPrep 24 5G homogenizer (MP Biomedicals, Solon, OH) and genomic DNA was extracted using the DNeasy Blood & Tissue extraction kit (Qiagen, Germantown, MD). ATL solution (180 µl) and 20 µl of proteinase-K were added to the homogenized samples and incubated at 56°C for 4 h. The rest of the protocol followed the manufacturer's instructions. DNA was eluted in 50 µl sterile nuclease-free water and stored at -20°C until further use.

A 245-bp genomic fragment of the GABA receptor gene that includes the *Rdl* mutation site was amplified with the primers BG-Rdl-F (5′- GTGCGGTCCATGGGATACTA -3′) and BG-Rdl-R (5′- AACGACGCGAAGACCATAAC -3′) designed by Hansen et al. (2005). The reactions were conducted in 20 µl reaction mix comprising 10 µl of AmpliTaq Gold 360 2X Master mix (Applied Biosystems, Waltham, MA), 1 µl of 10 µM of each primer, 0.2 µl BSA (bovine serum albumin; Sigma-Aldrich) (20 mg/ml) and 2 µl of German cockroach genomic DNA as template for the PCR reaction. A negative control with no template DNA was included in every PCR run. The following thermal cycle program was used for amplification: Initial activation at 95°C for 10 min followed by 40 cycles of 94°C for 30 s, 64.3°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min (Gondhalekar and Scharf 2012). Two microliters of each PCR product were used to verify proper sized bands on 1.2% agarose gel. The remaining PCR product was purified by ExoSAP-IT (Applied Biosystems) and direct-sequenced at the Genomic Sequencing Laboratory (North Carolina State University, Raleigh, NC) with BG-Rdl-R as

sequencing primer. Each sequence was determined by manually checking for the GCC to TCC mutation that results in the A302S substitution.

Statistical Analysis

The LD_{50} for each cockroach population was determined using log-dose probit-mortality analysis based on a spreadsheet template (Lei and Sun 2018). The values were in agreement with analysis in PoloPlus (LeOra Software, Petaluma, CA). Abbott's correction (Abbott 1925) was used to correct for control mortality, as needed. The effects of enzyme inhibitors on fipronil toxicity were determined using Chi-square analysis in SAS 9.4 (SAS Institute, Cary, NC), comparing mortality of cockroaches with and without inhibitor application.

Results

Fipronil and Cypermethrin Resistance

Adult males from four of the strains were individually weighed. Their mean body mass ± SEM were as follows: Orlando Normal = 52.1 ± 0.9 mg, n = 10; VS101 = 57.8 ± 1.6 mg, n = 10; DR2820B = 52.5 ± 1.3 mg, n = 10; CC29 = 56.4 ± 0.7, n = 10. A one-way ANOVA was highly significant (F = 5.2583, df = 3, 36, p = 0.0041), and Dunnett's test with Orlando Normal as the control group indicated that the body masses of VS101 and CC29 were significantly higher than Orlando Normal (p = 0.0065 and 0.0460, respectively), whereas DR2820B was not (p = 0.9865).

The LD_{50} values were determined from dose-response curves 4 d after topical applications of fipronil to cockroaches collected from five apartments (VS101, DR2800, DR2820B, CC29, PR515F; **Fig. 1**); these values were compared with the insecticide-susceptible Orlando Normal strain. The Orlando Normal males had 100% mortality at the highest dose of 3.5 ng fipronil per insect, whereas 90 ng/insect killed 73–93% of the field-collected cockroaches.

The LD_{50} of the Orlando Normal males was 1.55 ng/insect, whereas PR515F, the strain with the highest fipronil resistance, had an LD_{50} of 57.6 ng/male (**Table 2**). The resistance ratio values based on their respective LD_{50} values ranged from 22.4 (DR2800) to 37.3 (PR515F), and all five strains significantly differed from the Orlando Normal reference strain ($p < 0.05$). The relatively steep dose-response curves (slopes, 1.56–3.92) for the five populations suggest homogeneity in the insects' responses to fipronil within each population. Male body mass values were available for four strains. Correcting for body mass, the respective RR values were 22.9 (VS101, 7.3% lower than 24.7), 29.9 (DR2820B, 1.7% higher than 29.4), and 31.0 (CC29, 5.2% lower than 32.7).

We found relatively high levels of cypermethrin resistance in the three populations tested (**Table 3**). The LD_{50} of the Orlando Normal strain was 0.112 µg/male. At a diagnostic dose of 10 µg/male, representing ~100-fold the Orlando Normal LD_{50}, only 40% of adult males of strain DR2820B died, indicating a resistance ratio >100. At the same dose of 10 µg/male we found 80% and 83% mortality in strains CC29 and VS101, respectively. These results indicate resistance ratios to cypermethrin between 10 and >100.

Effect of Enzyme Inhibitors on Fipronil Toxicity

The toxicity of fipronil in the presence of the enzyme inhibitors PBO, DEF, DEM and TPP 4 d after treatment is shown in **Fig. 2**. Because the fipronil LD_{50} varied among the six strains, we used the strain-specific LD_{50} dose with and without the addition of the inhibitor. When pretreated with PBO, neither the susceptible reference Orlando Normal strain nor PR515F, the most resistant strain, experienced a significant increase in fipronil toxicity ($p = 0.12$ and 0.06, respectively). However, fipronil mortality increased significantly in the presence of PBO in strain VS101 ($\chi^2 = 11.4$, $df = 1$, $p < 0.05$) and in strain DR2800 ($\chi^2 = 10.7$, $df = 1$, $p < 0.05$).

Fipronil toxicity also was significantly enhanced in strains DR2820B ($\chi^2 = 4.6$ $df = 1$, $p < 0.05$) and CC29 ($\chi^2 = 6.9$ $df = 1$, $p < 0.05$), but to a lesser extent than in VS101 and DR2800.

Pre-applications of DEF, an EST inhibitor, caused a significant increase in fipronil mortality only in cockroaches from the VS101 strain ($\chi^2 = 4.59$ $df = 1$, $p < 0.05$). DEM, a GST inhibitor, caused significant synergism only in the Orlando Normal susceptible strain and the VS101 strain. In contrast, pre-treatments with TPP, also an EST inhibitor, antagonized the toxicity of fipronil in the susceptible strain, reducing fipronil mortality from 37% to 0%. Our findings suggest an involvement of P450s in fipronil metabolism in most strains, but the activity of the other enzyme inhibitors varied among the strains, with significant antagonism observed with TPP in the susceptible strain (**Fig. 2**).

A302S Rdl Mutation

A 245 bp fragment of the GABA receptor gene targeting the position of the *Rdl* mutation that causes the A302S substitution was amplified and sequenced from the Orlando Normal susceptible strain ($n = 10$) and the five field-collected populations ($n = 10$ per population). Based on previous studies relating fipronil resistance to this mutation, and the moderate level of resistance we detected in some populations, we expected to detect haplotypes corresponding to homozygous susceptible wild-type (Ala302/Ala302; S/S), putatively homozygous resistant (Ser302/Ser302; R/R) and the heterozygous haplotype (Ala302/Ser302; S/R). However, we found the *Rdl* mutation in all five recently collected cockroach strains, but not in the reference insecticide susceptible strain (**Table 4**); 100% of the apartment-collected cockroaches were homozygous for the resistant genotype (A302S).

Discussion

It appears that fipronil resistance in German cockroach populations in North Carolina has increased over the last two decades from a range of 1.2–17-fold (Holbrook et al. 2003) to 22.4–37.3-fold. The fipronil LD_{50} values ranged from 34.6 ng/male (approximately 0.67 µg/g, RR = 22.4) to 57.6 ng/male (approximately 1.1 µg/g, RR = 37.3). The moderate increase in resistance, despite the prevalence of fipronil-containing baits in the U.S., is consistent with patterns in other U.S. and global populations investigated over the last two decades (**Table 1**). These findings suggest that fipronil resistance in German cockroach populations might have reached a plateau, possibly representing a level above which cockroaches experience substantial fitness costs. However, unlike the broad variation in resistance ratios documented in populations in the 1990s and 2000s, we observed low variation among the five German cockroach populations collected in homes in Raleigh, NC. These results support the idea that populations have converged on an upper limit of resistance, represented by RR values of 30–40.

Mechanisms of Fipronil Resistance

The involvement of metabolic enzymes in fipronil degradation appears to vary among cockroach strains. Piperonyl butoxide was reported by Valles et al. (1997) to have antagonistic effects on fipronil toxicity in three different populations, and by Ang et al. (2013) in six populations. On the other hand, Gondhalekar et al. (2012) showed that PBO synergized fipronil in one strain, and it did so in four of the five strains in our study. However, fipronil mortality in our most resistant strain (PR515F) increased by only 24% in the presence of PBO (at the LD_{50} level of fipronil), from 53% to 77%, and this increase was not statistically significant. These findings are consistent with findings in other species. Although P450s have been reported to metabolize fipronil in the white rot fungus *Trametes versicolor* Lloyd (Polyporales) (Wolfand et al. 2016),

responses to PBO vary among insect species, with synergism in *Musca domestica* L. (Diptera: Muscidae) (Liu and Yue 2000) and bed bug (González-Morales et al. 2021), but no apparent effect in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Scharf et al. 2000). It is important to note that fipronil would be oxidized by P450s to fipronil sulfone, which also binds strongly to GABA and GluCl receptors in insects, including the German cockroach (Zhao and Salgado 2010), and thus plays an important role in the toxicity of fipronil. *In vivo* studies have found that fipronil and its sulfone metabolite range from similar bioactivity in some insects (e.g., *D. virgifera*; Scharf and Siegfried 1999), to the sulfone being 4.6-fold more bioactive than fipronil (non-biting midge, *Chironomus dilutus*, Diptera; Weston and Lydy 2014). It is possible that populations of German cockroaches express polymorphisms in the affinities of fipronil and fipronil sulfone to GABA and GluCl receptors. Thus, in some cockroach populations the application of PBO blocks fipronil oxidation, but does not greatly affect toxicity because fipronil and fipronil sulfone are equally bioactive. In populations where the sulfone is more bioactive than fipronil, PBO would block the oxidation and thus antagonize fipronil. In yet other populations, P450s may further hydroxylate the sulfone to less active forms. In these populations, PBO might block these later transformations and both fipronil and the sulfone would be more toxic in the presence of PBO. However, the high synergism expected in the latter scenario has not been demonstrated in any German cockroach population.

Similarly, DEF, an EST inhibitor, has been reported to antagonize fipronil toxicity in multiple populations of the German cockroach (Valles et al. 1997, Ang et al. 2013), and as a synergist in one strain (Gondhalekar et al. 2012). Although all five of our field-collected strains had greater mortality after DEF treatment, only one (VS101) showed a significant increase in mortality after DEF application. The role of GSTs in fipronil metabolism has not been studied

before in the German cockroach. However, a study evaluating enzymatic activity in the western corn rootworm, has shown that GSTs metabolize fipronil (Scharf et al. 2000). Interestingly, DEM, a GST inhibitor, significantly synergized fipronil toxicity only in the susceptible strain (Orlando Normal) and the VS101 strain, and it slightly elevated mortality in two strains and depressed fipronil mortality in two other strains. The only significant antagonism in our assays was caused by TPP in the Orlando Normal susceptible strain, although TPP also reduced fipronil bioactivity slightly in two other strains.

The lack of clear patterns in the involvement of detoxification enzymes in fipronil metabolism support the hypothesis that detoxification enzymes play a relatively minor role in fipronil toxicity in the German cockroach, compared for example to the bed bug, and their variable involvement across populations may be related to the unique history of exposure to xenobiotics of each population. Moreover, it is possible that intensive selection with pyrethroids, which has selected for relatively high resistance to pyrethroids (e.g., cypermethrin) in these populations, might have contributed to fipronil resistance. Hu et al. (2020) also suggested that high deltamethrin resistance in some cockroach strains could affect the performance of fipronil through upregulation of general cytochrome P450 monooxygenases that can detoxify both classes of compounds.

German cockroaches were likely pre-adapted for fipronil resistance, as indicated by the high correlation (i.e., cross-resistance) between dieldrin and fipronil resistance in the late 1990s in the U.S. (Holbrook et al. 2003) and Denmark (Kristensen et al. 2005). Recurrent treatments with dieldrin have selected for a mutation in the *Rdl* gene that results in a A302S/G substitution in Rdl in several phylogenetically diverse insect species (ffrench-Constant et al. 1993). This mutation has been reported in both U.S. and European cockroach populations (Hansen et al.

2005, Ang et al. 2013) but although it confers high resistance to dieldrin, it appears to confer low cross-resistance to fipronil (Scott et al. 1997, Hansen et al. 2005, Kristensen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013). All of our field-collected cockroaches were homozygous for the *Rdl* mutation, regardless of their level of resistance. The combination of low resistance to fipronil and the presence of the *Rdl* mutation has been shown in other insects, including planthoppers (Nakao 2017) and flies (Gao et al. 2007). However, other mutations in the GABA receptor subunit that confer fipronil resistance, which have been studied in other insects (Nakao 2017, Garrood et al. 2017), need to be investigated in *B. germanica*. Moreover, using antibiotic treatments to reduce the gut microbiome, Wolfe and Scharf (2021) showed that microbial metabolism may contribute to fipronil resistance in the German cockroach. Overall, it appears that the A302S substitution in the *Rdl* gene confers relatively low fipronil resistance to German cockroaches, and detoxification mechanisms, mainly involving P450s, also impart relatively low resistance. Together, these two mechanisms appear to account for most of the resistance to fipronil in the German cockroach.

Why Has Resistance to Fipronil Not Increased?

Fipronil resistance in German cockroach populations has increased over the past three decades, but only marginally. Early findings in the late 1990s of fipronil resistance in the U.S. reported LD_{50} resistance ratios (RR) <2, and follow-up studies in the 2000s found some populations with RR >17 (**Table 1**). Later reports, including recent findings, vary across studies and geographic locations, with RR values remaining low (1.0–8.7) in some populations (e.g., Liang et al. 2017, Wu and Appel 2017), while others increased appreciably as high as 38 (Gondhalekar et al. 2012, DeVries et al. 2019b, Lee et al. 2022). The RR values we found (22.4–37.3) are thus consistent

with the latter three papers given slight variations in methodology, reference strains used, and body mass of males.

The global patterns of resistance to fipronil appear to track the U.S. pattern. An early study showed highly variable RRs (1–15) among seven populations in Denmark (Kristensen et al. 2005); unfortunately, there are no recent follow-up studies of European *B. germanica* populations. In Asia, resistance to fipronil is generally lower than in the U.S., likely because of later introduction of fipronil into the global indoor market, and slower adoption of baits and hence weaker selection pressure with fipronil. Among 24 field populations of *B. germanica* collected throughout Taiwan, fipronil RR values were 1.7–3.7, determined by applying fipronil to a surface and recording the LT_{50}, the time to 50% mortality (Hu et al. 2020).

The enigma of why fipronil resistance levels have remained low is particularly interesting because fipronil-containing baits have been used extensively to control indoor cockroach populations since 1996, and artificial selection readily results in high levels of fipronil resistance in other insect species. In a common rice pest in Asia, the whitebacked planthopper (*Sogatella furcifera* Horváth, Hemiptera: Delphacidae), artificial selection with fipronil for 11 generations doubled fipronil RRs up to 137.5 (Tang et al. 2010). Artificial selection with fipronil also increased the RR in the housefly, *M. domestica*, to 182 after five generations (Abbas et al. 2016) and in the cotton seed bug, *Ocycarenus hyalinipennis* Costa (Hemiptera: Lygaeidae), to 9,855 after 11 generations of selection (Wazir and Shad 2020). Moreover, fipronil RRs were 500 and 1,000 in two field-collected populations of the bed bug *C. lectularius* (González-Morales et al. 2021).

In contrast, when German cockroach populations were artificially selected with fipronil or fipronil-containing baits, RRs increased, but appeared to be somehow constrained, capping at

26 after five generations (Ang et al. 2013), 15.9 after 2 years of selection (Ko et al. 2016), and 25 after 4 years (Liang et al. 2017). Also, selection with a fipronil bait resulted in up to 4,000-fold increase in the RR to dieldrin but only 23.4-fold increase in the RR to fipronil (Ang et al. 2013), consistent with early observations that cross-resistance to fipronil was >2,000-fold lower than the level of resistance to dieldrin (Scott and Wen 1997). The limited effects of selection with fipronil appear to be evident in several other species. Populations of the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), selected with fipronil for eight generations, increased their RR to only 30.5 (Ling et al. 2009). Other examples come from field-collected arthropods that had been under intense selection with fipronil-containing pest management formulations. Field-collected cat flea populations, *Ctenocephalides felis* (Bouché) (Siphonaptera: Pulicidae), had low RRs, up to 2.2 (Rust et al. 2015), and fipronil RR values in brown dog tick populations, *Rhipicephalus sanguineus* Latreille (Ixodida: Ixodidae), topped at 13.8 (Becker et al. 2019).

It is likely that fipronil resistance in some arthropods, including *B. germanica*, carries significant fitness costs, even at low levels of resistance. Fitness costs, such as lower larval survival rate, lower adult emergence rate, lower copulation rate, lower fecundity and fewer offspring were associated with higher fipronil resistance in the brown planthopper (Ling et al. 2009, Zhang et al. 2016), for example. Likewise, in the housefly, *M. domestica*, artificial selection with fipronil resulted in longer larval duration, lower pupal weight, lower fecundity, lower hatchability, lower number of the next generation larvae, lower intrinsic rate of population increases and lower biotic potential (Abbas et al. 2016). Other indirect evidence of significant fitness costs associated with fipronil resistance comes from observations that high resistance levels were not sustained when fipronil selection was discontinued in several insect species including planthoppers and houseflies. We therefore suspect that the high fitness costs of fipronil

resistance in the German cockroach might explain the moderate levels of resistance despite strong selection through several decades. In contrast, resistance levels to pyrethroids are extremely high in *B. germanica* populations (Wei et al. 2001, DeVries et al. 2019b, Lee et al. 2022, review: Scharf and Gondhalekar 2021), including in the three populations that we examined, and may be sustained for years after selection is discontinued (M.A.G-M and C.S., personal observations), suggesting lower fitness costs than with fipronil resistance. Overall, these patterns suggest potentially significant trade-offs between elevated levels of fipronil resistance in *B. germanica* and fitness-related traits. If so, this complex interaction has important implications for the continued use of fipronil in cockroach baits.

The mode of action of fipronil and its sulfone metabolite may also contribute to the stalled resistance in *B. germanica*. Together, fipronil and its bioactive metabolite are unique in binding with high affinity to three target sites in the insect central nervous system: GABA-gated chloride channels and two types of glutamate-gated chloride channels. Further, Zhao and Salgado (2010) suggested that there might be two subtypes of GABA receptors in the German cockroach. They also demonstrated that the *Rdl* mutation and resistance to dieldrin did not affect the sensitivity of GluCl receptors to fipronil sulfone. Generally, the evolution of target site-based resistance to insecticides can be greatly slowed by "pyramiding" two or more distinct toxins, as in pyramided Bt crops (Carrière et al. 2016). By binding multiple CNS targets, fipronil and fipronil sulfone may act as a combination of active ingredients that target different receptors, which is an important strategy of resistance management. However, much more information is needed about the levels of cross-resistance between fipronil and the sulfone and their differential affinities to different receptor sites.

Would Resistance to Fipronil Affect its Field Efficacy?

There are no studies that quantitatively correlate fipronil resistance levels with the efficacy of fipronil baits in the field. We suspect that it is unlikely that 37-fold resistance to fipronil would affect the field efficacy of baits because of the high doses of fipronil ingested during bait consumption, which would overwhelm the relatively low resistance we observed. For example, Holbrook et al. (2003) supplemented rodent chow with fipronil and showed that concentrations much lower than in commercial baits killed all the resistant cockroaches. Moreover, based on consumption of 1 mg of bait per cockroach, Holbrook et al. (2003) calculated that each cockroach would ingest 100 ng of fipronil from a bait containing 0.01% fipronil (the standard in 2003 – Maxforce FC gel, Bayer Environmental Science, Montvale, NJ). Ko et al. (2016) conducted similar estimates. They determined the LD_{90} of fipronil in susceptible males to be 3.18 ng per male (2.44 ng in the present study). If in a day a susceptible male ingested 1.8 mg of bait (0.05% fipronil, the current standard in Maxforce FC Magnum, Bayer), it would consume 900 ng of fipronil, or 370-fold the LD_{90}. Because fipronil is more active at lower levels than most other insecticides, the high-dose strategy inherent in gel bait formulations, and especially those containing fipronil, could overcome the highest fipronil resistance reported thus far in any German cockroach population. Nevertheless, behavioral traits such as avoidance of the bait (e.g., glucose aversion and associative learning, Wada-Katsumata and Schal 2021) or physical changes in the formulation (aging, repellency) could result in lower bait consumption and compromise control efforts. The recent introduction of residual formulations of fipronil might have a similar effect. In 2019, fipronil was approved in the U.S. for use in residual sprays (0.65%, Fipronil-Plus-C, EPA Reg. No. 55431-15; maximum allowed fipronil concentration 0.0076%) for controlling crawling insects indoors, including cockroaches. Residual sprays that expose insects

to much lower doses than those found in baits might nullify the advantages of the high-dose approach with baits, and even the moderate ~30-fold present-day fipronil resistance might protect cockroaches from residual fipronil formulations. A case in point may be the dual use of indoxacarb in baits and spray formulations. Indoxacarb has been used in baits since 2006 and in spray formulations since 2010. The increase in resistance and control issues over the years may be due to the dual use, which compromises the high-dose strategy (Gondhalekar et al. 2013).

Conclusion

The accumulated evidence on fipronil resistance in *B. germanica* suggests that target site insensitivity in Rdl contributes to low-level resistance to fipronil, and metabolic detoxification processes are highly variable across populations and contribute marginally to fipronil resistance. The metabolic mechanisms might be related to low fipronil cross-resistance with pyrethroids, which select for significant upregulation of a wide range of P450 genes. Our results suggest that combining fipronil with PBO could reduce fipronil resistance in the most resistant populations. However, this might not be necessary if fitness costs associated with fipronil resistance, perhaps related to ensuring proper functioning of the GABA-gated channels and glutamate-gated chloride channels, will prevent the emergence of highly resistant German cockroach populations. In practical cockroach control, lower fitness of resistant cockroaches would lend strong support for a resistance management program based on the practice of rotation of active ingredients, because populations are expected to quickly recover sensitivity to fipronil when fipronil pressure is withdrawn.

It is also important to note that several studies have found highly variable performance of fipronil baits under laboratory conditions. For example, Hu et al. (2020) found that mortality of various field-collected strains in Taiwan ranged from 8.5% to 95.9%, apparently with no

correlation to resistance levels (RRs) which were <3.7. Likewise, Lee et al. (2022) found low performance of fipronil bait against five field-collected strains in California that exhibited fipronil RR of ~27. These findings appear to implicate avoidance, deterrence, repellence, or other behavioral mechanisms that might relate to formulation ingredients, rather than metabolic or target site resistance mechanisms.

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Table 3.1. Summary of studies that quantified fipronil resistance in field-collected *B. germanica*

Year	Authors	RR[a] range	No. of strains	Collection years	Location	Type of treatment	Assay used	Time of mortality assessment
1997	Scott and Wen	1.0 – 1.8, (1.8–7.7)[b]	7	1990–1992	U.S.	Topical	Dose-response	4 d
1997	Valles et al.	1.0 – 1.3	3	1989–1996	U.S.	Topical	Dose-response	1 d
2003	Holbrook et al.	1.2 – >17[c]	20	1997–1998	U.S.	Topical	Discriminating doses	3 d
2004	Wang et al.[d]	8.7 – 9.3	3	2003	U.S.	Topical	Dose-response	3 d
2005	Kristensen et al.	1 – 15	7	1996–2002	Denmark	Topical	Dose-response	3 d
2006	Nasirian et al.	1 – 2.6	11	unknown	Iran	Topical	Dose-response	3 d
2010	Chai and Lee	1.0 – 10.0	22	2005	Singapore	Topical	Dose-response	2 d
2012	Gondhalekar et al.	37.9	1	2006	U.S.	Topical	Dose-response	3 d
2013	Ang et al.	1.2 – 3.0 (10.8 – 25.8)[b]	6[e]	2005	Singapore	Topical	Dose-response	2 d
2016	Ko et al.	5.6 (15.9)[b]	1	2012	Puerto Rico	Topical	Dose-response	2 d
2017	Liang et al.	0.9 – 1.4 (2.5–25.0)[b]	3	1999–2004	U.S.	Topical	Dose-response	5 d
2017	Wu and Appel	2.0 – 8.7	6	2011–2012	U.S.	Topical	Dose-response	3 d
2019 b	DeVries et al.	6 – 23	7	2011–2014	U.S.	Topical	Dose-response	2 d
2020	Hu et al.	1.5 – 3.8	24	2017–2018	Taiwan	Surface contact	Time-course (LT_{50})	7 d
2022	Lee et al.	~27.7[f]	5	2018-2020	U.S.	Ingestion	Discriminating doses	3 d
2022	Present paper	22.4 – 37.3	5	2018–2019	U.S.	Topical	Dose-response	4 d

[a]RR is the resistance ratio, calculated as LD_{50} (or LT_{50}) of field-collected strain / LD_{50} (or LT_{50}) of a reference susceptible strain
[b]Artificially selected population(s)
[c]RR >17 is based on the observation that the LD_{50} of the susceptible strain was 2 ng, and 34.5 ng (10-fold the LD_{99}) failed to kill 50% of the cockroaches
[d]One of the strains (Cincy) also was used by Wang et al. (2006) and had a RR = 8.6
[e]The same 6 populations were examined by Chai and Lee (2010)
[f]RR >27.7 is based on the observation that the LD_{50} of the susceptible strain was 1.3 ng, and 36 ng (10-fold the LD_{99}) failed to killed 20–70% of the cockroaches.

Table 3.2. Fipronil dose-response results in the susceptible Orlando Normal reference strain and five *B. germanica* populations recently collected from apartments in Raleigh, NC

Strain[a]	*n*	Lethal dose LD_{50} ng/male (95% CI)[b]	Slope ± SE	χ^2 (*df*)	*t*-ratio[c]	RR[d] (95% CI)
Orlando normal	270	1.5 (1.35, 1.77)	5.73 ± 0.68	5.39 (4)	8.3*	-
VS101	120	38.2 (20.4, 71.6)	2.58 ± 0.60	0.61 (2)	4.3*	24.7* (22.9) (18.3, 33.4)
DR2800	150	34.6 (14.7, 81.7)	1.56 ± 0.36	9.81 (2)	4.4*	22.4* (15.0, 33.5)
DR2820B	200	45.4 (36.2, 56.9)	3.92 ± 0.46	8.88 (3)	8.5*	29.4* (29.9) (24.8, 34.7)
CC29	150	50.5 (29.2, 87.4)	2.32 ± 0.46	0.38 (2)	5.1*	32.7* (31.0) (25.0, 42.7)
PR515F	150	57.6 (14.1, 235.4)	2.92 ± 0.74	1.48 (1)	3.9*	37.3* (29.4, 47.2)

[a]The strain name was based on location. The five recently collected strains are from Raleigh, NC, collected between 2018 and 2019.

[b]Insects were topically treated with fipronil (in 1 µl acetone); LD_{50} was estimated for each strain from probit analysis. CI is confidence interval. The average mass of the Orlando Normal, VS101, DR2820B, and CC29 strains were 52.1, 57.8, 52.5, and 56.4 mg/male, respectively; hence, multiply by 19.2, 17.3, 19.0, and 17.7 to obtain approximate ng/g body mass for these strains.

[c]*t*-ratio of the slope. Values >1.96 denote a significant regression (*$p < 0.05$).

[d]Resistance Ratios (RR) and 95% confidence intervals. RR was calculated as LD_{50} of apartment-collected strain / LD_{50} of susceptible reference strain (Orlando Normal). RR values with (*) are considered significant when their 95% CIs do not include 1.0 (Robertson et al. 2017). RR values were corrected (in parenthesis) for body mass differences for the 3 strains for which male body mass was available.

Table 3.3. Cypermethrin dose-response results in the susceptible Orlando Normal reference strain and responses of five recently collected *B. germanica* populations to diagnostic doses of cypermethrin

Strain	*n*	Lethal dose LD_{50} µg/male (95% CI)[a]	Slope ± SE	χ^2 (*df*)	*t*-ratio[b]
Orlando normal	240	0.112 (0.090,0.169)	7.4 ± 0.9	14.3 (4)	7.5*

	Mean ± SEM % mortality at 48 h (*n* = 3)[c]		
Diagnostic dose (µg/insect)[d]	VS101	DR2820B	CC29
0.1 (LD_{50} of Orlando Normal)	3.3 ± 5.7	0 ± 0	0 ± 0
1 (10X LD_{50} of Orlando Normal)	6.7 ± 5.7	0 ± 0	3.3 ± 5.8
10 (100X LD_{50} of Orlando Normal)	83.3 ± 5.7	40.0 ± 10.0	80.0 ± 10.0

[a]Insects were topically treated with cypermethrin (in 1 µl acetone); LD_{50} was estimated for the Orlando Normal strain from probit analysis. CI is confidence interval. The average mass of *B. germanica* males is ~50 mg, hence multiply by 20 to obtain approximate µg/g body mass.
[b]*t*-ratio of the slope. Values >1.96 denote a significant regression (*$p < 0.05$).
[c]The mean represents the average of 3 replicates with 10 cockroaches assayed in each replicate.
[d]Three diagnostic doses were used, representing the LD_{50} value (0.1 µg), 10-fold the LD_{50} (1 µg), and 100-fold the LD_{50} value (10 µg) of the Orlando Normal strain.**Table 3.4.** *Rdl* haplotype of the fipronil-susceptible Orlando Normal reference strain and of fipronil-resistant apartment-collected *B. germanica.*

Table 3.4. *Rdl* haplotype of the fipronil-susceptible Orlando Normal reference strain and of fipronil-resistant apartment-collected *B. germanica*

Population	*n*	No. of cockroaches		
		A302/A302 (S/S)	A302/ S302 (S/R)	S302/S302 (R/R)
Orlando Normal	10	10	0	0
VS101	10	0	0	10
DR2800	10	0	0	10
DR2820B	10	0	0	10
CC29	10	0	0	10
PR515F	10	0	0	10

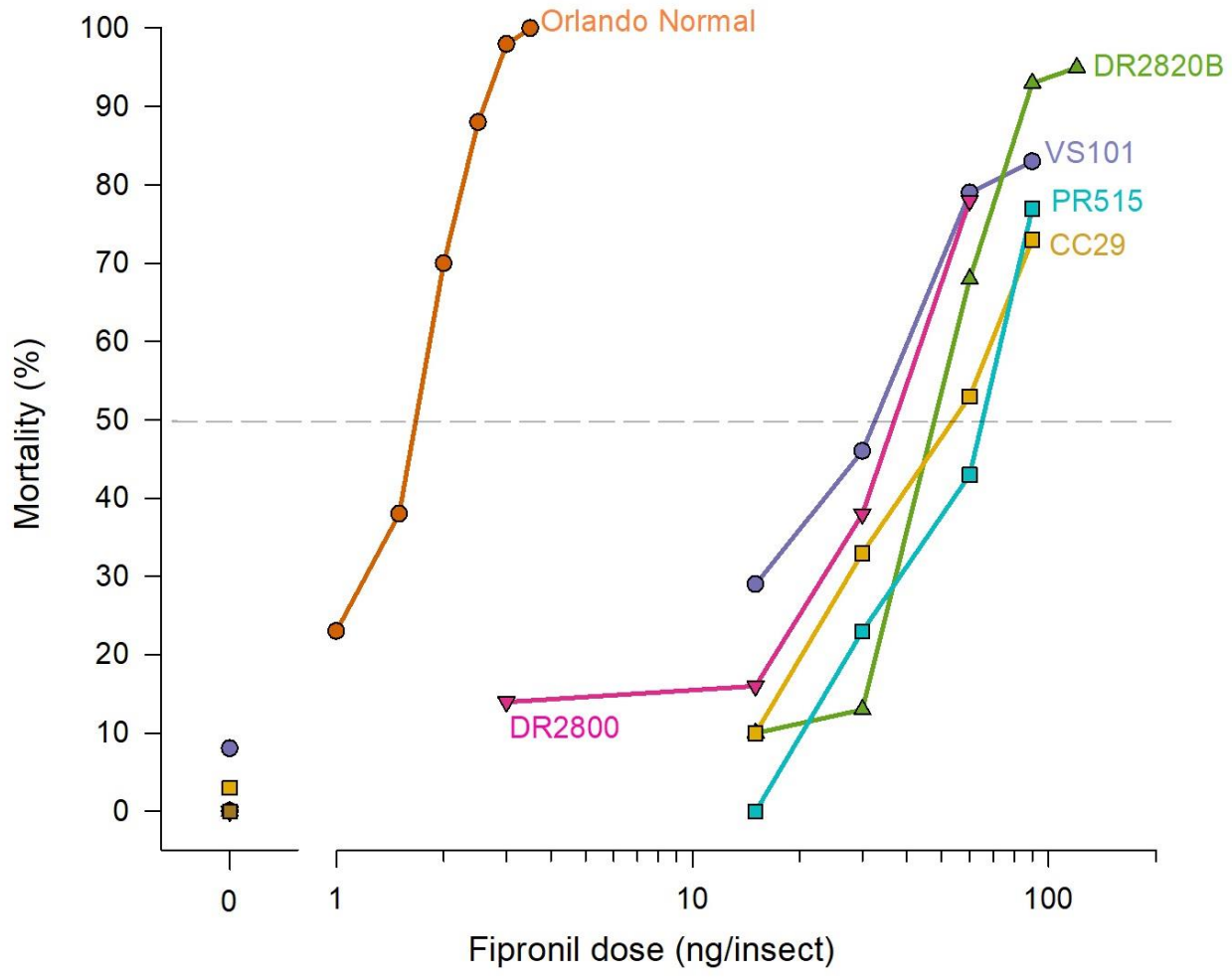

Fig. 3.1. Dose -response curves for fipronil-treated *B. germanica* adult males from a reference insecticide-susceptible strain (Orlando Normal) and five populations recently collected in apartments in Raleigh, NC. The lethal dose of fipronil that killed 50% of each population (LD_{50}) was determined by topical application. Mortality was assessed daily, and mortality at 4 d is reported. At least three replicates of 10 adult male cockroaches were performed per dose.

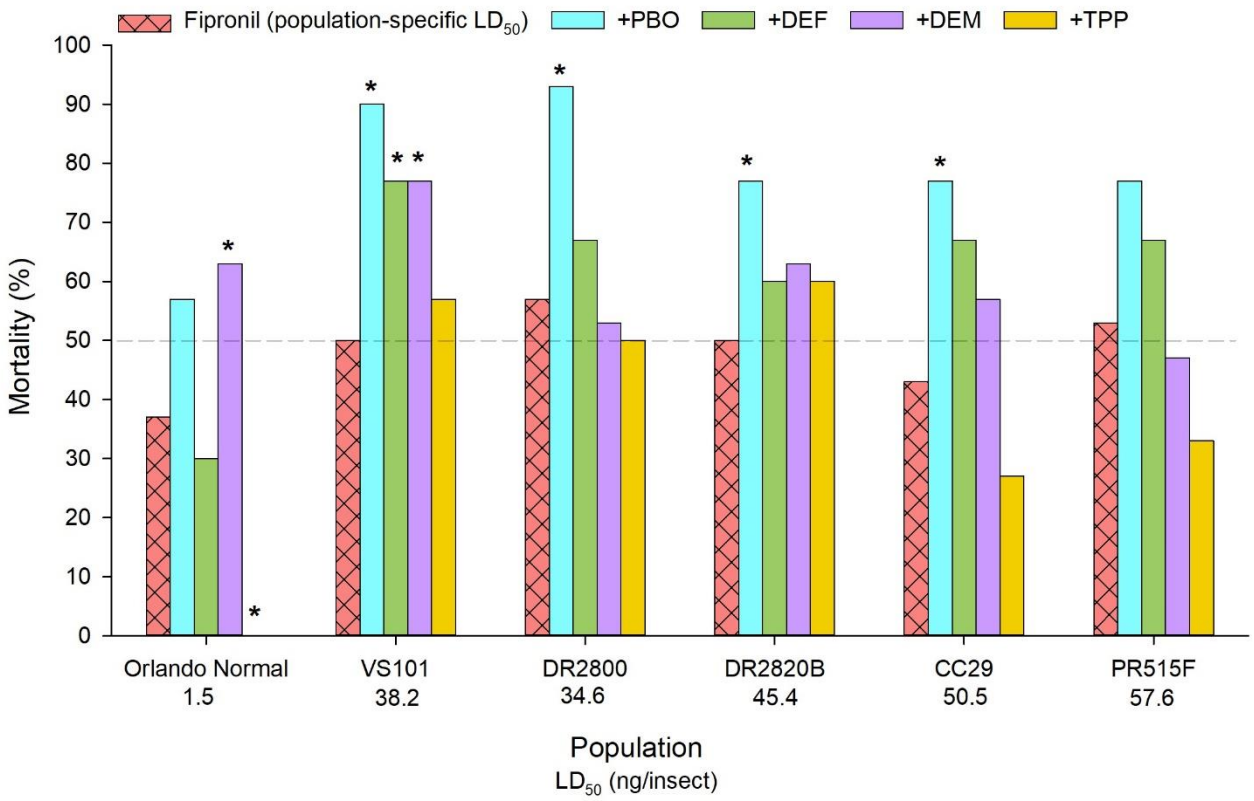

Fig. 3.2. Effects of four detoxification enzyme inhibitors (i.e., insecticide synergists) on fipronil toxicity in *B. germanica* adult males. Each enzyme inhibitor (piperonyl butoxide [PBO], *S*,*S*,*S*-tributyl phosphorotrithioate [DEF], diethyl maleate [DEM], and triphenyl phosphate [TPP]) was topically applied in 1 µl acetone 1 h prior to application of a population-specific LD_{50} (shown on the X-axis). Percent mortality was determined 4 d after treatment and mortality was corrected for control mortality (synergist only). The mean shown is of 3 replicates with 10 males each (n = 30 males per treatment). Significant differences between fipronil-only treatments and fipronil plus inhibitor treatments were determined using Chi-square analysis, with significance indicated by * ($p < 0.05$).

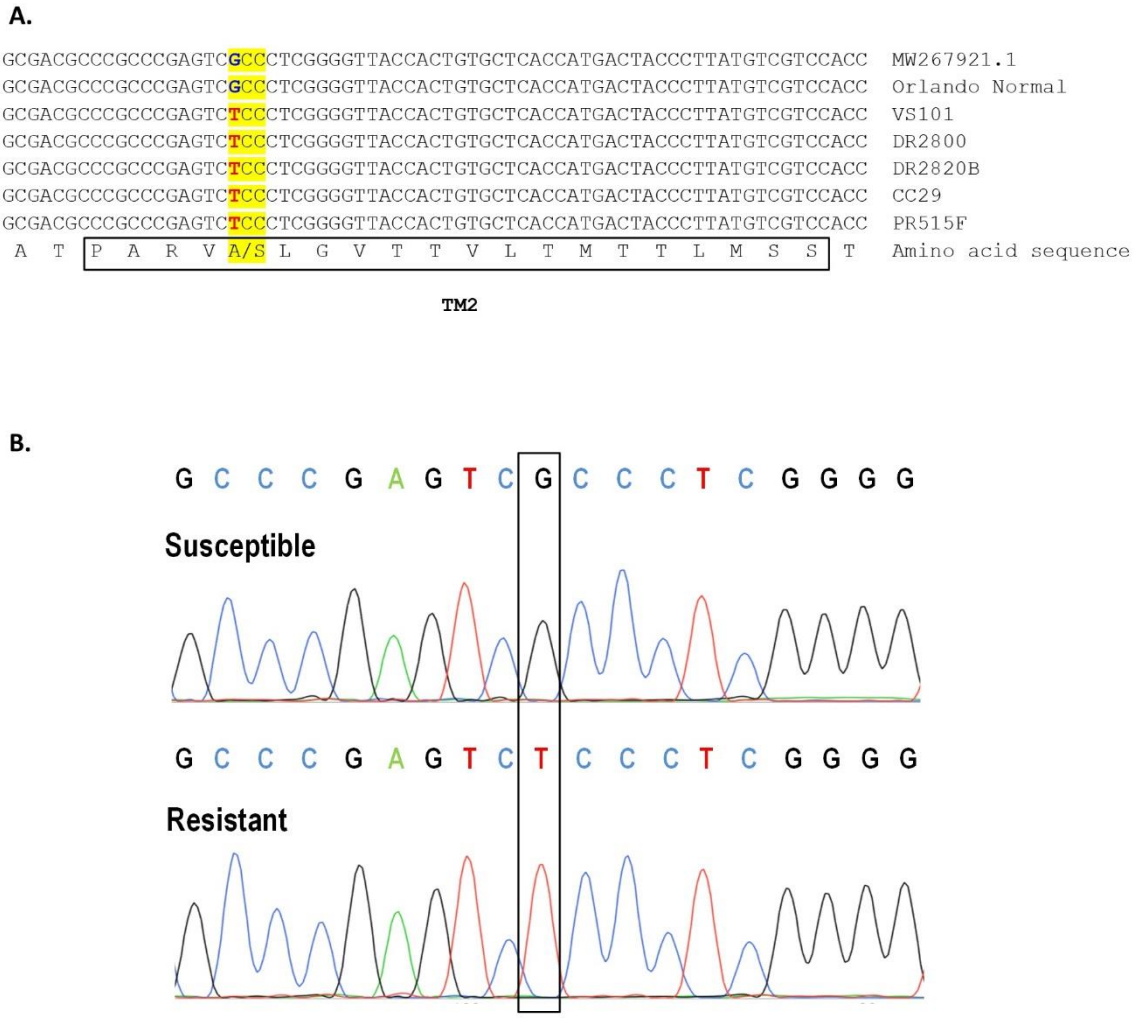

Fig. 3.3. Nucleotide sequences of the TM2 region of the *B. germanica Rdl* gene, which includes the point mutation that results in the A302S substitution. **A.** Representative sequences from the insecticide-susceptible Orlando Normal strain and five field-collected German cockroach populations were aligned against the reference sequence (MW267921.1), with the A302S region highlighted. **B.** Representative direct sequencing chromatograms of homozygous susceptible (G/G) and homozygous resistant (T/T) cockroaches. The G-to-T point mutation site is shown within a box. MW267921.1 is the GenBank accession number for the *B. germanica* GABA-gated chloride channel complete cds (Jones et al. 2021).

CHAPTER 4

Systemic veterinary drugs for control of the common bed bug, *Cimex lectularius*, in poultry farms

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Abstract

Background: The common bed bug, *Cimex lectularius,* is a hematophagous ectoparasite that was a common pest in poultry farms through the 1960s. DDT and organophosphates eradicated most infestations, but concurrent with their global resurgence as human ectoparasites, infestations of bed bugs have been reappearing in animal farms. Although the impact of bed bugs on chicken health has not been quantified, frequent biting and blood-feeding are expected to cause stress, infections, and even anemia in birds. Bed bug control options are limited due to the sensitive nature of the environment, limited products labeled for bed bug control, and resistance of bed bug populations to a broad spectrum of active ingredients. Veterinary drugs are commonly used to control ectoparasites, including mites and lice, on chickens. In this study, we evaluated the effects of two common veterinary drugs on bed bugs within the framework of xenointoxication treatment of hosts with systemic antiparasitic drugs.

Methods: We conducted dose-response studies of ivermectin and fluralaner using a membrane feeding system and several bed bug strains and compared our findings to *in vivo* treatments of birds. We evaluated the efficacy of ivermectin and fluralaner on bed bugs using different doses and delivery methods (topical treatment and ingestion) with chickens.

Results: Both ivermectin and fluralaner caused high mortality in insecticide-susceptible and pyrethroid resistant bed bugs using the artificial feeding system. Whereas ivermectin was ineffective in chickens either by topical treatment or ingestion, delivery of fluralaner to chickens by ingestion resulted in high mortality of bed bugs that fed on these chickens up to 28 days post treatment.

Conclusions: These findings suggest that systemic ectoparasitic drugs have great potential for practical use to control bed bug infestations in poultry farms. These findings also validate the

efficacy of fluralaner (and potentially other isoxazolines) as a potent new active ingredient for bed bug control.

Keywords: *Cimex lectularius*, fluralaner, ivermectin, xenointoxication, poultry, chicken

Introduction

The common bed bug (*Cimex lectularius*) is an obligate hematophagous ectoparasite that is commonly associated with humans as its main host; however, bed bugs will opportunistically parasitize other animals, including birds and bats [1]. Infestations of bed bugs in poultry farms were reported as early as the 1940s in North America [2] and Europe [Review: 3]. In the U.S., bed bugs were reported as major pests in poultry in 1985 [4]. Bed bugs are wingless, nocturnal, and cryptic insects that have limited dispersal capabilities, but are readily transported by humans [5, 6]; thus, it is likely that the introduction of bed bugs to poultry facilities is human-mediated [7]. Although the health effects of bed bugs on poultry are understudied and not clearly documented, it is reasonable to expect that as with other blood-sucking ectoparasites, skin reactions, itchiness or feather pecking, restlessness, anemia, overall decreased production and secondary infections could be consequences of bed bug infestations [7, 8].

Bed bug control in poultry

Bed bug infestations were largely eradicated from the poultry industry with the use of DDT and organophosphates [Review: 3], corresponding to similar outcomes in residential settings. However, DDT, most organophosphates, and other early approaches to bed bug control, such as turpentine, boiling water, kerosene and burning sulfur, have been discontinued due to health and safety concerns. Today, pyrethroids are the primary class of insecticides used in the poultry industry to control bed bug populations, along with some organophosphates, spinosyns and neonicotinoids. Although pyrethroid resistance has not been systematically evaluated in bed bugs collected from poultry farms, in preliminary assays we found that a bed bug population collected from an organic chicken farm was highly resistant to the pyrethroid deltamethrin (Schal, unpublished results). Pyrethroid resistance is widespread in bed bug populations worldwide [9],

and target-site resistance (*kdr*) has dramatically increased in bed bug populations in the last decade [10]. Therefore, bed bugs that are highly resistant to pyrethroids are expected to be introduced into poultry farms from residential infestations. Moreover, high levels of DDT resistance have been reported in populations of bed bugs collected in poultry houses [11]. Because DDT and pyrethroid insecticides target the voltage-gated sodium channels of insects, and P450 detoxification enzymes inactivate both groups of insecticides, historical resistance to DDT can confer cross-resistance to pyrethroids in bed bug populations [12].

Chemical control limitations

Limited availability of insecticides, and resistance to the most commonly used insecticides, appear to be major constraints in bed bug control. Permitted use of pyrethroids, neonicotinoids, and organophosphates is linked to the production cycle. For example, when birds are not present in the facility, allowed insecticides include lambda-cyhalothrin, cyfluthrin, and beta-cyfluthrin (all pyrethroids), imidacloprid (neonicotinoid), and two insect growth regulators [3]. When birds are present, however, chemical treatments are much more restricted: only dichlorvos, tetrachlorvinphos (organophosphates) and some permethrin formulations can be used for bed bug control. Some dust formulations of inorganic insecticides are also available, but their efficacy in the challenging poultry environment has been inconsistent [13]. Non-chemical interventions are limited to heat treatments. However, spatial heat treatments are particularly challenging in a poultry house, because high temperature of at least 118°F must be maintained for at least 90 minutes [14]. This procedure is expensive, highly demanding, and nearly impossible in such poorly insulated, porous, and very large structures.

Systemic treatments of hosts to control bed bugs

Systemic medication of the host, or xenointoxication, the systemic treatment of hosts to kill parasites, is widely used in human [15, 16] and veterinary medicine to control endoparasites (e.g., mosquito-borne pathogens) and ectoparasites, such as mosquitoes, ticks, and fleas. This strategy has been broadly accepted for use in pets and companion animals [Review: 17]. Studies that combined blood with insecticides in membrane-based artificial feeders, have demonstrated considerable mortality in the common bed bug; effective active ingredients include conventional insecticides such as abamectin and fipronil [18], and anti-parasitic drugs such as ivermectin and moxidectin [19] and fluralaner [20]. *In vivo* xenointoxication was also effective on bed bugs that were fed on ivermectin-treated mice [21] and rabbits [22].

Ivermectin and fluralaner

Ivermectin, first introduced as an antiparasitic drug in 1981 [17, 23] is a remarkably safe and effective, and commonly used in humans to control parasitic infections transmitted by mosquitoes (e.g., lymphatic filariasis), reduce malaria transmission, and to treat scabies, onchocerciasis and myasis [24]. Ivermectin is a macrocyclic lactone that acts on the glutamate-gated chloride (GluCI) ion channel of insects, causing hyperpolarization in nerves and muscles [25]. The wide variety of uses of ivermectin and other avermectins, include companion animals (dogs and cats) and livestock (cattle, horses, sheep, and swine) to control endoparasites such as heartworm and roundworm and ectoparasites like mites, fleas, and ticks [24]. Currently, ivermectin-based formulations are available for use in poultry only under prescription and with appropriate withdrawal periods [26].

Fluralaner, is a relatively new drug introduced to the market in 2014, as a flea treatment for dogs and in 2019 for cats. Fluralaner belongs to the isoxazolines class of insecticides that includes only parasiticide compounds. Isoxazolines have a dual mode of action as inhibitors of

the gamma-aminobutyric acid (GABA)-gated chloride channels (GABACls) and L-glutamate-gated chloride channels (GluCls) with insecticidal and acaricidal activity [27]. Several studies have evaluated the efficacy of fluralaner administered to hens to control the poultry red mite [28, 29] and the northern fowl mite [30]. Currently, there are no formulations of fluralaner for poultry use in North America. However, in Europe, fluralaner is approved for the use in chickens to control mites.

The ultimate goal of this project is to implement systemic veterinary drugs in chickens as an efficient and effective way to suppress and potentially eradicate bed bug infestations from poultry farms. Toward this end, we conducted ivermectin and fluralaner dose-response studies with bed bugs, using a membrane feeding system in which blood could be dosed with precise concentrations of active ingredients. We then transitioned to chicken flocks and tested the efficacy of these drugs by ingestion or topical treatment in chickens.

Materials and Methods

Experimental insects and rearing procedures

The Harold Harlan (Harlan) strain of *C. lectularius* was collected at Ft. Dix, NJ in 1973 and maintained on a human host until 2008. Between 2008 and 2021, the Harlan strain was maintained in our laboratory on defibrinated rabbit blood, and since 2021 on human blood (below). Since its collection, the Harlan population has not been challenged with insecticides, and therefore it was used in this study as an insecticide susceptible reference strain. Five other more recently field-collected strains (**Table 1**) were assayed in the *in vitro* dose-response feeding experiments.

Bed bug colonies were reared in 118 cm^3 plastic jars with cardstock paper substrate and plankton netting (BioQuip Products, Rancho Dominguez, CA, USA) to allow aeration and feeding. Bed bugs were maintained at 25°C, 50 ± 5% RH, and a photoperiod of 12:12 (L:D) h and fed weekly on human blood delivered through an artificial feeding system. Heparinized human blood was supplied by the American Red Cross (IRB #00000288 and protocol #2018-026). The artificial feeding system was modified after Montes et al. (2002) and Sierras and Schal (2017). It was housed in an NCSU-approved BSL-2 facility (Biological Use Authorization # 2020-09-836) and consisted of water-jacketed custom-fabricated glass feeders (**Fig. 1a**), each with an internal blood chamber within a circulating water chamber connected to a circulating water bath heated to maintain blood near human skin temperature (~32°C). Although each blood chamber had a 4 mL capacity, we used 2 mL, which was sufficient to eliminate air from the blood chamber. Plant grafting tape (A.M. Leonard Horticultural Tool and Supply Co., Piqua, OH, USA) was stretched across the bottom of the feeder and served to hold the blood within each feeder and as a membrane through which bed bugs could feed. Several feeders were connected in series to the water circulator, which allowed multiple colonies to be fed concurrently.

Pyrethroid resistance in bed bugs

Deltamethrin (98.9% purity, Chem Services, West Chester, PA, USA) was used in a dose-response study with the Harlan strain, to estimate the LD_{99} dose, which we then used as a diagnostic dose on the field-collected strains. Healthy 4 d post-feeding adult male bed bugs, were placed in plastic Petri dishes (diameter = 60 mm, Thermo Fisher Scientific, Waltham, MA, USA) lined with filter paper (Whatman No. 1, Sigma-Aldrich). Bed bugs where briefly anesthetized

with CO_2 and topical applications of deltamethrin (in acetone) were made with a microapplicator (Hamilton Co., Reno, NV, USA) equipped with a 25-µl glass syringe (Hamilton Co.) that delivered 0.5 µl of solution on the ventral thorax of each bed bug. We used eight doses between 0 (acetone control) and 10 ng, at least 25 adult male bed bugs per dose, for a total of approximately 240 bed bugs. Mortality was assessed every 24 h for 48 h by gently touching individual bed bugs with entomological forceps, categorizing them as alive (coordinated avoidance movement) or dead (no response or unable to right themselves after touching with forceps). The LD_{50}, LD_{90}, and LD_{99} were estimated from probit analysis (below) and the LD_{99} was used as a diagnostic dose on all field-collected bed bug populations.

Birds

Two flock of birds were used for this study. The first group consisted of thirty 3-year-old Rhode Island Red hens (*Gallus gallus domesticus*) (body weights ranged from 1.9 to 2.8 kg, average 2.4 kg) ranging in age from 2 to 3 years. The second group was comprised of 11 birds of the same breed, age range of 1 to 2 years, and weight averaging 2.5 kg (range 2.0 to 3.3). Birds were maintained on a 12:12 hour light/dark cycle and housed as a group in a climate-controlled facility (15.6 m^2) with wooden shaving substrate. Hens were obtained from a private supplier and maintained on a Layer NCSU mill diet with water available ad libitum through automatic waterers with nipple attachments. The flock was monitored to ensure optimal health conditions based on serial physical examinations, fecal floatation, serial packed cell volumes via microhematocrit tube and centrifugation, serial total solids via refractometer, and serial biochemical panels (VetScan Avian/Reptile Profile Plus, Abaxis Inc, Union City, CA, USA). All

study procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC # 21-152).

In vitro feeding assays using artificial feeders

To deliver the active ingredient effectively in blood, technical grade fluralaner (≥98%, Cayman Chemical, Ann Arbor, Michigan, USA) and ivermectin (≥98%, MilliporeSigma, Allentown, PA, USA) were dissolved in DMSO to make stock solutions of 10 mg AI/mL DMSO, from which we made serial dilutions to achieve the desired concentrations. Two µL of each AI in DMSO solution was added to 2 mL of blood (0.1% DMSO final concentration in blood) just before the assay commenced.

Healthy adult male bed bugs of unknown ages were separated from the colony and used 5–7 d post human blood feeding (**Fig. 1d**). For each replicate, 10 males were placed into 20 mL clear PET plastic containers with cardboard inserts for harborage and plankton netting on top to allow feeding. Each group of bed bugs was given 15 min to feed on human blood and only fully engorged individuals (**Fig. 1b**) were retained for further study. Fully fed bed bugs were transferred to individual wells of 24-well cell culture plates (Corning Inc., Corning, NY, USA) with tightly fitting filter paper at the bottom of each well. Mortality was assessed every 24 h for up to 7 d post feeding by touching the insects with entomological forceps and categorizing bed bugs as alive or dead, as described above. We conducted 3 replicates (total n = 30 bed bugs) for each insecticide concentration and two control groups consisted of human blood alone and human blood plus 0.1% DMSO. We used adult male bed bugs from six unique field-collected strains (**Table 1**).

In vivo feeding assays with chickens

Harlan strain adult male bed bugs (15 insects per replicate; **Fig. 1d**), 5–7 d post human blood feeding, were placed into 20 mL clear plastic containers, as described above. Each chicken was held by gently placing the bird on the lap, and to minimize stress, the bird was gently restrained in a towel. The plankton netting of the bed bug container (**Fig. 2b**) was gently placed on the lateral inguinal area of the chicken, and bed bugs were allowed to feed for 10 min (**Fig. 2a**). As in the *in vitro* assays, fully engorged bed bugs were transferred individually to each well of 24-well cell culture plates and maintained in an incubator at the same conditions as the rearing conditions. Each chicken received a single bed bug replicate (maximum 15 bed bugs) on a given day, and we alternated sides on the chicken at subsequent feeding sessions.

Ivermectin. Twelve chickens where randomly selected and divided into control and treatment groups. Ivermectin (Ivermax 1% sterile solution, **Aspen Veterinary Resources**, Liberty, MO, USA) was administered via subcutaneous injection in two birds, and the dose was adjusted to the weight of each bird (0.2 mg/kg). The same dose of ivermectin (0.2 mg/kg) was administered to six birds via oral gavage using a needleless syringe. The dose of 0.2 mg/kg was based on Cirak et al. [31]. The remaining four chickens were assigned to a control group that received only water gavage. Each chicken was assessed four times before and after treatment by allowing groups of bed bugs to feed on the bird as follows: (1) before treatment control (Pre-T1); (2) 30 min after the ivermectin administration (0.5 h Post-T1); (3) two days after treatment (2 d Post-T1); and (4) seven days after the initial treatment (7 d Post-T1). Mortality of each group of bed bugs was assessed every 24 h for up to 7 d post feeding.

Fluralaner. Twenty-four birds were randomly assigned to control and treatment groups. Since no fluralaner-containing products are labeled in the USA for use in poultry, we used

Bravecto™, a commonly used ectoparasitic drug for dogs and cats. Bravecto™ was administered orally via gavage (miniature dog formulation) or topically (kitten formulation). Topical administration was 2.5 mg/kg to the top of the neck of each chicken. We used two doses for oral administration of Bravecto™: In Experiment 1, 2.5 mg/kg administered once represented a high dose; in Experiment 2, 0.5 mg/kg administered at baseline (d 0; treatment 1 = T1) and again seven days later (d 7; treatment 2 = T2). The high dose was based on Prohaczik et al. [32], whereas the lower dose regime represented a protocol approved by regulatory authorities in Australia and the European Union for use of a fluralaner-containing product on chickens (Exzolt™, MSD Animal Health, Germany [33]). All doses were adjusted to the body mass of each bird. As with ivermectin, each chicken was assessed several times before and after treatment by allowing groups of bed bugs to feed on the bird. Chickens that received the high dose (2.5 mg/kg, Experiment 1) were assessed seven times, as follows: (1) before treatment control (Pre-T1); (2) 30 min after the fluralaner administration (0.5 h Post-T1); (3) two days after treatment (2 d Post-T1); (4) seven days after treatment (7 d Post-T1); (5) 14 d after treatment (14 d Post-T1); (6) 21 d after treatment (21 d Post-T1); and (7) 28 d after the initial treatment (28 d Post-T1). Birds that received the lower dose of fluralaner (0.5 mg/kg, Experiment 2) were assessed two more times (nine total), including 30 min after the administration of the second dose on day 7 (7 d Post-T1 = 0.5 h Post-T2), and two days later (9 d Post-T1 = 2 d Post-T2). Mortality of each group of bed bugs was assessed every 24 h for seven days after blood-feeding on a bird.

The aim of Experiment 3 was to replicate Experiment 2 (0.5 mg of fluralaner administered twice, seven days apart) with a different flock of six younger chickens. Moreover, blood was drawn from these six chickens, as well as five other chickens that were not exposed to

bed bugs. Thus, the time course of fluralaner plasma concentrations was quantified in 11 birds treated by oral gavage (0.5 mg of fluralaner administered twice, seven days apart).

Plasma concentrations and pharmacokinetics (*ongoing*)

All eleven birds from the second flock of chickens were used. Birds were divided into two groups of five and six chickens; both groups where treated twice with 0.5 mg/kg of fluralaner (at baseline = d 0, and at d 7) and blood was collected at the same time points indicated above for experiment 3 (Pre-T1, 0.5 h Post-T1, 2 d Post-T1, 7 d Post-T1, 0.5 h Post-T2 (d 7), 9 d Post-T1, 14 d Post-T1, 21 d Post-T1, and 28 d Post-T1). For the six chickens that were evaluated in bioassays with bed bugs, blood was collected immediately after bed bug feeding. Blood was collected using a 26-gauge needle and 1-mL syringe, alternating collections from a leg (medial metatarsal), wing (ulnar veins), and jugular of each bird. Birds were manually restrained during blood collection. Blood samples were centrifuged (3,500 × *g* for 6 min) within 1 h after collection and stored at −20°C until assayed.

Data analysis

The fluranalar LC_{50}, LC_{90}, and LC_{99} estimates for each bed bug population were determined using log-dose probit-mortality analysis based on a spreadsheet template [34]. The values agreed with analysis in PoloPlus (LeOra Software, Petaluma, CA). Abbott's correction (Abbott 1925) was used to correct for control mortality, as needed. The dose-response curve of each bed bug population was compared to that of the susceptible Harlan population. We also obtained ivermectin LC_{50}, LC_{90}, and LC_{99} estimates from log-dose probit-mortality analysis, but this analysis was restricted to the susceptible Harlan population. Likewise, log-dose probit-mortality

analysis of Harlan strain bed bugs was used to obtain an estimate of the LD_{99} value for deltamethrin. This dose was used as a diagnostic dose on the five field-collected strains. The effects of both ivermectin and fluralaner treatments in birds on Harlan bed bugs that fed on the treated birds were determined using ANOVA and Tukey's HSD test (JMP 2020). Means are presented with standard error of the mean (SEM). Fluralaner *in vitro* toxicity to each population was compared to the susceptible Harlan population using a resistance ratio (RR), calculated as (LC_{50} of the field-collected population)/(LC_{50} of the Harlan population). We used the lethal dose ratio significance test: the 95% confidence limits (CLs) of the RR were calculated, and if this confidence interval did not include the value of 1.0, then the RR at the LC_{50} was considered significant [35]

Results

Pyrethroid resistance in bed bugs

We topically applied deltamethrin to Harlan strain bed bugs to conduct a probit analysis of their log-dose-response. The LD_{50} was 1.4 ng (95% CI: 1.0, 2.1 ng; $n = 140$; slope = 2.5 ± 0.3 [SE]; $\chi^2 = 5.80$, df = 5; *t*-ratio: 7.2 [$P < 0.05$]) (**Table 1**). The estimated LD_{99} diagnostic dose was 11.8 ng deltamethrin (95% CI: 5.9, 51.5 ng), and this dose was topically applied to all field-collected populations to determine the percent mortality, as shown in **Table 1**. We found high levels of deltamethrin resistance in all five field-collected strains, including a strain (WS) maintained in the laboratory since 2008.

In vitro feeding assays using artificial feeders

We conducted a comparative dose-response analysis using technical grade fluralaner and ivermectin, each dissolved in DMSO and added to human blood in an artificial feeding system. A time-course study evaluated daily mortality of Harlan (susceptible) bed bugs fed various concentrations of fluralaner and ivermectin (**Fig. 3**). **Figure 4b** shows the relationship between cumulative mortality of Harlan strain bed bug, concentration of fluralaner in blood, and time since bed bugs fed (0 to 7 d). Based on this analysis, we chose to evaluate mortality 7 days after bed bugs fully fed on human blood. The results from the *in vitro* feeding experiments are shown in **Table 1** and **Fig. 3**. The LC_{50} value of fluralaner, tested on adult males of the Harlan susceptible bed bugs, was 15.3 ng/mL blood (95% CI: 11.7, 19.8 ng) and the estimated LC_{90} value was 38.6 ng/mL blood. We conducted a similar dose-response study using technical grade ivermectin and adult males of the Harlan strain. The LC_{50} value was 61.0 ng/mL (95% CI: 52.7, 69.9 ng) and the estimated LC_{90} value was 114.9 ng/mL blood (**Table 1**).

The flunalaner LC_{50} values of the five field-collected strains ranged from 18.0 to 23.2 ng/mL blood (**Table 1**, **Fig. 4a**); these values did not differ significantly from those of the Harlan strain bed bugs based on overlap of their 95% CI with the Harlan strain 95% CI. Although some significant resistance was found when FM, WS, and SH were compared with HA, the low resistance ratios (RR, based on LD_{50} values) of 1.2 to 1.5 in field-collected strains indicate minimal or no resistance to fluralaner. Further, the relatively steep slopes of all dose-response curves in all bed bug strains, are indicative of homogeneous populations in their response to ingestion of fluralaner. We found no evidence of any correlation between deltamethrin-caused mortality and fluralaner RR across the five strains (Spearman's $\rho = 0.2294$, $P = 0.7105$, $n = 5$). We did not evaluate the effect of ivermectin on the field collected strains.

In vivo feeding assays using chickens

At the dose of ivermectin we administered to chickens (0.2 mg/kg), treatments via injection did not result in any mortality in bed bugs (data not shown). However, when the same dose of ivermectin was administered via oral gavage, 5 ± 2.4% (SEM, range = 0–14%, n = 6 chickens) of the bed bugs that fully fed on the chickens two days after treatment died. This low mortality was significantly higher ($P < 0.05$) than at baseline (day 0), before ivermectin was administered to chickens. Still, because no bed bugs died after feeding on ivermectin-treated chickens 7 days after the treatment, we discontinued our studies with ivermectin.

We conducted three sequential experiments to elucidate the efficacy of fluralaner in chickens against bed bugs: Experiment 1 was a high dose of fluralaner (2.5 mg/kg) (**Fig. 5a**), and Experiments 2 (**Fig. 5b**) and 3 (**Fig. 5c**) were the label rate of fluralaner (two treatments of 0.5 mg/kg each, administered a week apart) with two different flocks of chickens. For each experiment we developed a time-course of mortality (days 0 to 28) and we monitored bed bugs daily for seven days after they fully fed on chicken blood.

Mortality was assessed over the time course of 7 days (day 0 to day 7) after bed bugs fed on chicken blood (**Fig. 6**). Quantitative analysis is based on cumulative percent mortality on day 7, when we obtained the highest level of mortality in all treatment groups with overall low mortality (<1.3%) of control bed bugs after they fully fed on untreated chickens (**Fig. 6a–c**).

In Experiment 1 (2.5 mg fluralaner/kg), 96.3 ± 2.6% (SEM, range = 84.6–100%, n = 6 chickens) of the bed bugs that fully fed on chickens died 0.5 h after gavage treatment (**Fig. 5a**). This high mortality was significantly higher than on day 0 (1.3 ± 1.3% mortality), before the gavage administration (ANOVA, Tukey's HSD, $P < 0.0001$; ANOVA results are shown in **Table 2a**). Mean mortality peaked on day 7 (100%) and remained >95% up to day 14. There was

higher variation and an overall decline in bed bug mortality on days 21 (66.8 ± 18.7%, range = 0–100%) and 28 (60.5 ± 19.6%, range = 0–100%) after treatment. Note that on day 21 only four of the six chickens were used as replicates due to technical constraints. Nevertheless, there were no significant differences in bed bug mortality across all times post gavage treatment ($P > 0.05$), and bed bug mortality was significantly higher at all times than on day 0 before fluralaner treatment (**Fig. 5a**). A graphical representation of the time-course of bed bug mortality after the chickens were fed fluralaner (days 0 to 28) and after bed bugs fully fed on chickens (days 0 to 7) is shown in **Fig. 6a**.

In Experiment 2, six chickens of the same flock as in Experiment 1 were treated by gavage with 0.5 mg fluralaner/kg on day 0 and again on day 7. The overall pattern of bed bug mortality was similar to that in Experiment 1 (**Fig. 5b**). However, bed bugs fed on chickens 0.5 h after the first treatment had lower mortality (72.5 ± 13.7%, $n = 6$ chickens; $P = 0.0296$) and higher variation across the six replicates (range = 7.7–100%) than mean bed bug mortality on 2 days after treatment (100%). Bed bug mortality remained >95% up to 21 days after treatment and there was no significant differences between days 2 and 21 ($P > 0.05$). By day 28, however, mean mortality significantly declined to 70.0 ± 8.1% ($P < 0.05$), and we observed higher variation among replicates (range = 38.5–92.9%) (**Figs. 5b, 6b**). Mortality was significantly higher at all time points than on day 0 (no mortality, before the gavage administration) (ANOVA, Tukey's HSD, $P < 0.0001$; ANOVA results are shown in **Table 2b**). Note again that in this experiment, a second gavage treatment was administered on day 7.

The design of Experiment 2 – chickens treated with 0.5 mg fluralaner/kg twice, on day 0 and day 7 – was replicated in Experiment 3 with 6 chickens from a younger flock. We also drew blood from these chickens for pharmacokinetic analysis. Overall, Experiments 2 and 3 yielded

similar results. However, in Experiment 3 bed bug mortality lagged somewhat, at only 55.8 ± 11.3% (range = 21.4–92.9%) (**Figs. 5c, 6c**). Bed bug mortality increased to 100% across all six replicates two days after the fluralaner treatment. As in Experiment 2, there was a slight, but statistically insignificant ($P > 0.05$) decline in mortality, to 80.7 ± 3.5% on day 7 after the first treatment. However, mortality quickly increased to 100% across all six replicates within 0.5 h after the second fluralaner treatment on day 7. Bed bug mortality remained >90% up to 21 days but declined to 66.7 ± 12.7% (range = 20.0–100%) by day 28 (**Fig. 5c**); this decline was significant relative to the peak mortality on days 2, 9, and 14 ($P = 0.0121$). Mortality was significantly higher at all time points than on day 0 (no mortality, before the first gavage administration) (ANOVA, Tukey's HSD, $P < 0.0001$; ANOVA results are shown in **Table 2c**).

We compared the three experiments, by day, after the first gavage treatment. We observed a significant difference (ANOVA, $F_{(2,15)} = 3.8424$, $P = 0.0449$) at 0.5 h after the first gavage treatment. As expected, post hoc analysis indicated significantly higher bed bug mortality with the higher dose of fluralaner in Experiment 1 (2.5 mg/kg of fluralaner) than in Experiment 3 (0.5 mg/kg administered twice) ($P = 0.0368$). However, mortality in Experiment 2 was intermediate and not significantly different from mortality in the other two experiments. No difference was observed 2 days after the respective gavage treatments (ANOVA, $F_{(2,15)} = 1.0000$, $P = 0.3911$). Seven days after treatments, we again observed a significant difference among the three experiments (ANOVA, $F_{(2,15)} = 8.6159$, $P = 0.0032$), and Tukey's HSD test indicated that Experiments 1 and 2 resulted in significantly greater mortality than Experiment 3 ($P = 0.0028$ and 0.00329, respectively); however, there was no difference ($P = 0.4533$) between Experiment 1 (100% mortality) and Experiment 2 (94.1% mortality). Experiments 2 and 3 differed only in the age of the chicken flocks and blood being drawn from chickens in Experiment 3. Although

bed bug mortality was high in both experiments 0.5 h after the second fluralaner treatment on day 7 (95.4 and 100%, respectively), these values were significantly different (*t*-test, *t*-ratio = 3.1583, $P = 0.0102$). However, 2 days after the second fluralaner treatment (day 9 of the experiment), mortality in both Experiments 2 and 3 was similar (98.4 and 98.7% mean mortality, respectively). Likewise, there were no differences among the three experiments on day 14 (range = 96.2–98.8%; $F_{(2,15)} = 0.4923$, $P = 0.6208$), day 21 (range = 66.8–97.5%; $F_{(2,13)} = 2.1643$, $P = 0.1544$), and day 28 (range = 60.5–69.6%; $F_{(2,15)} = 0.1024$, $P = 0.9033$). It is important to note, however, that at the termination of all experiments on day 28, variation across replicates was highest in Experiment 1 (highest dose administered once), ranging from 0% to 100% bed bug mortality.

Discussion

Bed bugs are obligatory blood-feeders. Unlike holometabolous blood-feeders (e.g., mosquitoes, fleas), all stages of the hemimetabolous bed bug must feed on blood to molt and reproduce. Moreover, unlike mosquitoes, where males feed on nectar and not on blood, both male and female bed bugs are obligatorily dependent on blood meals. Their dependency on host feeding make systemic veterinary antiparasite drugs particularly appropriate to consider for bed bug control. The use of veterinary drugs has been highly effective for controlling pests on companion and farm animals, including fleas, ticks, mosquitoes, and mites. Bed bugs have re-emerged in poultry farms throughout the US, and infestations represent a serious and increasing problem in the poultry industry (Gonzalez and Schal personal observation). A limited number of active ingredients is labeled for bed bug control in this challenging environment, and insecticide resistance is further limiting the suitability of some insecticides. This is the first study to explore

the systemic use of veterinary drugs (xenointoxication) to control bed bugs as ectoparasites of chickens.

Both ivermectin and fluralaner were highly effective against bed bugs under laboratory conditions. The ivermectin concentration that caused mortality in 90% of fully fed bed bugs from the insecticide-susceptible Harlan strain was 114.9 ng/mL human blood. The respective fluralaner concentration was significantly lower, at 38.6 ng/mL blood. Fluralaner was equally effective on five field-collected bed bug strains with a history of resistance to pyrethroid insecticides, including a strain we recently collected from an infested poultry farm.

We treated chickens with ivermectin or fluralaner either by topical application or by ingestion (gavage) and allowed unfed bed bugs to fully engorge on these chickens' blood. Despite its effectiveness in the membrane feeders, ivermectin was ineffective in these assays. Therefore, we discontinued testing ivermectin and did not conduct further dose-mortality assays with the field-collected strains. In three experiments with two chicken cohorts, we administered fluralaner to chickens (oral gavage). In all three experiments, 100% of the bed bugs that fully fed on chicken blood 2-days after fluralaner administration died. We observed high bed bug mortality up to 28 days after the initial fluralaner treatment of chickens. However, the single high dose of fluralaner (2.5 mg/kg) resulted in high variation in bed bug mortality at days 21 and 28 . In contrast, two successive 0.5 mg/kg treatments with fluralaner, 7 days apart, resulted in sustained high mortality up to at least 28 days. Importantly, we did not observe any allergic or other adverse reactions in fluralaner-treated chickens. Overall, our results strongly support the idea that fluralaner is an excellent candidate for bed bug control in infested poultry facilities.

Use of systemic veterinary drugs to control bed bugs: Ivermectin

Drug administration to control hematophagous insects has both advantages and disadvantages relative to the use of residual insecticides. Primary among the advantages is the potentially high bioavailability of the drug in the host's blood soon after its administration [36]. But a major disadvantage is that some drugs in some hosts are rapidly detoxified and/or cleared from the host plasma. Although we did not conduct pharmacokinetic analysis with ivermectin, using a membrane-based feeding apparatus with human blood, we determined that the ivermectin LC50 for bed bugs was 61.0 ng/mL, similar to that reported with heparinized mouse blood supplemented [37]. Therefore, a blood concentration >61.0 ng/mL, maintained for at least several days, would be desirable for effective control and ultimately elimination of bed bugs in chicken facilities.

However, multiple bioassays with bed bugs and pharmacokinetic studies in chickens suggest that ivermectin does not reach this target concentration in blood. For example, administration of ivermectin to laying hens by ingestion, at 0.2 mg/kg as in our study, resulted in it rapidly reaching a maximum concentration (Cmax) of only 10.2 ng/mL at 3.4 hrs after treatment, followed by a rapid decline, with an elimination half-life on only 0.23 days [31]. Thus, the maximum ivermectin concentration in blood at this dose, identical to the dose we used, was 6-fold lower than the concentration needed to kill 50% of the bed bugs, and likely a prerequisite for substantial impacts on bed bug infestations. Similar results were reported following administration of higher dosages of ivermectin – broiler chickens were treated with 0.4 mg/kg in drinking water on 2 consecutive days, and again 14 days later; ivermectin reached maximum serum concentrations of 145.5–182.7 ng/mL within 30-60 min after treatment, but only transiently, and rapidly declined to undetectable levels by 12–24 hrs [38]. Oddly, oral

administration of multiple doses of ivermectin to laying hens (0.4 mg/kg daily, in water, for 5 consecutive days) yielded peak concentrations of only 1.1 ng/mL at 1 day post treatment [39]. When ivermectin was injected intravenously at 0.2 mg/kg body mass, Cmax reached 316.0 ng/mL 6 hrs later, but with a short elimination half-life, it also fell below the target concentration for bed bugs in less than 1 day [31]. Finally, in a recent evaluation of the effects of ivermectin-treated backyard hens on Culex mosquitoes, chickens (average 1.95 kg) were fed ivermectin-supplemented feed for 72 consecutive days (200 mg ivermectin per kg feed and 0.151 kg feed per chicken daily) [40], representing a very high dose of 30.2 mg ivermectin per chicken per day). However, serum concentrations in treated chickens averaged only 33.1 ng/mL (range <5 to 155.2 ng/mL), and they peaked early in the study (54.9 ng/mL on day 11) and declined to much lower concentrations over the 72-day study (20.6 ng/mL on day 70), despite daily re-treatments [40]. Overall, these studies consistently show low bioavailability of ivermectin in chicken serum, likely related to its rapid detoxification and clearance, and chicken traits such as high metabolic rate and low amounts of fat in tissues [31]. It is important to note in this regard that bed bugs and other hematophagous insects ingest whole blood, including blood cells, whereas pharmacokinetic studies report plasma or serum concentrations. Thus, pharmacokinetic studies likely underestimate the actual concentration of the ectoparasitic drug ingested in a blood meal. We tentatively conclude that treatments with ivermectin might not be effective for elimination of bed bugs from infested poultry farms.

Nevertheless, because ivermectin has been used extensively in systemic treatments of various hosts to kill ectoparasites, we urge further studies of ivermectin, possibly considering different formulations and their potential sublethal effects on bed bugs. For example, <50% of bed bugs that fed on rabbits injected subcutaneously with 0.3 mg/kg of ivermectin died [22].

However, many bed bug nymphs that survived feeding on ivermectin-supplemented blood or on ivermectin-medicated rabbits, mice, or humans, failed to molt [22, 37]. Even bed bugs that survived after taking a blood meal containing only 2.5 ng ivermectin/mL blood exhibited long-term morbidity including lower fecundity, difficulty feeding, and incomplete molts [41]. In the sustained treatment of backyard chickens with ivermectin-supplemented feed, although the researchers found no changes in the mosquito population or in west Nile virus prevalence, mosquito parity declined despite the relatively low ivermectin concentrations observed in medicated chickens (described above) [40]. It is possible that fitness-related traits were adversely affected by sublethal concentrations of ivermectin and contributed to these effects.

A challenging approach to increase bed bug mortality on medicated chickens is to somehow achieve higher ivermectin concentrations in blood. The recommended dosage for chickens, 0.2–0.4 mg/kg, which can be repeated every 10–14 days, is clearly insufficient to sustain high ivermectin concentrations suitable to kill bed bugs. While specialized formulations could increase the blood titer and extend the ivermectin elimination half-life, perhaps a more realistic approach with chickens might be to use newer anti-ectoparasite drugs that are less rapidly degraded and cleared by chickens, such as the isoxazoline drug fluralaner.

Use of systemic veterinary drugs to control bed bugs: Fluralaner

Fluralaner is an isoxazoline (IRAC group 30) that has been extensively tested as a systemic insecticide against ectoparasitic insects, ticks, and mites, mainly on dogs and cats, but also on some birds, livestock, and zoo and feral animals. Recently, fluralaner has also been investigated as a broad-spectrum insecticide against a variety of agricultural and public health pests.

Bravecto™ (containing racemic fluralaner) is labeled for dogs and cats, and because of its long elimination half-life, it is administered every 3 months [42]. This unique property of fluralaner contrasts with ivermectin and prompted us to examine its effects on bed bugs. However, because there are no fluralaner-containing products labeled for use in chickens in the U.S., we used Bravecto™, but experimentally followed the dosage directions for Exzolt™, a racemic fluralaner-containing product approved in Australia and the European Union to control poultry ectoparasites, such as the poultry red mite and the northern fowl mite [33]. The 1% aqueous formulation of this product is designed as a drinking water treatment administered twice at a dose of 0.5 mg/kg, seven days apart, providing up to three months of effective mite control [33]. High efficacy of this approach in controlling mites on poultry [29] and its negligible health effects, and high safety to birds [32] make Exzolt™ and fluralaner particularly attractive for testing with bed bugs.

We first conducted a dose-response study with insecticide susceptible bed bugs feeding on fluralaner-supplemented human blood using an artificial membrane-based feeding system. Our results showed LC50 and LC90 values of 15.3 and 38.6 ng fluralaner/mL blood, respectively, and we obtained similar values for five field-collected bed bug strains known to possess high resistance to pyrethroid insecticides. These results suggest little to no resistance to fluralaner in field populations of bed bugs. Because our pharmacokinetic studies are still ongoing, we rely on several reports using chickens to infer serum concentrations in our medicated chickens. After oral administration of fluralaner to laying hens, as recommended (0.5 mg/kg twice, 7 days apart), high serum concentrations were reached, with a Cmax of 323.7 ng/mL at 36 hrs after the first treatment and 355.1 ng/mL at 12 hrs after the second treatment on day 7 [43]. Given an elimination half-life of fluralaner in chicken plasma of about 5 days [43], its

concentration in blood is expected to decline to 44 ng/mL 22 days after the first administration of fluralaner, still above the dose required to kill 90% of the bed bugs (38.6 ng fluralaner/mL blood).

We conducted a proof-of-principle experiment with a single high dose of Bravecto™ (2.5 mg/kg), and then two independent experiments with two chicken flocks, adjusting the Bravecto™ dose to match the Exzolt™ label dose (0.5 mg/kg twice, 7 days apart). It is important to note, however, that in all these experiments fluralaner was administered orally (by gavage) in tablet form (weighed sections of Bravecto™ tablets), whereas Exzolt™ is delivered in drinking water. In all three experiments, a single blood-meal on medicated chickens resulted in high bed bug mortality even 28 days after the experimental chickens were treated.

In a previous study with bed bugs fed fluralaner-supplemented sheep blood, high levels of bed bug incapacitation, defined as death or immobility, were observed in various life stages at ≥100 ng fluralaner/mL blood [20]. These surprisingly high concentrations compared to ours, might be due to at least two factors. First, the preliminary dose-response study [20] might not have had sufficient resolution at the critical concentrations between 1 and 100 ng/mL. Second, fluralaner is highly bound to plasma proteins, including in broiler chickens [43], and likely to blood cells. In Sheele's study [20], due to the artificial feeding system design, blood cells could readily settle away from the feeding bed bugs, reducing the effective blood concentration of fluralaner. Nevertheless, these studies indicate significant sublethal morbidity of bed bugs on low concentrations of fluralaner, adding to its value as an anti-parasite systemic agent.

Moreover, systemic treatments of the host to control bed bugs are known to be more effective to reduce populations than traditional spray contact formulations for bed bug harborage [18]. Systemic treatments to control ectoparasites are common in household pest (dogs and cats)

and livestock (cattle, horses, sheep, and swine) to control mites, fleas, and ticks [24], and a similar approach to treat pests has also been seen in urban indoor pest such as the use of baits to control the German cockroach [44]. Systemic ectoparasite control could facilitate control of nuisance pests specially in challenging environments such as extensive poultry house structures. It could also be argued that reoccurring populations would be less occurring due to the higher mortality. However, due to the fact that systemic drugs will not treat eggs laid before treatments, fluralaner should be considered as a tool of an Integrated Pest Management program (IPM).

Potential efficacy of fluralaner in bed bug eradication in poultry farms

All the field-collected bed bug strains that we tested showed high levels of resistance to deltamethrin, a pyrethroid commonly used to control bed bug infestations. However, bed bugs from the same strains were highly susceptible to fluralaner, administered in an artificial membrane-based apparatus using human blood. High efficacy of fluralaner on pyrethroid resistant bed bugs is particularly important in the poultry industry because most of the products labeled to control bed bugs in the farm environment are contain pyrethroid insecticides. Our findings are consistent with results showing higher performance fluralaner than deltamethrin against a wide range of arthropod pests, including *Stomoxys calcitrans* (stable fly), *Rhipicephalus sanguineus* (brown dog tick), *Aedes aegypti* larvae, and *Lucilia cuprina* (Australian sheep blowfly) [27].

Fluralaner, an isoxazoline (IRAC class 30), is a novel inhibitor of GABACl and GluCl channels. Fipronil, a phenylpyrazole (IRAC class 2B), also targets these channels and is commonly used in residential settings to control crawling insects, especially cockroaches. Insecticide resistance to fipronil involves metabolic detoxification and target-site insensitivity

associated with a specific mutation in the *Rdl* gene that results in A302S amino acid substitution; this substitution also confers high resistance to dieldrin, a cyclodiene (IRAC class 2A) [27]. The same field-collected bed bug strains used in this study exhibited variable, but high resistance levels to fipronil (4.4 to >492-fold) [45]. However, none of these bed bugs had the mutation in the *Rdl* gene associated with resistance to fipronil and dieldrin. Moreover, Gassel et al. [27] have shown that fluralaner efficacy is unaffected by dieldrin and fipronil resistance in the cat flea, ticks, and fruit fly, indicating a lack of cross-resistance is due to fluralaner targeting a site of GABACl channels distinct from the site targeted by cyclodienes and fipronil. These findings suggest that cross-resistance to fipronil and dieldrin is not likely to interfere with the efficacy of fluralaner on bed bugs in poultry farms. Nevertheless, consideration of fluralaner for bed bug control should proceed with caution because bed bug populations may be experiencing selection with fluralaner and afoxalaner through ongoing exposure to Bravecto™- and NexGard™-medicated dogs and cats.

Potential other uses of systemic veterinary drugs to control bed bugs

The administration of ectoparasitic drugs to host animals to control pest arthropods comes with significant ethical, regulatory, and bioactivity challenges. Most importantly, as highlighted with ivermectin in chickens, it is challenging to attain a sufficiently high concentration of the insecticide in host blood and maintain it long enough to target hematophagous pests that take blood-meals every 5 to 10 days (depending on temperature and life stage). Artificial liquid baiting systems may overcome these constraints and offer additional benefits. A baiting apparatus would consist of host odors and other cues to attract the target pest, a membrane through which the liquid bait is accessed, phagostimulants in an aqueous solution to stimulate

feeding, and an insecticide dissolved in the same solution. First, the amount of insecticide in solution can be maximized (or optimized), so long as it does not deter ingestion. Second, the insecticide can be stabilized in various protective formulations, and in the absence of cellular components and enzymes, it would remain active for days, weeks, or even months. Third, active ingredients that are not compatible with systemic use in animals can be used in baits. And finally, baits could be implemented in combination with medicated animals, possibly using multiple modes of action to slow the evolution of resistance. For example, while fluralaner could be used as an animal systemic agent, ivermectin could be used in an artificial baiting system at much higher concentrations than would be permitted in animals. A wide range of active ingredients that have been shown to be effective on bed bugs could be considered in liquid baits, including fipronil [18], spinosad [20], moxidectin [19], and even boric acid [46].

Study limitations

Foremost, it is important to reiterate that there are no fluralaner-containing products labeled for use in chickens in the U.S. Therefore, we used weighed portions of Bravecto™, which is labeled for use in dogs, and experimentally followed the dosage directions for Exzolt™, a fluralaner-containing product approved in Australia and the European Union for use in chickens. This "off label" use of Bravecto™ is strictly experimental and is not meant to condone its use in the field. Related to this approach, Exzolt™ is a 1% aqueous formulation, whereas we delivered a solid formulation of fluralaner by oral gavage. We suspect that the aqueous formulation might become more bioavailable than the solid formulation.

Related to the pharmacokinetics of systemic veterinary ectoparasitic drugs, we related our *in vitro* dose-response results and *in vivo* bioassays to previously reported pharmacokinetic

studies. As we cautioned already, pharmacokinetic studies analyze plasma or serum content, whereas hematophagous insects, including bed bugs, take whole blood meals and not plasma meals. Although our *in vitro* determined target concentrations (LC_{50} and LC_{90}) were remarkably predictive of *in vivo* results and previously reported plasma titers of fluralaner and ivermectin in chickens, we acknowledge that these associations need to be confirmed empirically with both plasma and whole blood pharmacokinetic studies.

We used only adult male bed bugs. Biosafety concerns related to transporting bed bugs between two laboratories 3.5 km apart dictated that we not use small nymphs and adult females. Although previous studies with membrane-based artificial feeders showed no major differences among different life stages of bed bugs [18], it is important that these findings be confirmed with nymphs and females. Also, we tested only five field-collected strains collected mainly in eastern U.S. Many more field-collected bed bug populations need to be sampled to confirm our results.

Finally, our study was conducted in controlled laboratory environments. Drug treatments, metabolic rates, bed bug behavior, and clearance rates of drugs are all bound to vary under field conditions. Therefore, studies with larger chicken flocks under more realistic field conditions are warranted.

Conclusions

The administration of fluralaner via drinking water at a dose of 0.5 mg/kg per chicken bodyweight, repeated in a seven-day interval, could be effective against the common bed bug. A combination of monitoring, education, heat treatments, and systemic insecticides could hold the key to eradication of bed bugs in poultry farms. This project is the first (post-DDT) to report a novel tool option to control bed bug infestations in the poultry industry. Moreover, the results

from this project validate the concept of xenointoxication to control bed bug infestations and the potential for bed bug bait development using fluralaner and ivermectin as active ingredients.

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Table 4.1. Fluralaner dose-mortality assays, resistance ratios, and deltamethrin diagnostic dose assays of five recently collected *C. lectularius* populations relative to an insecticide-susceptible (Harlan) population. Ivermectin dose-mortality assays are also shown for the Harlan population

Population, abbreviation (year collected)	Collection location	Fluralaner[a] *n*	LC_{50} ng/mL (95% CI)[b]	LC_{90} ng/mL (95% CI)[b]	Slope ± SEM	χ^2 (df)	*t*-ratio[c]	RR at LC_{50}[d] (95% CI)	Deltamethrin % mortality at HA LD_{99} dose (*n*)[e]
Harlan, HA (1973) (susceptible)	Fort Dix, NJ	237	15.3 (11.7, 19.8)	38.6 (28.3, 71.9)	3.2 ± 0.4	7.9 (5)	8.0*	-	-
Cincinnati, CIN (2012)	Cincinnati, OH	219	18.4 (16.2, 23.5)	51.7 (41.4, 72.5)	2.8 ± 0.3	3.5 (6)	8.2*	1.2	13 (45)
Fuller Miller, FM (2017)	High Point, NC	202	23.2 (13.8, 35.3)	67.5 (42.5, 216.6)	2.8 ± 0.3	12.6 (5)	7.8*	1.5*	5 (40)
Winston Salem, WS (2008)	Winston Salem, NC	226	22.0 (15.3, 30.6)	92.7 (58.4, 230.8)	2.0 ± 0.3	8.0 (6)	7.4*	1.4*	0 (48)
Shanda, SH (2017)	Raleigh, NC	220	21.1 (17.5, 25.4)	65.0 (48.7, 102.0)	2.6 ± 0.3	1.3 (5)	7.7*	1.4*	0 (46)
Poultry House, PH (2021)	PA	212	18.0 (15.3, 22.1)	43.6 (34.7, 61.6)	3.3 ± 0.4	4.9 (5)	7.8*	1.2	2 (50)

		Ivermectin[a]					
Harlan, HA (1973) (susceptible)	Fort Dix, NJ	231	61.0 (52.7, 69.9)	114.9 (95.4, 154.7)	4.7 ± 0.7	3.0 (4)	6.4*

Table 4.1 continues on next page

Table 4.1. (cont.)

[a] Only fully fed adult male bed bugs were included in these assays.

[b] Lethal concentration that killed 50% or 90% (LC_{90}) of the bed bugs estimated from probit analysis for each population. Values are in ng/mL of human blood.

[c] *t*-ratio of the slope. Values >1.96 denote a significant regression (*$P < 0.05$).

[d] Resistance Ratio (RR) was calculated as LC_{50} of field-collected strain / LC_{50} of susceptible reference strain (HA). RR values with (*) are considered significant when their 95% CIs do not include 1.0 (Robertson et al. 2017).

[e] The LD_{99} dose of deltamethrin estimated for the Harlan population from log dose-response probit analysis. This dose was used as discriminating dose topically applied to field-collected bed bugs. Deltamethrin was diluted in acetone and 0.5 µl was applied to each insect. Percentage mortality at 2 d and (*n*) are reported.

Table 4.2. Analysis of variance (ANOVA) results for three experiments in which fluralaner was administered to chickens by gavage. After each bed bug feeding, fully fed bed bugs were observed daily for 7 days. The ANOVAs show day 7 results.

A. Experiment 1: Dose = 2.5 mg fluralaner/kg on day 0 (*n* = 6 chickens; 7 time points, 0 to 28 days)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day of bed bug feeding	6	46627.427	7771.24	13.9307	<0.0001*
Error	33	18409.042	557.85		
C. Total	39	65036.469			

B. Experiment 2: Dose = 0.5 mg fluralaner/kg on day 0 and again on day 7 (*n* = 6 chickens; 9 time points, 0 to 28 days)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day of bed bug feeding	8	50401.119	6300.14	33.3020	<0.0001*
Error	45	8513.182	189.18		
C. Total	53	58914.301			

C. Experiment 2: Dose = 0.5 mg fluralaner/kg on day 0 and again on day 7 (*n* = 6 chickens; 9 time points, 0 to 28 days)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day of bed bug feeding	8	51448.803	6431.10	27.6944	<0.0001*
Error	45	10449.758	232.22		
C. Total	53	61898.561			

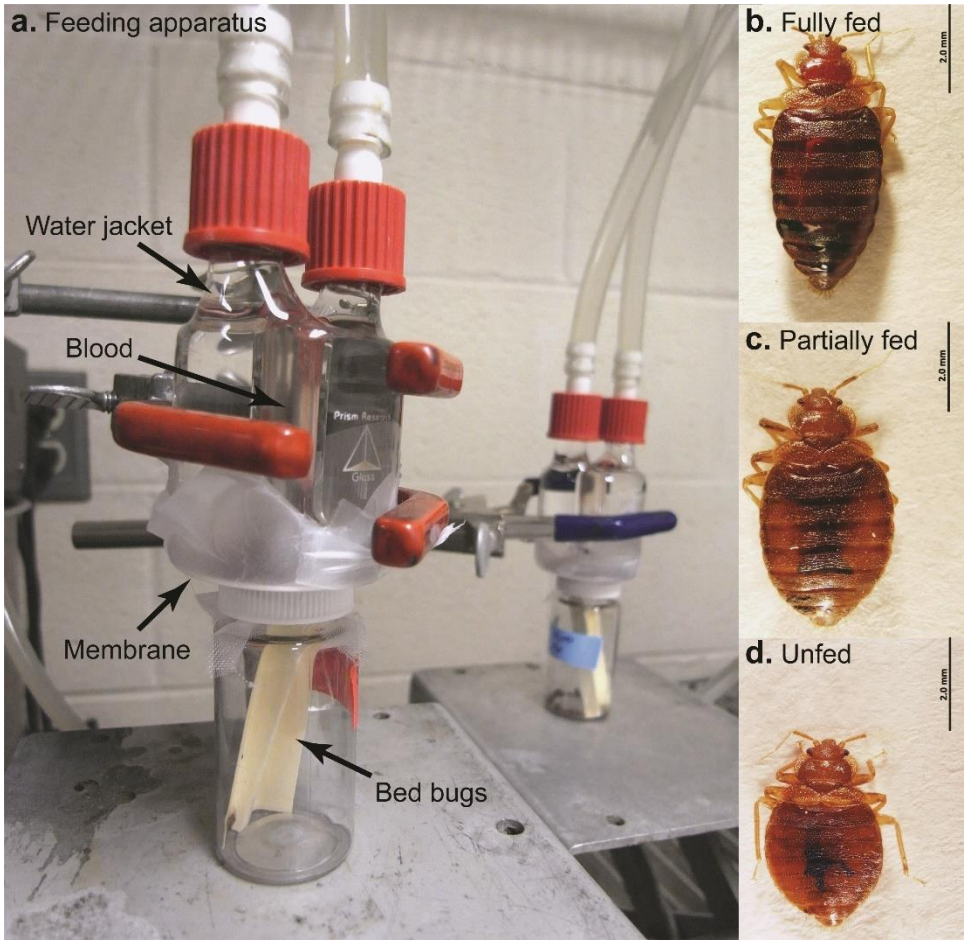

Figure 4.1. (a) Artificial feeding system used to feed bed bugs insecticide-supplemented human blood. Blood was placed into the internal chamber of a custom-fabricated glass feeder heated with a circulating water bath and held in place by plant grafting tape. Bed bugs were placed in PET plastic vials containing cardboard to provide harborage and capped with plankton screen that allowed them to insert their mouthparts and feed. Only fully engorged individuals were evaluated in our analyses (b), while partially fed (c) and unfed adult males (d) were discarded.

Figure 4.2. Birds where manually held in the lap (a). A PET plastic vial, containing a cardboard ramp and up to 15 adult male bed bugs, and capped with plankton screen (b) was placed on the bird's lateral inguinal contacting the skin to allow feeding. Each group of bed bugs was allowed to feed for 10 min.

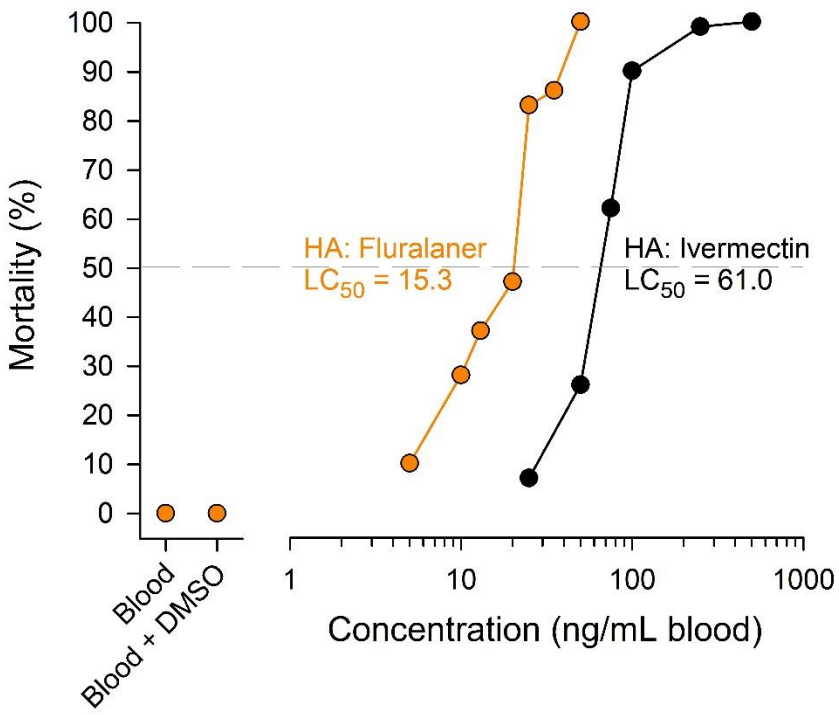

Figure 4.3. In vitro fluralaner and ivermectin log dose-response curves for the insecticide-susceptible Harlan strain (HA) adult male bed bugs. Fluralaner and ivermectin were separately dissolved in DMSO and mixed with human blood to obtain various concentrations of insecticides in 0.1% DMSO in blood. Blood and Blood + DMSO represent control treatments; there was no mortality in control bed bugs. Mortality at 7 days post-ingestion of chicken blood by bed bugs is reported. At least three replicates of 10 adult male bed bugs per replicate were performed per concentration. The LC50 estimates are based on probit analyses.

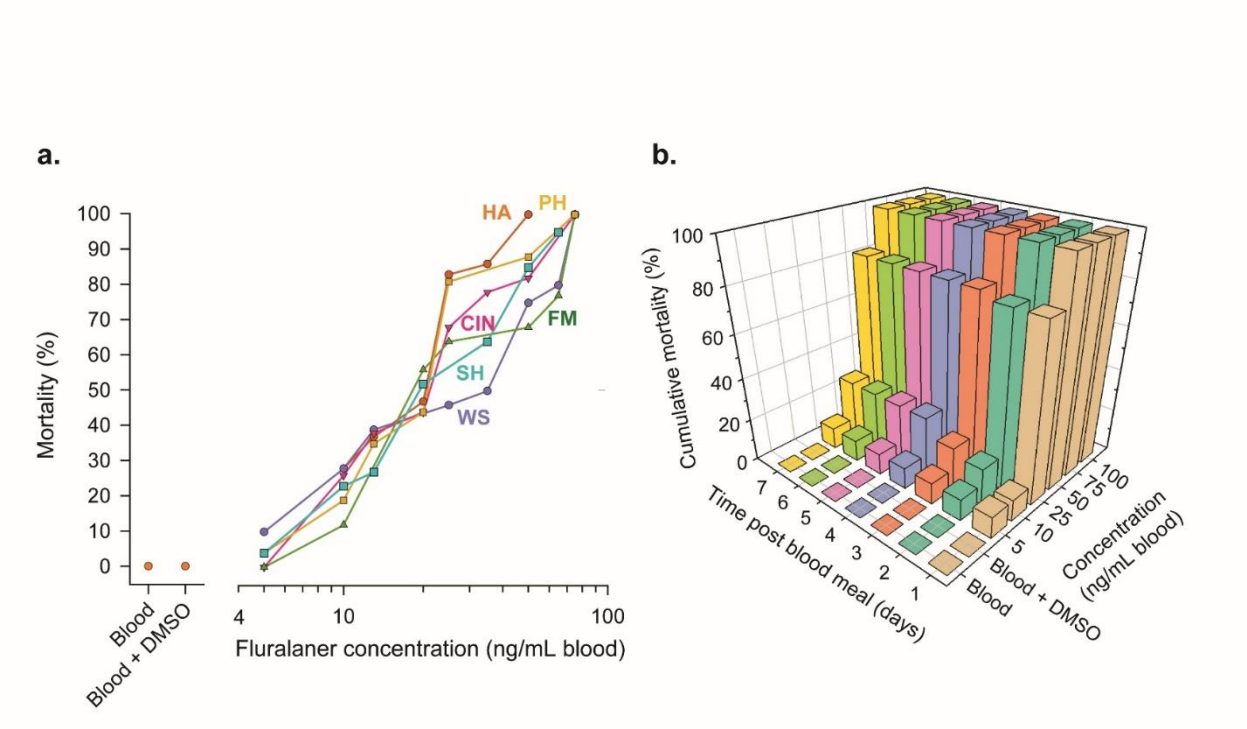

Figure 4.4 (a) In vitro fluralaner log dose-response curves for bed bug males from six populations, including five field-collected strains and the reference insecticide-susceptible strain (Harlan = HA). Abbreviations are detailed in Table 1. Fluralaner was dissolved in DMSO, as described in the Methods section. (b) 3-D representation of the time-course of mortality of Harlan (HA) strain bed bugs (1 to 7 days).

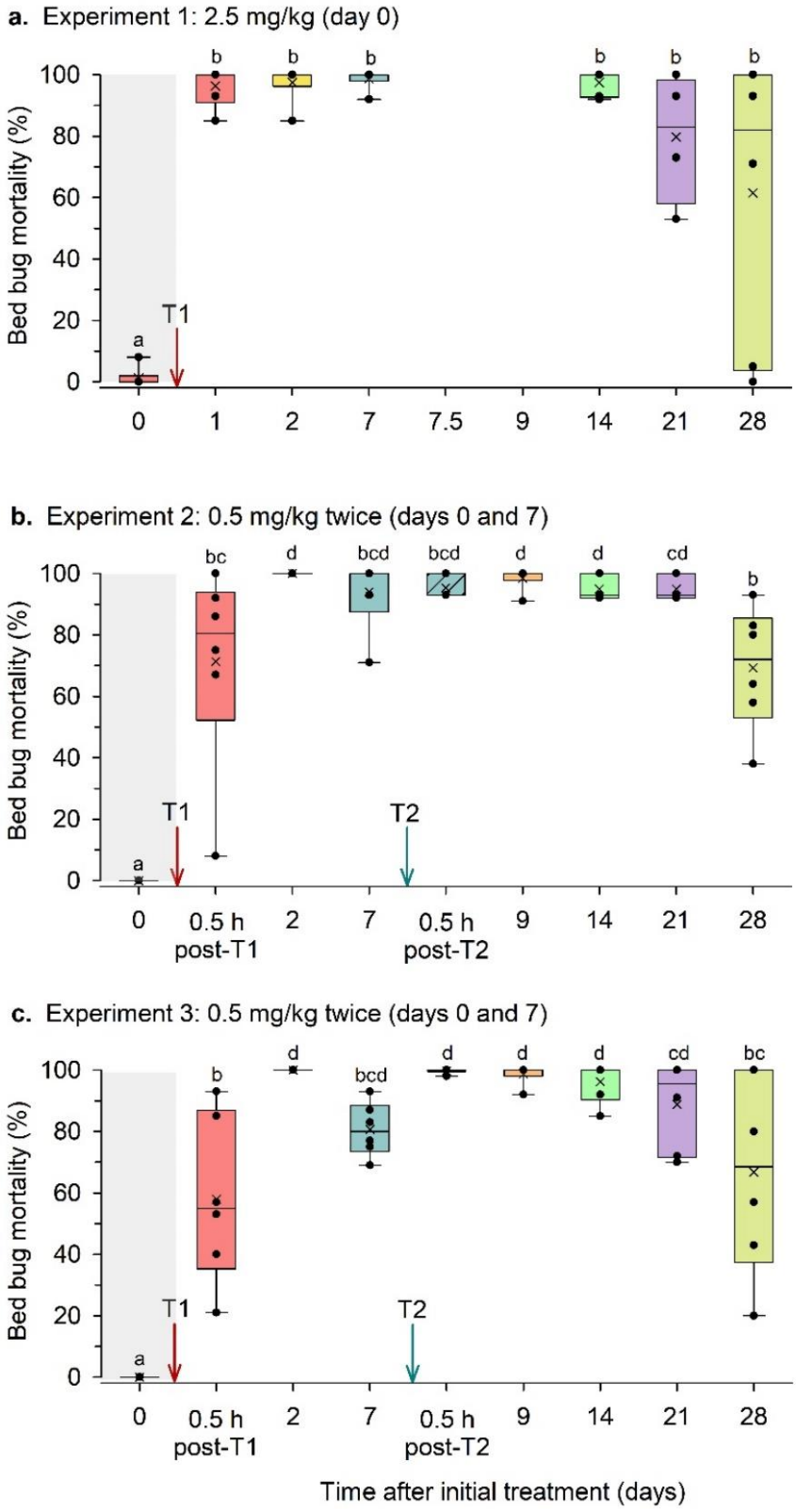

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Figure 4.5. (cont.) In vivo assays of chickens treated with fluralaner by oral gavage. (a) Experiment 1: Chickens treated with 2.5 mg/kg body mass on day 0; (b) Experiment 2: Chickens treated with 0.5 mg/kg body mass on day 0 and again on day 7; (c) Experiment 3: Chickens treated as in Experiment 2, but these birds came from a younger flock and were bled at the same time points for pharmacokinetic analysis of plasma concentrations of fluralaner. Each experiment consisted of six birds. At each time point a maximum of 15 bed bugs were fed on each bird. Thus, time points are represented by 78 to 87 bed bugs. We conducted an ANOVA within each experiment followed by Tukey's HSD test to separate means (represented within box plots by X). Means that do not share letters (above box plots) are significantly different ($P < 0.05$).

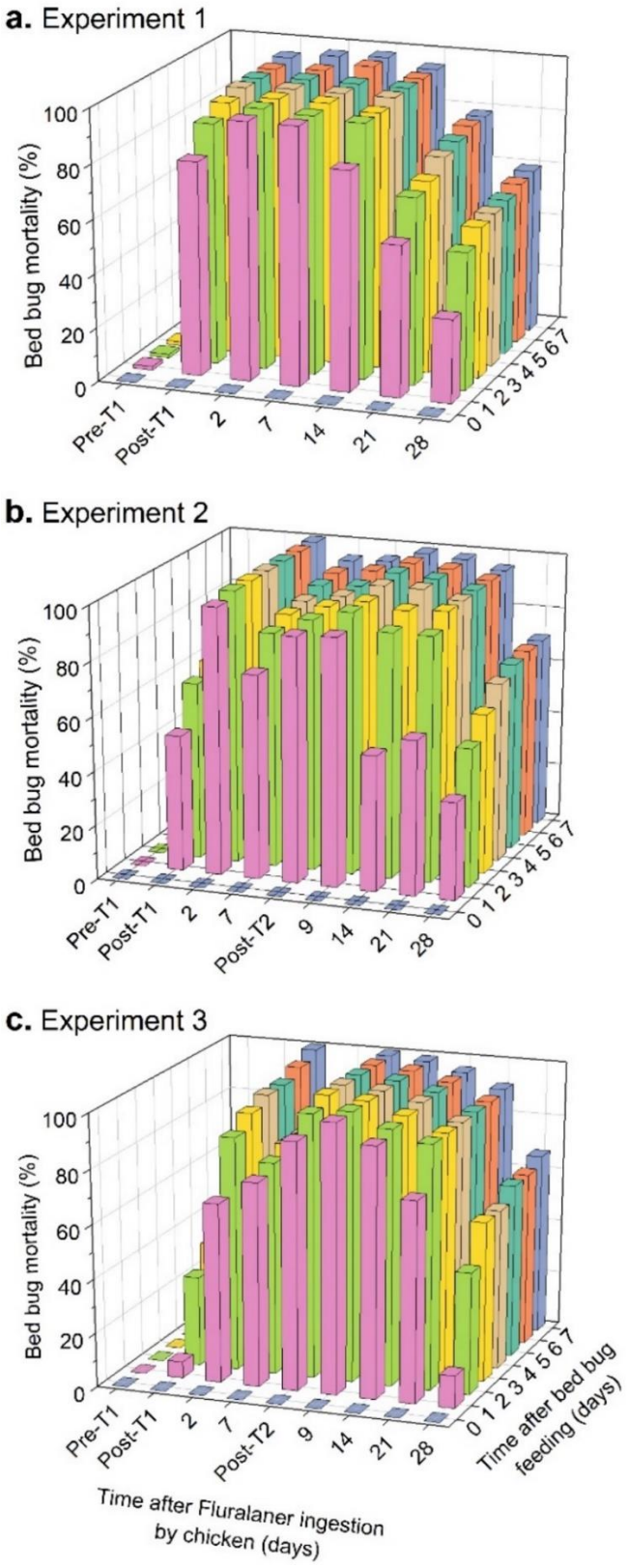

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Figure 4.6. (cont.) 3-D representation of the in vivo assays shown in Fig. 4.5. In addition to the day 7 cumulative mortality shown in Fig. 5, the time-course of mortality of Harlan (HA) strain bed bugs is shown 1 to 7 days after they fed on treated birds. (a) Experiment 1: Chickens treated with 2.5 mg/kg body mass on day 0; (B) Experiment 2: Chickens treated with 0.5 mg/kg body mass on day 0 and again on day 7; (C) Experiment 3: Chickens treated as in Experiment 2, but these birds came from a younger flock and were bled at the same time points for pharmacokinetic analysis of plasma concentrations of fluralaner. Each experiment included six birds. At each time point a maximum of 15 bed bugs were fed on each bird. Thus, time points are represented by 76 to 88 bed bugs.