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Resistance to Fipronil in the Common Bed Bug (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 lectularius*, fipronil, insecticide resistance, synergist

Over the past two decades, the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), has reestablished as a perennial indoor pest, causing global public health concerns (Boase 2001; Doggett et al. 2004, 2018; Potter 2006; Masetti and Bruschi 2007; Levy Bencheton et al. 2011). 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 et al. 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 labeled 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 United States (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 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 a noncompetitive 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* (F.) (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 reduced sensitivity 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% 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 postfeeding.

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 postfeeding, were placed in plastic Petri dishes (diameter = 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 posttreatment. The LD_{99} was used as a diagnostic dose on eight of the nine field-collected populations.

Table 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 per 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% CI 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.

Effects of Synergists on Fipronil Toxicity

The synergists we tested were: DEF (97.7%) (Chem Services), TPP (99%), PBO (99%), and 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 per 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 per 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′-GTGCGATC CATGGGCTACTA-3′) and CL-Rdl-R (5′-AGAGATGCGAAGACC ATGAC-3′). The PCRs 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. 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 (CLs) 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 Deltametrin 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 per male for the insecticide-susceptible Harlan-NCSU population, to >20 µg per 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 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).

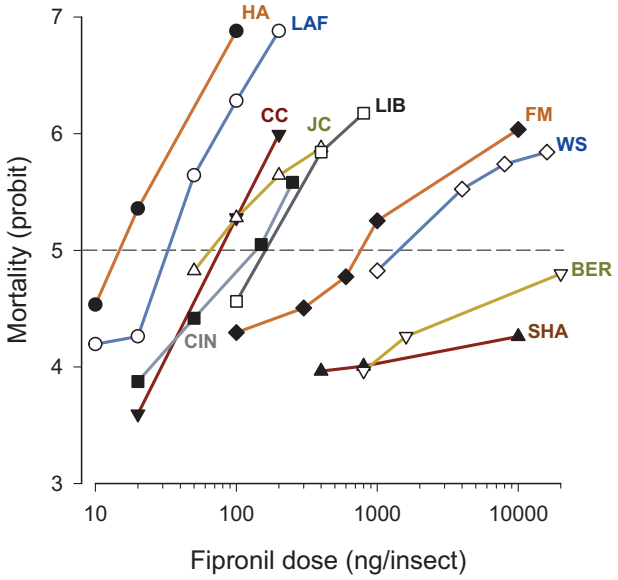

Fig. 1. Fipronil dose–response probit-transformed curves for *C. lectularius* adult males from 10 populations, including nine field-collected populations and our laboratory-reared population (Harlan-NCSU). Abbreviations are explained in Table 1. The lethal dose of fipronil that killed 50% of each population (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.

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) is 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). PBO, an inhibitor of P450s and ESTs, 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). DEF, an EST 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.

Table 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	58,336.7	6,481.8	64.8185	**<0.0001**
	Error	20	2,000.0	100.0		
	Total	29	60,336.7			
Cincinnati	Treatment	9	43,200.0	4,800.0	16.3636	**<0.0001**
	Error	20	5,866.7	293.3		
	Total	29	49,066.7			
Winston Salem	Treatment	9	49,950.0	5,550.0	138.7500	**<0.0001**
	Error	20	800.0	40.0		
	Total	29	50,750.0			
Shanda	Treatment	9	44,830.0	4,981.1	99.6222	**<0.0001**
	Error	20	1,000.0	50.0		
	Total	29	45,830.0			

[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. Significant differences among treatments within each population (ANOVA) are shown in bold. Tukey's HSD tests for comparisons of treatments within each population are shown in Fig. 2.

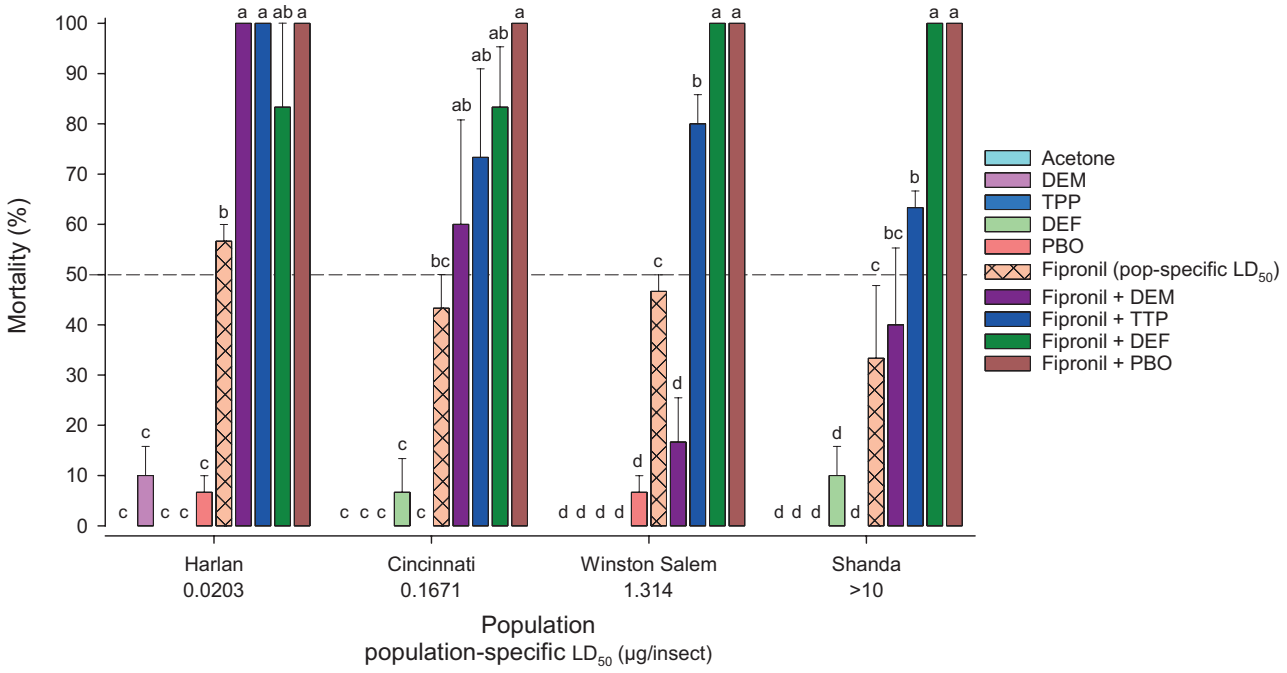

Fig. 2. Effects of four insecticide synergists on fipronil toxicity in *C. lectularius* adult males. Each synergist (PBO, DEF, DEM, and TPP) was topically applied in 0.5 µl acetone 2 h prior to application of a population-specific LD_{50} dose, as shown on the x-axis. Percent mortality was determined 4 d 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.

TPP, a CEST 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). 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.

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 per 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 per 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. Pretreatment 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 pretreatments with a combination of PBO and DEF further increased mortality in the Shanda population to 100%. Thus, suppression of major

Table 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	47,483.3	6,783.3	172.4576	**<0.0001**
	Error	17	668.7	39.3		
	Total	24	48,152.0			
Shanda	Treatment	7	38,473.8	5,496.3	92.5238	**<0.0001**
	Error	16	950.5	59.4		
	Total	23	39,424.3			

[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. Significant differences among treatments within each population (ANOVA) are shown in bold. Tukey's HSD tests for comparisons of treatments within each population are shown in Fig. 3.

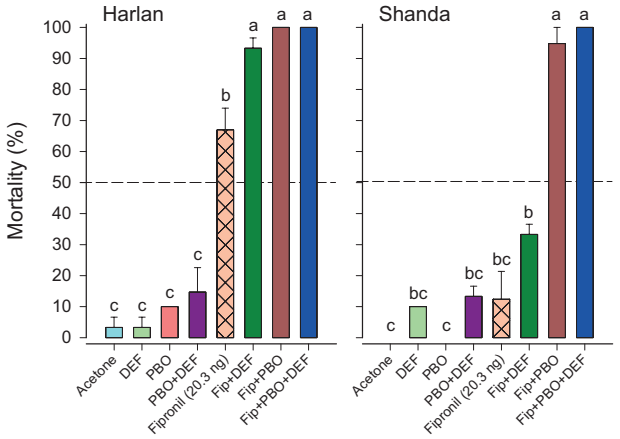

Fig. 3. A combination of PBO and DEF eliminates resistance in highly resistant adult male *C. lectularius*. PBO, DEF, or a mix of both (50 µg each) were topically applied, and 2 h later the Harlan-NCSU-specific LD_{50} dose of fipronil (20.3 ng) was applied. Mortality was assessed 4 d after treatment. 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, $P < 0.05$).

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 *C. 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).

Table 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.

Synergism in Two Susceptible Bed Bug Subpopulations

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 and 3), suggesting detoxification enzyme activity in the fipronil-susceptible population. Second, the fipronil LD_{50} values in this study (20.3 ng per 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 per male) increased mortality to 100% in both populations.

Discussion

Bed bug populations collected in the last 12 yr from various parts of the United States 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

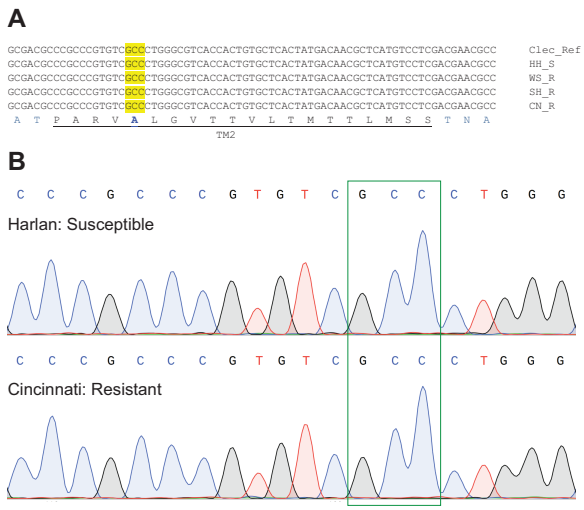

Fig. 4. Frequency of the *Rdl* mutation A302S in *C. lectularius* populations. Ten bed bug males from each of 10 populations were screened for the A302S mutation. Genomic DNA was extracted with the DNeasy Blood & Tissue extraction kit and a 245-bp genomic fragment of the GABA receptor gene that includes 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.

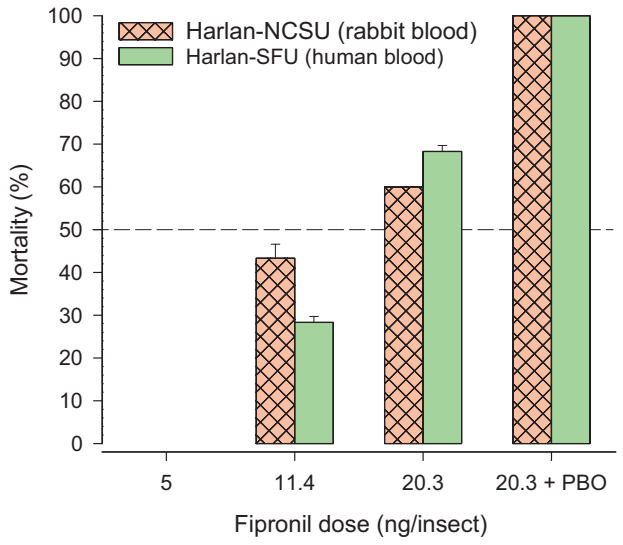

Fig. 5. Comparison of the responses of two related insecticide-susceptible populations of *C. lectularius* to treatment with fipronil. Both populations originated from a single collection in 1973. Harlan-NCSU has been fed defibrinated rabbit blood using an artificial feeding system since 2008. Harlan-SFU 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. In total, 92 bed bugs of each population were used.

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 1) is not labeled for any bed bug control products, 2) is unlikely to be used extensively in areas where bed bugs would commonly be found, and 3) its indoor use in Europe is even more restricted than in the United States.

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 nonmutually 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 United States (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 *C. hemipterus* 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 United States 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 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 nonhuman 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, ESTs 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 per 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 EST 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 ESTs) 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 per 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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