Research

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 monoxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases (CESTs), and esterases (ESTs) (Adelman et al. 2011, Bai et al. 2011), target-site insensitivity (e.g., knockdown resistance ldr) (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 phosphite (TPP) inhibits CESTs and S,S,S-tributyl phosphorotriothioate (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 (French-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-termite pests (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-aminobutyric 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 diel-drin) gene, is also the target of cyclodiene insecticides (Ghasiuddin and Matsumura 1982, French-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 (E) (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 defbrinated 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-cm³ 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 defbrinated 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-(trifluoromethylsulfonyl)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) 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-phenoxophenyl)methyl) (1R,3R)-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.
Effects of Synergists on Fipronil Toxicity

The synergists we tested were: DEF (97.7%) (Chem Services), TPP (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 ng of PBO, DEF, DEM, or TPP in 0.5 µl acetonitrile (ACN) and PBO was made up to 0.5 µl of ATL solution and 20 ng of ATL solution and 20 µl of proteinase-K was added to the solution. After the bed bugs recovered at room temperature for 2 h, they were briefly anesthetized with CO2 and topically treated with either acetonitrile alone (control) or the population-specific LD99 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 Shanda-specific fipronil LD99 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 acetonitrile alone (control) or the Harlan-NCSU population-specific fipronil LD99 (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

### Table 1. Fipronil dose–response assays, resistance ratios, and deltamethrin LD99 percentage mortality of recently collected C. lectularius populations relative to an insecticide-susceptible (Harlan-NCSU) population

<table>
<thead>
<tr>
<th>Population, abbreviation</th>
<th>Collection location</th>
<th>n</th>
<th>LD99 µg per male (95% CI)a</th>
<th>Slope ± SE</th>
<th>χ² (df)</th>
<th>t-ratioc</th>
<th>RR 0.5d</th>
<th>% mortality LD99 dose (n)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harlan-NCSU, HA (1973) (susceptible)</td>
<td>Fort Dix, NJ</td>
<td>124</td>
<td>0.0203 (0.0122–0.0310)</td>
<td>2.66 ± 0.62</td>
<td>0.5 (1)</td>
<td>4.40*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lafayette, LAF (2009)</td>
<td>Lafayette, IN</td>
<td>184</td>
<td>0.0289 (0.0171–0.0451)</td>
<td>2.24 ± 0.30</td>
<td>3.3 (3)</td>
<td>7.39*</td>
<td>1.4</td>
<td>88 (40)</td>
</tr>
<tr>
<td>Campus Courtyard, CC (2009)</td>
<td>Raleigh, NC</td>
<td>137</td>
<td>0.0765 (0.0566–0.1009)</td>
<td>2.47 ± 0.38</td>
<td>0.0 (1)</td>
<td>6.33*</td>
<td>3.8</td>
<td>0 (50)</td>
</tr>
<tr>
<td>Jersey City, JC (2008)</td>
<td>Jersey City, NJ</td>
<td>153</td>
<td>0.0880 (0.0252–0.1514)</td>
<td>1.28 ± 0.41</td>
<td>0.3 (2)</td>
<td>3.15*</td>
<td>4.4</td>
<td>20 (50)</td>
</tr>
<tr>
<td>Cincinnati, CIN (2012)</td>
<td>Cincinnati, OH</td>
<td>119</td>
<td>0.1671 (0.1231–0.2013)</td>
<td>6.48 ± 2.15</td>
<td>0.7 (1)</td>
<td>4.64*</td>
<td>8.4</td>
<td>13 (45)</td>
</tr>
<tr>
<td>Liberty, LIB (2017)</td>
<td>Liberty, NC</td>
<td>108</td>
<td>0.2147 (0.1017–0.3333)</td>
<td>2.15 ± 0.54</td>
<td>0.4 (1)</td>
<td>4.18*</td>
<td>10.7</td>
<td>ND</td>
</tr>
<tr>
<td>Fuller Miller, FM (2017)</td>
<td>High Point, NC</td>
<td>224</td>
<td>0.8877 (0.4807–1.576)</td>
<td>1.00 ± 0.21</td>
<td>1.5 (3)</td>
<td>5.04*</td>
<td>44.4</td>
<td>ND</td>
</tr>
<tr>
<td>Winston Salem, WS (2008)</td>
<td>Winston Salem, NC</td>
<td>161</td>
<td>1.343 (0.1703–2.648)</td>
<td>0.86 ± 0.26</td>
<td>0.6 (2)</td>
<td>3.33*</td>
<td>65.7</td>
<td>0 (48)</td>
</tr>
<tr>
<td>Shanda, SHA (2017)</td>
<td>Raleigh, NC</td>
<td>195</td>
<td>&gt;10 (23%)³</td>
<td>—</td>
<td>—</td>
<td>&gt;492</td>
<td>0 (46)</td>
<td></td>
</tr>
<tr>
<td>Beroun, BER (2014)</td>
<td>Czech Republic</td>
<td>183</td>
<td>&gt;20 (42%)³</td>
<td>—</td>
<td>—</td>
<td>&gt;985</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a The LD99 dose of deltamethrin, determined with the Harlan-NCSU population, was applied in 0.5 µl acetonitrile solution. Percentage mortality at 1 d and (n) is reported. ND = not determined.

b Adult male bed bugs were treated with 0.5 µl acetonitrile solution containing fipronil. Lethal dose that killed 50% of the bed bugs (LD50) 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 (RR50) was calculated as (LD50 resistant population)/(LD50 Harlan-NCSU population). RR values with (*) are considered significant when the 95% CI does not include 1.0 (Robertson et al. 2017).

³Maximum mortality is indicated in parenthesis. LD99 could not be estimated and therefore a formal test of the lethal dose ratios (RR50) could not be done.
followed the manufacturer’s instructions. DNA was eluted in 50 µl sterile nuclease-free H₂O 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’-GTGGGATCC CATGGGCTACTA-3’) and CL-Rdl-R (5’-AGAGATGGGAGGCC 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 LD₅₀ 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 LD₅₀ as a diagnostic dose. The toxicity of fipronil to each population was compared relative to the susceptible Harlan population using a resistance ratio (RR₅₀), calculated as (LD₅₀-resistant population)/(LD₅₀ susceptible 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 RR₅₀ was considered significant (Robertson et al. 2017). Abbott’s correction (Abbott 1925) was used to correct for mortality control, 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 LD₅₀ 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 (RR₅₀) for the recently field-collected populations, relative to the Harlan-NCSU population, ranged from 1.44- to >985-fold. However, from 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 RR₅₀ 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 (RR₅₀ = 44.4-fold) and Winston Salem (RR₅₀ = 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 RR₅₀ values ranging from 1.4- to 10.7-fold. With the exception of the Lafayette population (RR₅₀ = 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 LD₅₀ was 1.431 ng (95% CI: 1.00, 2.14; total n = 140; slope = 2.54 ± 0.353 [SE]; χ² = 5.80, df = 5; t-ratio = 7.193 [P < 0.05]). The estimated LD₅₀ diagnostic dose of 11.79 ng (95% CI: 5.91, 51.46) was applied to six populations, and the percentage 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.

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 LD₅₀ 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 LD₅₀ dose of fipronil significantly increased mortality to 100% in the four populations—Harlan-NCSU, Cincinnati, Winston Salem, and Shanda (Fig. 2). DEF, an EST inhibitor, significantly increased mortality caused by the LD₅₀ 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.
TPP, a CEST inhibitor, also significantly synergized the mortality caused by the population-specific LD50 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 LD50 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 LD50 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 metabolic resistance was achieved only if both PBO and DEF were used in conjunction. The synergistic effects of four enzyme inhibitors are shown in Table 2 and Fig. 2.

**Table 2. Synergistic effects of four enzyme inhibitors, assayed with four C. lectularius populations**

<table>
<thead>
<tr>
<th>Population</th>
<th>Source</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harlan-NCSU</td>
<td>Treatment</td>
<td>9</td>
<td>58,336.7</td>
<td>6,481.8</td>
<td>64.8185</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>20</td>
<td>2,000.0</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>29</td>
<td>60,336.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cincinnati</td>
<td>Treatment</td>
<td>9</td>
<td>43,200.0</td>
<td>4,800.0</td>
<td>16.3636</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>20</td>
<td>5,866.7</td>
<td>293.3</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td>29</td>
<td>49,066.7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Winston Salem</td>
<td>Treatment</td>
<td>9</td>
<td>49,950.0</td>
<td>5,550.0</td>
<td>138.7500</td>
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<tr>
<td></td>
<td>Error</td>
<td>20</td>
<td>800.0</td>
<td>40.0</td>
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<td></td>
<td>Total</td>
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<td>Shanda</td>
<td>Treatment</td>
<td>9</td>
<td>44,830.0</td>
<td>4,981.1</td>
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<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>Error</td>
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<td>1,000.0</td>
<td>50.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>29</td>
<td>45,830.0</td>
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</tbody>
</table>

**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 LD50 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.
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_014355500.2 [predicted *C. lectularius* gamma-aminobutyric acid receptor subunit beta [LOC706661790], 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 3. Synergistic effects of PBO, DEF, and a combination of both, assayed with two *C. lectularius* populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Source</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harlan-NCSU</td>
<td>Treatment</td>
<td>7</td>
<td>47,483.3</td>
<td>6,783.3</td>
<td>172.4576</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>17</td>
<td>668.7</td>
<td>39.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>24</td>
<td>48,152.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shanda</td>
<td>Treatment</td>
<td>7</td>
<td>38,473.8</td>
<td>5,496.3</td>
<td>92.5238</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>16</td>
<td>950.5</td>
<td>59.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>23</td>
<td>39,424.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.*

### Table 4. Frequency of Rdl mutations in fipronil-resistant and -susceptible populations of *C. lectularius*

<table>
<thead>
<tr>
<th>Population</th>
<th>RR$_{95}$</th>
<th>n</th>
<th>No. of bed bugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A302/</td>
<td>S302/</td>
<td>A302/</td>
</tr>
<tr>
<td></td>
<td>(S/S)</td>
<td>(R/R)</td>
<td>(S/R)</td>
</tr>
<tr>
<td>Harlan-NCSU</td>
<td>—</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Lafayette</td>
<td>1.4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Courtyard</td>
<td>3.8</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Jersey City</td>
<td>4.4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Cincinnati</td>
<td>8.4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Liberty</td>
<td>10.7</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Fuller Miller</td>
<td>44.4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Winston</td>
<td>65.7</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Salem</td>
<td>&gt;492</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Shanda</td>
<td>&gt;985</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Beroun</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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-resistant 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
To our knowledge, this is the first investigation of Rdl mutations 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. 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.

Collecting 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 (French-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, in 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 ectoparasiticide 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 (LD₉₀ 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 LD₉₀ 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 LD₉₀ 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 pyrethrroids (Romero 2018). Nevertheless, combining the fipronil dose that killed 50% (LD50) 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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