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

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Abstract

Populations of *Blattella germanica* (L.) (German cockroach) have been documented worldwide to be resistant to a wide variety of insecticides with multiple modes of action. The phenylpyrazole insecticide fipronil has been used extensively to control German cockroach populations, exclusively in baits, yet the highest reported fipronil resistance is 38-fold in a single population. We evaluated five populations of German cockroaches, collected in 2018–2019 in apartments in North Carolina and assayed in 2019, to determine the status of fipronil resistance in the state. Resistance ratios in field-collected strains ranged from 22.4 to 37.2, indicating little change in fipronil resistance over the past 20 yr. In contrast, resistance to pyrethroids continues to escalate. We also assessed the roles of detoxification enzymes in fipronil resistance with four synergists previously shown to diminish metabolic resistance to various insecticides in German cockroaches—piperonyl butoxide, *S,S,S*-tributyl phosphorotrithioate, diethyl maleate, and triphenyl phosphate. These enzymes appear to play a variable role in fipronil resistance. We also sequenced a fragment of the *Rdl* (*resistant to dieldrin*) gene that encodes a subunit of the GABA receptor. Our findings showed that all field-collected strains are homozygous for a mutation that substitutes serine for an alanine (A302S) in *Rdl*, and confers low resistance to fipronil. Understanding why cockroaches rapidly evolve high levels of resistance to some insecticides and not others, despite intensive selection pressure, will contribute to more efficacious pest management.

Key words: urban IPM, German cockroach, *Blattella germanica*, fipronil, insecticide resistance, *Rdl*, target site insensitivity

The German cockroach (*Blattella germanica* L., Blattodea: Ectobiidae) is arguably the most prevalent and harmful indoor pest in the United States, and often the most difficult to eradicate, especially from multi-family apartment complexes. Chronic cockroach infestations produce potent aeroallergens that trigger allergies and asthma in atopic children (Rosenstreich et al. 1997, Pomés and Schal 2020). Cockroaches also harbor a diverse microbial community in their digestive system and feces (Kakumanu et al. 2018) and have been implicated in pathogen transmission in hospitals and residential settings (Schal and DeVries 2021). Controlling and eradicating cockroach infestations is the most effective strategy to mitigate potential health risks (Schal and Hamilton 1990, Schal and DeVries

2021). However, German cockroach eradication strategies are seriously constrained by the rapid evolution of resistance to numerous active ingredients across a wide array of modes of action (Scharf and Gondhalekar 2021).

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

in sprays, and the dose that insects receive from ingesting baits is typically higher than in sprays, representing a more effective 'high dose' strategy.

Fipronil has been used in baits for cockroach control for nearly three decades (Kaakeh et al. 1997). It is a member of the broad-spectrum phenylpyrazole class of insecticides that act as antagonists of gamma-aminobutyric acid (GABA)-gated chloride channels (Gant et al. 1998). Fipronil blocks both GABA-gated channels that mediate synaptic inhibition in the insect central nervous system (Gant et al. 1998) and glutamate-gated chloride (GluCl) channels involved in locomotion, feeding and sensory input (Zhao et al. 2004, Narahashi et al. 2010). It was first registered in the United States for cockroach control in 1996, but 1 yr later, resistance was reported in the German cockroach (Scott et al. 1997, Valles et al. 1997). Both reports documented low levels of resistance ranging from a resistance ratio based on the LD_{50} value (RR) of 1.3 in three cockroach strains (Valles et al. 1997) to 7 in seven strains (Scott et al. 1997) (Table 1). Holbrook et al. (2003) collected cockroaches from 20 populations in central North Carolina before fipronil baits were introduced in commercial products and found moderate and highly variable levels of resistance, ranging from 1.2 to >17-fold (Table 1). However, despite intensive selection pressure with baits over the past three decades, resistance levels have increased only marginally, to 17–38-fold (Wang et al. 2004, Gondhalekar et al. 2012, Lee et al. 2022a).

Similar patterns of relatively low fipronil resistance were also reported in Europe (Kristensen et al. 2005) and Malaysia (Ang et al. 2013).

Two major mechanisms have been proposed for fipronil resistance in the German cockroach—metabolic resistance and target site insensitivity. Metabolic mechanisms usually involve upregulation of detoxification of enzymes such as cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs), carboxylesterases (CESTs), and esterases (ESTs). Activity of these enzymes in vivo can be inferred with specific enzyme inhibitors such as piperonyl butoxide (PBO) that inhibits P450s and ESTs (Bergé et al. 1998), triphenyl phosphate (TPP) and *S,S,S*-tributyl phosphorotrithioate (DEF), which inhibit the activity of ESTs (Plapp 1963), and diethyl maleate (DEM), an inhibitor of GSTs (Motoyama and Dauterman 1974). Application of PBO to cockroaches affects the efficacy of fipronil, but results have been inconsistent, with some researchers finding synergism, while others found antagonism (Scott et al. 1997, Valles et al. 1997, Gondhalekar et al. 2012, Ang et al. 2013, Lee et al. 2022b). A recent study found that DEF synergized fipronil in four out of five strains, whereas DEM had no effect in any of the strains (Lee et al. 2022b). The enzyme inhibitor TPP has not been evaluated in combination with fipronil in the German cockroach.

Single-nucleotide polymorphisms (SNPs) in the binding sites of insecticides can result in target site insensitivity. The GABA-gated chloride channel is encoded by the *Rdl* (Resistant to dieldrin) gene.

Table 1. Summary of studies that quantified fipronil resistance in field-collected *B. germanica*

Year	Authors	RR[a] range	No. of strains	Collection years	Location	Type of treatment	Assay used	Time of mortality assessment
1997	Scott and Wen	1.0–1.8, (1.8–7.7)[b]	7	1990–1992	United States	Topical	Dose-response	4 d
1997	Valles et al.	1.0–1.3	3	1989–1996	United States	Topical	Dose-response	1 d
2003	Holbrook et al.	1.2–>17[c]	20	1997–1998	United States	Topical	Discriminating doses	3 d
2004	Wang et al.[d]	8.7–9.3	3	2003	United States	Topical	Dose-response	3 d
2005	Kristensen et al.	1–15	7	1996–2002	Denmark	Topical	Dose-response	3 d
2006	Nasirian et al.	1–2.6	11	Unknown	Iran	Topical	Dose-response	3 d
2010	Chai and Lee	1.0–10.0	22	2005	Singapore	Topical	Dose-response	2 d
2012	Gondhalekar et al.	37.9	1	2006	United States	Topical	Dose-response	3 d
2013	Ang et al.	1.2–3.0 (10.8–25.8)[b]	6[e]	2005	Singapore	Topical	Dose-response	2 d
2016	Ko et al.	5.6 (15.9)[b]	1	2012	Puerto Rico	Topical	Dose-response	2 d
2017	Liang et al.	0.9–1.4 (2.5–25.0)[b]	3	1999–2004	United States	Topical	Dose-response	5 d
2017	Wu and Appel	2.0–8.7	6	2011–2012	United States	Topical	Dose-response	3 d
2019b	DeVries et al.	6–23	7	2011–2014	United States	Topical	Dose-response	2 d
2020	Hu et al.	1.5–3.8	24	2017–2018	Taiwan	Surface contact	Time-course (LT_{50})	7 d
2022a	Lee et al.	~27.7[f]	5	2018-2020	United States	Ingestion	Discriminating doses	3 d
2022	Present paper	22.4–37.2	5	2018–2019	United States	Topical	Dose-response	4 d

[a]RR is the resistance ratio, calculated as LD_{50} (or LT_{50}) of field-collected strain/ LD_{50} (or LT_{50}) of a reference susceptible strain.

[b]Artificially selected population(s)

[c]RR >17 is based on the observation that the LD_{50} of the susceptible strain was 2 ng, and 34.5 ng (10-fold the LD_{99}) failed to kill 50% of the cockroaches.

[d]One of the strains (Cincy) also was used by Wang et al. (2004) and had an RR = 8.6.

[e]The same 6 populations were examined by Chai and Lee (2010) and Ang and Lee (2011).

[f]RR >27.7 is based on the observation that the LD_{50} of the susceptible strain was 1.3 ng, and 36 ng (10-fold the LD_{95}) killed 20–70% of the cockroaches.

Table 2. Fipronil dose-response results in the susceptible Orlando Normal reference strain and five *B. germanica* populations recently collected from apartments in Raleigh, NC

Strain[a]	n	Lethal dose LD$_{50}$ ng/male (95% CI)[b]	Slope ± SE	χ^2 (df)	z-test[c]	RR[d] (95% CI)
Orlando Normal	270	1.55 (1.32, 1.82)	5.40 ± 0.73	4.00 (3)	7.4*	-
VS101	120	38.2 (20.4, 71.6)	2.58 ± 0.61	0.61 (2)	4.3*	24.7* (18.3, 33.4)
DR2800	150	34.6 (14.7, 81.7)	1.56 ± 0.36	9.81 (2)	4.4*	22.4* (15.0, 33.5)
DR2820B	200	45.4 (36.2, 56.9)	3.92 ± 0.46	8.88 (3)	8.5*	29.3* (24.7, 34.8)
CC29	150	50.5 (29.2, 87.4)	2.32 ± 0.46	0.38 (2)	5.1*	32.6* (24.9, 42.7)
PR515F	150	57.6 (14.1, 235.4)	2.92 ± 0.74	1.48 (1)	3.9*	37.2* (29.3, 47.2)

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

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

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

[d]Resistance Ratios (RR, lethal dose ratio) and 95% confidence intervals. RR was calculated as LD$_{50}$ of apartment-collected strain/ LD$_{50}$ of susceptible reference strain (Orlando Normal). RR values with (*) are considered significant when their 95% CIs do not include 1.0 (Robertson et al. 2017).

A mutation that results in substitution of serine or glycine in place of an alanine residue (A302S/G) in *Rdl* confers different levels of resistance in various insect species, and the response to various phenylpyrazole insecticides varies across species and populations of the same species (Zhao et al. 2003, Nakao 2017). In *B. germanica*, the *Rdl* mutation appears to contribute more to dieldrin resistance than to fipronil resistance (Scott et al. 1997, Hansen et al. 2005, Kristensen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013, Lee et al. 2022b).

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

Materials and Methods

Experimental Insects

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

were conducted in 2019, two to four generations after the field collections.

Fipronil Toxicity

Fipronil ((RS)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile; CAS 120068-37-3), at 98.7% purity, was obtained from Sigma-Aldrich (St. Louis, MO). The lethal dose of fipronil that killed 50% of each population (LD$_{50}$) was determined by topical application. Groups of 10 adult male cockroaches were briefly anesthetized with CO$_2$ in a plastic Petri dish (90 × 15 mm). Fipronil was topically applied in acetone with a micro-applicator (Hamilton, Reno, NV) equipped with a 50 µl glass syringe (Hamilton Co.) that delivered 1 µl on the ventral thorax of each cockroach. We chose this area, between the coxae, because it is not groomed as frequently as other regions of the body. Fipronil concentrations ranged from 0 (acetone control) to 120 ng/µl acetone and varied by population tested. Mortality was assessed every 24 h for 96 h post-application by gently touching individuals with forceps, with morbid cockroaches (unable to right themselves after touching with forceps) considered dead. We report mortality at 96 h.

Metabolic Enzyme Inhibitors in Fipronil Resistance

We used the following enzyme inhibitors: DEF (97.7% purity, Chem Services, West Chester, PA), TPP (99%), PBO (99%), and DEM (97%) (Sigma-Aldrich). We evaluated the effects of detoxification enzyme inhibitors on fipronil toxicity in six populations: The susceptible population (Orlando Normal), and the five strains collected in North Carolina. Each adult male was topically treated with a non-lethal dose of one of the inhibitors in 1 µl acetone: 100 µg PBO, 30 µg DEF, 100 µg DEM, or 30 µg TPP. These doses were based on previously reported values. After the cockroaches recovered at room temperature for one h, they were briefly anesthetized again with CO$_2$ and treated topically with either acetone (control) or the strain-specific LD$_{50}$. Three replicates of 10 adult male German cockroaches were performed for each population-inhibitor combination. Mortality was recorded every 24 h for 96 h, as described above, and mortality at 96 h is reported.

Pyrethroid Resistance

Cypermethrin (98% purity, Sigma-Aldrich) resistance was evaluated in the Orlando Normal strain and three apartment-collected strains DR2820B, CC29, and VS101. We topically treated 240 adult males

of the Orlando Normal strain, as described for fipronil, to generate a dose-response curve and estimate the LD_{50} from Probit analysis. The field-collected cockroaches were then treated with the estimated LD_{50} of the Orlando Normal strain, as well as 10-fold and 100-fold the LD_{50}. Mortality was assessed 48 h post-treatment. We also weighed individual males of these strains, as well as the Orlando Normal strain.

Detection of *Rdl* Mutation

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

A 245-bp genomic fragment of the GABA receptor gene that includes the *Rdl* mutation site was amplified with the primers BG-Rdl-F (5′-GTGCGGTCCATGGGATACTA-3′) and BG-Rdl-R (5′-AACGACGCGAAGACCATAAC-3′) designed by Hansen et al. (2005). The reactions were conducted in 20 µl reaction mix comprising 10 µl of AmpliTaq Gold 360 2X Master mix (Applied Biosystems, Waltham, MA), 1 µl of 10 µM of each primer, 0.2 µl BSA (bovine serum albumin; Sigma-Aldrich) (20 mg/ml), and 2 µl of German cockroach genomic DNA as template for the PCR reaction. A negative control with no template DNA was included in every PCR run. The following thermal cycle program was used for amplification: Initial activation at 95°C for 10 min followed by 40 cycles of 94°C for 30 s, 64.3°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min (Gondhalekar and Scharf 2012). Two microliters of each PCR product were used to verify proper sized bands on 1.2% agarose gel. The remaining PCR product was purified by ExoSAP-IT (Applied Biosystems) and direct-sequenced at the Genomic Sequencing Laboratory (North Carolina State University, Raleigh, NC) with BG-Rdl-R as sequencing primer. Each sequence was determined by manually checking for the GCC to TCC mutation that results in the A302S substitution.

Statistical Analysis

The LD_{50} for each cockroach population was determined using log-dose probit-mortality analysis based on a spreadsheet template (Lei and Sun 2018). The values were in agreement with analysis in PoloPlus (LeOra Software, Petaluma, CA). Abbott's correction (Abbott 1925) was used to correct for control mortality, as needed. The *z*-test was used to determine if the slope of the dose–response regression was significant ($P < 0\ 0.05$); results were similar to the *t*-ratio in PoloPLus. The lethal dose ratio at LD_{50} was used to generate resistance ratios (RR). The effects of enzyme inhibitors on fipronil toxicity were determined using Chi-square analysis in SAS 9.4 (SAS Institute, Cary, NC), comparing mortality of cockroaches with and without inhibitor application.

Results

Fipronil and Cypermethrin Resistance

Adult males from four of the strains were individually weighed. Their mean body mass ± SEM were as follows: Orlando Normal = 52.1 ± 0.9 mg, *n* = 10; VS101 = 57.8 ± 1.6 mg, *n* = 10; DR2820B

= 52.5 ± 1.3 mg, *n* = 10; CC29 = 56.4 ± 0.7, *n* = 10. A one-way ANOVA was highly significant (*F* = 5.2583, *df* = 3, 36, *P* = 0.0041), and Dunnett's test with Orlando Normal as the control group indicated that the body masses of VS101 and CC29 were significantly higher than Orlando Normal (*P* = 0.0065 and 0.0460, respectively), whereas DR2820B was not (*P* = 0.9865).

The LD_{50} values were determined from dose-response curves 4 d after topical applications of fipronil to cockroaches collected from five apartments (VS101, DR2800, DR2820B, CC29, PR515F; Fig. 1); these values were compared with the insecticide-susceptible Orlando Normal strain. The Orlando Normal males had 97.5% mortality at the highest dose of 3 ng fipronil per insect, whereas 90 ng/insect killed 73–93% of the field-collected cockroaches. The LD_{50} of the Orlando Normal males was 1.55 ng/insect, whereas PR515F, the strain with the highest fipronil resistance, had an LD_{50} of 57.6 ng/male (Table 2). The resistance ratio values based on their respective LD_{50} values ranged from 22.4 (DR2800) to 37.2 (PR515F), and all five strains significantly differed from the Orlando Normal reference strain (*P* < 0.05). The slopes of the dose–response curves (range, 1.56–3.92) for the five populations were less steep than for the Orlando Normal population (slope = 5.40), and indeed, the RR values at LD_{90} were 1.23–3.83 greater than at LD_{50}, indicating a more heterogeneous response of the field-collected cockroaches. Male body mass values were available for four strains. Their body masses were 0.8%–10.9% greater than the Orlando Normal cockroaches.

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

Effects of Enzyme Inhibitors on Fipronil Toxicity

The toxicity of fipronil in the presence of the enzyme inhibitors PBO, DEF, DEM, and TPP 4 d after treatment is shown in Fig. 2. Because the fipronil LD_{50} varied among the six strains, we used the strain-specific LD_{50} dose with and without the addition of the inhibitor. When pretreated with PBO, neither the susceptible reference Orlando Normal strain nor PR515F, the most resistant strain, experienced a significant increase in fipronil toxicity (*P* = 0.12 and 0.06, respectively). However, fipronil mortality increased significantly in the presence of PBO in strain VS101 (χ^2 = 11.4, *df* = 1, *P* < 0.05) and in strain DR2800 (χ^2 = 10.7, *df* = 1, *P* < 0.05). Fipronil toxicity also was significantly enhanced in strains DR2820B (χ^2 = 4.6 *df* = 1, *P* < 0.05) and CC29 (χ^2 = 6.9 *df* = 1, *P* < 0.05), but to a lesser extent than in VS101 and DR2800.

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

A302S *Rdl* Mutation

A 245 bp fragment of the GABA receptor gene targeting the position of the *Rdl* mutation that causes the A302S substitution was
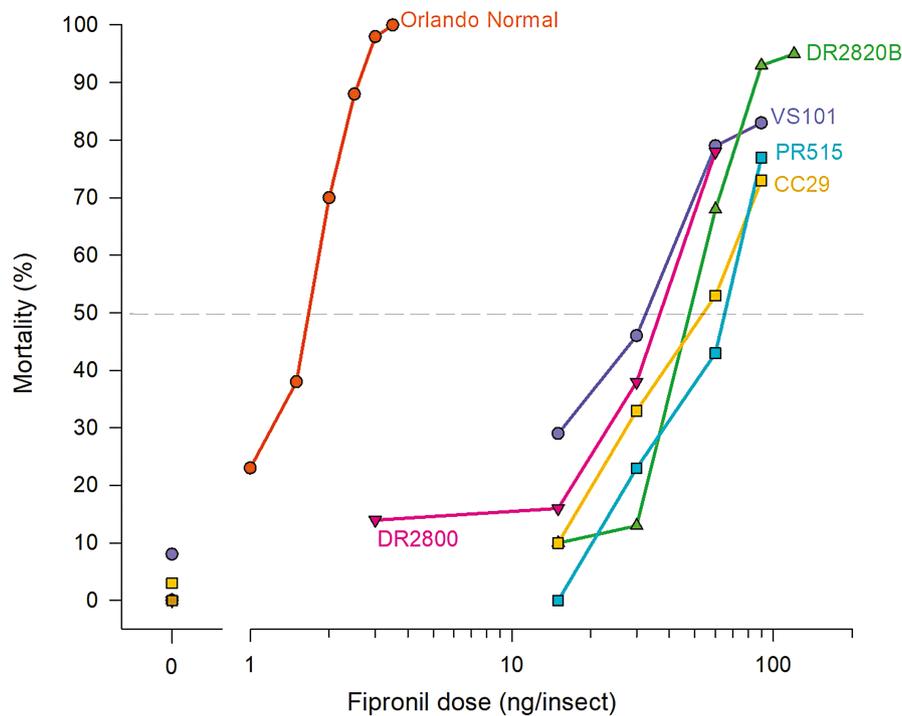
Fig. 1. Dose–response curves for fipronil-treated *B. germanica* adult males from a reference insecticide-susceptible strain (Orlando Normal) and five populations recently collected in apartments in Raleigh, NC. The lethal dose of fipronil that killed 50% of each population (LD_{50}) was determined by topical application. Mortality was assessed daily, and mortality at 4 d is reported. At least three replicates of 10 adult male cockroaches were performed per dose.

Table 3. Cypermethrin dose-response results in the susceptible Orlando Normal reference strain and responses of five recently collected *B. germanica* populations to diagnostic doses of cypermethrin

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

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

[a]Insects were topically treated with cypermethrin (in 1 µl acetone); LD_{50} was estimated for the Orlando Normal strain from probit analysis. CI is confidence interval. The average mass of *B. germanica* males is ~50 mg, hence multiply by 20 to obtain approximate µg/g body mass.

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

[c]The mean represents the average of 3 replicates with 10 cockroaches assayed in each replicate.

[d]Three diagnostic doses were used, representing the LD_{50} value (0.1 µg), 10-fold the LD_{50} (1 µg), and 100-fold the LD_{50} value (10 µg) of the Orlando Normal strain.

amplified and sequenced from the Orlando Normal susceptible strain (n = 10) and the five field-collected populations (n = 10 per population). Based on previous studies relating fipronil resistance to this mutation, and the moderate level of resistance we detected in some populations, we expected to detect haplotypes corresponding to homozygous susceptible wild-type (Ala302/Ala302; S/S), putatively homozygous resistant (Ser302/Ser302; R/R) and the heterozygous haplotype (Ala302/Ser302; S/R). However, we found the *Rdl* mutation in all five recently collected cockroach strains, but not in the reference insecticide susceptible strain (Table 4); 100% of the apartment-collected cockroaches were homozygous for the resistant genotype (A302S) (Fig. 3).

Discussion

It appears that fipronil resistance in German cockroach populations in North Carolina has increased over the last two decades from a range of 1.2–17-fold (Holbrook et al. 2003) to 22.4–37.2-fold. The fipronil LD_{50} values ranged from 34.6 ng/male (approximately 0.69 µg/g) to 57.6 ng/male (approximately 1.15 µg/g). The moderate increase in resistance, despite the prevalence of fipronil-containing baits in the United States, is consistent with patterns in other United States and global populations investigated over the last two decades (Table 1). These findings suggest that fipronil resistance in German cockroach populations might have reached a plateau, possibly representing a level above which
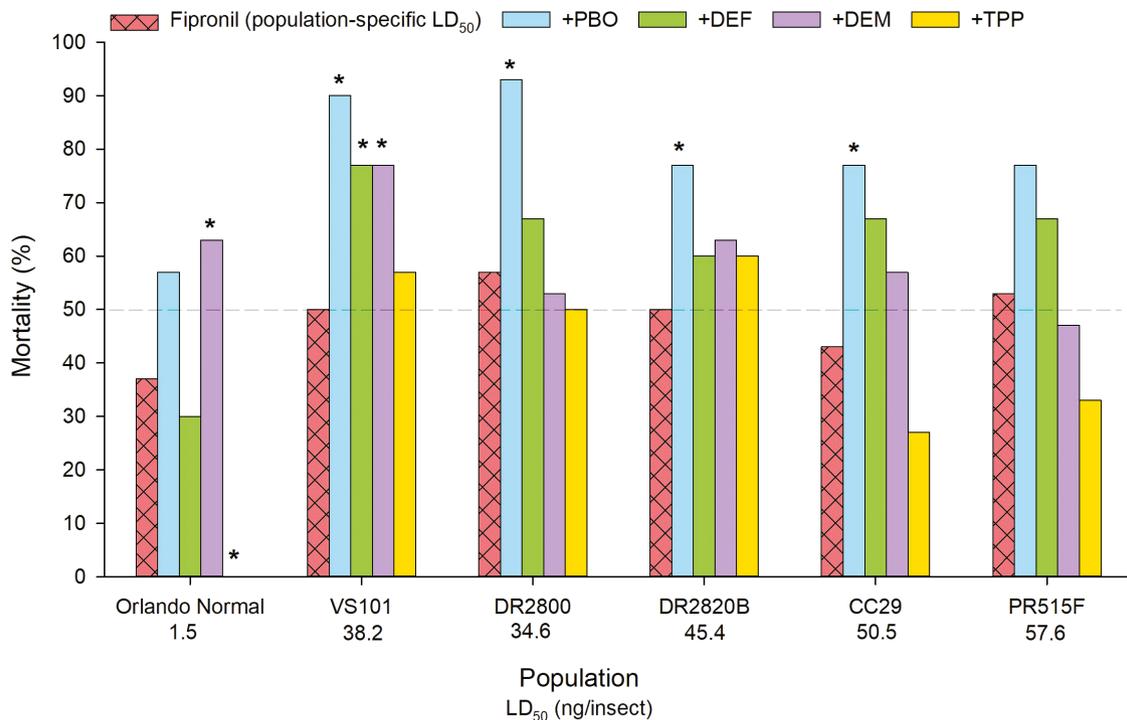
Fig. 2. Effects of four detoxification enzyme inhibitors (i.e., insecticide synergists) on fipronil toxicity in *B. germanica* adult males. Each enzyme inhibitor (piperonyl butoxide [PBO], *S,S,S*-tributyl phosphorotrithioate [DEF], diethyl maleate [DEM], and triphenyl phosphate [TPP]) was topically applied in 1 µl acetone 1 h prior to application of a population-specific LD_{50} (shown below the population name). Percent mortality was determined 4 d after treatment and mortality was corrected for control mortality (synergist only). The mean shown is of 3 replicates with 10 males each (*n* = 30 males per treatment). Significant differences between fipronil-only treatments and fipronil plus inhibitor treatments were determined using Chi-square analysis, with significance indicated by * (*P* < 0.05).

Table 4. *Rdl* haplotype of the fipronil-susceptible Orlando Normal reference strain and of fipronil-resistant apartment-collected *B. germanica*

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

cockroaches experience substantial fitness costs. However, unlike the broad variation in resistance ratios documented in populations in the 1990s and 2000s, we observed low variation among the five German cockroach populations collected in homes in Raleigh, NC. These results support the idea that populations might have reached an upper limit of resistance, represented by RR values of 30–40.

Mechanisms of Fipronil Resistance

The involvement of metabolic enzymes in fipronil degradation appears to vary among cockroach strains. Piperonyl butoxide was reported by Valles et al. (1997) to have antagonistic effects on fipronil toxicity in three different populations, and by Ang et al. (2013) in six populations. On the other hand, Gondhalekar et al. (2012) showed that PBO synergized fipronil in one strain and Lee et al. 2022b showed similar results in four out of five strains, as in our study. However, fipronil mortality in our most resistant strain (PR515F)

increased by only 24% in the presence of PBO (at the strain-specific LD_{50} level of fipronil), from 53% to 77%, and this increase was not statistically significant. Responses to PBO vary among insect species, with synergism in *Musca domestica* L. (Diptera: Muscidae) (Liu and Yue 2000) and bed bug (González-Morales et al. 2021), but no apparent effect in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Scharf et al. 2000). It is important to note that fipronil would be oxidized by P450s to fipronil sulfone, which also binds strongly to GABA and GluCl receptors in insects, including the German cockroach (Zhao and Salgado 2010), and thus plays an important role in the toxicity of fipronil. In vivo studies have found that fipronil and its sulfone metabolite range from similar bioactivity in some insects (e.g., *D. virgifera*; Scharf and Siegfried 1999), to the sulfone being 4.6-fold more bioactive than fipronil (non-biting midge, *Chironomus dilutus* Shobanov, Kiknadze & Butler, Diptera; Weston and Lydy 2014). It is possible that populations of German cockroaches express polymorphisms in the affinities of fipronil and fipronil sulfone to GABA and GluCl receptors. Thus, in some cockroach populations the application of PBO blocks fipronil oxidation, but does not greatly affect toxicity because fipronil and fipronil sulfone are equally bioactive. In populations where the sulfone is more bioactive than fipronil, PBO would block the oxidation and thus antagonize fipronil. In yet other populations, P450s may further hydroxylate the sulfone to less active forms. In these populations, PBO might block these later transformations and both fipronil and the sulfone would be more toxic in the presence of PBO. However, the high synergism expected in the latter scenario has not been demonstrated in any German cockroach population.

Similarly, DEF, an EST inhibitor, has been reported to antagonize fipronil toxicity in multiple populations of the German cockroach (Valles et al. 1997, Ang et al. 2013), but synergize fipronil

A.

```
GCGACGCCCGCCCGAGTCGCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  MW267921.1
GCGACGCCCGCCCGAGTCGCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  Orlando Normal
GCGACGCCCGCCCGAGTCTCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  VS101
GCGACGCCCGCCCGAGTCTCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  DR2800
GCGACGCCCGCCCGAGTCTCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  DR2820B
GCGACGCCCGCCCGAGTCTCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  CC29
GCGACGCCCGCCCGAGTCTCCCTCGGGGTTACCACTGTGCTCACCATGACTACCCTTATGTCGTCCACC  PR515F
 A  T  P  A  R  V  A/S  L  G  V  T  T  V  L  T  M  T  T  L  M  S  S  T   Amino acid sequence
```

TM2

B.
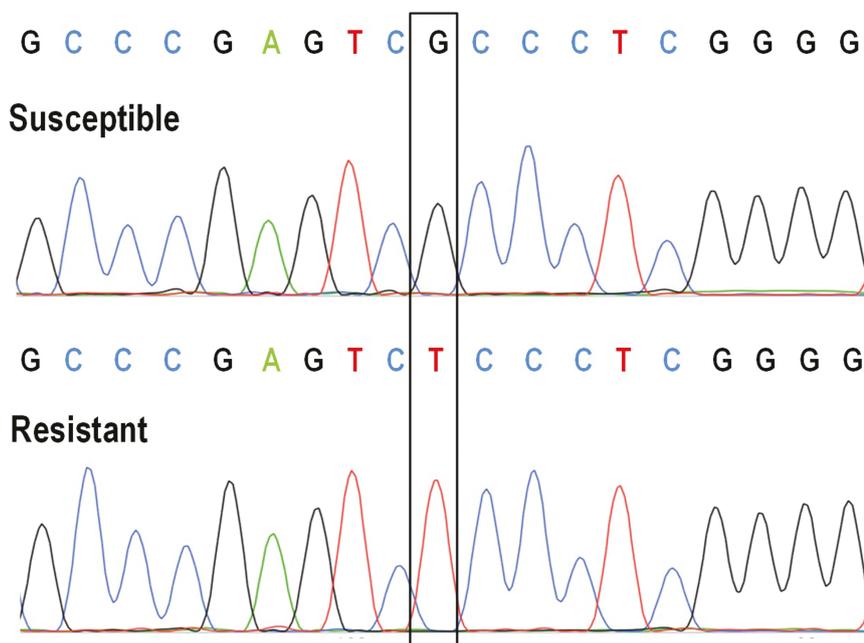
Fig. 3. Nucleotide sequences of the TM2 region of the *B. germanica Rdl* gene, which includes the point mutation that results in the A302S substitution. (A) Representative sequences from the insecticide-susceptible Orlando Normal strain and five field-collected German cockroach populations were aligned against the reference sequence (MW267921.1), with the A302S region highlighted. (B) Representative direct sequencing chromatograms of homozygous susceptible (G/G) and homozygous resistant (T/T) cockroaches. The G-to-T point mutation site is shown within a box. MW267921.1 is the GenBank accession number for the *B. germanica* GABA-gated chloride channel complete cds (Jones et al. 2021).

in the one investigated strain (Gondhalekar et al. 2012) and four out of five strains (Lee et al. 2022b). Although all five of our field-collected strains had greater mortality after DEF treatment, only one (VS101) showed a significant increase in mortality after DEF application. There was no apparent effect of DEM, a GST inhibitor, on fipronil metabolism in five strains of the German cockroach (Lee et al. 2022b). Interestingly, DEM significantly synergized fipronil toxicity only in the susceptible strain (Orlando Normal) and the VS101 strain, and it slightly elevated mortality in two strains and depressed fipronil mortality in two other strains. The only significant antagonism in our assays was caused by TPP in the Orlando Normal susceptible strain, although TPP also reduced fipronil bioactivity slightly in two other strains. It is important to note that some studies with *B. germanica* use the LD_{50} dose of the susceptible strain on field-collected cockroaches, which would result in low mortality of field-collected strains and high sensitivity to the effects of synergists. We used strain-specific LD_{50} doses of fipronil, resulting in approximately 50% mortality, and relatively low sensitivity to the synergistic effects of enzyme inhibitors.

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

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

1993). This mutation has been reported in both U.S. and European cockroach populations (Hansen et al. 2005, Ang et al. 2013) but although it confers high resistance to dieldrin, it appears to confer relatively low cross-resistance to fipronil (Scott et al. 1997, Hansen et al. 2005, Kristensen et al. 2005, Gondhalekar and Scharf 2012, Ang et al. 2013, Lee et al. 2022b). All of our field-collected cockroaches were homozygous for the *Rdl* mutation, regardless of their level of resistance, similar to a recent report with cockroaches collected in California (Lee et al. 2022b). The combination of low resistance to fipronil and the presence of the *Rdl* mutation has been shown in other insects, including planthoppers (Nakao 2017) and flies (Gao et al. 2007). However, other mutations in the GABA receptor subunit that confer fipronil resistance, which have been studied in other insects (Nakao 2017, Garrood et al. 2017), need to be investigated in *B. germanica*. Moreover, using antibiotic treatments to disrupt the gut microbiome, Wolfe and Scharf (2021) showed that microbial metabolism may contribute to fipronil resistance in the German cockroach. Overall, it appears that the A302S substitution in the *Rdl* gene confers relatively low fipronil resistance to German cockroaches, and detoxification mechanisms, mainly involving P450s and esterases, also impart relatively low resistance. Together, these two mechanisms appear to account for most of the resistance to fipronil in the German cockroach.

Why Has Resistance to Fipronil Not Increased More?

Fipronil resistance in German cockroach populations has increased over the past three decades, but only marginally. Early findings in the late 1990s of fipronil resistance in the U.S. reported LD_{50} resistance ratios (RR) <2, and follow-up studies in the 2000s found some populations with RR >17 (Table 1). Later reports, including recent findings, vary across studies and geographic locations, with RR values remaining low (1.0–8.7) in some populations (e.g., Liang et al. 2017, Wu and Appel 2017), while others increased appreciably as high as 38 (Gondhalekar et al. 2012, DeVries et al. 2019b, Lee et al. 2022a). The RR values we found (22.4–37.2) are thus consistent with the latter three papers given slight variations in methodology, reference strains used, and body mass of males.

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

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

field-collected populations of the bed bug *C. lectularius* (González-Morales et al. 2021).

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

It is likely that fipronil resistance in some arthropods, including *B. germanica*, carries significant fitness costs. For example, lower larval survival rate, lower adult emergence rate, lower copulation rate, lower fecundity and fewer offspring were associated with higher fipronil resistance in the brown planthopper (Ling et al. 2009, Zhang et al. 2016). Likewise, in the housefly, *M. domestica*, artificial selection with fipronil resulted in longer larval duration, lower pupal weight, lower fecundity, lower hatchability, lower number of the next generation larvae, lower intrinsic rate of population increases and lower biotic potential (Abbas et al. 2016). Other indirect evidence of significant fitness costs associated with fipronil resistance comes from observations that high resistance levels were not sustained when fipronil selection was discontinued in several insect species including planthoppers and houseflies. We therefore suspect that high fitness costs of fipronil resistance in the German cockroach might explain the moderate levels of resistance despite strong selection through several decades. Nevertheless, a study of fitness-related traits in *B. germanica* found no evidence of fitness costs; however, resistance to fipronil in the six field-collected strains ranged from 1.2- to 2.8-fold (Ang and Lee 2011), leaving this question unresolved. In contrast, resistance levels to pyrethroids are extremely high in *B. germanica* populations (Wei et al. 2001, DeVries et al. 2019b, Lee et al. 2022a, Scharf and Gondhalekar 2021), including in the three populations that we examined, and may be sustained for years after selection is discontinued (M.A.G-M and C.S., personal observations), suggesting lower fitness costs than with fipronil resistance. Overall, these patterns suggest potentially significant trade-offs between elevated levels of fipronil resistance in *B. germanica* and fitness-related traits. If so, this complex interaction has important implications for the continued use of fipronil in cockroach baits.

The mode of action of fipronil and its sulfone metabolite may also contribute to the stalled resistance in *B. germanica*. Together, fipronil and its bioactive metabolite are unique in binding with high affinity to three target sites in the insect central nervous system: GABA-gated chloride channels and two types of glutamate-gated chloride channels. Further, Zhao and Salgado (2010) suggested that there might be two subtypes of GABA receptors in the German cockroach. They also demonstrated that the *Rdl* mutation and resistance to dieldrin did not affect the sensitivity of GluCl receptors to fipronil sulfone. Generally, the evolution of target site-based resistance to

insecticides can be greatly slowed by 'pyramiding' two or more distinct toxins, as in pyramided Bt crops (Carrière et al. 2016). By binding multiple CNS targets, fipronil and fipronil sulfone may act as a combination of active ingredients that target different receptors, which is an important strategy of resistance management. However, much more information is needed about the levels of cross-resistance between fipronil and the sulfone and their differential affinities to different receptor sites.

Is Resistance to Fipronil Expected to Affect Its Field Efficacy?

There are no studies that quantitatively correlate fipronil resistance levels with the efficacy of fipronil baits in the field. We suspect that it is unlikely that 37-fold resistance to fipronil would affect the field efficacy of baits because of the high doses of fipronil ingested during bait consumption, which would overwhelm the relatively low resistance we observed. For example, Holbrook et al. (2003) supplemented rodent chow with fipronil and showed that concentrations much lower than in commercial baits killed all the resistant cockroaches (RR = 1.2 to >17). Moreover, based on consumption of 1 mg of bait per cockroach, Holbrook et al. (2003) estimated that each cockroach would ingest 100 ng of fipronil from a bait containing 0.01% fipronil (Maxforce FC gel – the standard in 2003, Bayer Environmental Science, Montvale, NJ). Ko et al. (2016) conducted more recent, but similar estimates. They determined the LD_{90} of fipronil in susceptible males to be 3.18 ng per male (2.67 ng in the present study). If a susceptible male ingested 1.8 mg of bait containing 0.05% fipronil (Maxforce FC Magnum, Bayer), it would consume 900 ng of fipronil, or 370-fold the LD_{90}. Because fipronil is more active at lower levels than most other insecticides, the high-dose strategy inherent in gel bait formulations, and especially those containing fipronil, could overcome the highest fipronil resistance reported thus far in any German cockroach population.

However, the foregoing estimates assume highly palatable baits that are consumed in large quantities and thus deliver a high dose of fipronil. Behavioral traits such as avoidance of the bait (e.g., glucose aversion) or physical changes in the formulation (aging, repellency) could result in lower bait consumption and compromise control efforts. Glucose aversion has become more prevalent since it was discovered 3 decades ago (Silverman and Bieman 1993, Wang et al. 2004, Wada-Katsumata et al. 2013). Moreover, recent research showed that cockroach saliva rapidly degrades various sugars, releasing glucose, which interrupts feeding by glucose-averse cockroaches (Wada-Katsumata and Schal 2021, Wada-Katsumata et al. 2022). Thus, bait formulations that contain sugars, not just glucose, would deter glucose-averse cockroaches. We underscore that bait palatability, resistance to the active ingredient, and expected performance in the field are intricately linked. Highly palatable baits deliver enough fipronil to overcome ~30–40-fold resistance that we observe in the field. However, less palatable baits would deliver less fipronil, and ~30–40-fold resistance might offer ample protection to field cockroaches.

The recent introduction of residual formulations of fipronil might have a similar effect. In 2019, fipronil was approved in the United States for use in residual sprays (0.65%, Fipronil-Plus-C, EPA Reg. No. 55431-15; maximum allowed fipronil concentration 0.0076%) for controlling crawling insects indoors, including cockroaches. Residual sprays that expose insects to much lower doses than those found in baits might nullify the advantages of the high-dose approach with baits, and even the moderate ~30–40-fold present-day fipronil resistance might protect cockroaches from residual fipronil

formulations. A case in point may be the dual use of indoxacarb in baits and spray formulations. Indoxacarb has been used in baits since 2006 and in spray formulations since 2010. The increase in resistance and control issues over the years may be due to the dual use, which compromises the high-dose strategy (Gondhalekar et al. 2013).

Conclusion

The accumulated evidence on fipronil resistance in *B. germanica* suggests that target site insensitivity in *Rdl* contributes to low-level resistance to fipronil, and metabolic detoxification processes are highly variable across populations and contribute marginally to fipronil resistance. The metabolic mechanisms might be related to low fipronil cross-resistance with pyrethroids, which select for significant upregulation of a wide range of P450 genes. Our results suggest that combining fipronil with PBO or DEF could reduce fipronil resistance in the most resistant populations. However, this might not be necessary if fitness costs associated with fipronil resistance, perhaps related to ensuring proper functioning of the GABA-gated channels and glutamate-gated chloride channels, will prevent the emergence of highly resistant German cockroach populations. In practical cockroach control, lower fitness of resistant cockroaches would lend strong support for a resistance management program based on the practice of rotation of active ingredients, because populations are expected to quickly recover sensitivity to fipronil when fipronil pressure is withdrawn. Highly palatable baits can deliver high doses of fipronil that can overcome present-day resistance levels. However, less palatable baits (e.g., poor quality, rancid), and taste aversions in cockroaches (e.g., glucose-aversion) would result in less fipronil ingested, and ~30–40-fold resistance to fipronil might protect cockroaches from such baits.

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