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Author(s) :Ameya D. Gondhalekar and Michael E. Scharf

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Mechanisms Underlying Fipronil Resistance in a Multiresistant Field Strain of the German Cockroach (Blattodea: Blattellidae)

AMEYA D. GONDHALEKAR¹ AND MICHAEL E. SCHARF^{1,2}

Entomology and Nematology Department, University of Florida, Gainesville, FL

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ABSTRACT German cockroaches (*Blattella germanica* L.) have significant impacts on human health, most notably they are implicated as causes of childhood asthma. Gel bait formulations of fipronil, a phenylpyrazole insecticide, have been in use for German cockroach control in the United States since 1998. Previously, dieldrin resistant German cockroach strains were shown to have 7- to 17-fold cross-resistance to fipronil. More recently, a field-collected strain (GNV-R) displayed $\approx$ 36-fold resistance to topically applied fipronil at the LD₅₀ level, which is the highest level of fipronil resistance reported to date in the German cockroach. The aim of the current research was to identify mechanism(s) responsible for high-level fipronil resistance in the GNV-R strain. Synergist bioassays conducted using topical and injection application methods implicated cytochrome P450-mediated detoxification in resistance. Electrophysiological recordings using the suction-electrode technique revealed the nervous system of the GNV-R strain is insensitive to fipronil. In agreement with electrophysiology results, the alanine to serine (A302S) mutation encoded by the γ -amino butyric acid-gated chloride channel subunit gene *resistance to dieldrin*, which confers limited cross-resistance to fipronil, was detected in 95% of GNV-R strain individuals. Logistic regression analysis showed that A302S mutation frequency correlates with neurological insensitivity as shown by electrophysiology data. Overall, results of synergism bioassays, electrophysiological recordings, and A302S mutation frequency measurements suggest that fipronil resistance in the GNV-R strain is caused by the combined effects of enhanced metabolism by cytochrome P450s and target-site insensitivity caused by the A302S-encoding mutation in the *resistance to dieldrin* gene.

KEY WORDS *Blattella germanica*, German cockroach, fipronil resistance, enhanced metabolism, target-site insensitivity

German cockroaches (*Blattella germanica* L.) are synanthropic pests that contribute significantly to childhood asthma, enteric pathogen transmission, and diminished psychological well-being (Ash and Greenberg 1980, Kopanic et al. 1994, Rosentreich et al. 1997, Institute of Medicine [IOM] 2000, Zurek and Schal 2004, Arbes et al. 2004). The most efficient way to overcome these issues associated with German cockroaches is through effective cockroach control (Schal and Hamilton 1990, Arbes et al. 2004, Miller and Meek 2004, Nalyanya et al. 2009). However, insecticide resistance is a major limiting factor in efficient control of German cockroaches and it has been an area of active investigation for decades. As in many other insect species, the major mechanisms of insecticide resistance in the German cockroach include target-site insensitivity caused by point mutations in ion channels (Kaku and Matsumura 1994, Dong 1997) and enhanced metabolism of insecticides by detoxification

enzymes like cytochrome P450 monooxygenases (P450s) and general esterases (Siegfried et al. 1990, Prabhakaran and Kamble 1995, Valles et al. 1996, Scharf et al. 1998, Wu et al. 1998, Pridgeon et al. 2002).

In recent years, German cockroach control in the United States has been mainly based on use of bait formulations of insecticides like abamectin, fipronil, hydramethylnon, imidacloprid, and indoxacarb (Buczowski et al. 2001, 2008; Appel 2003; Harbison et al. 2003; Wang et al. 2004, Gondhalekar et al. 2011). In general, lower levels of resistance to newer bait active ingredients (AIs) have been reported in the German cockroach (Cochran 1994; Valles et al. 1997; Kaakeh et al. 1997; Valles and Brenner 1999; Wang et al. 2004, 2006). An exception to this generalization was recently reported wherein the field-collected Gainesville-Resistant (GNV-R) strain was found to have 36-fold resistance to topically applied fipronil and significant tolerance (2.3-fold) to orally fed Maxforce FC Select Roach Killer Bait Gel (0.01% fipronil) (Gondhalekar et al. 2011).

Like cyclodiene insecticides, the phenylpyrazole insecticide fipronil acts antagonistically with the *re-*

¹ Present address: Department of Entomology, Purdue University, West Lafayette, IN.

² Corresponding author, e-mail: mscharf@purdue.edu.

sistance to dieldrin (*Rdl*) subunit of the γ -amino butyric acid (GABA) receptor (Cole et al. 1993, Gant et al. 1998). Cyclodiene resistance in several insect species is known to be caused by an alanine to serine substitution (A302S; commonly known as the *Rdl* mutation) at position 302 (*Drosophila melanogaster* Meigen numbering) in the *Rdl* gene that encodes for the *Rdl* subunit of the GABA receptor (ffrench-Constant et al. 1993, 2000). The cyclodiene resistance-associated *Rdl* mutation is also known to confer limited cross-resistance to fipronil (Cole et al. 1993, 1995; ffrench-Constant et al. 2000; Kristensen et al. 2005; Le Goff et al. 2005; Li et al. 2006).

Cockroach gel baits containing fipronil have been available in the United States for more than a decade. When the GNV-R strain was collected in 2006, deposits of Avert Cockroach Gel Bait (0.05% abamectin) were detected in the apartment and the inhabitants confirmed the use of pyrethroid aerosols. Although clear evidence on use of fipronil for GNV-R control is lacking, given their widespread use, it is reasonable to suspect that the GNV-R strain must have been exposed to fipronil baits in the field. Consistent with this hypothesis, the GNV-R strain has 36-fold resistance to fipronil at the LD₅₀ level (Gondhalekar et al. 2011), which is the highest level of fipronil resistance reported to date in the German cockroach. Previous reports on fipronil resistance ratios of 7- to 17-fold in various cockroach strains (Valles et al. 1997; Scott and Wen 1997; Holbrook et al. 2003; Wang et al. 2004, 2006; Kristensen et al. 2005; Chai and Lee 2010) and resistance observed in the recently field-collected GNV-R strain (Gondhalekar et al. 2011) suggest that fipronil resistance in German cockroach field populations is on the rise.

Holbrook et al. (2003) and Kristensen et al. (2005) predicted that wide-spread use of fipronil for cockroach control could possibly select for fipronil resistance mechanisms other than *Rdl*. To test the hypothesis that fipronil resistance in the GNV-R strain is caused by multiple mechanisms, our specific objectives were as follows: 1) perform synergist bioassays to investigate enzyme systems potentially involved in mediating fipronil resistance, 2) conduct electrophysiological recordings using the suction recording electrode technique to ascertain the involvement of target-site modification in resistance, and 3) sequence a portion of the *Rdl* gene to detect the presence and determine the frequency of the resistance associated A302S-encoding mutation. Our findings show fipronil resistance in German cockroaches can result from multiple mechanisms that include metabolic detoxification and target site insensitivity.

Materials and Methods

Insects. Two German cockroach strains were used in this study: Johnson Wax susceptible (JWax-S) and GNV-R. JWax-S is a standard susceptible laboratory strain, while the GNV-R strain is a multi-insecticide resistant field strain collected from an apartment in Gainesville, FL, after reported control failures (Gond-

halekar et al. 2011). Rearing was conducted in 3.8 liters plastic containers that were held in a reach-in environmental chamber at $25 \pm 1^\circ\text{C}$ temperature and 12:12 h light:dark photoperiod. The inner top portion of the rearing units was lightly coated with a mixture of petroleum jelly and mineral oil (2:3) to keep the cockroaches from escaping. Each rearing unit contained corrugated cardboard harborages, a water source, and rodent diet (No. 8604; Harlan Teklad, Madison, WI).

Chemicals. Piperonyl butoxide (PBO; 98.3% pure), S,S,S-tributylphosphorothioate (DEF; 98% pure), and fipronil (97.1% AI) were purchased from Chem Service (West Chester, PA). HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) sodium salt (99.5% pure) was obtained from Sigma-Aldrich (Milwaukee, WI). All other chemicals and solvents were either purchased from Fisher (Pittsburgh, PA) or other local distributors.

Topical Bioassays. Topical bioassays were performed by application of 1 μl volumes of fipronil or synergists in acetone to first abdominal sternites of adult males (1- to 4-wk old) anesthetized with carbon dioxide (CO_2) or ice. Topical applications were made using a 50 μl 80501 syringe attached to a PB-600-1 dispenser (Hamilton, Reno, NV). The insecticide synergists PBO (100 $\mu\text{g}/\text{insect}$) or DEF (30 $\mu\text{g}/\text{insect}$) were applied 1 h before fipronil application. A minimum of 3-4 fipronil concentrations providing mortality between 10-90% were included for each treatment. Control insects were treated with acetone or synergists alone. Each bioassay consisted of 10 insects per dose and was replicated 3-5 times. Treated cockroaches were held in 100×15 mm plastic petri dishes (Fisher, Pittsburgh, PA) with screened lids, corrugated cardboard, water, and rodent food. The petri dishes were held in environmental chambers where temperature and photoperiod conditions were the same as those used for rearing. Mortality was evaluated every 24 h up to 72 h. Cockroaches lying on their backs and unable to right themselves upon disturbance were scored as dead.

Injection Bioassays. Pretreatment with insecticide synergists (PBO, DEF, etc.) may affect the rate of insecticide penetration (Sun and Johnson 1972, Scott and Georgiou 1986, Bull and Pryor 1990, Sanchez-Arroyo et al. 2001). Such altered penetration rates may lead to "quasi-synergism" or antagonism of insecticide toxicity. To check if PBO or DEF preapplication was causing such an effect on fipronil toxicity, injection bioassays were conducted. Fipronil injections were performed using a 50 μl Hamilton syringe with a 28-gauge beveled edge needle attached to a PB-600-1 dispenser (Hamilton). For injections, the intersegmental membrane between the second and third abdominal sternites was pierced and 1 μl fipronil in acetone was injected into anesthetized adult male cockroaches (Valles et al. 1997). Fipronil doses of 0.05 $\mu\text{g}/\text{insect}$ (for GNV-R) and 0.0016 $\mu\text{g}/\text{insect}$ (for JWax-S) were tested in injection bioassays. The fipronil doses used here displayed highest mortality differences in topical dose-response bioassays with and without synergists. Synergists were applied topically

to first abdominal sternites 1 h before fipronil injections. Controls received acetone or synergists alone. Positive controls included insects treated with synergists followed by topical fipronil treatment with doses mentioned above (0.05 or 0.0016 $\mu\text{g}/\text{insect}$). Each treatment consisted of ten insects and was replicated three times. All other procedures and mortality evaluations were performed as explained above under topical bioassays.

Neurophysiology Equipment. Suction electrode holders (Cat. No. 64–1035) were used to hold recording and reference electrodes (Warner Instruments, Hamden, CT), which were fabricated from ≈ 4 cm lengths of 0.5-mm-diameter gold wire (World Precision Instruments; Sarasota, FL). Recording and reference electrodes were fitted within 1.0-mm borosilicate capillary tubing (World Precision Instruments) pulled to a fine point with a PUL-1 capillary puller (World Precision Instruments). Recording and reference electrodes were connected via a model 4001 capacitance compensation headstage (Dagan Inc., Minneapolis, MN) and a Hum Bug 50/60 Hz Noise Eliminator (Quest Scientific Instruments Inc., North Vancouver, BC, Canada) to a model EX-1 differential amplifier (Dagan). The amplifier was interfaced with computerized digitizing hardware (PowerLab/4SP; ADInstruments, Milford, MA) and software designed to function as an eight-channel chart recorder (Chart version 3.5.7; ADInstruments). Recording chambers consisted of 35 mm tissue culture dishes filled to half capacity with Sylgard silicone elastomer (Dow Corning, Midland, MI). Recording chambers were used for only a single recording and then discarded.

Dissections, Neurophysiological Recordings, and Analyses. Adult male German cockroaches (1- to 4-wk old) were used for all neurophysiological recordings. Dissections and recordings were performed in physiological saline (185 mM sodium chloride, 10 mM potassium chloride, 5 mM calcium chloride, 5 mM magnesium chloride, 5 mM HEPES sodium salt, and 20 mM glucose; pH 7.1) (Bloomquist et al. 1991, Salgado et al. 1998, Scharf and Siegfried 1999). After removal of legs and wings, cockroaches were dissected longitudinally along the dorsal abdomen and thorax. Gut and fat body tissues were removed to expose the ventral nerve cord. The cockroach carcass was pinned open and the abdomen filled with physiological saline. To position the pulled capillary tubing containing the recording electrode in contact with the first abdominal ganglion a micromanipulator (model MNJR; World Precision Instruments) was used. The reference electrode was placed into the physiological saline within the abdomen and activity was observed until a steady, reference-subtracted baseline was achieved.

Spontaneous electrical activity was recorded for 10 min following established protocols (Bloomquist et al. 1991, 1992; Scharf and Siegfried 1999; Durham et al. 2001; Song and Scharf 2008). Recordings of spontaneous baseline electrical activity were achieved by setting a threshold with the “counter” function on the Chart software to obtain ≈ 100 threshold-surpassing

electrical bursts per minute. Baseline recordings continued for 5 min and were then stopped momentarily to allow 5 μl of physiological saline containing 10 μM fipronil to be gently pipetted onto the preparation. Spontaneous electrical activity was then recorded for an additional 5 min. Cockroach preparations in which antennal movement was not detected at the end of a recording were discarded and were not used for statistical analysis. Twenty individual recordings were obtained for both the JWax-S and GNV-R strains. Physiological saline containing 0.4% vol:vol of the solvent carrier DMSO was tested as the experimental control and found to elicit no changes in neurological activity ($n = 5$ per strain).

For each preparation ($n = 20$), average baseline activity was determined for the first 5 min. Next, departures from average baselines were determined per minute during the 5 min fipronil recordings. From these data, an average departure from baseline was determined for each preparation, whereby values >1 indicate neurological excitation and <1 indicate neurological inhibition (Scharf 2008). Average departure data were compared statistically between the JWax-S and GNV-R strains by nonparametric Mann-Whitney U test at the $P < 0.05$ significance level in JMP 8.0. A similar analysis was used to determine the statistical significance of average departure values from the baseline for each strain.

Detection of the A302S Mutation. After each electrophysiology recording, cockroach heads from the JWax-S and GNV-R strains ($n = 20$) were cut off and stored individually in 90% ethanol. These heads were later used individually for genomic DNA extraction using the DNazol Genomic DNA isolation kit (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. A 245-bp genomic region of the *B. germanica* *Rdl* gene encompassing the resistance associated A302S-encoding mutation was polymerase chain reaction (PCR) amplified using the forward (5'-GTGCGGTCCATGGGATACTA-3') and reverse (5'-AACGACGCGAAGACCATAAC-3') primers designed by Hansen et al. (2005). Each 20 μl PCR reaction contained SybrGreen PCR Master Mix (2x SensiMix Sybr and Fluorescein Kit; Quantace, Norwood, MA), *ca.* 50 ng genomic DNA, 10 μM of forward and reverse primers, and nuclease free water. Temperature cycles for PCR amplification were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 64.3°C for 30 s, and 72°C for 30 s followed by a final extension step at 72°C for 10 min. A higher annealing temperature of 64.3°C was used to avoid primer dimer formation. The PCR products were purified using sodium acetate-ethanol precipitation (Sambrook et al. 1989) and sequenced directly at the University of Florida Genomics core facility. The resulting sequences were then compared by BLASTn and BLASTx against the NCBI nr database to detect the presence of nucleotide and amino acid substitutions, respectively. *Rdl* genotypes were scored by comparing the sequence chromatograms (Li et al. 2006).

Data Analyses. Probit analyses of topical dose-mortality data with and without synergists were performed in the SAS software package (SAS Institute, Cary, NC) using PROC PROBIT. If necessary, the dose-mortality data were corrected for control mortality using Abbott's formula (Abbott 1925). As the difference between average strain weight of the JWax-S and GNV-R strains was ≈ 12 mg, lethal dose (LD_{50} and LD_{90}) values were corrected for body weight and expressed as micrograms per gram body weight (Table 1). Resistance ratios were determined by dividing LD values of the GNV-R strain with LD values of JWax-S strain and were considered significant based on nonoverlap of corresponding 95% confidence intervals (CI). Synergism ratios and their 95% CIs were calculated using the procedure given by Robertson and Preisler (1992). Synergism ratios were considered significant if their confidence intervals did not include one ($P < 0.05$). Synergism ratios < 1 indicate synergism of fipronil toxicity and ratios > 1 indicate antagonism. Percent mortality data obtained from the injection bioassays and topical bioassays with and without synergists were arcsine transformed and analyzed nonparametrically by the Kruskal-Wallis test ($P < 0.05$) in JMP 8.0. Logistic regression analysis in JMP 8.0 was used to determine if the continuous independent variable, "average departure of electrical activity from baseline" had significant predictive effect on occurrence of the A302S mutation in the *Rdl* subunit.

Results

Effect of Synergists on Fipronil Toxicity. Topical toxicity of fipronil at 72 h in the presence or absence of PBO or DEF is presented in Table 1. In the absence of any synergist, the fipronil LD_{50} resistance ratio for the GNV-R strain was similar to that reported previously, that is, ≈ 36 –37-fold (Gondhalekar et al. 2011). At the LD_{50} level, preapplication of PBO or DEF reduced the magnitude of fipronil resistance in the GNV-R strain to 18- to 20-fold. Although decreases in fipronil resistance ratios in the GNV-R strain were seen after PBO and DEF pretreatment, the responses of the JWax-S and GNV-R strains to synergist application were completely opposite. Both synergists non-significantly antagonized fipronil toxicity in the JWax-S strain. In contrast, in the GNV-R strain, fipronil toxicity was synergized by both PBO and DEF but only PBO caused significant synergism. Fipronil slope values and LD_{90} synergist ratios also reflected a similar trend of antagonism and synergism in JWax-S and GNV-R, respectively.

The effects of fipronil injections on antagonism/synergism profiles in the JWax-S and GNV-R strains are shown in Fig. 1. In agreement with topical bioassays, PBO and DEF pretreatments caused significant antagonism of fipronil toxicity in JWax-S (Kruskal-Wallis one-way analysis: $\chi^2 = 6.77$, $P = 0.033$ for injection bioassays; $\chi^2 = 6.77$, $P = 0.033$ for topical bioassays) and synergism of fipronil toxicity in GNV-R ($\chi^2 = 7.32$, $P = 0.025$ for injection bioassays; $\chi^2 = 6.94$,

Table 1. Effects of insecticide synergists DEF and PBO on fipronil toxicity to the JWax-S and GNV-R strains (72 h) in topical bioassays

Strain	Treatment	n	χ^2 (df)	Slope	LD_{50} (95% CI) ^a	SR_{50} ^c (95% CI) ^b	RR_{50} ^c	LC_{50} (95% CI) ^a	SR_{90} ^c (95% CI) ^b	RR_{90} ^c
JWax-S	Fipronil	220	5.20 (3)	6.45 (0.85)	0.028 (0.026–0.030)	—	—	0.043 (0.038–0.053)	—	—
	+ DEF ^b	180	3.42 (3)	4.92 (0.72)	0.038 (0.034–0.045)	1.35 (0.99–2.11)	—	0.070 (0.060–0.091)	1.62 (0.91–3.02)	—
GNV-R	+ PBO	180	1.35 (3)	4.93 (0.73)	0.034 (0.030–0.038)	1.21 (1.00–1.68)	—	0.062 (0.051–0.085)	1.44 (0.78–2.56)	—
	Fipronil	290	5.75 (5)	3.7 (0.48)	1.02 (0.85–1.19)	—	36.42*	2.25 (1.78–3.27)	—	52.32*
	+ DEF ^b	240	1.40 (5)	7.45 (1.05)	0.75 (0.68–0.81)	0.73 (0.51–1.05)	19.73*	1.10 (0.98–1.32)	0.48* (0.25–0.96)	15.71*
	+ PBO	330	4.90 (6)	8.86 (1.70)	0.63 (0.57–0.67)	0.65* (0.45–0.94)	18.53*	0.86 (0.79–0.98)	0.38* (0.21–0.78)	13.87*

^a LD_{50} and LD_{90} values with 95% confidence intervals (CI). Values are expressed in micrograms per gram body wt (Strain weights: JWax-S = 47 mg/male, and Gainesville-R = 59 mg/male).

^b Synergist ratios at LD_{50} and LD_{90} : SR_{50} or SR_{90} = LD value with synergist $\div$ LD value without synergist. Confidence intervals (CIs) for synergists were calculated according to the procedure described by Robertson and Preisler (1992). Synergist ratios are considered significant if the 95% CIs did not include 1 ($P < 0.05$).

^c Resistance ratios at LD_{50} and LD_{90} : RR_{50} or RR_{90} = LD value of GNV-R $\div$ LD value of JWax-S. RR_{50} and RR_{90} ratios are considered significant based on nonoverlap of LD_{50} or LD_{90} CIs.

* SR_{50} or SR_{90} and RR_{50} or RR_{90} ratios with asterisks (*) are considered significant (see description in Footnotes ^b and ^c).

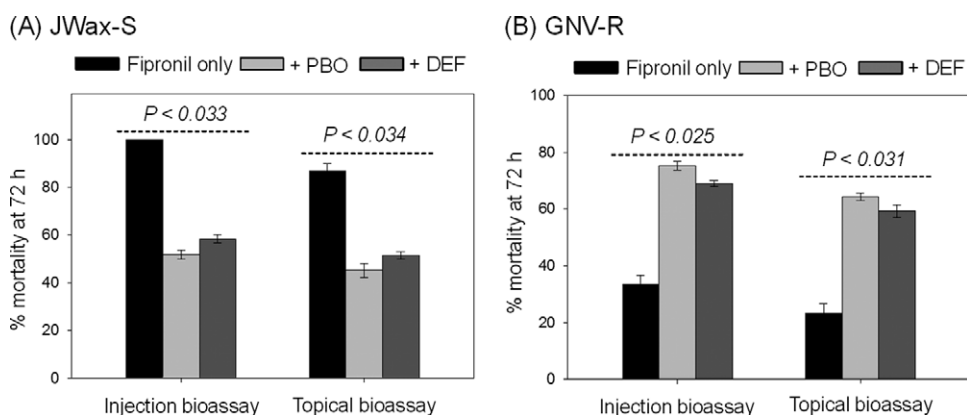


Fig. 1. Effect of bioassay method on synergism profiles in the JWax-S (A) and GNV-R (B) cockroach strains. Vertical bars indicate percent mortality values at 72 h posttreatment. For the JWax-S strain a concentration of 0.0016 $\mu\text{g}/\text{insect}$ was tested whereas a concentration of 0.05 $\mu\text{g}/\text{insect}$ was tested against the GNV-R strain ($n = 30$). The synergists PBO and DEF were topically administered 1 h before fipronil injections or topical applications. $P < 0.05$ indicate significant differences among treatments (Kruskal-Wallis test).

$P = 0.031$ for topical bioassays). The findings of injection bioassays indicate that reduced penetration is not involved as a resistance mechanism and that synergist preapplication does not impact fipronil penetration.

Neurological Impacts of Fipronil. The suction electrode technique was used for recording neurophysiological activity from first abdominal ganglia of adult male German cockroaches ($n = 20$ per strain). Figure 2A depicts an electrophysiological recording of a JWax-S strain individual that displayed the most pronounced neuroexcitatory effect upon application of 10 μM fipronil. The other JWax-S and GNV-R strain recordings did not produce such visually evident neuroexcitatory effects. Overall the average departure of electrical activity from baseline levels was higher in the JWax-S strain (Mann-Whitney U test: $Z = -4.62$; $P < 0.0001$), but in the GNV-R strain it was similar to baseline activity levels ($Z = -0.56$, $P = 0.57$; Fig. 2B). Additionally, comparison of average departure values

for the susceptible and resistant strains using a non-parametric Mann-Whitney U test revealed that the mean responses of the two strains were significantly different ($Z = -3.69$, $P = 0.002$; Fig. 2B). These results are consistent with the hypothesis that target site modifications are responsible for the lack of fipronil-induced neuroexcitation in the GNV-R nervous system.

Detection of the A302S Mutation and Its Relationship With Nervous System Insensitivity to Fipronil. A 245-bp region of the *Rdl* gene of the GABA receptor (corresponding to exon seven of *D. melanogaster*) was PCR amplified and sequenced. BLASTn/ BLASTx analysis and alignment of the wildtype *Rdl* cDNA sequence of *B. germanica* (GenBank S76552) with the *Rdl* DNA sequences of the JWax-S and GNV-R strains revealed that the known A302S-encoding mutation was present only in GNV-R (alignments not shown). The A302S amino acid substitution resulted from a single G to T nucleotide polymorphism and no other

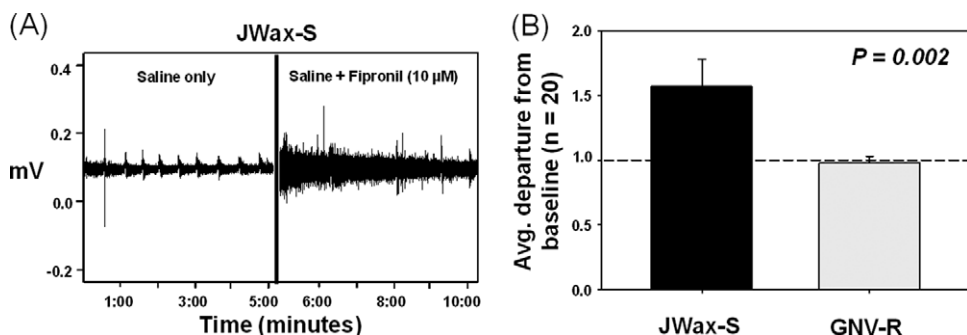


Fig. 2. Summary of findings from electrophysiology studies. (A) Representative neurophysiological recording showing effects of 10 μM fipronil on spontaneous nervous/electrical activity of the susceptible (JWax-S) strain. Solid vertical black line indicates the time point (5 min) when recordings were stopped momentarily and 10 μM fipronil was applied to nerve preparations. (B) The average departure of electrical activity from baseline levels after fipronil application in the JWax-S and GNV-R strains. The baseline (1.0) is indicated by the dashed horizontal line. The responses of two strains were compared using a nonparametric Mann-Whitney U test ($Z = -3.69$; $P = 0.002$).

Table 2. *Rdl* genotypes in fipronil susceptible (JWax-S) and resistant (GNV-R) strains of the German cockroach

Strain	n	No. genotypes			Frequency of resistant (S302) allele (%) ^a
		A302/A302 (S/S)	A302/S302 (S/R)	S302/S302 (R/R)	
JWax-S	20	20	0	0	0
GNV-R	20	1	19	0	48

^a Twenty insects were used for genotyping, thus, the no. of alleles sampled for each strain is 40.

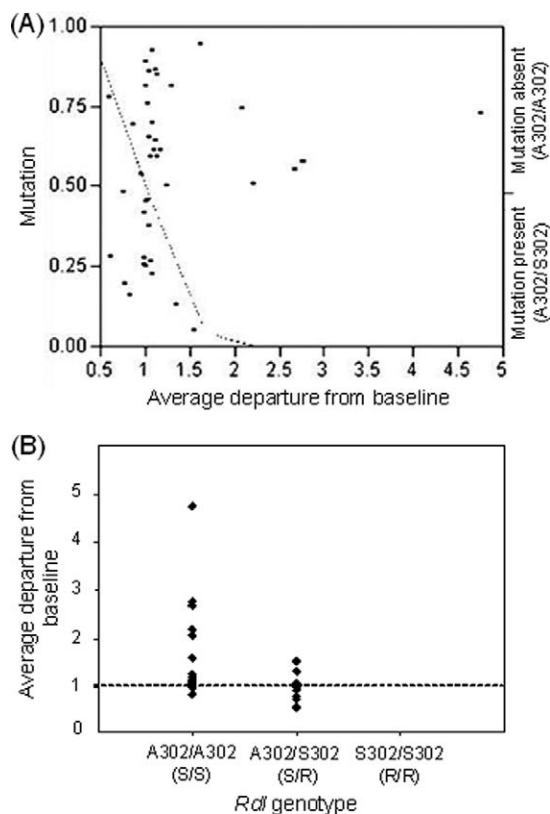


Fig. 3. Correlation between the alanine to serine (A302S) *Rdl* mutation and insensitivity of the cockroach nervous system to fipronil. (A) Logistic plot of average departure of electrical activity from baselines determined in neurophysiological recordings (X-axis) versus *Rdl* mutation frequency (Y-axis). The binary (miss/hit) data for the *Rdl* mutation and the corresponding values for the independent variable, that is, average departure of electrical activity from baselines for both strains ($n = 40$) were analyzed using logistic regression. Higher values of the independent variable are associated with the absence of the mutation; whereas, lower values are associated with presence of the *Rdl* mutation ($\chi^2 = 4.10$; $df = 1$; $P = 0.04$). (B) Scatter plot of average departure of electrical activity from baseline (Y-axis) by *Rdl* genotypes of sampled insects from the JWax-S and GNV-R strains (X-axis) ($n = 20$ insects per strain). The dashed horizontal line indicates baseline electrical activity. Data points for average departures that fall in the A302/A302 (susceptible homozygote) category correspond to all JWax-S strain individuals and one GNV-R strain insect ($n = 21$); whereas, all data points in the heterozygote category (A302/S302) correspond to the GNV-R strain ($n = 19$). No resistant *Rdl* homozygotes (S302/S302) were identified.

polymorphisms were detected within this 245-bp region. In the GNV-R strain, the A302S mutation was found in 95% (19 of 20) of the sampled insects and the frequency of the resistant allele (S302) was 48% (Table 2). GNV-R strain insects homozygous for the resistant allele were not detected in the current study.

Because DNA isolated from the heads of the same individual insects used in electrophysiology experiments was used for determining frequency of the A302S mutation, we were able to perform a logistic regression of the presence or absence of the mutation (binary data) versus neurological responses to fipronil (Fig. 3A). The logistic fit indicated that the independent variable, that is, *average departure of electrical activity from baseline* had a significant predictive effect on the occurrence of the A302S mutation ($\chi^2 = 4.04$; $df = 1$; $P = 0.04$). In addition, a scatter plot of *Rdl* genotype (A302/A302, A302/S302, and S302/S302) versus average departure data from neurophysiology experiments (Fig. 3B) illustrates that higher average departure values were associated with susceptible homozygotes (A302/A302) and lower values were associated with heterozygotes (A302/S302). Lower average departure values are indicative of reduced sensitivity of the nervous system to fipronil and thus the presence of A302S mutation or S302 allele renders the nervous system of the heterozygotes less sensitive to fipronil.

Discussion

Role of Metabolic Mechanisms in Fipronil Resistance. Topical bioassay data presented here showed that fipronil toxicity is synergized by PBO in the GNV-R strain and antagonized in the JWax-S strain. Antagonism of fipronil toxicity in JWax-S is consistent with previous observations on two susceptible and seven resistant strains of the German cockroach (Scott and Wen 1997, Valles et al. 1997). However, synergism of fipronil toxicity as observed in the GNV-R strain has not been previously reported in the German cockroach. Because oxidative conversion of fipronil to its bioactive sulfone metabolite is the major metabolic conversion pathway of fipronil in insects (Scharf et al. 2000, Durham et al. 2002), the synergistic effect of PBO on fipronil toxicity in the GNV-R strain was unexpected. Although metabolic biotransformation pathways of fipronil in the GNV-R strain remain to be elucidated these results indicate that metabolic detoxification of fipronil is likely occurring in GNV-R. In the house fly, *Musca domestica* L. and the tropical bed bug *Cimex hemipterus* F., PBO and/or DEF were also found to significantly synergize fipronil toxicity (Cole

et al. 1993, Scott and Wen 1997, Wen and Scott 1999, How and Lee 2011). Additional studies with the LPR strain of *M. domestica* further revealed reduced penetration and P450-mediated detoxification as the major mechanisms of 15-fold fipronil cross-resistance (Wen and Scott 1999).

The chemical structure of fipronil does not possess any ester linkages on which insect esterase enzymes are known to act (Oppenoorth 1985). Despite this, the stereotypical esterase inhibitor DEF caused antagonism and synergism of fipronil toxicity in the JWax-S and GNV-R strains, respectively. In vitro inhibition studies conducted in German cockroaches have demonstrated that DEF can inhibit cytochrome P450s at micro molar concentrations (Valles et al. 1997). Hence, the antagonistic and synergistic effects of DEF on fipronil noted here may have been caused by non-specific inhibition of P450s involved in fipronil activation or detoxification. In this regard, DEF could be competitively inhibiting P450s that act on the thioether group of fipronil to potentially limit sulfone formation.

In general, low magnitudes of fipronil antagonism/synergism ratios at the LD₅₀ level (1.2- to 1.5-fold) were obtained in topical bioassays. The low level of antagonism observed in the JWax-S strain was expected because fipronil and its sulfone metabolite are known to be equally toxic (Hainzl et al., 1998, Scharf and Siegfried 1999) and thus, inhibition of sulfone production may or may not result in large differences in toxicity. Although a lower degree of fipronil synergism was observed at the LD₅₀ level in the GNV-R strain, higher synergism ratios at LD₉₀ (2- to 3-fold) and higher slope values in treatments receiving synergist preapplication clearly support that significant fipronil synergism and detoxification are occurring in the GNV-R strain. Moreover, injection of fipronil did not change the observed synergism profiles in the susceptible and resistant strains, indicating that synergism of fipronil toxicity observed in GNV-R is indeed true-synergism and not "quasi-synergism" caused by altered penetration of fipronil because of synergist pretreatment (Sun and Johnson 1972).

Treatment with PBO and DEF reduced fipronil resistance ratios in the GNV-R strain from 36-fold to $\approx$ 18- to 20-fold at the LD₅₀ level and this decrease was even more pronounced at LD₉₀. However, these synergist-induced changes in resistance levels should be interpreted cautiously because to a certain degree they were caused by differential synergism profiles in the JWax-S and GNV-R strains. Nonetheless, synergist preapplication did reduce resistance levels in the GNV-R strain; thus, our results support the idea that enhanced detoxification is responsible for at least low-level fipronil resistance in the GNV-R strain.

Correlation Between the A302S Mutation and Target-Site Insensitivity to Fipronil. Electrophysiological studies provided definitive evidence that the GNV-R strain nervous system is insensitive to fipronil. In contrast, rapid neuroexcitation after fipronil application was observed in the susceptible JWax-S strain, which corresponds with previous findings and the estab-

lished mode of action of fipronil (Cole et al. 1993, Gant et al. 1998, Scharf and Siegfried 1999, Zhao et al. 2003). As shown previously in *Drosophila* spp. (Cole et al. 1995, Hosie et al. 2001, Le Goff et al. 2005), we hypothesized that the insensitivity of the GNV-R nervous system to fipronil could be caused by the dieldrin resistance-associated A302S substitution in the *Rdl* subunit of the GABA receptor. In strong agreement with our neurophysiological findings, the resistance associated allele (S302) was found at a frequency of 48% (95% resistant heterozygotes) in the GNV-R strain while it was undetectable in the JWax-S strain. Logistic regression analysis also provided significant statistical support for the association between the A302S mutation and target-site insensitivity to fipronil in the GNV-R strain. Although binding sites and mechanisms of action of fipronil and dieldrin on insect GABA receptors appear to be different (Deng et al. 1993, Gant et al. 1998, Zhao et al. 2003), heterologously expressed A302S or A301G mutants were less sensitive to fipronil than wild-type receptors (Cole et al. 1995, Hosie et al. 2001, Le Goff et al. 2005). Similarly, whole-cell patch clamp recordings with dieldrin resistant German cockroaches revealed that GABA receptors of resistant cockroaches were 15-fold less sensitive to fipronil sulfone (Zhao and Salgado 2010). In insects, apart from GABA-gated chloride channels, fipronil and its sulfone metabolite are also known to act on glutamate-gated chloride channels (GluCl) in the central nervous system (Zhao et al. 2005). However, a recent study by Zhao and Salgado (2010) showed that GluCl may not be an important target of fipronil and fipronil sulfone in the German cockroach. Thus, based on our current understanding about interaction between GluCl and fipronil in the German cockroach, it does not appear that insensitive GluCl channels play a role in fipronil resistance in the GNV-R strain.

Evidence for Multiple Mechanisms of Fipronil Resistance in the GNV-R Strain. Insecticide resistance-associated target site mutation(s) can interact either synergistically or additively with metabolic resistance factors (Raymond et al. 1989, Hardstone and Scott, 2010). In the current study, the GNV-R strain with an S302 allele frequency of 48%, displayed 36-fold fipronil resistance. Interestingly, however, German cockroach strains from Denmark that possessed a higher frequency (97%) of the resistant allele (S302) were only 15-fold cross-resistant to fipronil (Hansen et al. 2005, Kristensen et al. 2005). These differences suggest strongly that additional factors other than the *Rdl* mutation are responsible for high-level fipronil resistance in the GNV-R strain.

Fipronil cross-resistance in United States German cockroach strains from the prefipronil era was not affected by synergists (Valles et al. 1997, Scott and Wen, 1997) pointing toward the involvement of the *Rdl* locus in resistance. In contrast, in the GNV-R strain, resistance to fipronil decreased because of apparent inhibition of cytochrome P450s, which to our knowledge is the first report of fipronil synergism in the German cockroach. Previously, cytochrome P450s

have been implicated in fipronil resistance in *M. domestica*, *C. hemipterus*, and the rice stem borer, *Chilo suppressalis* (Walker) (Wen and Scott 1999, Li et al. 2007, How and Lee 2011).

The GNV-R strain, which was collected 8 yr after wide-spread use of fipronil began in the United States, had likely undergone fipronil selection in the field. Direct selection by fipronil or other insecticides in the field may have further selected the GNV-R strain for resistance mechanisms in addition to *Rdl*. For example, the laboratory-selected Eyguieres 42 strain of *D. simulans* showed 20,000-fold fipronil resistance, which was associated with A301G and T350M point mutations in the *Rdl* subunit and an over expressed "D1" glutathione-S-transferase (Le Goff et al. 2005 and references cited therein). In addition, a lab-selected strain of the diamond back moth, *Plutella xylostella*, displayed 300-fold fipronil resistance that was only partially associated with the A302S mutation, and it was hypothesized that other mutations in the *Rdl* GABA receptor subunit might be contributing to fipronil resistance (Li et al. 2006). Although the possibility of occurrence of the T350M mutation in GNV-R cannot be ruled out, it is unlikely that this mutation is present in the GNV-R strain because the observed fipronil resistance in GNV-R strain appears to be associated with the *Rdl* mutation and cytochrome P450s. Moreover, the T350M mutation has not been reported in any other insect species and it is not known if this mutation can be selected for under field conditions (Le Goff et al. 2005).

In conclusion, from the evidence obtained in the current study, high-level fipronil resistance in the GNV-R strain is likely caused by the combined effects of target-site modification (A302S mutation in GABA receptor *Rdl* subunit) and enhanced metabolism by cytochrome P450 monooxygenases. Because PBO pretreatment reduced fipronil resistance in GNV-R from 36-fold to 18-fold at the LD₅₀ level, enhanced fipronil detoxification by P450s appears to account only for about half of observed fipronil resistance in the GNV-R strain. The remaining 50% of resistance is likely caused by the *Rdl* mutation. Additional studies to elucidate metabolic pathways for fipronil, to determine the interaction among fipronil resistance loci, and to understand the genetics of fipronil resistance in the GNV-R strain are now warranted.

Finally, it should be noted that, despite displaying high-level resistance to topically applied fipronil, the GNV-R strain only displays low-level tolerance (2- to 3-fold) to an older bait formulation containing 0.01% fipronil (Maxforce FC Select Roach Killer Bait Gel) (Gondhalekar et al. 2011). This relative susceptibility of the GNV-R strain to fipronil gel baits is attributable to: 1) a high concentration of active ingredient in gel baits, and 2) a highly palatable bait matrix that causes repeated toxicant ingestion (Valles and Brenner 1999, Holbrook et al. 2003, Wang et al. 2004, Gondhalekar et al. 2011). Moreover, it is also expected that the GNV-R strain would essentially appear susceptible to newer bait formulations that contain a higher concentration (0.05%) of fipronil (e.g., Maxforce FC Magnum Roach

Killer Bait Gel). Thus, despite the presence of resistance mechanisms in field populations, the combination of high fipronil concentrations and bait palatability would appear to ensure ingestion of toxicant in amounts sufficient to overwhelm resistance mechanisms. However, with increasing gel bait active ingredient concentrations, the potential for selection of high-level resistance also increases (Scharf et al. 1998). Thus, once resistance mechanisms are detectable as seen in the current study, "high dose" resistance management strategies are only likely to provide short-term solutions.

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