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Combined metabolic and target-site resistance mechanisms confer fipronil and deltamethrin resistance in field-collected German cockroaches (Blattodea: Ectobiidae)

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ABSTRACT

Despite insecticide resistance issues, pyrethroids and fipronil have continued to be used extensively to control the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae) for more than two decades. We evaluated the physiological insecticide resistance in five German cockroach populations collected from 2018 to 2020 and measured the extent of metabolic detoxification and target-site insensitivity resistance mechanisms. Topically applied doses of the 3 x LD_{95} of deltamethrin, fipronil, DDT, or dieldrin of a susceptible strain (UCR, Diagnostic Dose) failed to cause >23% mortality, and the 10 x LD_{95} of deltamethrin or fipronil failed to cause >53% mortality. All field-collected strains possessed a combination of metabolic and target-site insensitivity mechanisms that cause reduced susceptibility. Elevated activities of esterase and glutathione S-transferase were measured, and the synergists piperonyl butoxide or *S,S,S*-tributyl phosphorotrithioate increased topical mortality up to 100% for deltamethrin and 93% for fipronil 10 x LD_{95}. The target-site mutations L993F of the *para*-homologous sodium channel and A302S of the GABA-gated chloride channel associated with pyrethroid and fipronil resistance, respectively, were found at ~80–100% frequency in field populations. Pyrethroid and fipronil spray formulations also were ineffective in a choice box assay against field-collected strains suggesting that these treatments would fail to control cockroaches under field conditions.

1. Introduction

The German cockroach (*Blattella germanica* [L.] [Blattodea: Ectobiidae]) is one of the most important urban insect pests worldwide (Wang et al., 2021). Insecticide application is the primary method to manage this species (Lee and Rust, 2021), often leading to widespread insecticide resistance towards many compounds used in its control (Scharf and Gondhalekar, 2021). Pyrethroids and synergized pyrethrin, commonly used in residual sprays, aerosols, and fogs, have been frequently documented as ineffective due to resistance development (Chai and Lee, 2010; Hu et al., 2020; Lee and Rust, 2021; Lee et al., 2022). The phenylpyrazole fipronil has been incorporated in baits, but spray formulations are recently available. Although severe resistance towards this compound has not been widely documented, a recent study reported that fipronil was ineffective as a bait against all German cockroach populations collected from the field (Lee et al., 2022).

In theory, resistance can be overcome by a systematic combination of multiple management strategies. However, this is often unrealistic in areas where German cockroach infestations are prevalent, such as low-income housing where minimal time, funding, and resources are allocated (Miller and Smith, 2020; Miller et al., 2021). Thus, reliance on one or a few commonly available, off-patent insecticides with previous documentation of resistance such as pyrethroids or fipronil remains a standard management approach. A current understanding of the resistance levels and mechanisms is necessary to consider resistance management options and predict future resistance development.

A main physiological mechanism associated with insecticide resistance is the activity of cytosolic and microsomal enzymes such as cytochrome P450 monooxygenases (P450), esterases, and glutathione S-transferases (GST) that are known to detoxify xenobiotics. Measurable increases in activity or concentration of these enzymes are associated with resistant German cockroach populations (Anspaugh et al., 1994; Scharf et al., 1997a, 1997b; Valles, 1998; Lee et al., 2000; Limoee et al., 2007), and synergism of insecticides using enzymatic inhibitors can be

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used to confirm the detoxification of specific insecticides. In particular, piperonyl butoxide (PBO) and *S,S,S*-tributyl phosphorotrithioate (DEF) have been shown to synergize pyrethroids (Bull and Patterson, 1993; Valles, 1998; Wei et al., 2001; Limoee et al., 2007; DeVries et al., 2019; Hu et al., 2020). The detoxification of fipronil is less studied, with PBO or DEF causing synergistic, antagonistic, or zero effect across different populations (Valles et al., 1997; Gondhalekar and Scharf, 2012; Ang et al., 2013).

Additionally, several target site mutations in the German cockroach maintain normal channel function in the presence of insecticides, resulting in decreased susceptibility (Scharf and Gondhalekar, 2021). Resistance towards pyrethroids, which are sodium channel modulators, is associated with several mutations of the *para*-homologous sodium channel, known as knockdown resistance, *kdr,* or *kdr*-type mutations. A nucleotide substitution on the 6th segment of domain II (G^{2979} to C) that results in a lysine to phenylalanine amino acid substitution (L993F) has been demonstrated to decrease susceptibility towards pyrethroids (Dong, 1997; Valles et al., 2003) and confer cross-resistance towards dichlorodiphenyltrichloroethane (DDT) (Scott and Matsumura, 1981). Two other mutations, T^{2290} to C (C764R) and G^{1300} to A (E434K) can reduce sodium channel sensitivity to deltamethrin *in vitro* when L993F is simultaneously present (Tan et al., 2002) and have been found at low frequencies in field populations of German cockroach (Liu et al., 2000; DeVries et al., 2019). Resistance towards fipronil is conferred by an alanine to serine substitution (A302S) on the *Rdl* subunit of the γ-aminobutyric acid (GABA)-gated chloride channel (Gondhalekar and Scharf, 2012; Ang et al., 2013). The *Rdl* mutation, named after the phrase "resistance-to-dieldrin," also confers resistance to cyclodienes due to their similar target on the GABA receptor (Hansen et al., 2005).

Lee et al. (2022) found significant contact resistance towards deltamethrin and fipronil in all German cockroach strains collected from residential sites between 2018 and 2020. The current study evaluates deltamethrin and fipronil resistance in these strains using a topical diagnostic dose method and a choice bioassay with residual products to estimate treatment efficacy and determine if repellency contributes towards reduced susceptibility. To examine the roles of detoxification and target-site resistance, *in vivo* and *in vitro* methods were used. The average activities of the esterase and GST enzymatic families were measured using biochemical assays with cockroach homogenates. Live cockroaches were treated with P450, esterase, and GST inhibitors and insecticides to observe any synergistic effects. Resistance towards DDT and dieldrin was evaluated with the diagnostic dose method due to their association with *kdr* and *Rdl* target-site mutations, respectively. The frequency of mutations was determined for every strain by sequencing each region of interest. We found evidence of multiple resistance mechanisms for deltamethrin and fipronil in all strains.

2. Methods

2.1. Cockroach populations

The German cockroach strains WM, RG386, Ryan, CDR, and SY were collected from the field from 2018 to 2020 (Lee et al., 2022) and maintained in the laboratory under ambient conditions (24 ± 2 °C, 30–50% RH, and 12:12 h L:D) with dog food (Purina Dog Chow, Nestlé Purina Petcare, St. Louis, MO), cardboard harborages, and water provided *ad libitum*. A susceptible strain, UCR, was included in this study to compare resistance in field-collected strains. The UCR strain was established from the Orlando normal strain over 40 yrs. ago and has never been exposed to insecticides.

2.2. Insecticides

The following compounds were used in insecticide resistance and synergism assays: deltamethrin (≥ 98%), fipronil (≥ 95%), DDT (≥ 98%), dieldrin (≥ 95%), diethyl maleate (DEM) (97%), piperonyl butoxide (PBO) (90%) (Sigma Aldrich Corporation, St. Louis, MO), and *S,S,S*-tributyl phosphorotrithioate (DEF) (100%, Chem Service Inc., West Chester, PA).

2.3. Topical assay

Field-collected cockroach strains were assessed for resistance towards deltamethrin, fipronil, DDT and dieldrin using the diagnostic dose (DD) method described in Lee et al. (2022). A range of serial dilutions of DDT and dieldrin in acetone causing ~10–90% mortality in the UCR strain was topically applied in 0.5 μl quantities to the abdominal sternites of adult males (anesthetized with CO_2) using a microapplicator (Burkard Manufacturing Co Ltd., Rickmansworth, England). Treated cockroaches were provided with dog food and water, and mortality was scored at 72 h. The data were used to generate the lethal dose to kill 95% of the cockroaches at 72 h (LD_{95}) with the susceptible UCR strain.

Ten adult males of each field-collected strain were anesthetized with CO_2, and a DD (3 x LD_{95} of deltamethrin, fipronil, DDT, or dieldrin) was applied to the abdominal sternites using a microapplicator. Treated individuals were provided with food and water, and mortality was recorded at 72 h. Controls were treated with acetone only. Each dose was replicated three times per field-collected strain. Strains that had >10% survival from the DD of deltamethrin or fipronil were considered resistant and were treated with the 10 x LD_{95} to estimate the extent of resistance.

2.4. Choice box assays

Insecticide sprays were applied to rectangular panels of unpainted plywood (30.8 by 15.2 by 0.8 cm) with an airbrush (Master Hi-Flow All-Purpose, TCP Global, San Diego, CA). The treatments were applied according to label directions. The panels were sprayed with 3 ml of 0.0038% fipronil (2.4 mg/m^2) (Fipronil-Plus-C, Arizona Chemical Group, LLC, Meza, AZ) and 0.03% deltamethrin (19.2 mg/ m^2) (Suspend® Polyzone, Bayer Environmental Science, Research Triangle Park, NC). The treated panels were allowed to dry for 24 h before being tested.

The 1-day-old residual deposits were tested in Ebeling choice boxes to determine their repellency and potential field efficacy (Ebeling et al., 1966). The boxes were constructed from white pine drawer siding (30.5 by 9.5 cm) with a tempered Masonite floor. The box was divided into two equal compartments (light and dark) with a panel. A hole in the top center allowed the cockroaches to move between the light and dark compartments. Both compartments were covered with a piece of plexiglass. The treated dark compartment was covered with a piece of Masonite.

Twenty adult male cockroaches were confined in the light compartment for 5–6 h before being allowed access to the dark compartment that contains the treated surfaces. Three replicates were set up for each treatment and the untreated controls. The choice boxes were placed in a room on a photoperiod of 12:12 h (L:D). The number of dead and live cockroaches in the light and dark compartment was counted for 14 d. The location of the live cockroaches (dark or light compartment) was recorded.

The performance index (PI) combines the effects of mortality and repellency in the choice box and estimates potential field performance (Rust and Reierson, 1978). The PI is calculated as follows:

$$PI = 1 - \left(\frac{Number\ alive + Number\ alive\ in\ light\ side}{Number\ dead + Initial\ total\ number}\right) \times 100$$

Complete repellency and no mortality provide a PI of −100. Complete mortality and no repellency provide a PI of +100. No mortality and no repellency provide a PI of 0.

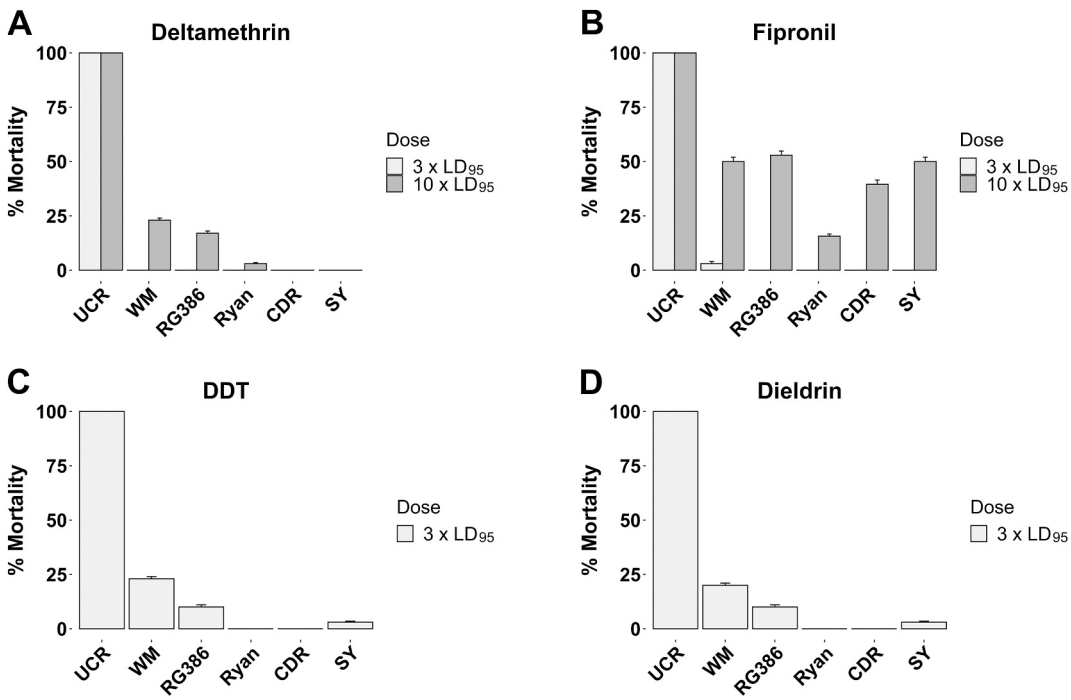

Fig. 1. Mortality of cockroach strains 72 h after treatment with topically applied (A) deltamethrin, (B) fipronil, (C) DDT, and (D) dieldrin.

Table 1
The topical doses used against *B. germanica* in this study.

Insecticide	3 x LD_{95} (μg/insect)	10 x LD_{95} (μg/insect)	Source
deltamethrin	0.034	0.113	Lee et al. (2022)
fipronil	0.011	0.036	Lee et al. (2022)
DDT	55.056	–	Supplementary Table S1
dieldrin	0.624	–	Supplementary Table S1

2.5. Synergism of insecticides

The synergists were diluted separately in acetone and treated at a rate of 100 μg (PBO), 30 μg (DEF), and 100 μg (DEM) per 0.5 μl. The synergist doses were previously reported to be sublethal and/or tested on the UCR susceptible strain to confirm minimal mortality (Valles et al., 1997; Chai and Lee, 2010; Hu et al., 2020). Adult male cockroaches were anesthetized with CO_2, and one synergist was applied to the abdominal sternites with a microapplicator. A second application of the 3 x LD_{95} or 10 x LD_{95} of deltamethrin or fipronil was applied using the same procedure an hour later. Treated cockroaches were provided food and water, and total mortality was recorded at 72 h. Controls were treated with acetone. The experiment was replicated three times.

Table 2
Primers used to amplify regions containing target-site mutations for *kdr* and *Rdl*.

Category	Mutation	Forward 5′-3′	Reverse 5′-3′	Amplicon Size
	L993F	ATGATTGTGTTCCGAGTGTTG	TCCCTGACCAACCTGTGAAA	230
kdr	C764R	AAAGCATTGCACAGTCTCCA	AAGGAGGGCGACGTATTCTT	166
	E434K	GCCCTGGCATATGCTGTT	TGAGAAGAACTGCGTAATGATCT	255
Rdl	A302S	GTGCGGTCCATGGGATACTA	AACGACGCGAAGACCATAAC	245

Table 3
Mean activity levels of esterases and glutathione S-transferase (GST) in field strains of the German cockroach.

Strain	Mean activities ± SE							
	n	1-NA esterase[a]	n	2-NA esterase[b]	n	PNPA esterase[c]	n	GST[d]
UCR	95	91.88 ± 3.67	95	109.16 ± 3.39	76	519.10 ± 15.56	96	0.37 ± 0.02
WM	96	118.6 ± 4.62*	96	142.60 ± 4.47*	48	633.60 ± 17.30	96	1.18 ± 0.06*
RG386	64	219.21 ± 11.60*	64	349.50 ± 19.93*	84	1494.31 ± 84.53*	64	1.74 ± 0.14*
Ryan	64	236.8 ± 7.06*	64	258.9 ± 7.99*	56	688.0 ± 26.97*	64	2.04 ± 0.10*
CDR	56	206.52 ± 7.54*	56	205.3 ± 7.56*	64	916.0 ± 40.63*	55	1.02 ± 0.07*
SY	50	160.2 ± 6.24*	50	183.64 ± 5.19*	50	800.9 ± 23.35*	50	0.53 ± 0.03

[a] 1-naphthol (ng/min/mg protein) ± SE.
[b] 2-naphthol (ng/min/mg protein) ± SE.
[c] *p*-nitrophenyl (nmol/min/mg protein) ± SE.
[d] GST conjugate (mmol/min/mg protein) ± SE.
* Indicates significant difference from the UCR strain (Dunnett's test; α = 0.01).

Table 4
Distribution of *kdr* and *Rdl* mutations in susceptible and field strains of the German cockroach.

Strain	n	*kdr*			*Rdl*		
		L993F			A302S		
		R/R[a]	R/S[b]	S/S[c]	R/R	R/S	S/S
WM	15	9	5	1	14	1	0
RG386	15	6	8	1	15	0	0
Ryan	15	13	2	0	15	0	0
CDR	15	9	5	1	15	0	0
SY	15	11	4	0	15	0	0
UCR	8	0	0	8	0	0	8

[a] R/R: number of individuals homozygous for point mutation.
[b] R/S: number of individuals heterozygous for point mutation.
[c] S/S: number of individuals with susceptible genotype.

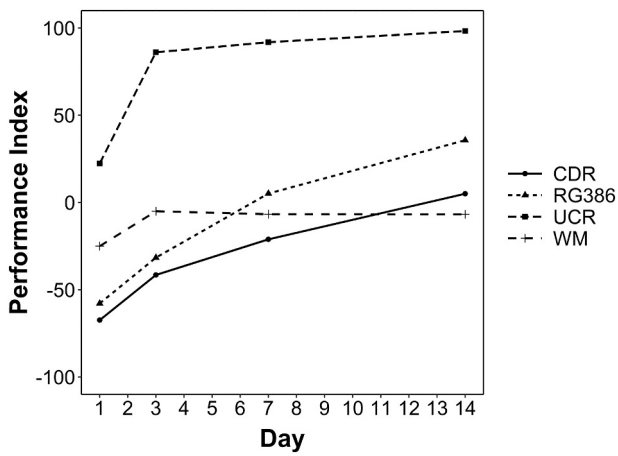

Fig. 2. Performance of deltamethrin sprays against UCR, WM, RG386, and CDR strains of the German cockroach.

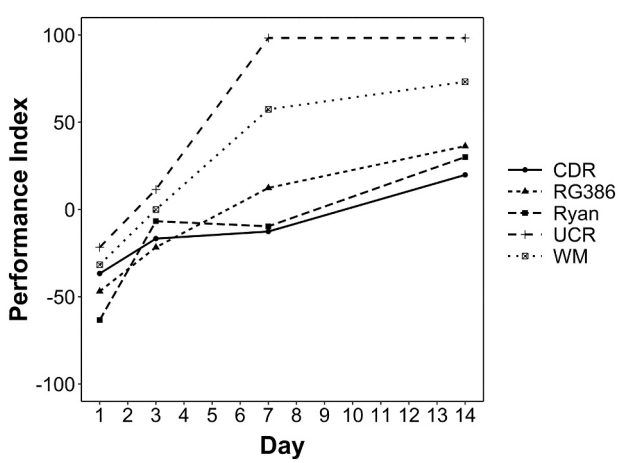

Fig. 3. Performance of fipronil sprays against UCR, WM, RG386, Ryan, and CDR strains of the German cockroach.

2.6. Measurement of metabolic detoxification enzymes

2.6.1. Materials

All biochemical methods are modifications of the World Health Organization insecticide resistance mechanism detection manual (Hemingway, 1998). The following materials were used in biochemical assays: 1-naphthyl acetate (≥ 98%, Sigma Aldrich Corporation, St. Louis, MO), 2-naphthyl acetate (≥ 98%, Sigma Aldrich Corporation, St. Louis, MO), 1-naphthol (≥ 99%, Spectrum Chemical Mfg. Corp., Gardena, CA), 2-naphthol (≥ 99%, Spectrum Chemical Mfg. Corp., Gardena, CA), fast blue B salt (MP Biomedicals, LLC, Irvine CA), sodium lauryl sulfate (≥ 99% MP Biomedicals, LLC, Irvine CA), *p*-nitrophenyl acetate (≥ 98%, Sigma Aldrich Corporation, St. Louis, MO), 1-chloro-2,4-dinitrobenzene (CDNB) (99%, Acros Organics, Carlsbad, CA), and reduced glutathione (GSH) (99%, Chem-Impex International, Inc., Wood Dale, IL).

2.6.2. Homogenization

Adult male cockroaches were randomly selected and individually homogenized or stored at −80 °C before homogenization. Individuals without heads were homogenized in 500 μl of distilled water and spun at 10000 *g* for 1 min. The supernatant was diluted five times with distilled water before being spun again at 10000 *g* for 2 min. The final homogenate was aliquoted and immediately used in each assay or stored at −80 °C.

2.6.3. Naphthyl acetate esterase assay

Two replicates of homogenate (20 μl) per sample were added to separate microplate wells. In one replicate, 200 μl of 1-naphthyl acetate working solution (0.2 ml 0.03 M 1-naphthyl acetate in acetone +19.8 ml 0.02 M sodium phosphate buffer pH 7.2) was added. In the other, 200 μl of 2-naphthyl acetate working solution (0.2 ml 0.03 M 2-naphthyl acetate in acetone +19.8 ml 0.02 M sodium phosphate buffer pH 7.2) was added. Both were incubated at room temperature for 5 min. After incubation, 50 μl of Fast Blue B solution [0.06 g Fast Blue B salt (tetrazotized *o*-dianisidine) in 6 ml distilled water +14 ml 5% sodium lauryl sulfate] was added to each replicate. The plate was incubated at room temperature for 5 min. Controls were prepared with distilled water instead of homogenate. The products were read at an endpoint of 570 nm using an Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT). The activity was calculated based on the standard curves of 1-naphthol and 2-naphthol.

2.6.4. Esterase assay – PNPA rate reaction

Homogenates (10 μl per sample) were added into separate microplate wells. Each well received 200 μl of *p*-nitrophenyl acetate (PNPA) working solution (300 μl 0.1 M PNPA in acetonitrile +29.7 ml 0.05 M sodium phosphate buffer pH 7.4). Controls were prepared with distilled water instead of homogenate. The plate was read at 405 nm for 2 min, and activity was calculated after converting with Beer's Law ($A = \varepsilon lc$) using an extinction coefficient of 6.53 μM^{-1} and pathlength of 0.6 cm.

2.6.5. Glutathione S-transferase assay

Homogenates (2 μl per sample) were added to separate microplate wells. Then, 8 μl of distilled water was added to dilute, followed by 200 μl of 1-chloro-2,4-dinitrobenzene (CDNB)/reduced glutathione (GSH) working solution (48.6 μg of GSH in 15 ml 0.1 M sodium phosphate buffer pH 6.5 + 750 μl of 0.063 M CDNB in methanol). Controls were prepared with distilled water instead of homogenate. The plate was read at 340 nm for 5 min, and activity was calculated with Beer's Law ($A = \varepsilon lc$) using an extinction coefficient of 4.39 mM^{-1} and pathlength of 0.6 cm.

2.6.6. Protein assay

Protein concentration was measured for each sample to correct for variations in cockroach size. Homogenates (10 μl per sample) were added to separate microplate wells, followed by 200 μl of Bio-Rad protein dye reagent. After 5 min of incubation at room temperature, the plate was read at 570 nm. Protein concentration was calculated based on the standard curve generated with bovine serum albumin provided with the Bio-Rad kit.

2.7. Molecular detection of target-site mutations

Adult male individuals from each strain were collected and

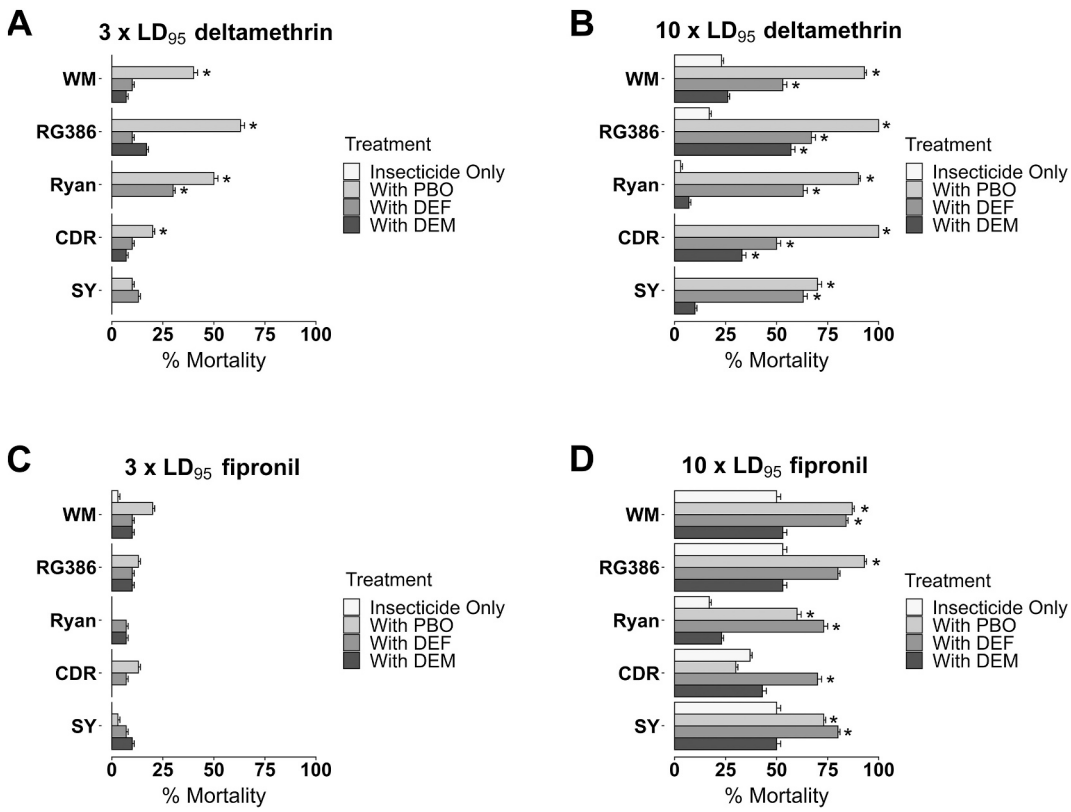

Fig. 4. Effect of synergists PBO, DEF, and DEM on (A) 3 x LD_{95} deltamethrin, (B) 10 x LD_{95} deltamethrin, (C) 3 x LD_{95} fipronil, and (D) 10 x LD_{95} fipronil toxicity at 72 h. Asterisks indicate a significant difference in mortality compared to insecticide only (Mantel-Haenszel test; $\alpha = 0.05$).

immediately used or stored at −20 °C. Genomic DNA was extracted from the thorax and legs following the manufacturer's protocol from the DNeasy Blood and Tissue kit (Qiagen LLC, Germantown, MD). Primer pairs used to amplify the L993F, C764R, and E434K regions for *kdr* mutations were designed from the genomic sequence of the *para* sodium channel (GenBank accession number: PYGN0100236; Harrison et al., 2018) using Primer3 (Untergasser et al., 2012); and an additional primer pair was used for the A302S region for the *Rdl* mutation (Hansen et al., 2005) (Table 2).

PCR reactions were prepared in 50 μl total volumes with 25 μl of Taq PCR Master Mix (Qiagen LLC, Germantown, MD), 2 μl of forward and reverse primers (10 μM), 2 μl of template DNA (100–200 ng), and 21 μl of nuclease-free water. The L993F region was amplified with PCR parameters of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. The C764R region was amplified with 95 °C for 5 min, a 10-step touchdown cycle of 95 °C for 30 s, 60 °C to 50 °C (decreasing by increments of 1 °C) for 30 s and 72 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. The E434K region was amplified with 95 °C for 5 min, a 10-step touchdown cycle of 95 °C for 15 s, 60 °C to 55 °C (decreasing by increments of 0.5 °C) for 15 s and 72 °C for 15 s followed by 35 cycles of 95 °C for 15 s, 50 °C for 15 s, 72 °C for 15 s, and a final extension of 72 °C for 10 min. The PCR parameters for *Rdl* followed that of Gondhalekar and Scharf (2012): 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 64.3 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min.

PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific Inc., Waltham, MA) and sent for Sanger sequencing in the Genomics Core facility at the University of California, Riverside. Sequences were aligned with the corresponding region in the *para* sodium channel to confirm correct amplification, and genotypes of mutations were determined from the sequence chromatograms.

2.8. Statistical analysis

Topical mortality data of the UCR strain was analyzed with probit analysis using PoloPlus (LeOra Software LLC, Petaluma, CA). The difference in mortality from insecticides with and without synergists was analyzed with Mantel-Haenszel tests using R version 3.5.1. Enzymatic activities were averaged and compared with Dunnett's test in R version 3.5.1.

3. Results

3.1. Topical assay

Field-collected strains had incomplete or no mortality when treated with the diagnostic dose (DD) of 0.034 μg deltamethrin, 0.011 μg fipronil, 55.056 μg DDT, and 0.624 μg dieldrin (Table 1, Fig. 1A-D; Supplementary Table S1). All field-collected strains had 0% mortality when treated with the DD of deltamethrin and 0–23% mortality with the 0.113 μg dose (Fig. 1A). Mortality against fipronil was 0–3% with the DD of fipronil and 17–53% against the 0.036 μg dose (Fig. 1B). All field-collected strains were resistant to DDT and dieldrin, having 0–23% mortality against the DD of DDT and 0–20% against the DD of dieldrin (Fig. 1C and D).

3.2. Choice box

The performance indices (PI) were lower in the field-collected strains *versus* the UCR strain in the deltamethrin and fipronil choice boxes (Fig. 2; Fig. 3). No field strains had PIs > 90 for either fipronil or deltamethrin. The initial negative PIs in the deltamethrin choice boxes and shallow slope suggest that cockroaches actively avoided the treated panels (Fig. 2). In the fipronil choice boxes, the slopes of most of the strains were similar for the first three days suggesting that the treatment

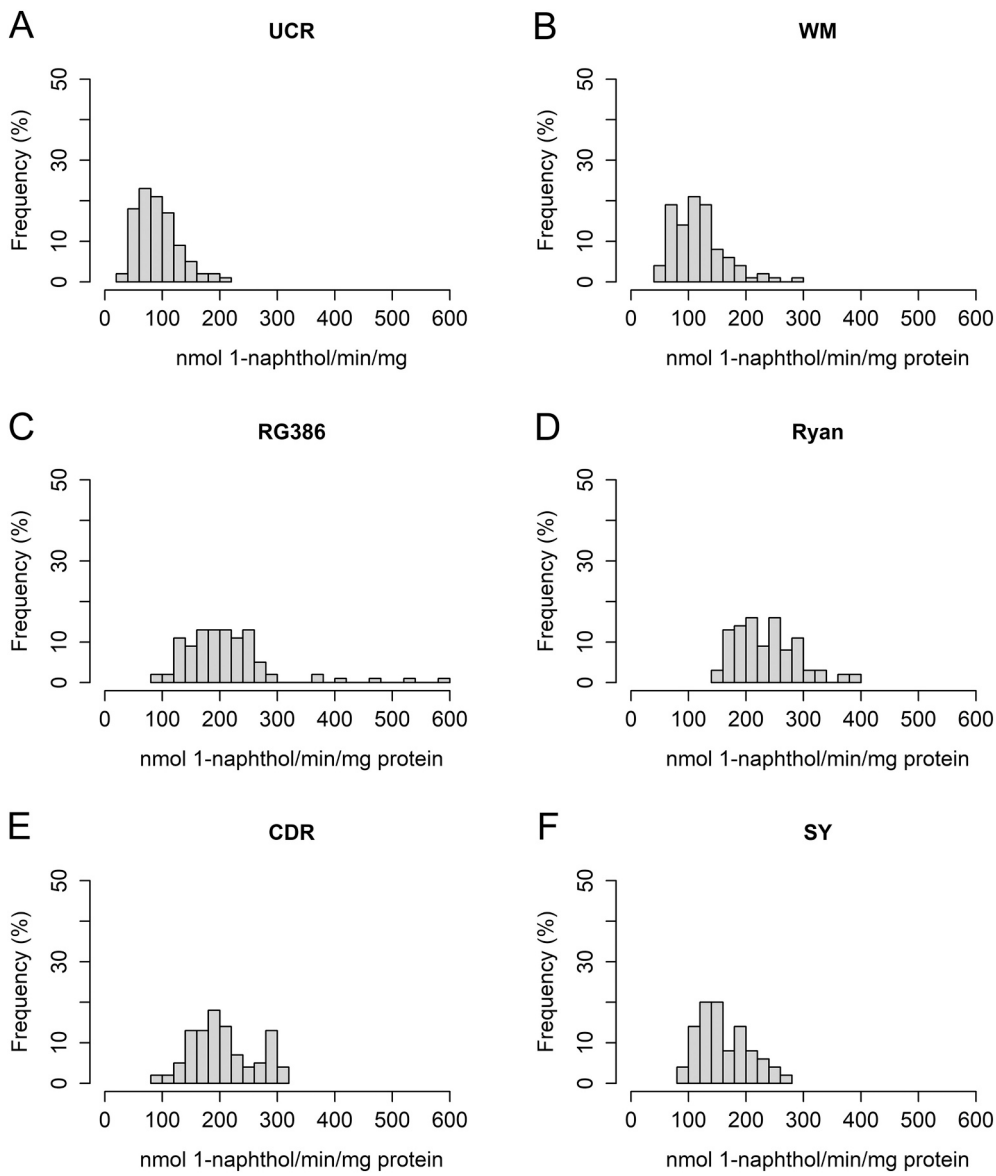

Fig. 5. The activity of 1-NA esterase in (A) UCR, (B) WM, (C) RG386, (D) Ryan, (E) CDR, and (F) SY strains.

was not repellent (Fig. 3).

3.3. Effect of synergism

Synergists were more impactful when combined with the 10 x LD_{95} *versus* deltamethrin DD (Fig. 4A and B). PBO increased the toxicity of deltamethrin for all strains from 0% mortality to 10–63% for the DD and from 0–23% to 70–100% for the 0.113 μg dose. Mortality with DEF was 10–30% for the DD and 50–67% for the 0.113 μg dose. DEM had a marginal effect on deltamethrin toxicity for most strains, causing an increase in mortality for RG386 and CDR against the 0.113 μg dose (17 to 57% and 0 to 33%, respectively) but did not significantly impact mortality in other strains.

PBO and DEF did not significantly affect mortality of the DD of fipronil for all strains (Fig. 4C). At the 0.036 μg dose, PBO increased average mortality of WM from 50 to 87%, RG386 from 53 to 93%, and Ryan from 17 to 60% (Fig. 4D). DEF also caused overall increases in mortality from 17–53% to 70–84%. DEM had minimal impact on mortality at both doses of fipronil (Fig. 4C and D).

3.4. Biochemical measurement of enzymatic activity

Mean enzymatic activities of each strain are reported in Table 3, and frequency distributions are presented in Figs. 5–8. All field-collected strains were found to have significantly elevated esterase activity (~2-fold increase on average) compared to the UCR strain based on 1-NA, 2-NA, and PNPA assays ($P < 0.01$) except PNPA activity in the WM strain (Table 3; Fig. 5; Fig. 6; Fig. 7). GST activity was ~3–6 times greater in all field-collected strains except SY (Table 3; Fig. 8).

3.5. Target-site mutations

The L993F *kdr* mutation was present in all field-collected strains with only one individual sampled from the WM, RG386, and CDR strains with a homozygous susceptible genotype (Table 4). No susceptible individuals were found in the Ryan and SY strains. Both strains had the highest degree of homozygosity for the mutation (13 and 11 individuals out of 15, respectively) out of all strains. The C764R and E434K mutations were absent in all strains.

The *Rdl* mutation was present at high frequency in all field-collected strains (Table 4). Every individual was homozygous for the mutation

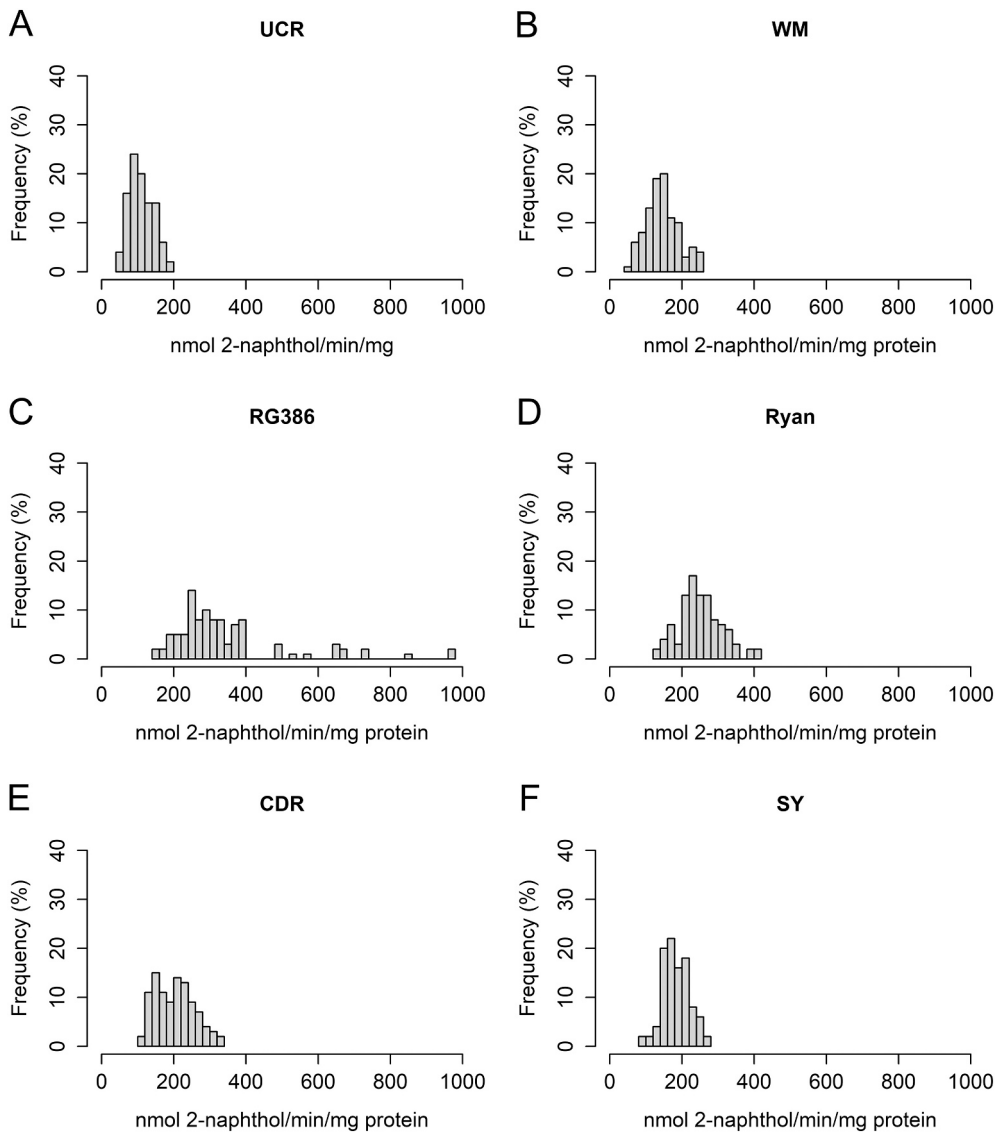

Fig. 6. The activity of 2-NA esterase in (A) UCR, (B) WM, (C) RG386, (D) Ryan, (E) CDR, and (F) SY strains.

except for one heterozygous individual from the WM strain.

4. Discussion

All field-collected German cockroach strains were resistant towards deltamethrin and fipronil in laboratory assays, and almost all treated cockroaches survived the diagnostic dose (DD; 3 x LD_{95}) of both insecticides. The 10 x LD_{95} doses caused higher overall mortality but did not exceed 23% with deltamethrin and 53% with fipronil. This was comparable to the previous data from Lee et al. (2022), reporting ≤20% mortality from deltamethrin and 20–70% mortality from fipronil in these strains.

The Ebeling choice box was designed to examine the relationship between insecticide repellency and the potential efficacy of sprays and dust against cockroaches (Ebeling et al., 1966). Its utility was expanded to include field-collected strains (Rust and Reierson, 1978; Rust and Reierson, 1991; Rust et al., 1993; Wu and Appel, 2018) to examine the relationship of repellency and insecticide resistance on potential field efficacy. The deposits of deltamethrin and fipronil killed all UCR susceptible strain individuals and provided PIs of 100. There were substantial declines in the PIs for both deltamethrin and fipronil for all the other strains tested because the applications failed to eliminate the remaining cockroaches. Furthermore, the lack of strongly negative PIs indicates that repellency was not a major factor in the survival of field-collected strains. Physiological resistance prevented the initial exposures from being lethal, and cockroaches avoided the toxic deposits. Incomplete mortality in forced exposure and spatially restrictive laboratory assays are strong indicators that field populations with similar resistance will survive against treatments when they are not forced to be in proximity. This represents a nucleus for re-infestation and proliferates resistance development (Fardisi et al., 2019). The Ryan strain was not evaluated against deltamethrin nor SY against deltamethrin and fipronil in the choice box experiments due to low colony populations at the time of testing, but we expect similar responses due to comparable resistance among all strains in the topical assessment.

Measurement of the major metabolic detoxification enzyme families through biochemical assays on cockroach homogenate samples showed elevated esterase and GST activities in most strains, suggesting an increased baseline detoxification capacity across these populations (Table 3). Strains had skewed distributions or outliers in the upper range of readings (RG386 for all enzymes; WM for 1NA and GST), indicating heterogeneity of resistance and potential survivors from treatments that would eliminate most of the population (Figs. 5–8). Distributions with low kurtosis comparable to the UCR strain (SY for 1NA, 2NA, PNPA and

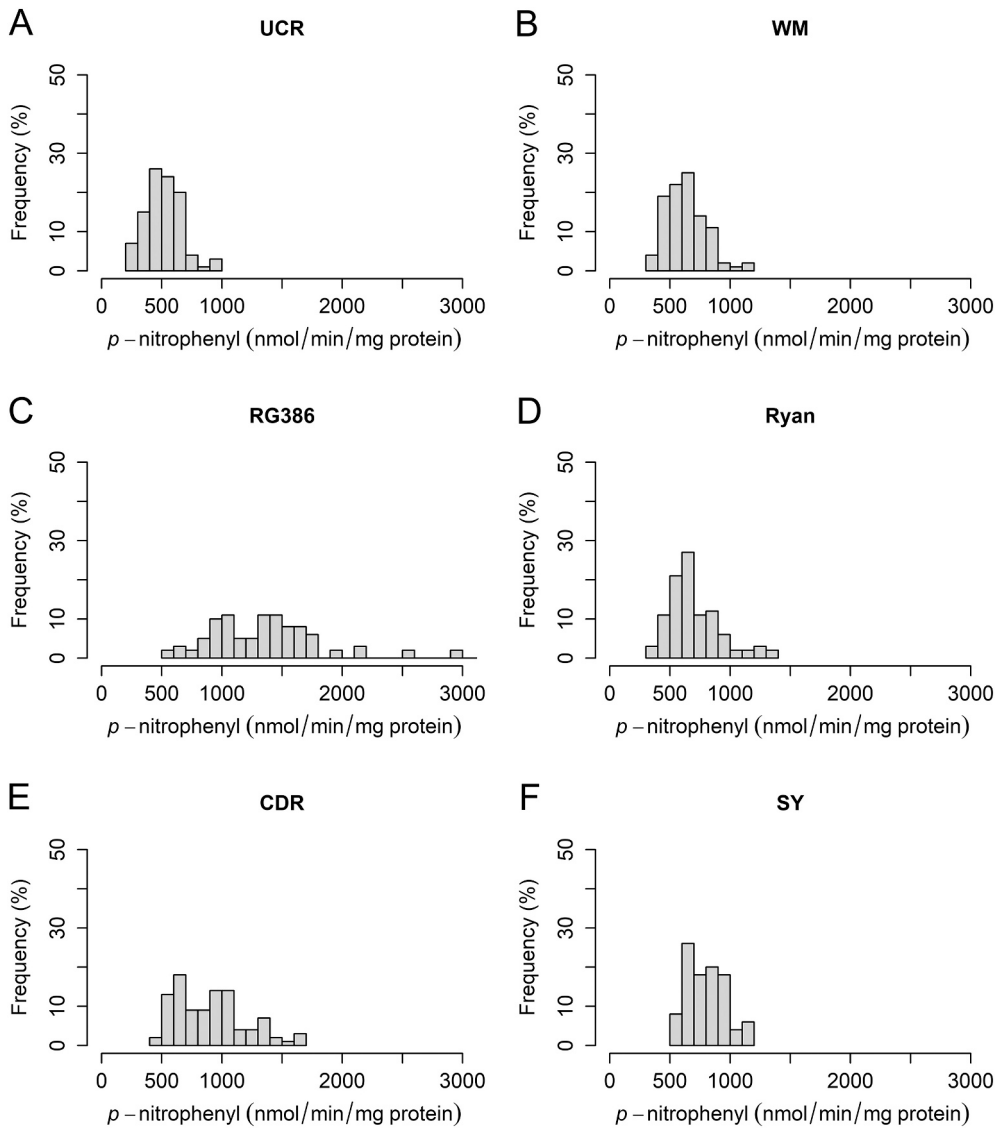

Fig. 7. The activity of PNPA esterase in (A) UCR, (B) WM, (C) RG386, (D) Ryan, (E) CDR, and (F) SY strains.

GST; CDR for 1NA and 2NA; WM for 2NA and PNPA) indicate strain homozygosity.

Pre-treatment with the P450 inhibitor PBO synergized the 0.113 μg dose of deltamethrin, increasing mortality from 0–23% to 70–100% in the field-collected strains, confirming that P450 monooxygenase plays a crucial role in detoxifying deltamethrin (Fig. 4 B). Similarly, ester linkage hydrolysis is another important pathway based on increased mortality from deltamethrin from 0 to ~50% after pretreatment with DEF. Both compounds synergize other pyrethroids such as permethrin, beta-cyfluthrin, cypermethrin, and lambda-cyhalothrin in *B. germanica* (Bull and Patterson, 1993; Valles, 1998; Wei et al., 2001; Limoee et al., 2007; Chai and Lee, 2010; DeVries et al., 2019; Hu et al., 2020). Despite the elevation of GST activity in WM, RG386, CDR, and SY strains, the impact of DEM was less than the other synergists, and synergism was only detected in RG386 and CDR. Previous studies described a lack of association between GST activity and detoxification of pyrethroids in the German cockroach, which appears to be strain-specific (Scharf et al., 1997b; Valles, 1998).

The effect of PBO on fipronil toxicity can be synergistic or antagonistic, possibly due to the degree to which P450-mediated conversion of fipronil to fipronil-sulfone is the dominant pathway in the insect (Scharf et al., 2000; Zhao and Salgado, 2010). Synergism or antagonism may also correspond with the innate fipronil resistance level in German cockroach strains; Ang et al. (2013) and Valles et al. (1997) reported PBO antagonism in strains with no greater than 4.1-fold resistance, whereas Gondhalekar and Scharf (2012) reported synergism in one strain with 36.42-fold resistance. In the present study, most cockroaches survived both fipronil topical doses; many also survived the choice box treatments. The pre-treatment of PBO increased mortality in 4 of 5 strains, demonstrating that P450 was detoxifying fipronil (Fig. 4 D). DEF also caused a synergistic effect on the 0.036 μg dose of fipronil despite the insecticide containing no ester linkages. These findings corroborate Gondhalekar and Scharf (2012), who reported the synergism of fipronil by DEF in their field strain attributed to the unintended inhibition of mixed-function oxidases at high concentrations of DEF (Scott, 1990).

None of the synergists increased the sensitivity of field-collected strains to susceptible levels (*i.e.*, 100% mortality at both diagnostic doses of an insecticide), indicating the involvement of other mechanisms besides metabolic-based resistance towards the total resistance level. All strains possessed *kdr* (L993F) and *Rdl* mutations and were simultaneously resistant towards DDT, dieldrin, deltamethrin, and fipronil that these mutations are expected to affect, which is characteristic of populations with target-site mutation mediated resistance (Scott and Matsumura, 1981; Kristensen et al., 2005).

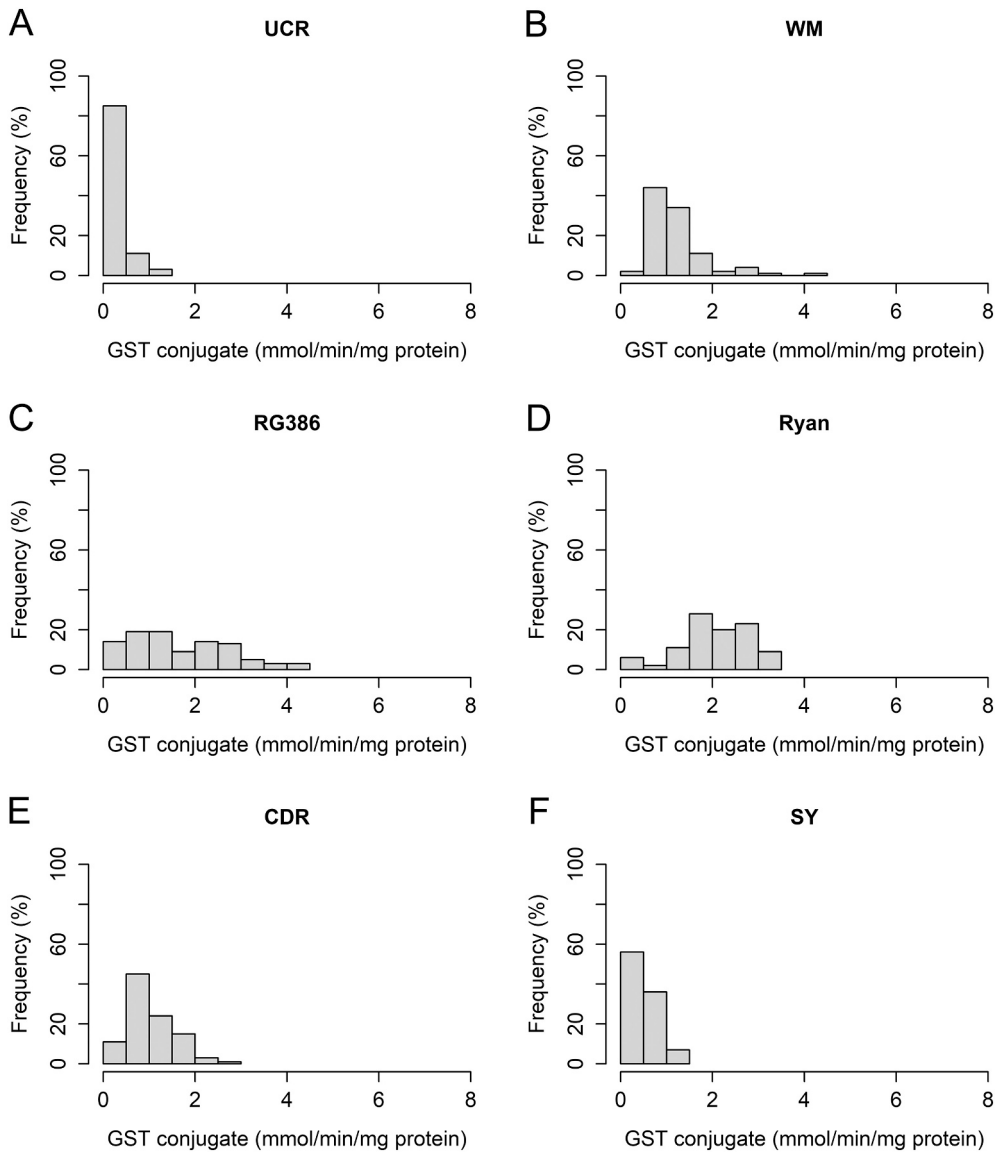

Fig. 8. The activity of glutathione S-transferase (GST) in (A) UCR, (B) WM, (C) RG386, (D) Ryan, (E) CDR, and (F) SY strains.

The L993F *kdr* mutation, common in pyrethroid-resistant German cockroaches, was found in all field-collected strains in this study (Dong et al., 1998; Gholizadeh et al., 2014; DeVries et al., 2019). Every strain was also resistant towards DDT; the apparent cross-resistance is a consequence of *kdr* affecting both insecticides due to their similar modes of action on the sodium channel (Scott and Matsumura, 1981; Dong, 1997). Specifically, the L993F mutation has been shown to cause low sodium channel desensitization to pyrethroids by itself (Tan et al., 2002) and it is unlikely solely responsible for the high survival observed in the deltamethrin bioassays. However, the secondary mutations C764R and E434K that further decrease sensitivity were not present. Both mutations have only been found at low or zero frequencies in field populations (Liu et al., 2000; DeVries et al., 2019); in the present study, this can be explained by a lack of selection pressure to maintain or select for additional target-site alterations. The L993F mutation was not fixed in any of the strains (all of them had heterozygous or susceptible individuals), indicating that they were not recently exposed to pyrethroid doses that eliminate susceptible genotypes and maximize the proportion of homozygous individuals. Given the extent to which pyrethroids have been available and used in the field, this is not an issue of infrequent exposure. The combination of metabolic resistance with incomplete *kdr* already provides significant resistance to realistic exposures of pyrethroids based on the deltamethrin choice boxes and topical assay experiments. High concentrations that sufficiently kill the resistant strains are typically repellent and poorly bioavailable in non-forced field settings (Wu and Appel, 2018). With few or none of the individuals dying from treatments, selection for greater resistance would have stagnated.

The *Rdl* mutation of the GABA-gated chloride channel was completely homozygous in all field-collected strains, except for a heterozygous individual in the WM strain. The frequency of *Rdl* may not accurately infer fipronil resistance level in German cockroaches. Kristensen et al. (2005) and Ang et al. (2013) reported moderate resistance towards fipronil in *Rdl* homozygous strains. In contrast, Gondhalekar and Scharf (2012) found greater resistance in a population with ~50% mutation frequency. Low resistance observed in earlier populations with no history of fipronil exposure is likely an artifact of dieldrin-selected *Rdl*, which by itself is suspected to only moderately reduce susceptibility to fipronil due to its different binding site from dieldrin and its sulfone metabolite having an alternative target-site (Holbrook et al., 2003; Kristensen et al., 2005; Ang et al., 2013). Because this mutation alone does not strongly affect fipronil resistance, higher resistance levels are contingent on additional mechanisms that complement pre-existing *Rdl*.

The population studied by Gondhalekar and Scharf (2012) and the presently investigated strains possess fipronil detoxification capabilities not observed in low and moderately resistant populations. The evolution of metabolic mechanisms appears to be integral in higher-level fipronil resistance.

Alternatively, there could be other novel point mutations not screened in this investigation. Although no other mutation has been directly shown to affect sodium channel sensitivity in the German cockroach, other single-nucleotide polymorphisms such as P1880L and D58G were found in pyrethroid-resistant strains (Liu et al., 2000). Similarly, additional resistance conferring mutations on the GABA-gated chloride channel have not been documented in the German cockroach but have been found in other insects (Le Goff et al., 2005; Nakao et al., 2011; Zhang et al., 2016). More research is warranted to determine the presence and function of novel mutations in these strains. Regardless of their presence, target-site insensitivity coexists with metabolic resistance contributing to a portion of the total resistance in these strains.

Because of the prevalence of P450-mediated detoxification in the German cockroach, incorporating PBO into pyrethroid sprays could potentially enhance their performance against field populations. Deltamethrin was ineffective against all the field-collected strains, but their detoxification ability was compromised by PBO, indicating the effectiveness of synergism on similarly resistant populations. However, such methods must be approached carefully because of possible limitations on PBO usage due to environmental and health concerns (EPA, 2021). Substituting PBO with other compounds that can inhibit metabolism, such as plant essential oils, may offer a more sustainable alternative (Norris et al., 2018; Gaire et al., 2021). Still, exposure to synergist mixtures may select for generalized resistance mechanisms (Comont et al., 2020) and constant metabolic inhibition could isolate target-site insensitivity as the remaining major mechanism and facilitate selection for additional mutations. The combination of L993F, C764R, and E434K *kdr* mutations may substantially increase protection against pyrethroids, potentially leading to complete negation of synergized pyrethroids (Tan et al., 2002).

In contrast, PBO is not mixed with fipronil, likely due to the typical usage of fipronil exclusively in baits, and treating populations not reliant on enzymatic detoxification with PBO may result in antagonistic effects. However, all currently investigated strains were weakened by the addition of synergists, and the continued exposure to fipronil treatments can further this dependency on metabolic systems. Unlike the near-complete survival against deltamethrin, resistance was less severe in the fipronil bioassays, translating to faster removal of susceptible cockroaches and expediting resistance selection (Scharf et al., 1997a, 1997b; Fardisi et al., 2019). Also, with *Rdl* fixed or near fixation, resistance cannot increase through the frequency change of this mutation alone. Thus, metabolic resistance may be prioritized, increasing the consistency of enzyme inhibition effects. The usage of fipronil as an indoor spray against cockroaches, unprecedented until the introduction of Fipronil-Plus-C in 2020, offers a possibility to incorporate enzymatic inhibitors together with fipronil. Future resistance studies should prioritize documenting the extent of high metabolic resistance towards fipronil in field populations and examine the viability of fipronil-synergist mixtures.

In conclusion, deltamethrin and fipronil were ineffective against all field-collected German cockroaches in this study due to combined metabolic and target-site resistance mechanisms. Despite the pre-existing high resistance, no strain possessed the upper-most extent of the screened mechanisms such as complete *kdr* homozygosity, multiple sodium channel mutations, and involvement of all major detoxification enzyme families. The continued use of these compounds should be reexamined since treatments may leave many survivors and select for even greater resistance levels through these mechanisms.

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Declaration of Competing Interest

None.

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