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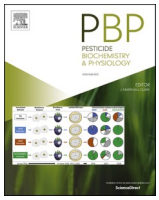

Multiple mechanisms associated with deltamethrin and imidacloprid resistance in field-collected common bed bug, *Cimex lectularius* L.

Jin-Jia Yu [a], Shao-Hung Lee [b], Chow-Yang Lee [b], Changlu Wang [a,*]

[a] Department of Entomology, Rutgers University, 96 Lipman Drive, New Brunswick, NJ 08901, USA
[b] Department of Entomology, University of California, Riverside, 165 Citrus Drive, Riverside, CA 92521, USA

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ABSTRACT

Pyrethroids and neonicotinoids are commonly used to manage the common bed bug (*Cimex lectularius* L.) infestations. However, the effectiveness of these insecticides is often challenged due to insecticide resistance. We investigated the mechanisms of deltamethrin and imidacloprid resistance in eight *C. lectularius* strains collected from New Jersey, U.S. Piperonyl butoxide (PBO), S,S,S-tributyl phosphorotrithioate (DEF), and diethyl maleate (DEM) were topically applied on bed bugs before deltamethrin or imidacloprid treatments (deltamethrin: 115 ng per adult; imidacloprid: 67 ng per adult). The results showed that PBO and DEF had a greater synergistic effect with deltamethrin treatments than DEM based on the significantly increased 72 h mortality of Aberdeen, Bayonne 2015, Cotton, Irvington, and Irvington 624-5G strains. With imidacloprid alone, seven out of eight strains experienced 100 % mortality except for the Linden 2019 strain. The Linden 2019 strain had mean mortalities of 93, 97, and 47 % from imidacloprid after receiving PBO, DEF, and DEM, respectively. The activities of glutathione S-transferase and general esterase in all strains were enhanced compared to a susceptible strain. Molecular detection of voltage-gated sodium channel (VGSC) mutations revealed homozygous V419L and L925I resistance mutations in all strains at 20–100 % and 30–100 % frequency, respectively. The presence of both V419L and L925I was found in 20–100 % of the individuals from each resistant strain. The results indicate a combination of metabolic and target site insensitivity mechanisms confers resistance to deltamethrin and imidacloprid in *C. lectularius*.

1. Introduction

The common bed bug (*Cimex lectularius* L.) has resurged as a common indoor pest that causes global public concerns. Chemical control is the most popular method for managing bed bug infestations (Doggett and Lee, 2023). According to the United States Environmental Protection Agency, pyrethrins and pyrethroids are the most common compounds used to control bed bugs (EPA, 2024). Additionally, pyrethroid-neonicotinoid mixtures are available to pest control professionals and can be more successful in controlling bed bugs compared to pyrethroids-only insecticides (Doggett and Lee, 2023; Wang et al., 2015; Wang et al., 2016). However, failures of pyrethroid-neonicotinoid mixture products against pyrethroid-resistant bed bugs have been reported (Gordon et al., 2015; Yu et al., 2023). High resistance to neonicotinoid insecticides is expected to become more common among bed bug populations as these insecticides continue to be widely used by professionals (Romero and Anderson, 2016).

To increase the efficacy of insecticides, piperonyl butoxide (PBO) is often mixed with insecticides to counter the effect of increased enzyme activities. PBO mainly inhibits P450s and other esterases. Two less-known synergists are S,S,S-tributyl phosphorotrithioate (DEF) and diethyl maleate (DEM). The former is a general esterase inhibitor, and the latter is a putative inhibitor for glutathione S-transferase (GST) enzymes (Feyereisen, 2015; Horowitz et al., 1988; Wu et al., 2007). In *C. lectularius*, the synergistic effect of PBO on deltamethrin has been reported for over a decade (Cáceres et al., 2023; Cáceres et al., 2019; Gonzalez-Morales and Romero, 2019; Lilly et al., 2016; Romero et al., 2009) while Gonzalez-Morales and Romero (2019) and Gaire et al. (2020) studied the synergistic effect of other synergists, such as DEF and DEM, on deltamethrin. Meanwhile, *C. lectularius* populations in the U.S. have been documented to have resistance to neonicotinoids, including imidacloprid and acetamiprid (Romero and Anderson, 2016; Yu et al., 2023). However, there are no reports investigating the effect of synergists on neonicotinoids.

* Corresponding author at: Department of Entomology, Rutgers University – New Brunswick, 96 Lipman Drive, New Brunswick 08901, USA.
E-mail address: changluw@rutgers.edu (C. Wang).

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Cytochrome P450 monooxygenase (P450s), general esterase, and GST activity can metabolize endogenous and xenobiotic substrates (Casida, 1970; Grant and Matsumura, 1989; Hemingway, 2000; Scott, 1999). Under high frequency of insecticide applications, the selection pressure of insecticides can elicit abnormal expression of detoxifying enzymes. Overexpressed P450s, esterase, and GST have been correlated with pyrethroid resistance (Kasai et al., 2014; Scharf et al., 1997; Somwang et al., 2011; Vontas, 2001) and neonicotinoid resistance (Bao et al., 2016; Bass et al., 2015; Li et al., 2012; Zewen et al., 2003) in insects.

Point mutations on the alpha subunit of the voltage-gated sodium channel (VGSC) confer resistance to pyrethroids through target-site insensitivity, resulting in knockdown resistance (*kdr*) in insects (Dong et al., 1998; Feyereisen, 1995; Reimer et al., 2014). Yoon et al. (2008) identified two single nucleotide polymorphisms, V419L and L925I, related to *kdr* in *C. lectularius*. Later reports found V419L and L925I widely distributed among the common bed bug populations worldwide (Balvín and Booth, 2018; Cho et al., 2020; Cho et al., 2024; Dang et al., 2015; Lewis et al., 2023; Porras-Villamil et al., 2025; Vander Pan et al., 2020). A new mutation in the pyrethroid target site, F1524C, was recently reported in *C. lectularius* (Porras-Villamil et al., 2025).

Our previous study showed that field-collected *C. lectularius* strains exhibited various resistance levels to acetamiprid, imidacloprid, and deltamethrin (Yu et al., 2023). The current study investigated both metabolic resistance and target site insensitivity in eight *C. lectularius* strains to provide a comprehensive profile of mechanisms of deltamethrin resistance. This study is also the first report to understand the upregulated enzymes activities and *kdr* mutations in *C. lectularius* strains in New Jersey, USA. The results show that deltamethrin resistance in field strains is driven by increased activities of detoxifying enzymes and the presence of V419L and L925I mutations. Increased activities of detoxifying enzymes also contribute to imidacloprid resistance in one *C. lectularius* strain, indicating that this must be considered in future bed bug management.

2. Materials and methods

2.1. Bed bug strains

This study used one susceptible *C. lectularius* strain (Fort Dix, also known as Harlan strain) and eight field-collected strains from New Jersey, USA. (collection year: 2012 to 2021). Bed bug strains were maintained in plastic containers (5.0 cm diameter and 4.7 cm height, Consolidated plastics, Stow, OH, USA). Defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) was provided as a food resource every 2–4 weeks using a Hemotek membrane-feeding system (Discovery Work- shops, Accrington, UK). All strains were colonized in an environmental chamber at 25 ± 1 °C, 45 ± 10 % RH, and a photoperiod of 12:12 (L:D) h. All strains showed high resistance to deltamethrin and low resistance to imidacloprid, except the Linden 2019 strain which had very high resistance to imidacloprid (Yu et al., 2023). Hence, the eight strains were named as resistant strains in this study.

2.2. Chemicals

To evaluate the synergistic effect on deltamethrin and imidacloprid (section 2.3), technical grade deltamethrin (93.2 %) and imidacloprid (98.9 %) were used in this study. Three synergists, including PBO, DEF, and DEM, were used as detoxifying enzyme inhibitors. All chemicals were purchased from Chem Service Inc., West Chester, PA, USA.

Materials used in biochemical assays include Triton X-100 from Bio-Rad Laboratories Inc. (Hercules, CA, USA), 1-naphthyl acetate (≥ 98 %) and 2-naphthyl acetate (≥ 98 %) from Sigma Aldrich Corporation (St. Louis, MO, USA), 1-naphthol (≥ 99 %) and 2- naphthol (≥ 99 %) from Spectrum Chemical Mfg. Corp. (Gardena, CA, USA), Fast Blue B Salt and sodium lauryl sulfate (≥ 99 %) from M.P. Biomedicals, LLC (Irvine, CA, USA), *p*-nitrophenyl acetate (PNPA) (≥ 98 %) from Sigma Aldrich Corporation (St. Louis, MO, USA), 1-chloro-2,4-dinitrobenzene (CDNB) (99 %) from Acros Organics (Carlsbad, CA, USA), and reduced glutathione (GSH) (99 %) from Chem-Impex International, Inc. (Wood Dale, IL, USA).

2.3. Topical assay of insecticide synergists

2.3.1. Topical assay

The topical assay followed the same method as described in a previous study (Yu et al., 2023). Briefly, 10 adult males of unknown age, fed 5–7 d before the assay, were anesthetized in a Petri dish on ice for 1 min. PBO, DEF, or DEM was diluted with acetone, and 1 μl of one synergist was applied to the dorsal surface of the abdomen at a concentration of 50 μg/μl (Gonzalez-Morales and Romero, 2019) using a micro-applicator (Burkard Manufacturing Co. Ltd., Rickmansworth, U.K.). Bed bugs were then moved to a clean Petri dish lined with filter paper (Grade P8, Fisher Scientific, Pittston, PA, USA). After 2 h, bed bugs were anesthetized on ice again for 1 min then received 1 μl of a discriminating dose of either deltamethrin or imidacloprid solution diluted with acetone (deltamethrin: 115 ng per adult; imidacloprid: 67 ng per adult). The discriminating doses were determined as 10 times of LD_{90} of each insecticide tested on a susceptible strain (Yu et al., 2023). The control group was treated with acetone only. The numbers of knocked down bed bugs were recorded every 5 min for the first hour followed by every 10 min until 2 h or until 90 % of tested bed bugs were knocked down. Mortality was observed daily until 72 h post-treatment. Each strain was treated with insecticide and synergist with three replications.

2.4. Measurement of metabolic detoxification enzymes

The biochemical analysis method followed the WHO protocol (Hemingway, 1998) and Lee et al. (2022) with some modifications.

2.4.1. Homogenization

To prevent influence of blood meals on the biochemical results, a total of 96 unfed, 14–17-d-old mix-sexed adults (M:F = 1:1 to demonstrate the enzyme activity in a population) was collected from laboratory-maintained colonies and used in biochemical assays. Whole bed bugs were homogenized individually in 600 μl of 0.1 M sodium phosphate buffer (pH 7.0) with 0.3 % Triton X-100 using a pestle (Corning Inc., Corning, NY, USA) until their bodies were completely crushed. The homogenate was then centrifuged at 10000*g* at 4 °C for 10 min. The supernatant (~ 500 μl) was aliquoted into five PCR tubes and stored at −70 °C for further assays. Homogenized bed bugs were not pooled and were analyzed individually for a total of 50–95 biological replicates across each enzyme assay. Sodium phosphate buffer was used in blank controls in each enzyme assay.

2.4.2. Protein assay

A 10 μl volume of homogenate from each sample was added to separate wells on a 96-well microplate. Bio-Rad protein dye reagent (200 μl) was added to each well, and the plate was incubated at room temperature (25 ± 1 °C) for 5 min. The plate read at 570 nm using an Epoch 2 Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Bovine serum albumin was used to generate the standard curve to calculate protein concentration. Protein concentration was measured to correct for the variation of different bed bug sizes.

2.4.3. Naphthyl acetate esterase assay

The 1-naphthyl acetate (1-NA), 2-naphthyl acetate (2-NA) working solutions (0.3 ml of 0.03 M 1-NA or 2-NA in acetone +29.7 ml of 0.02 M sodium phosphate buffer pH 7.2), and Fast Blue B solution (0.075 g Fast Blue B Salt in 7.5 ml distilled water +17.5 ml of 5 % sodium lauryl sulfate) were made before the assay.

Homogenate of each sample was added to two 96-well microplates

Table 1
Mean percent mortality (± SE) of *C. lectularius* strains at 72 h treated with piperonyl butoxide (PBO), S,S,S-tributyl phosphorotrithioate (DEF), and diethyl maleate (DEM) 2 h prior to discriminating doses of deltamethrin (115 ng/adult) and imidacloprid (67 ng/adult) treatments.

Strain	Collection year	Treatment							
		Deltamethrin				Imidacloprid			
		Insecticide only*	PBO	DEF	DEM	Insecticide only*	PBO	DEF	DEM
Fort Dix	1973	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Aberdeen	2018	17 ± 7	47 ± 9**	43 ± 19**	33 ± 9	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Bayonne 2015	2015	13 ± 9	67 ± 13**	57 ± 12**	100 ± 0**	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Canfield	2018	20 ± 6	33 ± 9	40 ± 10	23 ± 15	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Cotton	2018	73 ± 9	100 ± 0**	100 ± 0**	87 ± 3	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Irvington	2012	27 ± 7	100 ± 0**	77 ± 9**	47 ± 9	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Irvington 624-5G	2013	0	57 ± 9**	43 ± 3**	23 ± 3**	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Linden 2019	2019	3 ± 3	20 ± 10	20 ± 6	10 ± 10	60 ± 6	93 ± 7**	97 ± 3**	47 ± 7
New Brunswick	2021	7 ± 7	17 ± 9	20 ± 12	33 ± 3**	100 ± 0	100 ± 0	100 ± 0	100 ± 0

* The insecticide-only treatment data are from Yu et al. (2023).
** The mortality is significantly different from that in the insecticide-only treatment (chi-square test). There was no mortality in the control.

(20 μl in each well). In one plate, 200 μl of 1-NA working solution was added to each well, and 200 μl of 2-NA working solution was added to each well in the other plate. Both plates were incubated at room temperature for 5 min, then 50 μl of Fast Blue B solution was added. The plates were incubated at room temperature for 5 min again and read at an endpoint of 570 nm. The activity was calculated according to the standard curves of 1-naphthol and 2-naphthol.

2.4.4. Esterase assay – p-nitrophenyl rate reaction

The PNPA working solution (0.3 ml 0.1 M PNPA in acetonitrile +29.7 ml of 0.05 M sodium phosphate buffer pH 7.4) was made before the assay.

Homogenate (10 μl) of each sample was added to a microplate. PNPA working solution was added to the plate and then read at 405 nm at 1 min intervals for 4 min. The activity was calculated after converting with Beer's Law ($A = \varepsilon lc$) using an extinction coefficient of 6.53 μM^{-1} and a path length of 0.6 cm.

2.4.5. Glutathione S-transferase assay

The glutathione S-transferase (GST) 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) was made before the assay.

Homogenate (10 μl) of each sample was added to a microplate. Each well received 200 μl of GST working solution and was incubated at room temperature for 20 min. The product was read at 340 nm for 5 min at 1 min intervals. The activity was calculated after converting with Beer's Law ($A = \varepsilon lc$) using an extinction coefficient of 4.39 μM^{-1} and a path length of 0.6 cm.

2.5. Detection of target site mutation

A total of 10 5-d-old male adults from each strain were taken out from the laboratory-maintained colony and stored in 95 % ethanol at −20 °C for DNA extraction. Whole bed bugs were homogenized individually to extract genomic DNA using the DNeasy Blood and Tissue kit (Qiagen LLC, Germantown, MD, USA) following the manufacturer's protocol.

PCR reactions were conducted with 1 μl of template DNA in a 25 μl total volume using a Taq PCR Master Mix kit (Qiagen LLC, Germantown, MD, USA). Primers used to amplify the V419L *kdr* mutation were BBParaF1 (5'-AACCTGGATATACATGCCTTCAAGG-3′) and BBParaR1 (5′-TG ATGGAGATTTTGCCACTGATG-3′); L925I regions were amplified using primers of BBparaF3 (5'-GGAATTGAAGCTGCCATGAAGTTG-3′) and BBparaR3 (5'-TGCCTATTCTGTCGAAAGCCTCAG-3′) (Zhu et al., 2010). The cycle conditions for both pairs of primers were 95 °C for 2 min; 40 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min. The PCR products were visualized on a 1 % agarose gel and then purified with ExoSAP-IT (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sanger sequencing was performed in the University of California, Riverside Genomics Core facility. Sequence results were checked for *kdr* mutation using SnapGene software (www.snapgene.com).

2.6. Data analysis

Synergistic effect on survivorship was analyzed with Kaplan-Meier analysis using SPSS Statistics version 29.0 (IBM Corporation, Armonk, NY). Mortality of *C. lectularius* strains treated with insecticide in a previous study (Yu et al., 2023) was compared with the mortality after treatment with a synergist and insecticide in the current study using chi-square tests. Mean activity levels of enzymes of field strains were compared with the Fort Dix strain using Welch's *t*-test. Enzyme activities were visualized with histograms to show the distribution frequency for each strain. The chi-square test, Welch's *t*-test, and visualization of enzyme activities were conducted using R version 4.3.1.

3. Results

3.1. Insecticide synergist assay

The mortality data at 72 h post-treatment were used to evaluate the synergistic effect of three synergists. In all assays, the control groups had no mortality at 72 h. Mean survival time and the statistical value in synergist assays were reported in Tables S1 and S2. For PBO or DEF + deltamethrin treatments, Aberdeen ($\chi^2 = 4.9$, df = 1, $p = 0.026$; $\chi^2 = 3.9$, df = 1, $p = 0.048$), Bayonne 2015 ($\chi^2 = 15.6$, df = 1, $p < 0.001$; $\chi^2 = 10.6$, df = 1, $p < 0.001$), Cotton ($\chi^2 = 7.1$, df = 1, $p = 0.008$, $\chi^2 = 7.1$, df = 1, $p = 0.008$), Irvington ($\chi^2 = 31.6$, df = 1, $p < 0.001$; $\chi^2 = 13.1$, df = 1, $p < 0.001$), and Irvington 624-5G ($\chi^2 = 21.0$, df = 1, $p < 0.001$; $\chi^2 = 14.1$, df = 1, $p < 0.001$) experienced significantly increased mortality compared to insecticide only treatments (Tables 1 and S2). Bayonne 2015 ($\chi^2 = 42.4$, df = 1, $p < 0.001$), Irvington 624-5G ($\chi^2 = 5.8$, df = 1, $p = 0.016$), and New Brunswick ($\chi^2 = 5.1$, df = 1, $p = 0.024$) experienced significantly increased mortality after treatment with DEM + deltamethrin versus deltamethrin alone.

Only the Linden 2019 strain was utilized for imidacloprid to observe synergistic effects because all other strains reached 100 % mortality with insecticide alone. PBO and DEF significantly increased mortality by 93 % ($\chi^2 = 7.5$, df = 1, $p = 0.006$) and 97 % ($\chi^2 = 9.8$, df = 1, $p = 0.002$), respectively (Tables 1 and S2). DEM did not synergize with imidacloprid. The synergistic effect of all three synergists in other strains can be seen from the decreased mean survival time with synergist treatments compared to those without synergist treatments (Table S1).

Table 2
Mean enzyme activities of glutathione S-transferase (GST), general esterase, *p*-nitrophenyl (PNPA) in *C. lectularius* strains and their ratios to Fort Dix strain.

Srtrain	GST (mmol/min/mg)			General esterase (nmol/min/mg)						PNPA (mmol/min/mg)		
	n	Mean (± SE)	Ratio	n	1-naphthol	Ratio	n	2-naphthol	Ratio	n	Mean (± SE)	Ratio
Fort Dix	89	0.29 ± 0.03	–	95	8.52 ± 0.56	–	95	11.74 ± 1.20	–	92	26.66 ± 2.86	–
Aberdeen	93	0.47 ± 0.07*	1.6	94	11.55 ± 0.72*	1.4	94	21.90 ± 2.38*	1.9	87	49.96 ± 5.28*	1.9
Bayonne 2015	50	0.81 ± 0.20*	2.8	82	37.76 ± 5.40*	4.4	82	46.77 ± 7.80*	4.0	65	154.59 ± 33.93*	5.8
Canfield	77	0.30 ± 0.03	1.1	88	16.85 ± 2.60*	2.0	80	26.77 ± 3.71*	2.3	62	334.07 ± 37.33*	12.5
Cotton	87	0.47 ± 0.07*	1.6	90	11.52 ± 1.36*	1.4	90	16.63 ± 2.53	1.4	83	94.05 ± 9.46*	3.5
Irvington	81	0.37 ± 0.07	1.3	94	8.98 ± 0.87	1.1	89	14.98 ± 1.77	1.3	74	49.95 ± 8.15*	1.9
Irvington 624-5G	80	0.27 ± 0.03	0.9	89	10.22 ± 0.95	1.2	89	19.62 ± 2.40*	1.7	75	235.64 ± 54.28*	8.8
Linden 2019	88	0.54 ± 0.06*	1.9	90	16.73 ± 1.34*	2.0	90	39.75 ± 3.45*	3.4	82	125.70 ± 11.60*	4.7
New Brunswick	84	0.41 ± 0.07	1.4	88	26.52 ± 8.12*	3.1	82	25.21 ± 2.63*	2.1	76	53.03 ± 5.52*	2.0

* The enzyme activity is significantly different from the Fort Dix strain (Welch's *t*-test, $p < 0.05$).

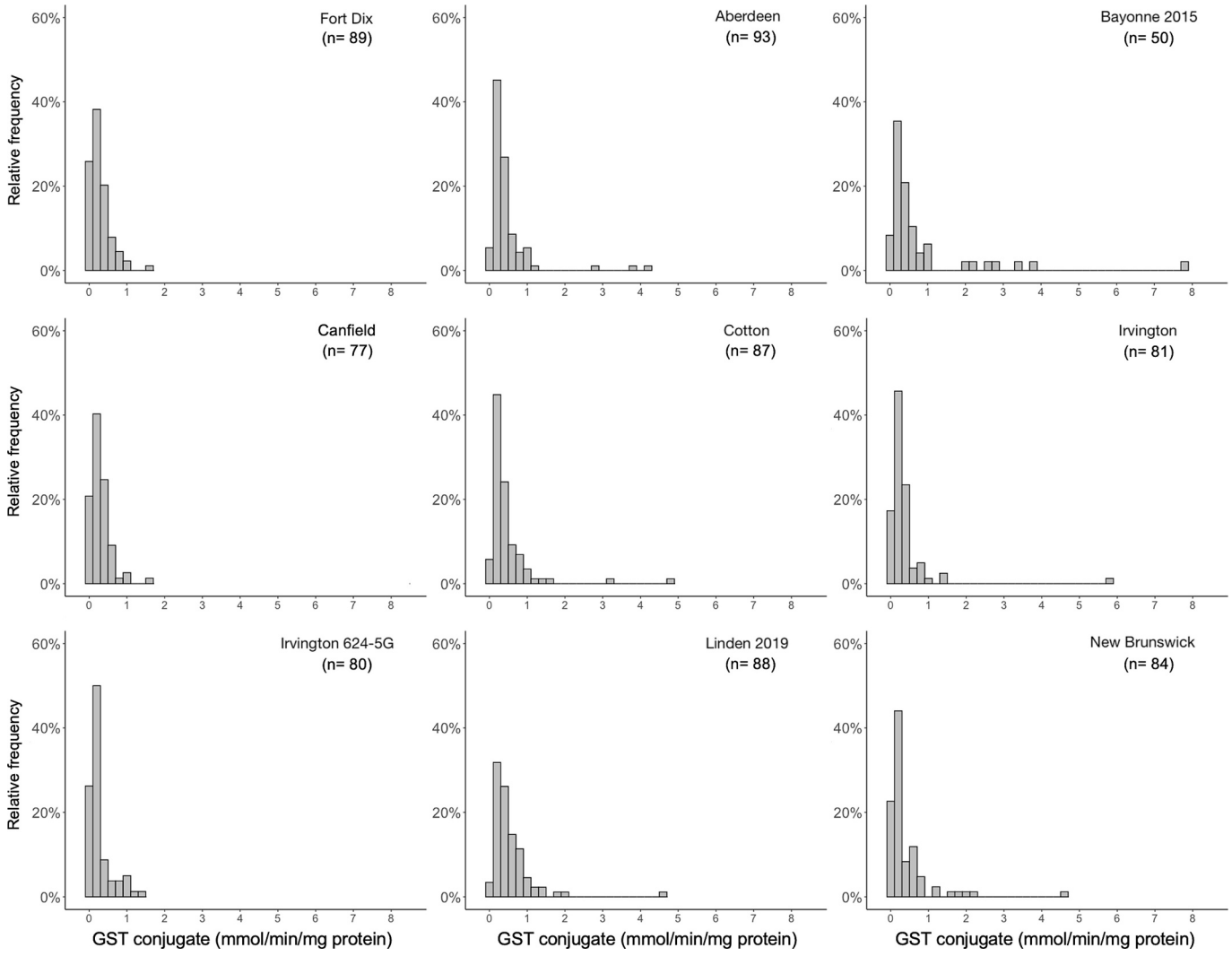

Fig. 1. The distribution of glutathione S-transferase (GST) activity in *C. lectularius* samples from nine strains.

3.2. Enzyme activities

Compared to the Fort Dix strain, GST activity was significantly higher (1.6 to 2.8-fold) in Aberdeen, Bayonne 2015, Cotton, and Linden 2019 strains (Tables 2 and S3). General esterase activities in Aberdeen, Bayonne 2015, Canfield, Linden 2019, and New Brunswick strains were higher by 1.4 to 4.4-fold based on 1-naphthol and 1.7 to 4.0-fold based on 2-naphthol (Table 2). The Cotton strain only had increased activity of 1-naphthol, and Irvington 624-5G only had increased 2-naphthol activity. In addition, all strains showed significantly elevated PNPA activities (1.9 to 12.5-fold increase) compared to the Fort Dix strain. The frequency of distribution of the enzyme activities are shown in Figs. 1–4.

3.3. Target site mutation

The susceptible Fort Dix strain of bed bugs had no *kdr* mutation (Table 3). All resistant strains had 2–10 individuals homozygous resistant for the V419L mutation, while Bayonne 2015 and Cotton strains had

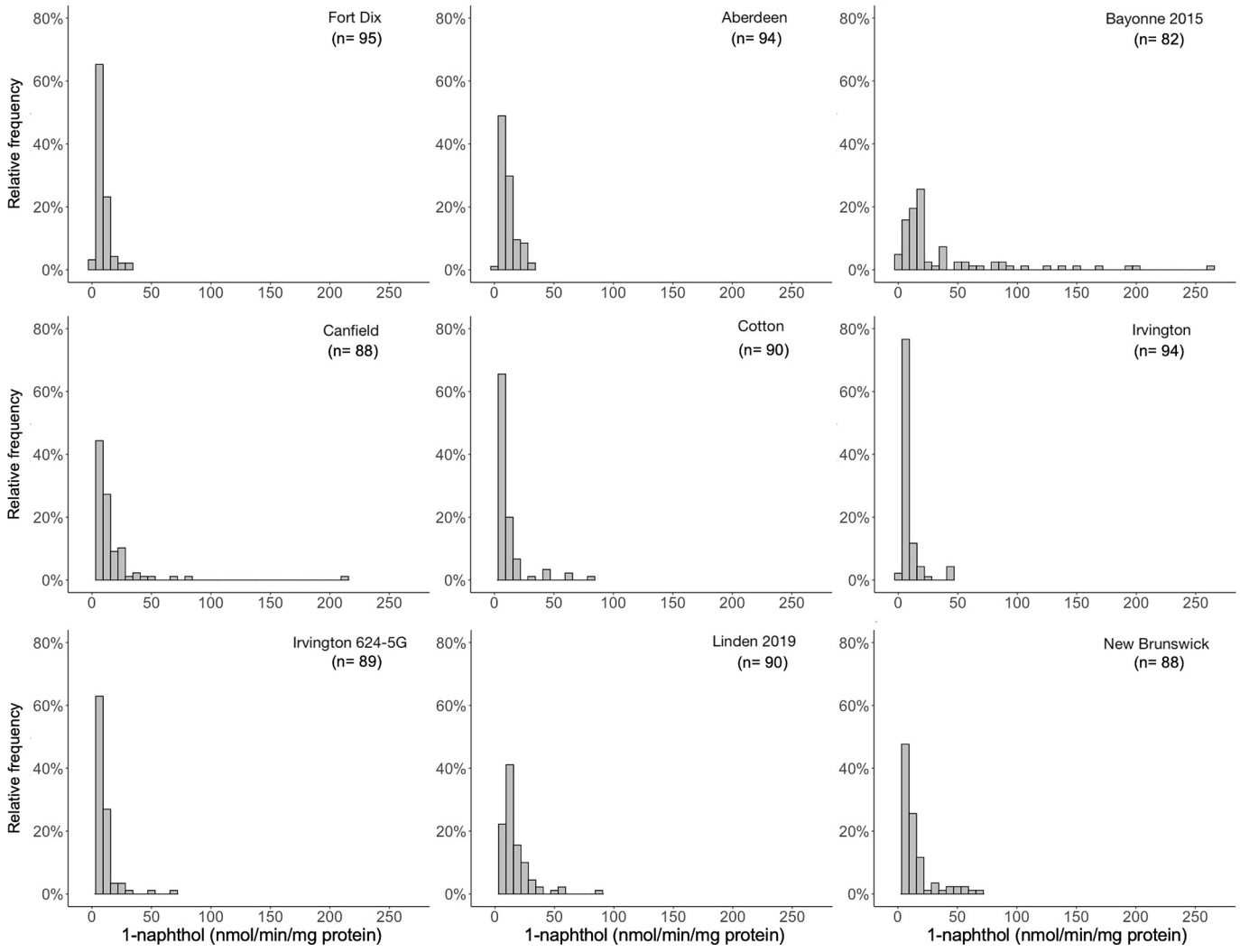

Fig. 2. The distribution of 1-naphthol esterase activity in *C. lectularius* samples from nine strains.

one heterozygous individual for the V419L mutation (Table 3). Aberdeen, Bayonne 2015, Cotton, Irvington, and New Brunswick strains had 2–8 individuals with the susceptible genotype for the V419L mutation. L925I homozygous resistance was detected in all resistant strains in 3–10 individuals (Table 3). Bayonne 2015, Cotton, Irvington, and New Brunswick strains had 1–6 heterozygous individuals for L925I mutation. Both Cotton and New Brunswick strains had one individual with the susceptible genotype for the L925I mutation.

Zhu et al. (2010) designated *kdr* mutations of *C. lectularius* into four haplotypes: A- no mutation at both 925 and 419 amino acids (aa); B- with mutation at 925 aa but not at 419 aa; C- with mutations at both 925 and 419 aa; D- with mutation at 419 aa but no mutation at 925 aa. All bed bugs from Fort Dix strain belonged to Haplotype A (Fig. 5). Among all resistant strains, Haplotype A was found in 10 % of Cotton and New Brunswick strains and absent in the other strains. Haplotype B was found in 10–80 % of Aberdeen, Bayonne 2015, Cotton, Irvington, and New Brunswick strains (Fig. 5). Haplotype C was the major haplotype in 80–100 % of Bayonne 2015, Canfield, Irvington 624-5G, Linden 2019, and New Brunswick strains; while it was only found in 20–40 % of Aberdeen, Cotton, and Irvington strains (Fig. 5). Haplotype D was absent in all strains in this study.

4. Discussion

Mechanisms of insecticide resistance, such as upregulated detoxification, *kdr*-related gene mutation, and enhanced cuticular thickness, have been studied in *C. lectularius* (Dang et al., 2017). We measured the activities of detoxification enzymes and analyzed *kdr*-type mutations in eight field-collected bed bug strains from New Jersey. Results confirm a combination of metabolic and target-site insensitivity mechanisms contributed to deltamethrin resistance in *C. lectularius* strains. Metabolic mechanisms also contributed to imidacloprid resistance in our *C. lectularius* from Linden 2019 strain.

We tested the synergistic effect of three synergists on deltamethrin in each resistant *C. lectularius* strain and evaluated their enzyme activities simultaneously. PBO and DEF synergized with deltamethrin more than DEM based on higher mortality in five out of eight resistant strains (Table 1). Among the five strains, significantly elevated general esterase was observed in Aberdeen, Bayonne 2015, Cotton, and Irvington 624-5G (Table 2) as well as the elevated PNPA reaction in Irvington strain. In addition, the right-skewed curves of 1-naphthol, 2-naphthol, and PNPA of these five strains indicate a subset of the strain possessing enhanced enzyme activities compared to the Fort Dix strain (Figs. 1–4). P450s play an essential role mediating deltamethrin resistance in bed bugs (Zhu et al., 2012). However, we did not show P450 data due to improper testing methods. PBO or DEF-synergized deltamethrin toxicity has been reported in resistant *C. lectularius* (Cáceres et al., 2019; Cáceres et al., 2023; Gaire et al., 2020; Gonzalez-Morales and Romero, 2019; Lilly et al., 2016) while Cáceres et al. (2023) and Gaire et al. (2020) also evidenced elevated activities of P450s and esterases in those resistant

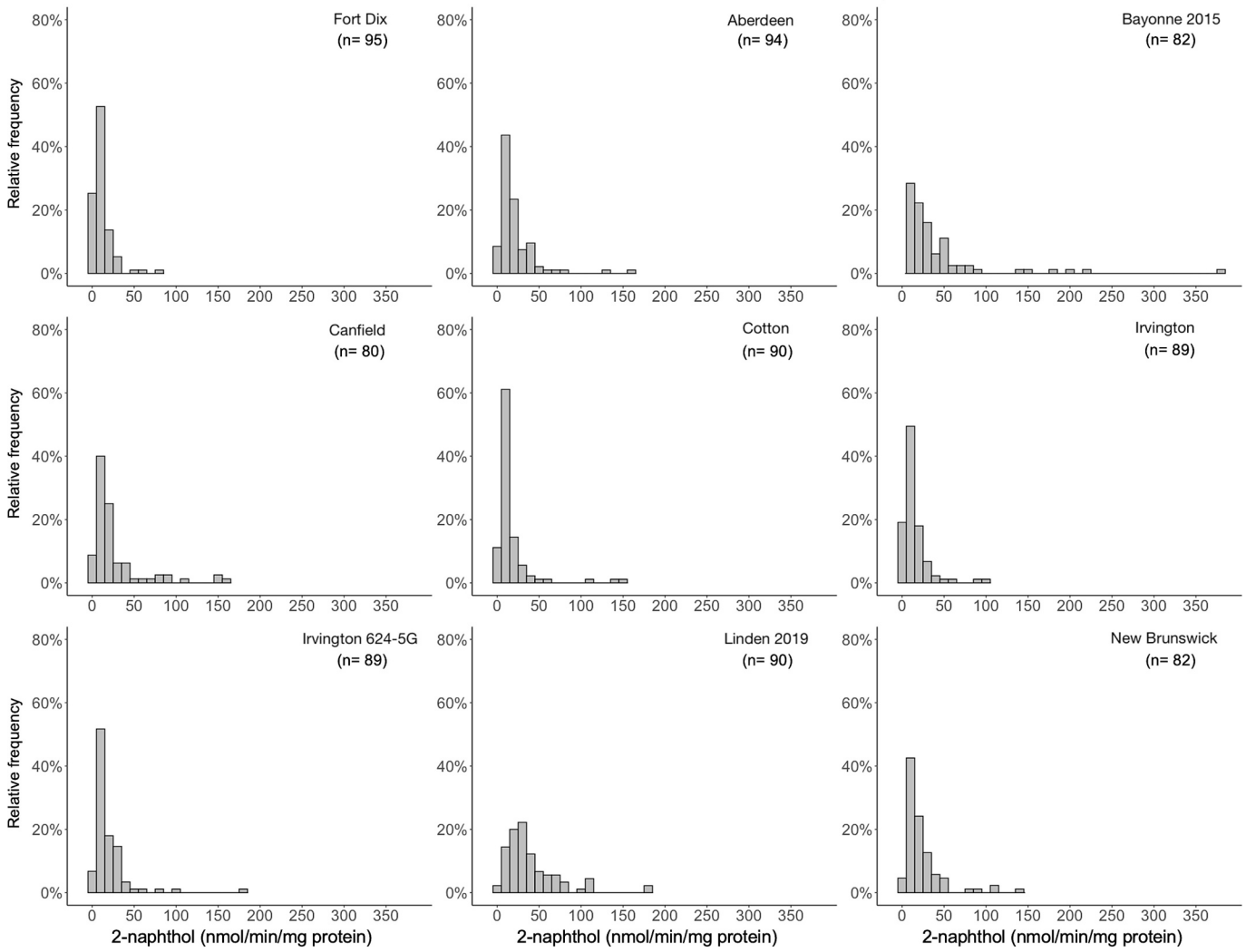

Fig. 3. The distribution of 2-naphthol esterase activity in *C. lectularius* samples from nine strains.

strains. Gaire et al. (2021) demonstrated P450s and esterases of *C. lectularius* were significantly decreased by PBO and DEF treatments. Similarly, high levels of esterase activity were found in resistant *C. hemipterus* strains (Dang et al., 2021; Karunaratne et al., 2007). Meanwhile, the synergistic effect of DEM on deltamethrin was only found in the three strains (Table 1). Among these strains, only Bayonne 2015 experienced 100 % mortality (Table 1) and had a 2.8-fold increase in GST activity (Table 2). Compared to P450s and esterase, GST has been reported as having less impact on pyrethroid resistance in *C. lectularius* (Yoon et al., 2008) and *C. hemipterus* (Soh and Veera Singham, 2021) strains. In the current study, the results demonstrated that both synergistic effect and the enzymatic activity varied among the resistant strains.

Except for the Linden 2019 strain, the field-collected *C. lectularius* in this study were documented with low resistance and complete mortality after being treated with a discriminating dose of imidacloprid (Yu et al., 2023). We demonstrate synergism of imidacloprid with PBO and DEF in the Linden 2019 strain, but the synergistic effect in other strains could not be determined because they reached 100 % mortality when treated with imidacloprid only. However, evidence of synergistic effect in these strains can be seen in the decreased mean survival time after being treated with PBO, DEF, or DEM (Table S1). To date, upregulation of detoxifying enzymes was the only mechanism attributed to neonicotinoid resistance in *C. lectularius* (Dang et al., 2017; Romero and Anderson, 2016). Toga et al. (2024) reported mutations in candidate genes related to nicotinic acetylcholine receptors in *C. lectularius*. Nevertheless, they cannot confirm neonicotinoid resistance because the mutations may not associate with neonicotinoid reception.

In the current study, most strains did not reach complete mortality when treated with synergists + deltamethrin. The results imply that other mechanisms, such as target-site insensitivity (*kdr*-type mutation), may be involved in deltamethrin resistance. The results showed that all resistant *C. lectularius* strains were detected having at least one *kdr* mutation. In particular, L925I was found at high frequencies of homozygous resistance in all strains, with over 70 % in each strain except Cotton (Table 3). Homozygous resistant V419L mutation was found at over 80 % in five out of eight strains. A high frequency of homozygous resistant L925I in *C. lectularius* strains was also documented in Australia (Dang et al., 2015). The authors detected 25 out of 33 strains with 100 % homozygous resistant L925I. However, only one strain had 100 % homozygous resistant V419L and two strains with 10–20 % heterozygous V419L. The frequency of *kdr*-related alleles is influenced by natural fitness and could decrease without insecticide application (Freeman et al., 2021; Rinkevich et al., 2007). However, the current study shows that most resistant strains still contained a high frequency of homozygous resistant V419L and L925I mutations after a long period of rearing. Dang et al. (2015) detected 30 % of a 9-y maintained *C. lectularius* strain carrying homozygous resistant L925I mutations. The stability of the *kdr* mutations suggests that our *C. lectularius* strains may have carried a high frequency of mutations at the time of collection, or that the fitness costs

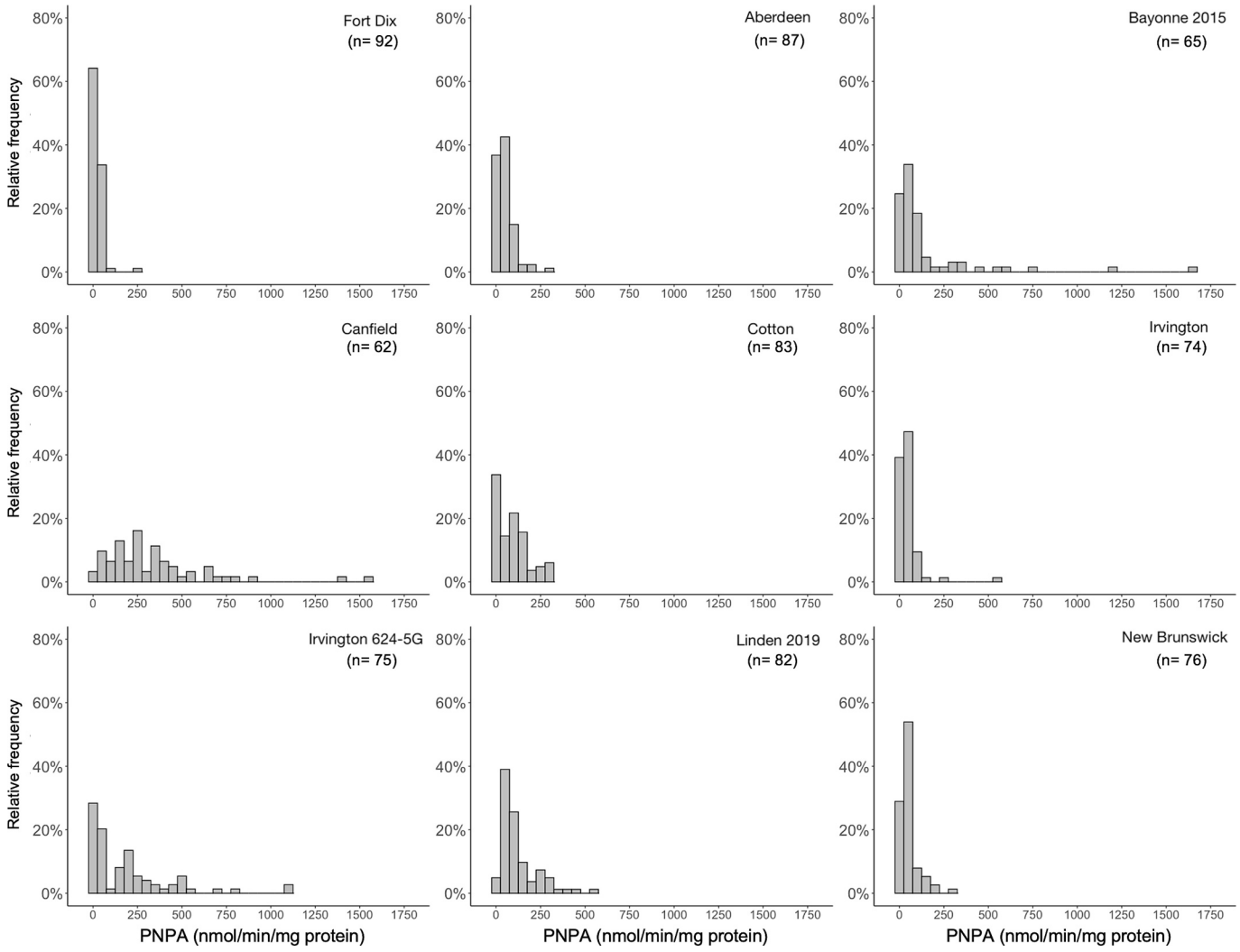

Fig. 4. The distribution of *p*-nitrophenyl (PNPA) esterase activity in *C. lectularius* samples from nine strains.

Table 3
Number of *C. lectularius* identified with V419L and L925I *kdr* mutations in the Fort Dix strain and eight resistant strains.

Strain	n	*kdr*					
		V419L			L925I		
		SS	RS	RR	SS	RS	RR
Fort Dix	10	10	0	0	10	0	0
Aberdeen	10	6	0	4	0	0	10
Bayonne 2015	10	2	1	8	0	1	9
Canfield	10	0	0	10	0	0	10
Cotton	10	7	1	3	1	6	3
Irvington	10	8	0	2	0	3	7
Irvington 624-5G	10	0	0	10	0	0	10
Linden 2019	10	0	0	10	0	0	10
New Brunswick	10	2	0	8	1	1	8

SS: Susceptible genotype.
RS: Heterozygous point mutation.
RR: Homozygous point mutation.

is not significant in our strains.

In a previous survey in the U.S., Haplotype C was found mainly in the northeastern states, in which 43 % of New Jersey strains (three out of seven strains) were reported as Haplotype C (Zhu et al., 2010). The authors pooled three bed bugs from each location to detect the *kdr* mutations. In the current study, 10 bed bugs were extracted individually to represent each strain. The result showed that Haplotype C dominated among all strains with 80–100 % occurrence in five out of eight strains (Fig. 5). The high occurrence of Haplotype C is consistent with a recent study indicating a temporal acceleration of *kdr* alleles in field *C. lectularius* strains (Lewis et al., 2023). Interestingly, Haplotype B was recorded in five out of eight strains with 10–80 % occurrence (Fig. 5). In the U.S., Haplotype B was also reported as the second dominant haplotype in previous surveys (Holleman et al., 2019; Lewis et al., 2023; Zhu et al., 2010).

None of the *C. lectularius* strains showed Haplotype D in this study. Similarly, no record of Haplotype D has been published in Australia (Dang et al., 2015), Korea (Cho et al., 2024; Seong et al., 2010), the Republic of Korea (Cho et al., 2020), Japan (Tomita et al., 2012), Israel (Palenchar et al., 2015), and Europe (Balvín and Booth, 2018; Booth et al., 2015; Durand et al., 2012; Porras-Villamil et al., 2025). Furthermore, only 10 % of both Cotton and New Brunswick strains were found with Haplotype A in this study. As pyrethroid insecticides continue to be used by residents (in the form of over-the-counter products) and pest management professionals, losing susceptible genotypes in the field is inevitable.

In conclusion, this study showed multiple mechanisms were involved in deltamethrin and imidacloprid resistance in field-collected *C. lectularius* strains. The synergist bioassays and the biochemical results demonstrate a relationship between enhanced detoxifying enzymes

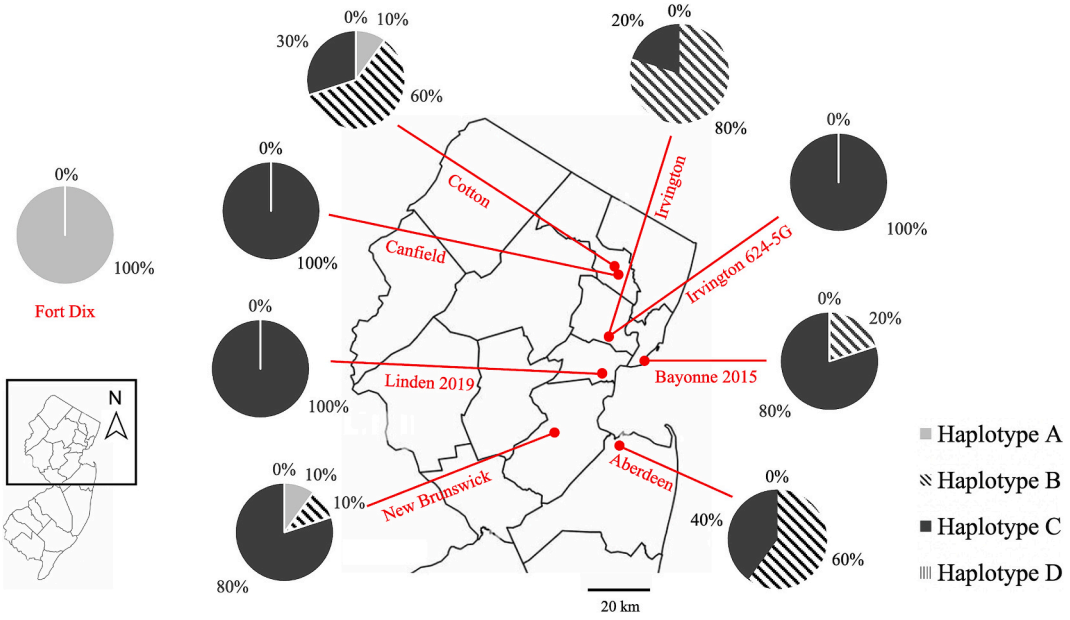

Fig. 5. Haplotypes of *kdr* mutation in one susceptible strain and eight resistant *C. lectularius* strains. Haplotype A: No mutation at both 419 and 925 aa; Haplotype B: mutation at 925 but no mutation at 419 aa; Haplotype C: mutations at both 419 and 925 aa; Haplotype D: mutation at 419 but no mutation at 925 aa.

and resistance to deltamethrin. In addition, the presence of *kdr* mutations further provides evidence of pyrethroid resistance in our *C. lectularius* strains. In the U.S., pyrethroid is the main class of active ingredient used for bed bug control (EPA, 2024). The results indicate that efficacy of pyrethroids or pyrethroid-neonicotinoid mixtures to *C. lectularius* strains in New Jersey will be reduced as a result of high levels of insecticide resistance.

CRediT authorship contribution statement

Jin-Jia Yu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shao-Hung Lee:** Writing – review & editing, Supervision, Resources, Methodology. **Chow-Yang Lee:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Changlu Wang:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2025.106357.

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