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## Short Communication

## High Levels of Resistance in the Common Bed Bug, *Cimex lectularius* (Hemiptera: Cimicidae), to Neonicotinoid Insecticides

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### Abstract

The rapid increase of bed bug populations resistant to pyrethroids demands the development of novel control tactics. Products combining pyrethroids and neonicotinoids have become very popular for bed bug control in the United States, but there are concerns about evolution of resistance to these compounds. Laboratory assays were used to measure the toxicity of topical applications of four neonicotinoids to a susceptible population and three pyrethroid-resistant populations. Activity of esterases, glutathione *S*-transferases, and cytochrome P450s of all strains was also evaluated. High levels of resistance to four neonicotinoids, acetamiprid, imidacloprid, dinotefuran, and thiamethoxam, relative to the susceptible Fort Dix population, were detected in populations collected from human dwellings in Cincinnati and Michigan. Because activity of detoxifying enzymes was increased in these two populations, our results suggest that these enzymes have some involvement in neonicotinoid resistance, but other resistance mechanisms might be involved as well. Detection of high levels of resistance to neonicotinoids further limits the options for chemical control of bed bugs.

**Key words:** bed bug, neonicotinoid, insecticide combination, insecticide resistance, detoxifying enzyme

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), is an obligate hematophagous insect that has resurged worldwide in the past 15 yr (Doggett et al. 2004, Potter 2006). Bed bugs were a part of everyday life before DDT (dichloro-diphenyl-trichloroethane) and other broad-spectrum insecticides became widely used in the 1940s and 1950s (Usinger 1966). Broad application of these insecticides effectively controlled infestations and caused bed bug populations to steeply decline for decades (Potter 2011a). However, insecticide resistance of bed bugs to DDT and other compounds were reported about a decade after they were widely used (Busvine 1958, Mallis and Miller 1964). One of the hypotheses that have been proposed to explain the sudden resurgence of bed bugs is the evolution of insecticide resistance to pyrethroids (Romero et al. 2007). Evaluations of populations from across the United States indicate that resistance to pyrethroid insecticides is widespread (Romero et al. 2007, Zhu et al. 2010). Several studies have demonstrated that some pyrethroid-resistant bed bugs have multiple resistance mechanisms including target site insensitivity (*kdr*-type), metabolic detoxifying enzymes, and cuticular penetration resistance (Yoon et al. 2008, Zhu et al. 2010, Adelman et al. 2011, Koganemaru et al. 2013).

A number of strategies have been proposed to manage pyrethroid resistance in bed bug populations. The addition of the synergist

piperonyl butoxide (PBO) to pyrethroids has been attempted to control pyrethroid-resistant bed bugs (Romero et al. 2009). The pyrrole chlorfenapyr (Phantom, BASF, Research Triangle Park, N.C.) is increasingly being used commercially, although some researcher reports a relative slow killing action and high variable efficacy of this insecticide against bed bugs (Moore and Miller 2009, Wang et al. 2009, Romero et al. 2010, Doggett et al. 2012). Insect growth regulators (IGRs) are potential alternatives for managing bed bugs, but they are also slow-acting and are generally used by the pest control industry in combination with other effective fast-acting insecticides (Goodman et al. 2013). Neonicotinoids have become the most widely used group of insecticides, with large scale application for plant and human protection (Jeschke et al. 2011). In the past years, several neonicotinoid insecticide combined with pyrethroids have been introduced in the US market for bed bug control (Potter et al. 2011b, 2012). Intensive use of these combinations by pest management professionals in the United States for bed bug control have raised concerns about the increase of pyrethroid resistance as well as the evolution of resistance to neonicotinoid compounds. The results presented here show evidence of resistance to various neonicotinoids in recently collected bed bugs with increased levels of enzymes associated with insecticide detoxification.

## Materials and Methods

### Insects

Three populations were used in this study, Jersey City (collected in 2008 in Jersey City, New Jersey) maintained in the laboratory for 5 yr, Michigan (collected in 2012 in Troy, Michigan) and Cincinnati (collected in 2012 in Cincinnati, Ohio) both maintained in the laboratory for 1 yr. The insects were maintained at 25°C, 65 ± 5% RH, and a photoperiod of 14:10 (L:D) h. These populations were determined to be resistant to deltamethrin following method proposed by Romero et al. (2007) (discriminating doses of 0.13 mg/cm<sup>2</sup> technical grade deltamethrin; 0% mortality in 20 third-to-fifth instar nymphs). A fourth population, Fort Dix (susceptible population), had not been exposed to insecticides for ~30 yr. Insects were fed in the laboratory through a parafilm-membrane feeder with defibrinated rabbit blood (Quad five, Ryegate, Montana) which was heated to 39°C with a circulating water bath (Montes et al. 2002). Evaluations began 8–10 d after adult emergence, and they had been fed as adults three days before the initiation of the experiment.

### Insecticides

All chemicals were purchased from Chem. Service (West Chester, PA). Test amounts of insecticide ranged from 1.0 × 10<sup>-5</sup> to 10 µg for acetamiprid (99% purity); from 5.0 × 10<sup>-3</sup> to 10 µg for dinotefuran (99% purity); from 1.0 × 10<sup>-3</sup> to 10 µg for imidacloprid (99% purity); and from 1.0 × 10<sup>-3</sup> to 10 µg for thiamethoxam (99% purity).

### Topical Assay

Adults (1:1 sex ratio; 60 insects) were treated topically with 1 µl of insecticide solution in acetone. Topical applications were made onto the dorsal surface of the abdomen with a microapplicator (Hamilton Co., Reno, NV) equipped with a 25-µl glass syringe (Hamilton Co.). Control insects received 1 µl of acetone alone and insects were maintained at 25°C. Mortality was assessed after 72 h by gently touching each individual with a fine paint brush and categorizing it as alive (coordinated avoidance movement) or dead (no response). Dose-mortality data were analyzed by using probit analysis (Finney 1971, Minitab Inc 2005). The resistance ratio (RR = LC<sub>50</sub> of the field population divided by LC<sub>50</sub> of the susceptible population) was calculated for each strain.

### Detoxification Enzyme Activity Assays

α-Naphthol, α-naphthyl acetate (α-NA), β-naphthol, β-naphthyl acetate (β-NA), reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), bicinchoninic acid solution (BCA), bovine serum albumin (BSA), 1-chloro-2, 4-dinitrobenzene (CDNB), 7-ethoxycoumarin (7-EC), fast blue B salt (O-dianisidine, tetrazotized), glutathione, glutathione reductase, sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO). General esterase activity was determined in adult bed bugs according to the method described by Zhu and Gao (1998), with slight modification. Each sample (*n* = 4) was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.3% (v/v) Triton X-100. After the homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C, the supernatants were used as the enzyme source for measuring general esterase activities with α-NA and β-NA as substrates. The absorbance was read using a SpectraMax M2 multimode microplate reader (Molecular Devices, Inc., Sunnyvale, CA) at 600 and 560 nm for α-NA and β-NA, respectively. Glutathione S-transferase activity was determined according to Zhu et al.

(2000), with slight modification, using CDNB as a substrate. The conjugation of glutathione toward CDNB was determined by recording the change in absorbance at 340 nm for CDNB for 1 min at 10-s intervals using a multimode microplate reader. Nonenzymatic controls were performed in parallel to correct for nonenzymatic conjugation. Cytochrome P450 monooxygenase activity was determined according to the method of Anderson and Zhu (2004), with slight modification, using 7-ethoxycoumarin (7-EC) as a substrate. The relative fluorescence units were measured using a multimode microplate reader at 465 nm while exciting at 390 nm. Total protein in each sample preparation was determined using the method of Smith et al. (1985) with BSA as a standard. The measurement was performed on a multimode microplate reader at 560 nm. Data were subjected to analysis of variance (ANOVA) using Mixed Procedure (SAS Institute 2002) and Tukey's pairwise comparison (at 5% level of significance).

## Results

### Susceptibility to Neonicotinoids

Recently collected populations Michigan and Cincinnati, maintained 1 yr in the laboratory and evaluated in 2013, were more resistant to neonicotinoids than populations that have been maintained in the laboratory for 5 yr (Jersey City) or ~30 yr (the susceptible population Fort Dix). Jersey City was susceptible to imidacloprid and thiamethoxam with only 2.0- and 2.4-fold difference in the LD<sub>50</sub>, respectively, when compared to the reference susceptible population Fort Dix (Table 1). However, Jersey City was moderately resistant to the neonicotinoids acetamiprid (RR = 31.7) and dinotefuran (RR = 46.8) (Table 1). Higher levels of resistance to neonicotinoids were observed in recently collected populations. Michigan was resistant to all neonicotinoids tested, including thiamethoxam (RR = 546.0), imidacloprid (RR = 462.6), and dinotefuran (RR = 198) (Table 1). Similarly, Cincinnati was resistant to dinotefuran (RR = 358.6), thiamethoxam (RR = 226.2), and imidacloprid (RR = 163.3) (Table 1). In both populations, there were dramatic levels of resistance to the neonicotinoid acetamiprid and the magnitude of resistance to this compound can be inferred when the susceptible Fort Dix population suffered 100% mortality at 10 ng of acetamiprid, while concentrations 1,000 times larger (1,000 ng, the highest concentration tested) only killed a small portion of recently field collected bed bugs (Cincinnati, 28.3%; Michigan, 26.7%). The resistance ratio of these two populations relative to Fort Dix was >33,333.

### Characterization of Detoxification Enzyme Activities

General esterase activities of Michigan and Jersey City, measured with α-NA as substrate, were significantly increased (by 16.7 and 22.3%, respectively) compared to the insecticide-susceptible Fort Dix (Fig. 1). Similarly, general esterase activities were significantly enhanced in all field-collected populations when β-NA was used as substrate (Cincinnati, 33.6%; Jersey City, 74.7%; Michigan 79.8%). The glutathione S-transferase activities of all populations were also significantly enhanced (Cincinnati, 12.7%; Jersey City, 20.0%; Michigan, 27.7%) compared to the insecticide-susceptible bed bug population (Fig. 1). In contrast, only the cytochrome P450 monooxygenase activity of Michigan was significantly enhanced by 19.1% when compared to the insecticide-susceptible bed bug population (Fig. 1).

**Table 1.** Log-dose probit-mortality data for a susceptible strain (Fort Dix) and three pyrethroid-resistant populations tested with several neonicotinoid insecticides

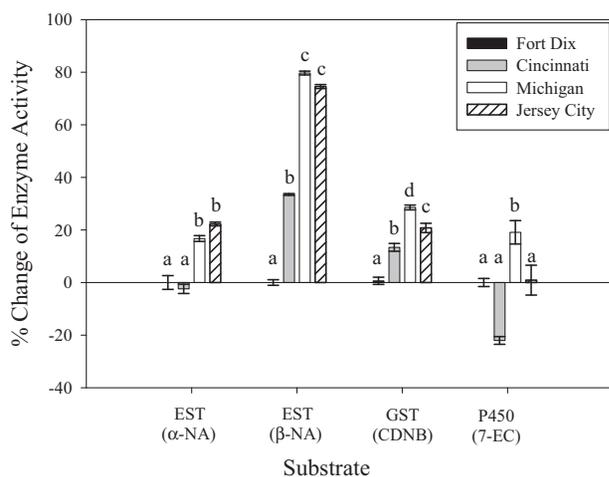
A.I.	<i>n</i> <sup>a</sup>	Population	Slope ± SE	LD <sub>50</sub> <sup>b</sup> ng (95% CI)	χ <sup>2c</sup> (df)	<i>P</i> <sup>c</sup>	RR <sup>d</sup>
Imidacloprid	60	Fort Dix	0.98 ± 0.11	2.3 (1.9–2.8)	1.54 (2)	0.46	—
	60	Jersey City	0.54 ± 0.05	4.6 (3.1–6.8)	1.44 (2)	0.48	2.0
	60	Michigan	0.70 ± 0.07	1,064.1 (788.3–1,400.0)	2.25 (2)	0.32	462.6
	60	Cincinnati	0.22 ± 0.03	375.6 (175.1–823.2)	0.62 (2)	0.73	163.3
Acetamiprid	60	Fort Dix	0.54 ± 0.06	0.3 (0.2–0.4)	4.22 (2)	0.12	—
	60	Jersey City	0.50 ± 0.04	9.5 (6.6–13.7)	6.94 (2)	0.07	31.7
	60	Michigan	0.31 ± 0.07	>10,000	1.97 (2)	0.37	>33,333
	60	Cincinnati	0.21 ± 0.04	>10,000	2.71 (2)	0.25	>33,333
Thiamethoxam	60	Fort Dix	0.92 ± 0.11	1.9 (1.5–2.3)	0.04 (2)	0.97	—
	60	Jersey City	0.81 ± 0.11	4.7 (3.8–6.2)	2.29 (2)	0.31	2.4
	60	Michigan	0.63 ± 0.07	1,037.5 (717.0–1,503.8)	1.79 (2)	0.40	546.0
	60	Cincinnati	0.27 ± 0.04	429.8 (226.5–820.8)	1.26 (2)	0.53	226.2
Dinotefuran	60	Fort Dix	0.97 ± 0.09	14.5 (11.7–17.7)	3.52 (2)	0.17	—
	60	Jersey City	0.41 ± 0.05	679.6 (440.1–1,034.3)	0.87 (2)	0.64	46.8
	60	Michigan	0.51 ± 0.06	2,872.3 (1,973.9–4,596.5)	5.23 (2)	0.07	198.0
	60	Cincinnati	0.43 ± 0.06	5,200.0 (3,122.4–10,268.3)	3.88 (2)	0.14	358.6

<sup>a</sup> Total number of insects used.

<sup>b</sup> LD<sub>50</sub> in ng per insect 72 h post-treatment.

<sup>c</sup> Larger values of χ<sup>2</sup> for goodness-of-fit and *P* value < 0.05 indicate a poorer fit on the probit regression line.

<sup>d</sup> Resistance Ratio: LD<sub>50</sub> of resistant population/LD<sub>50</sub> of susceptible population (Fort Dix).



**Fig. 1.** Comparison of general esterase (EST), glutathione *S*-transferase (GST), and cytochrome P450 monooxygenase (P450) activities of insecticide-susceptible bed bugs (Fort Dix) and three pyrethroid- and neonicotinoid-resistant bed bugs. Bars indicate percentage change in enzyme activity of resistant bed bug population relative to the susceptible strain Fort-Dix. Vertical bars indicate standard errors of the mean (*n* = 4). The bars within substrates with the same letter are not significantly different from one another (ANOVA, *P* > 0.05).

## Discussion

Our study is the first report of resistance in bed bugs to the neonicotinoids acetamiprid, imidacloprid, and thiomethoxam, active ingredients of various pyrethroid–neonicotinoid combination products currently registered for bed bug control in the United States. We also detected resistance to dinotefuran, a neonicotinoid that is used in combination with diatomaceous earth (Alpine Dust Insecticide, BASF, Research Triangle Park, N.C.). Jersey City showed susceptibility to imidacloprid and thiamethoxam, and moderate levels of resistance to acetamiprid and dinotefuran. Jersey City is a pyrethroid-resistant population that has been maintained in the laboratory for five years without insecticide exposure since it was collected in 2008, before the use of insecticide

combinations containing neonicotinoids against bed bugs in the United States. Therefore, resistance mechanisms of Jersey City that confer resistance to previously used insecticides (e.g. pyrethroids) might also be conferring resistance to neonicotinoids. Pre-existing insecticide resistance mechanisms in bed bug populations could jeopardize the effectiveness of neonicotinoids or new active ingredients.

Two populations of bed bugs that were collected in 2012, the pyrethroid-resistant Cincinnati and Michigan, were resistant in various degrees to all neonicotinoids, with resistance ratios ranging from 163.3 to >33,333. This variation in resistance among populations could be due to the background of detoxifying enzymes induced by previously used insecticides, different metabolic pathways that each neonicotinoid is subjected to, the type of resistance mechanisms involved in each strain, and intensive selection with neonicotinoids (Rauch and Nauen 2003, Jeschke and Nauen 2008). Neonicotinoid resistance in our bed bugs could be associated with selection pressure caused by the continuous use of products containing neonicotinoids in the last years. In general, neonicotinoid resistance has been attributed to mutations in nicotinic acetylcholine receptor (*nAChR*) subunits which alter the binding ability of neonicotinoid compounds to their target site (Liu et al. 2015). In order to know if target-site mutations account for neonicotinoid resistance in our bed bug populations, comparison of nucleotide sequences from a susceptible population needs to be accomplished. Additionally, ligand competition experiments that evaluate the binding affinity of neonicotinoids to nicotinic acetylcholine receptors would confirm that target site insensitivity plays a role in neonicotinoid resistance (Rauch and Nauen 2003, Liu et al. 2015).

Dramatic levels of resistance in Michigan and Cincinnati to acetamiprid (>33,333) could be associated with either the presence of multiple mechanisms of resistance and/or intense enzyme-mediated metabolism of acetamiprid (Thany 2010). Neonicotinoids are metabolized by detoxifying enzymes in several insect species (Nauen and Denholm 2005, Simon-Delso et al. 2015). A more rapid enzymatic biotransformation of acetamiprid, revealed by the production of at least five metabolites with reduced affinity to the *nAChRs* (Simon-Delso et al. 2015), might explain the high levels of resistance to this neonicotinoid detected in our study (RR > 33,333).

Interestingly, both Michigan and Cincinnati were also resistant to thiamethoxam and dinotefuran, two active ingredients that had not been available for bed bug control at the time these populations were collected from the field. These results indicate that cross resistance among neonicotinoids might occur, but further research is needed to clarify this phenomenon. While analysis of the enzymatic profile showed increased activities of general esterases, glutathione S-transferases in all neonicotinoid-resistant populations, relative to the insecticide-susceptible Fort Dix, activity of cytochrome P450s was only increased in the Michigan populations. The increased levels of detoxifying enzymes in neonicotinoid-resistant populations indicate that these enzymes might be involved in detoxifying neonicotinoid insecticides. Genes of these enzymes are generally overexpressed in insecticide-resistant bed bugs, suggesting their involvement in metabolic resistance (Adelman et al. 2011, Bai et al. 2011). However, the high resistance ratios calculated for Cincinnati and Michigan implies that target site resistance or other resistance mechanisms are involved in neonicotinoid resistance.

Future investigations might include the sequencing of nAChRs to determine the presence or absence of target-site resistance to neonicotinoids, and if present, the extents of this phenomenon in bed bug populations. Similarly, detailed studies with insecticide synergists will determine the relative importance of detoxifying enzymes in neonicotinoid resistance. The frequency and the relative importance of such resistance mechanisms will determine the type of strategies for the management of neonicotinoid resistance in bed bugs.

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