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Bed bugs, *Cimex lectularius* L., exhibiting metabolic and target site deltamethrin resistance are susceptible to plant essential oils

Sudip Gaire[a,b,**], Cari D. Lewis[c], Warren Booth[c], Michael E. Scharf[a], Wei Zheng[d], Matthew D. Ginzel[a], Ameya D. Gondhalekar[a,*]

[a] *Department of Entomology, Purdue University, West Lafayette, IN 47907, USA*
[b] *Current address: Department of Entomology, University of Kentucky, Lexington, KY 40546, USA*
[c] *Department of Biological Science, The University of Tulsa, Tulsa, OK 74104, USA*
[d] *School of Health Sciences, Purdue University, West Lafayette, IN 47907, USA*

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ABSTRACT

Pyrethroid resistance has been a major hurdle limiting the effective control of bed bugs (*Cimex lectularius* L.). Alternative approaches that include the use of plant essential oils (EOs) have been proposed for effective management of bed bugs. However, EO resistance level comparisons between pyrethroid susceptible and resistant bed bug populations have not been previously conducted. The goal of this study was twofold: (i) determine deltamethrin resistance levels and associated resistance mechanisms in the field-collected Knoxville strain and (ii) quantify resistance levels of the Knoxville strain to five EOs (thyme, oregano, clove, geranium and coriander), their major insecticidal constituents (thymol, carvacrol, eugenol, geraniol and linalool) and an EO-based product (EcoRaider®). First, we found that the Knoxville strain was 72,893 and 291,626 fold more resistant to topically applied deltamethrin in comparison to the susceptible Harlan strain at the LD_{25} and LD_{50} lethal dose levels, respectively. Synergist bioassays and detoxification enzyme assays revealed significantly higher activity of cytochrome P450 and esterase enzymes in the resistant Knoxville strain. Further, Sanger sequencing revealed the presence of the L925I mutation in the voltage-sensitive sodium channel α subunit gene. The Knoxville strain that possesses both enzymatic and target site deltamethrin resistance, however, did not show any resistance to EOs, their major insecticidal constituents and EcoRaider® in topical bioassays (resistance ratio of ~1). In conclusion, this study demonstrated that a deltamethrin-resistant strain of bed bugs is susceptible to EOs and their insecticidal constituents.

1. Introduction

Resistance to pyrethroids (e.g., deltamethrin, beta-cyfluthrin and d-allethrin) has been documented in two species of bed bugs, *Cimex lectularius* L. (bed bugs) and *C. hemipterus* F. (tropical bed bugs) (Karunaratne et al., 2007; Romero et al., 2007; Adelman et al., 2011; Zhu et al., 2013; Gonzalez-Morales and Romero, 2019). The highest deltamethrin (a type II pyrethroid class insecticide) resistance ratio reported in *C. lectularius* was 20,000-fold (Gonzalez-Morales and Romero, 2019), whereas in *C. hemipterus* it was 370,000-fold (Lilly et al., 2015). Populations of both bed bug species may possess multiple mechanisms that confer resistance to deltamethrin, including the elevation of detoxification enzyme activity (cytochrome P450s, esterases and glutathione transferases), knockdown resistance (*kdr*-type) associated point mutations in the voltage-sensitive sodium channel α subunit gene, and reduced cuticular penetration (Yoon et al., 2008; Zhu et al., 2010a; Adelman et al., 2011; Zhu et al., 2013; Dang et al., 2015; Lilly et al., 2016a).

Given the array of mechanisms by which pyrethroid insecticides may fail to control *C. lectularius* infestations, alternative management strategies are required for their control, which include treatments involving heat (Kells and Goblirsch, 2011; Ashbrook et al., 2019), cold (Olson et al., 2013), steam (Puckett et al., 2013; Wang et al., 2018), desiccant dusts (Romero et al., 2009b), carbon dioxide or dry ice (Singh et al., 2013), insecticide-treated mattress encasements (Jones et al., 2015), fumigants (Lehnert et al., 2011; Phillips et al., 2014; Feston

* Correspondence to: A. D. Gondhalekar, Center for Urban and Industrial Pest Management, Department of Entomology, Purdue University, 901 W. State St., West Lafayette, IN 47907, USA.
** Correspondence to: S. Gaire, Department of Entomology, University of Kentucky, S-205 Ag. Science Center North Lexington, KY 40546, USA.
E-mail addresses: sgaire@uky.edu (S. Gaire), ameyag@purdue.edu (A.D. Gondhalekar).

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et al., 2020), and plant essential oils or EOs (Singh et al., 2014; Zha et al., 2018; Gaire et al., 2019, 2020). The public demand for EO-based or natural products is currently on the rise for the control of several insect pests, including bed bugs (Regnault-Roger et al., 2012; Singh et al., 2014; Isman, 2020).

EOs are extracts from aromatic plants that contain several insecticidal constituents (also referred as EO components or monoterpenoids), with various functional groups such as phenol, ketone, aldehyde, and alcohol (Guenther, 1949). Among the different functional groups, phenolic EO constituents such as thymol, eugenol and carvacrol exhibit superior toxicity against different urban pests including bed bugs, cockroaches, and kissing bugs (Phillips et al., 2010; Moretti et al., 2013; Gaire et al., 2017, 2019; Oladipupo et al., 2020). More than 20 EOs and their constituents are considered low risk insecticides and are exempt from EPA (Environmental Protection Agency) registration (https://www.epa.gov/minimum-risk-pesticides). Because of this low risk status, many EO-based products targeted for bed bug and/or general urban insect pest control are available in the market (Singh et al., 2014; Isman, 2020). Some of the efficacious EO-based products (e.g., EcoRaider® and Bed Bug Patrol®) tested by Singh et al. (2014) were effective in controlling field-collected *C. lectularius* (Indy strain) that were presumptively resistant to pyrethroid-containing insecticide products. Zha et al. (2018) later found that 18 pure EOs exhibited topical toxicity against the same bed bug strain used by Singh et al. (2014). However, because Singh et al. (2014) and Zha et al. (2018) did not use an insecticide susceptible bed bug strain in their experiments, firm conclusions on comparative susceptibility of field-collected bed bugs to EOs, their insecticidal constituents and EO-based products could not be drawn. Furthermore, when used as stand-alone treatments in bed bug infested apartments, EcoRaider® and one of the neonicotinoid and pyrethroid mixture insecticide provided equivalent efficacy (> 90% reduction in bed bug count); however, complete bed bug elimination was not achieved in most of the apartments (Wang et al., 2014).

Several EOs and their constituents possess contact and fumigant activity against insecticide susceptible strains of *C. lectularius* (Feldlaufer and Ulrich, 2015; Gaire et al., 2019, 2020). However, as explained above, none of the previous studies have compared EO resistance levels between pyrethroid susceptible and resistant bed bugs. Generating data to bridge this knowledge gap is important for confirming the susceptibility of field-collected or pyrethroid resistant bed bugs to various EOs, as well as informing the development of new and effective EO-based formulations for use in bed bug and urban IPM. Therefore, the goals of this study were to (i) determine deltamethrin resistance levels and mechanisms in a field-collected *C. lectularius* strain (Knoxville), and (ii) assess resistance levels to EOs, their major constituents and a commercial EO product in the Knoxville strain.

2. Materials and methods

2.1. Bed bug strains

Experiments were performed on two strains of *C. lectularius*, the Harlan laboratory strain, and the field-collected Knoxville strain. The Harlan strain was collected from the field in 1973 and is susceptible to all insecticides, including pyrethroids (Doggett et al., 2018). As such, the Harlan strain was used as a baseline susceptible strain for all bioassay and enzyme activity determination experiments. Additionally, it has been previously shown to lack the pyrethroid resistance-associated *kdr*-type target site mutations (Adelman et al., 2011). The Knoxville strain was collected from apartments in Knoxville, TN in 2013 and has a history of exposure to chlorfenapyr (a pyrrole insecticide) and imidacloprid (a neonicotinoid insecticide)/beta-cyfluthrin (a pyrethroid insecticide) treatments prior to its collection (Ashbrook et al., 2017). Furthermore, the Knoxville strain showed reduced susceptibility to bifenthrin (a type I pyrethroid insecticide) containing products (Ashbrook et al., 2017). The Knoxville strain was chosen for this study because it exhibited the highest level of deltamethrin-resistance among six field strains in preliminary topical application bioassays (Fig. S1). Strains were maintained at 25 °C, 50 ± 15% relative humidity, and a photoperiod of 12:12 h (L: D) in environmental chambers (Percival Scientific, Perry, IA). Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using the membrane feeding method (Chin-Heady et al., 2013).

2.2. Chemicals

Deltamethrin (purity 99.3%) was obtained from Chem Service Inc. (West Chester, PA). The insecticide synergists piperonyl butoxide (PBO), *S,S,S*-tributyl phosphorotrithioate (DEF) and diethyl maleate (DEM) were purchased from Sigma-Aldrich (St. Louis, MO). The solvent carrier acetone (analytical grade) and the surfactant Triton X 100 were purchased from Fisher Scientific (Hampton, NH). Substrates and reagents used in enzyme assays were procured either from Sigma-Aldrich or Fisher Scientific.

Five pure EOs: thyme oil (*Thymus vulgaris* L.), oregano oil (*Origanum vulgare* L.), clove oil (*Eugenia caryophyllata* Thunb.), coriander oil (*Coriandrum sativum* L.) and geranium oil (*Pelargonium graveolens* L'Heritier) were purchased from Frontier Natural Products (Norway, IA). The EO constituents that are known to possess insecticidal activity; carvacrol, thymol, eugenol, geraniol and linalool were purchased either from Sigma-Aldrich or from Alfa Aesar (Haverhill, MA). The EO-containing commercial product EcoRaider® (active ingredients: geraniol (1%), cedar extract (1%), and sodium lauryl sulfate (2%)) was purchased from Reneotech, Inc. (North Bergen, NJ).

2.3. Deltamethrin topical application bioassays

For toxicity evaluation, 7–10 days old adult males were used. These insects were fed defibrinated rabbit blood 3 days before performing bioassays. Identical feeding status of experimental insects was maintained for all topical application bioassays to ensure that they had similar body weights. Topical application bioassays followed methods outlined in Gaire et al. (2019, 2020). In brief, deltamethrin was serially diluted in acetone to prepare a range of more than five dilutions (Table S1). Topical applications of different concentrations (volume range 0.5–1 μL) were made on the ventral metathorax using a 25 μL microsyringe attached to a PB-600-1 repeating dispenser (Hamilton, Reno, NV). Insects were immobilized by attaching them dorsally to the labeling tape (Fisher Scientific, Pittsburg, PA) during topical treatments (Gaire et al., 2019, 2020). Control groups were treated with acetone only. After treatment, insects (in groups of 10) were held in 35 × 10 mm Petri dishes (Greiner Bio-One, Frickenhausen, Germany) and placed in an environmental chamber under conditions identical to those used for rearing. Mortality scoring for all treatments was performed 24 h post-treatment. Insects that were lying on their backs and/or were unable to move upon prodding were scored as dead. In total, three replicates were performed for each concentration ($n = 30$). The deltamethrin dose-mortality data generated for the Harlan and Knoxville adult males were subjected to probit analysis in SAS version 9.4 (SAS Institute, Cary, NC) to determine the lethal dose (LD) values (Finney, 1971). The topical LD values are reported as μg/mg body weight based on the average mass of each strain (Harlan average weight = 5.35 mg per insect; Knoxville average weight = 3.81 mg per insect). Resistance ratios were calculated by dividing the deltamethrin LD_{25} or LD_{50} values for the Knoxville strain by LD_{25} or LD_{50} value for the Harlan strain.

2.4. Synergist bioassays

PBO, DEF and DEM are known synergists that inhibit detoxifying enzymes; specifically, cytochrome P450s, esterases, and glutathione

transferases, respectively (Bernard and Philogene, 1993). All three synergists were diluted individually in acetone to prepare a 100 mg/mL concentration (Gonzalez-Morales and Romero, 2019). They were topically applied to bed bugs (0.5 µL or 50 µg per insect) following the method described in section 2.3. Two hours after synergist or acetone application, insects of each strain (Harlan and Knoxville) were topically treated with their respective LD_{25} dose of deltamethrin (0.5 µL volume). Control bed bugs that were pre-treated with 0.5 µL of acetone or synergists received a second topical treatment (0.5 µL) of acetone to ensure that the application of either two acetone treatments (i.e., acetone control) or synergist followed by acetone (i.e., synergist control) did not cause mortality. Mortality was scored after 24 h as previously described. Six replicates were performed for each treatment (10 insects per rep, $n = 60$). Independent samples *t*-test were performed to compare the effects of synergist pre-treatment on deltamethrin toxicity in both *C. lectularius* strains using SPSS Version 25 (Armonk, NY). Figures were created using the SigmaPlot software (Version 10.10; Systat Software, Inc., San Jose, CA).

2.5. Detoxification enzyme assays

2.5.1. Protein preparations

Ten adult male bed bugs (10–15 days old) that were starved (i.e., not fed rabbit blood) post-eclosion to adulthood, were homogenized in 1 mL ice-cold 0.1 M sodium phosphate buffer (pH = 7.0). Using starved bed bugs for protein extractions ensured that they did not have undigested rabbit blood in their gut, which could have interfered with colorimetric and fluorescent enzyme assays. The homogenization buffer used for measuring cytochrome P450 activity also contained 0.3% Triton (vol./vol. basis) (Yoon et al., 2008; Romero and Anderson, 2016). Next, the insect homogenate was centrifuged at 10,000*g* for 20 min at 4 °C in a 5424 R centrifuge (Eppendorf North America, Hauppauge, NY). Resulting supernatants were used as the enzyme source for measuring detoxification enzyme activity. Bradford assays were performed to measure the protein concentration of each sample using bovine serum albumin (BSA) as a standard (Bradford, 1976). Protein concentration was measured in a PowerWave 340 spectrophotometer (BioTek Instruments Inc., Winooski, VT) at 595 nm.

2.5.2. Cytochrome P450 activity

The 7-ethoxycoumarin (7-EC) O-deethylase (ECOD) activity was determined according to the method described by Anderson and Zhu (2004), Yoon et al. (2008) and Romero and Anderson (2016) with some modifications. Assays were conducted in black walled 96-well micro plates (Corning Inc., Corning, NY) to prevent cross talk between wells. Reaction mixture (120 µL) in each well included protein obtained from the Harlan or Knoxville strains (40 µL), 7-EC (50 mM) and the co-factor, reduced NADPH (β-nicotinamide dinucleotide phosphate; 62.5 mM). For control reactions, protein was replaced by an equal volume of sodium phosphate buffer (Stumpf and Nauen, 2001). Relative fluorescence units were measured using a Spectramax m2e instrument (Molecular Devices, LLC, San Jose, CA) at an emission wavelength of 460 nm and excitation wavelength of 380 nm (Valles and Yu, 1996). The extinction coefficient for the end product, 7-hydroxycoumarin (4.44 M^{-1} cm^{-1}), was used for calculating specific activity, which was expressed as nmol/min/mg protein (Fang et al., 1997). Four replicates were performed for each *C. lectularius* strain. An independent samples *t*-test was used to compare the cytochrome P450 activity between strains. All figures were drawn with SigmaPlot Version 10.10 (Systat Software, Inc., San Jose, CA).

2.5.3. Esterase activity

Esterase activity was measured using para-nitrophenyl acetate (pNPA) as a substrate following Wu et al. (1998). Initially, the reaction mixture was prepared by adding 50 µL of pNPA (0.2 M in acetonitrile) in 10 mL sodium phosphate buffer (pH = 7.0). Assays were conducted in clear 96-well microplates (Corning Inc., Corning, NY). Each treatment well received 10 µL of protein from either Harlan or Knoxville bed bugs and the same volume of sodium phosphate buffer was used in control or blank wells. Reactions were initiated by adding 225 µL of sodium phosphate buffer containing 1 mM pNPA to all wells. Immediately thereafter reactions were monitored at a wavelength of 405 nm every 20 s for 5 min in a PowerWave 340 spectrophotometer. The extinction coefficient for the end product p-nitrophenol (6.53 mM^{-1} cm^{-1}) was used for calculating specific activity, which was expressed as nmol/min/mg protein (Wu et al., 1998). Six replicates were performed for each *C. lectularius* strain and, an independent samples *t*-test was used to determine differences in activity between the two strains.

2.5.4. Glutathione transferase activity

Glutathione transferase activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate following Wu et al. (1998). Assays were conducted in clear 96-well microplates. First, bed bug protein samples (10 µL) from either the Harlan or Knoxville strains were added to both treatment and control wells. Next, two reaction mixtures were freshly prepared in 10 mL sodium phosphate buffer (pH 7.0). The first mixture (reaction mix 1) contained 5 mM reduced glutathione and 1 mM CDNB, whereas the second mix (reaction mix 2) contained everything except the co-factor reduced glutathione. Reaction mix 1 (225 µL) was then added to all treatment wells and reaction mix 2 (225 µL) was added to control wells. Reactions were monitored every 20 s for 5 min at 344 nm wavelength in a PowerWave 340 spectrophotometer. The extinction coefficient of 9.5 mM^{-1} cm^{1} for the end product, *S*-(2,4-dintrophenyl) glutathione, was used for calculating specific activity (nmol/min/mg protein) (Wu et al., 1998). Six replicates were performed for each *C. lectularius* strain. Statistical differences in enzyme activity between strains were determined by performing an independent samples *t*-test.

2.6. DNA extraction and voltage-sensitive sodium channel mutation detection

Genomic DNA was extracted from 10 specimens per bed bug strain using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). DNA was stored at −20 °C until use. PCR amplification of two genomic fragments of the voltage-sensitive sodium channel α subunit gene, previously shown to possess *kdr*-type mutations (Yoon et al., 2008; Dang et al., 2015) was performed using primer combinations BBParaF1/BBParaR1 (V419L) and BBParaF3/BBParaR3 (L925I, I936F) (Zhu et al., 2010a). PCR conditions used for amplification of genomic fragments were identical to those described by Zhu et al. (2010a). PCR products were purified using Exo-SAP-IT (Affymetrix Inc., Santa Clara, CA) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Each fragment was unidirectionally sequenced, as the mutation sites were positioned such that base calling was unambiguous. Primers BBparaF1 sequenced the V419L region, and BBparaR3 sequenced the L925I and I936F regions. Sequencing was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and the resulting chromatograms visualized using CLC Genomic Workbench (https://www.qiagenbioinformatics.com). The presence or absence of mutations was scored visually.

Individuals were identified as susceptible or resistant following Yoon et al. (2008) and Dang et al. (2015). Specifically, V419L – <u>G</u>TC = valine, <u>C</u>TC = leucine; L925I – <u>C</u>TT = leucine, <u>A</u>TT = isoleucine; I936F – <u>A</u>TT = isoleucine, <u>T</u>TT = phenylalanine. For each, the former amino acid represents the wild type (susceptible) state and the latter the mutant (resistant) state. Heterozygotes were identified by overlapping peaks at the respective position. Haplotype designations followed the methods of Zhu et al. (2010a) and Balvin and Booth et al. (2018).

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2.7. Assessment of resistance to EOs, their insecticidal constituents and EcoRaider® in the Knoxville strain

Thymol, carvacrol, eugenol, geraniol and linalool were the most toxic EO constituents against the insecticide susceptible Harlan strain in a previous study (Gaire et al., 2019). The bioactivity of these five constituents were tested against the Knoxville strain by conducting topical dose-response bioassays and compared with previous data for the Harlan strain (Gaire et al., 2019). Topical toxicity of five EOs (thyme, oregano, clove, geranium and coriander) was also determined against both Harlan and Knoxville strains by conducting dose-response bioassays. These five EOs were selected because they are known to contain a high abundance of above mentioned insecticidal constituents (i.e., thymol, carvacrol, eugenol, linalool and geraniol; https://phytochem.nal.usda.gov/phytochem/search) that are most toxic to the Harlan strain (Gaire et al., 2019). The chemical composition of the five EOs used in this study were also analyzed using gas chromatography–mass spectrometry (GC–MS) according to Gaire et al. (2017) with slight modification to re-verify that the respective insecticidal constituents were present in high abundance (Table S2; refer to footnotes of this table for GC–MS methods). Lastly, an EO product (EcoRaider®) was also evaluated in topical dose-response bioassays against both strains. This product was chosen because it was previously shown to be efficacious against bed bugs in both laboratory and field studies (Singh et al., 2014; Wang et al., 2014; Zha et al., 2018). Topical application of EOs, their major constituents and EcoRaider®, and all data analysis methods (e.g., probit analysis and resistance ratio determination) were conducted as described for deltamethrin topical bioassays in section 2.3.

3. Results

3.1. Deltamethrin resistance levels in the Knoxville strain

The field-collected Knoxville strain was 72,893, and 291,626-fold resistant to topically applied deltamethrin in comparison to the susceptible Harlan strain, at the LD_{25} and LD_{50} levels, respectively (Table 1). Due to the high level of deltamethrin resistance in the Knoxville strain, mortality achieved with the highest dose (300 μg/insect) of deltamethrin was only 30%. Therefore, the probit estimated LD_{25} value and corresponding resistance ratios are relatively more accurate than the LD_{50} value and its associated resistance ratios. Bed bug mortality in the control treatments was less than 6% in both strains.

3.2. Effects of synergists on deltamethrin toxicity

In both strains, less than 5% control mortality was observed following treatment with acetone or a synergist alone (PBO, DEF and DEM). However, in the insecticide-treated groups, pretreatment with the synergists PBO and DEF significantly increased deltamethrin toxicity (> 90% mortality at the LD_{25} dose) in the resistant Knoxville strain in comparison to the deltamethrin-only treatment ($P < .01$, independent samples *t*-test) (Fig. 1). Pretreatment with DEM did not cause an increase in toxicity of deltamethrin in the Knoxville strain ($P > .05$, independent samples *t*-test) (Fig. 1). In the Harlan strain, pretreatment with PBO significantly decreased deltamethrin toxicity ($P < .05$, independent samples *t*-test, Fig. 1), which represented an antagonistic effect. Neither DEF nor DEM caused a statistically significant change in deltamethrin toxicity in the Harlan strain ($P > .05$, independent samples *t*-test) (Fig. 1).

3.3. Detoxification enzyme activity

The cytochrome P450 ECOD activity in the deltamethrin resistant Knoxville strain was significantly higher (~3.9-fold) compared to the susceptible Harlan strain ($P < .01$, independent samples *t*-test) (Fig. 2). The pNPA hydrolysis esterase activity was ~1.5-fold significantly higher in the Knoxville strain than in Harlan ($P < .01$, independent samples *t*-test) (Fig. 2). Lastly, glutathione transferase CDNB conjugation activity was also significantly higher (~1.25-fold) in the Knoxville strain relative to the Harlan strain ($P < .05$, independent samples *t*-test) (Fig. 2).

3.4. Pyrethroid resistance-associated mutation frequency in the voltage-sensitive sodium channel

Unambiguous gene sequences were produced for both amplified fragments of the sodium channel gene through Sanger sequencing. As expected, analysis of these gene fragments revealed only haplotype A (100% frequency of genotype SS) (susceptible at both V419L and L925I *kdr*-type mutation sites) in the deltamethrin susceptible Harlan Strain (Table 2). In contrast, the deltamethrin resistant Knoxville strain exhibited haplotypes A and B (susceptible at V419L, resistant at L925I) (Table 2). Specifically, for the L925I mutation site, 10% of the Knoxville population or sequenced individuals represented a susceptible (SS) genotype, 30% of the bed bugs were heterozygous (RS) and 60% were homozygous (RR) (Table 2). In both populations, all samples were susceptible for the I936F mutation (100% SS frequency) (Table 2).

3.5. Resistance to EOs, their insecticidal constituents and EcoRaider® in the deltamethrin-resistant Knoxville strain

Treatment of the deltamethrin resistant Knoxville strain with five different EOs and EcoRaider® revealed no evidence of resistance in comparison to the susceptible Harlan strain at the LD_{25} and LD_{50} levels (resistance ratio range 0.34 to 1.37) (Table 3). For the Knoxville strain, EOs with descending order of toxicity were as follows, thyme > oregano > clove > coriander > geranium > EcoRaider® (Table S3; Robertson et al., 2007). In the Harlan strain, thyme and oregano oils were equally active followed by coriander > clove > EcoRaider® > geranium (Table S3; Robertson et al., 2007). With respect to EO constituents, carvacrol, eugenol, and thymol were equally active against

Table 1
Resistance to deltamethrin in the field-collected Knoxville strain in comparison to the susceptible Harlan strain.

Strains	N	LD_{25}[I], μg/mg body weight (FL95%)[II]	LD_{50}[I], μg/mg body weight (FL95%)[II]	Slope ± SE	χ^2	Df	*P* value	LD_{25} resistance ratio[III]	LD_{50} resistance ratio[III]
Harlan	270	5.36×10^{-5} (2.24×10^{-5}–8.41×10^{-5})	9.90×10^{-5} (5.79×10^{-5}–1.72×10^{-4})	2.51 ± 0.51	20.73	6	0.002	–	–
Knoxville	180	3.91 (ND)[IV]	288.71 (ND)[IV]	0.36 ± 0.20	2.383	3	0.496	72,893	291,626

[I] LD_{25} and LD_{50} = lethal dose necessary to kill 25% and 50% of individuals, respectively.
[II] FL = 95% fiducial limits.
[III] Resistance ratio was calculated by dividing the LD_{25} or LD_{50} value of deltamethrin for Knoxville by LD_{25} or LD_{50} value of deltamethrin for Harlan. LD_{25} resistance ratio is relatively more accurate than the LD_{50} resistance ratio in this case because > 30% mortality was never observed in the Knoxville strain even when using the highest possible dilution or dose of deltamethrin (i.e., 300 μg/insect).
[IV] The acronym ND in parenthesis next to the deltamethrin LD_{25} and LD_{50} explains that 95% FLs were "not determinable".

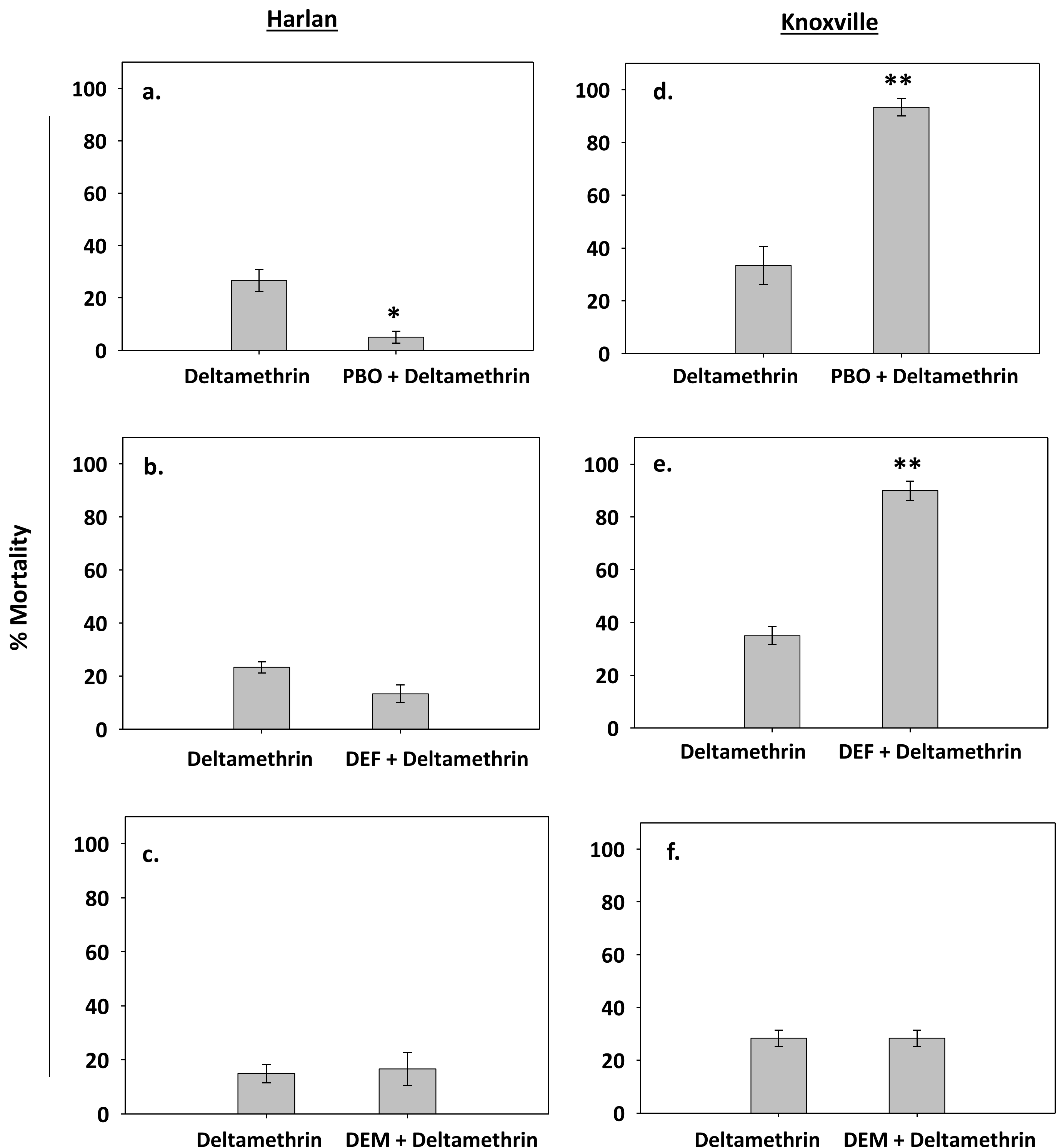

Fig. 1. Effects of synergist on deltamethrin toxicity at respective LD_{25} doses for the susceptible Harlan and resistant Knoxville strains. (a-c) There was an antagonistic effect of PBO pretreatment on deltamethrin toxicity in the Harlan strain. However, DEF and DEM pre-application did not cause significant change in deltamethrin toxicity. (d-f) PBO and DEF significantly increased toxicity of deltamethrin in the resistant Knoxville strain, however, the effect of DEM was non-significant. Double asterisk (**) indicates statistical significance at $P < .01$ and single asterisk (*) indicates significance at $P < .05$ (independent samples *t*-test).

the Knoxville strain, followed by geraniol and linalool (Table S4; Robertson et al., 2007). Further comparison of the EO constituent (e.g., thymol, carvacrol, eugenol, linalool and geraniol) LD_{25} and LD_{50} estimate data for the Knoxville strain with previously determined LD_{25} and LD_{50} values for the Harlan strain (Gaire et al., 2019) revealed resistance ratios close to 1 (Table 4). The mortality in the control group (acetone-treated) was less than 6%.

4. Discussion

This study revealed the presence of high-level deltamethrin resistance, influenced by both metabolic and target-site mechanisms, in the field-collected Knoxville strain. However, this strain exhibited susceptibility to various EOs, their major insecticidal constituents, and the EO-based product EcoRaider®. In the following subsections, findings on

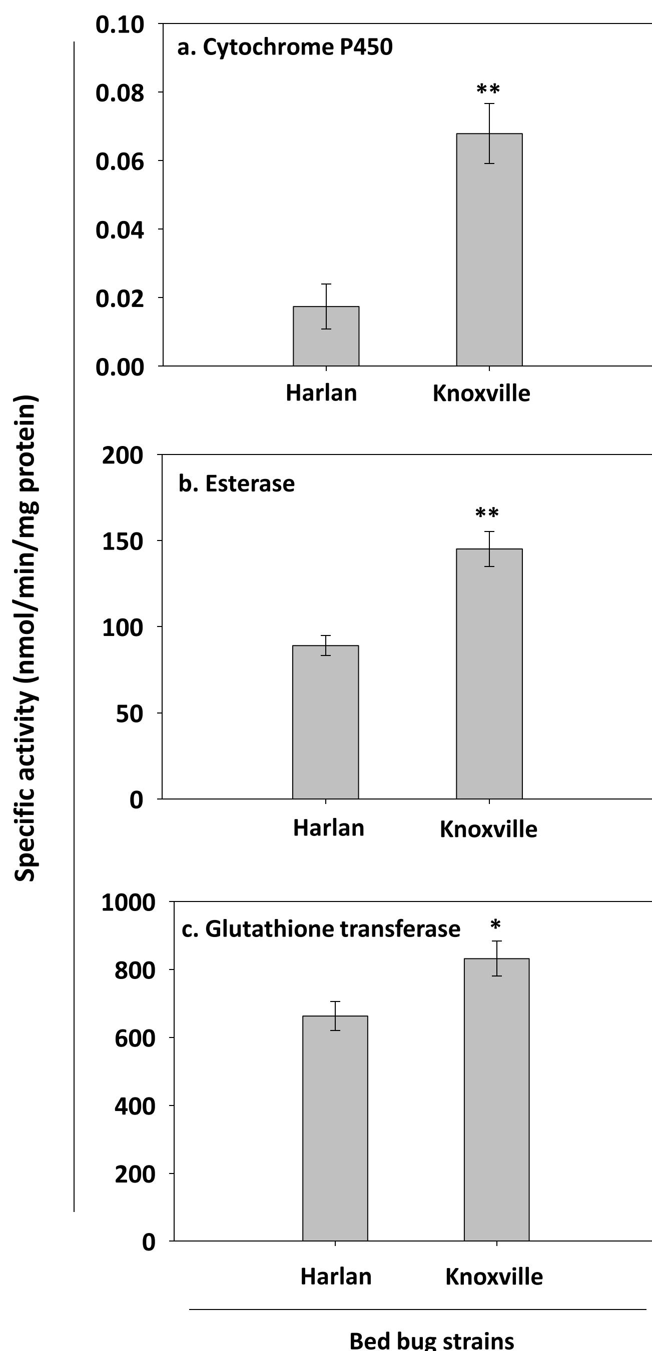

Fig. 2. Detoxifying enzyme activities of the susceptible (Harlan) and deltamethrin resistant (Knoxville) strains. (a) Shows data for cytochrome P450 ECOD activity, (b) represents data for pNPA hydrolysis activity of carboxylesterases and (c) depicts glutathione transferase CDNB conjugation activity. Significantly higher activities of all three detoxifying enzymes were observed in the deltamethrin resistant Knoxville strain in comparison to the susceptible Harlan strain. Double asterisk (**) indicates significance at $P < .01$ and single asterisk (*) indicates significance at $P < .05$ (independent samples *t*-test).

the magnitude of deltamethrin resistance and its mechanisms in the Knoxville strain are discussed along with potential factors that may have caused EO susceptibility in the deltamethrin resistant Knoxville strain bed bugs.

4.1. Deltamethrin resistance in the Knoxville strain

Pyrethroid resistance is reported as a primary reason for the resurgence of bed bugs or *C. lectularius* in the early 2000s (Myamba et al., 2002; Boase et al., 2006; Moore and Miller, 2006; Romero et al., 2007; Yoon et al., 2008). Since then, several studies have reported field-collected strains of bed bugs exhibiting pyrethroid resistance (Romero, 2018). In the present study, the field-collected Knoxville strain was 72,000 and 290,000 fold more resistant to topically applied deltamethrin at LD_{25} and LD_{50} levels, respectively, which is relatively high in comparison to previous studies with *C. lectularius*. Deltamethrin resistance ratio range of 5000–20,000 fold at the LD_{50} or LC_{50} level have been previously reported in various *C. lectularius* field populations collected across the United States (Romero et al., 2007; Adelman et al., 2011; Gonzalez-Morales and Romero, 2019). The Knoxville strain used in this study also exhibits cross-resistance to another pyrethroid insecticide, bifenthrin, and multiple resistance to the pyrrole class insecticide chlorfenapyr (Ashbrook et al., 2017). Various studies have shown that bed bugs possess multiple mechanisms that allow them to resist the insecticidal effects of deltamethrin and other pyrethroid class insecticides (Romero et al., 2007; Yoon et al., 2008; Adelman et al., 2011; Zhu et al., 2013). Because the Knoxville strain was highly resistant to deltamethrin, we further determined the presence of metabolic and target site-based resistance mechanisms in this strain.

Table 2
Frequency of *kdr*-associated mutations in the Harlan susceptible and Knoxville resistant strains across three previously identified mutation sites.

Mutation sites	Genotype[1]	Harlan	Knoxville
V419L	SS (V419/V419)	100%	100%
(Valine to Leucine at amino acid	RS (L419/V419)	–	–
position 419)	RR (L419/L419)	–	–
L925I	SS (L925/L925)	100%	10%
(Leucine to Isoleucine at amino acid	RS (I925/L925)	–	30%
position 925)	RR (I925/I925)	–	60%
I936F	SS (I936/I936)	100%	100%
(Isoleucine to Phenylalanine at	RS (F936/I936)	–	–
position 936)	RR (F936/F936)	–	–

[1] SS indicates susceptible homozygotes or haplotype A. RS refers to resistant heterozygotes and RR refers to resistant homozygotes. Both RS and RR represent haplotype B.

4.2. Mechanisms of deltamethrin resistance

The use of synergists such as PBO, DEF and DEM allows initial identification of the possible involvement of detoxification enzymes in insecticide resistance (Bernard and Philogene, 1993; Romero et al., 2009a; Lilly et al., 2016a; Dehkordi et al., 2017; Gonzalez-Morales and Romero, 2019). These synergists (PBO, DEF and DEM) are known to inhibit detoxifying enzymes that respectively include cytochrome P450s, esterases and glutathione transferases (Bernard and Philogene, 1993). Romero et al. (2009a) and Lilly et al. (2016a) showed that pre-application of PBO significantly reduced deltamethrin resistance in field strains of *C. lectularius*. The synergist, DEF, has been successfully used in evincing the role of esterase enzymes in deltamethrin resistant bed bugs, and carbamate resistant German cockroaches (Dehkordi et al., 2017; Gonzalez-Morales and Romero, 2019). In *C. lectularius*, Gonzalez-Morales and Romero (2019) showed that pre-treatment of insects with DEM significantly increased deltamethrin toxicity in the field-collected strains. In this study, we found that pre-application of PBO and DEF significantly increased deltamethrin toxicity in the Knoxville strain at the LD_{25} level, indicating the role of cytochrome P450s and esterases in resistance. In contrast, PBO pre-treatment significantly reduced or antagonized deltamethrin toxicity in the susceptible Harlan strain. The antagonistic effects of PBO pretreatment on deltamethrin toxicity could be due to induction in expression of certain P450 and glutathione transferase genes as shown in a susceptible strain of *Drosophila melanogaster* Meigen (Willoughby et al., 2007) or due to changes in pharmacokinetic factors that decrease the penetration of insecticide through

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Table 3
Status of resistance to EOs and EcoRaider® in the deltamethrin resistant Knoxville strain in comparison to the susceptible Harlan strain.

EOs	Strains	N	LD_{25}[I], µg/mg body weight (FL95%)[II]	LD_{50}[I], µg/mg body weight (FL95%)[II]	Slope ± SE	χ^2	Df	P value	LD_{25} resistance ratio[III]	LD_{50} resistance ratio[III]
Thyme oil	Harlan	270	22.80 (20–25.42)	30.84 (27.85–34.20)	2.26 ± 0.25	5.56	6	0.474	–	–
	Knoxville	270	21.78 (14.43–27.55)	33.07 (25.98–42.25)	3.70 ± 0.62	12.34	6	0.054	0.955	1.07
Oregano oil	Harlan	240	19.25 (6.72–27.47)	30.65 (19.06–45.79)	3.33 ± 0.81	18.01	5	0.002	–	–
	Knoxville	210	26.50 (22.30–29.92)	35.17 (31.49–39.10)	5.52 ± 0.74	2.05	4	0.725	1.37	1.14
Clove bud oil	Harlan	270	37.94 (26.54–48.59)	98.31 (74.95–155.32)	1.63 ± 0.30	7.97	6	0.239	–	
	Knoxville	210	23.62 (18.63–27.82)	34.12 (29.39–38.84)	4.23 ± 0.55	3.36	4	0.498	0.622	0.34
Coriander oil	Harlan	240	55.70 (27.85–77)	86.91 (60.74–137.38)	3.51 ± 0.80	16.88	5	0.0047	–	
	Knoxville	240	64.56 (29.65–84.25)	85.82 (56.95–113.64)	5.49 ± 1.35	20.80	5	< 0.001	1.15	0.98
Geranium oil	Harlan	270	79.25 (65.04–95.51)	150.65 (121.3–213.08)	2.42 ± 0.39	1.71	6	0.944	–	
	Knoxville	270	52.23 (42.25–60.89)	87.92 (76.37–102.88)	2.99 ± 0.39	5.28	6	0.507	0.659	0.58
EcoRaider®	Harlan	270	63.36 (27.10–93.08)	104.11 (69.90–216.82)	3.14 ± 0.82	18.91	5	0.002	–	
	Knoxville	270	69.55 (47.50–88.97)	121.25 (94.75–166.66)	2.79 ± 0.46	13.11	7	0.069	1.09	1.16

[I] LD_{25} and LD_{50} = lethal dose necessary to kill 25% and 50% of individuals respectively.
[II] FL = 95% fiducial limits.
[III] Resistance ratio was calculated by dividing the LD_{25} or LD_{50} value of EOs or EcoRaider® for Knoxville by LD_{25} or LD_{50} value of EOs or EcoRaider® for Harlan.

Table 4
Status of resistance to EO constituents in the deltamethrin resistant Knoxville strain in comparison to the susceptible Harlan strain.

EO constituents	Strain	N	LD_{25}[I], µg/ mg body weight (FL 95%)[II]	LD_{50}[I], µg/ mg body weight (FL 95%)[II]	Slope ± SE	χ^2	Df	P value	LD_{25} resistance ratio[III]	LD_{50} resistance ratio[III]
Carvacrol	Harlan[IV]		16.12	27.5					–	–
	Knoxville	240	8.31 (6.48–9.50)	11.62 (10.28–12.90)	4.63 ± 0.57	8.85	5	0.115	0.51	0.42
Thymol	Harlan[IV]		19.86	32.5					–	–
	Knoxville	240	15.21 (13.71–16.46)	18.85 (17.49–20.36)	7.21 ± 0.89	4.31	5	0.504	0.76	0.58
Eugenol	Harlan[IV]		28.82	52					–	–
	Knoxville	270	11.69 (9.86–13.29)	16.67 (14.82–18.57)	4.37 ± 0.46	9.78	6	0.134	0.40	0.32
Geraniol	Harlan[IV]		32.9	64					–	–
	Knoxville	210	27.84 (23.88–31.08)	35.33 (31.71–39.13)	6.52 ± 0.90	3.26	3	0.353	0.84	0.55
Linalool	Harlan[IV]		57.82	112					–	–
	Knoxville	300	92.32 (79.30–111.90)	141.92 (115.58–271.34)	3.61 ± 1.04	1.91	4	0.751	1.59	1.26

[I] LD_{25} and LD_{50} = lethal dose necessary to kill 25% and 50% of individuals within a population.
[II] FL = 95% fiducial limits.
[III] Resistance ratio was calculated by dividing the LD_{25} or LD_{50} value of EO constituents for Knoxville by LD_{25} or LD_{50} value of essential oil constituents for Harlan.
[IV] The Harlan strain LD_{25} and LD_{50} values for EO constituents reported in this table are adapted from a previous study (Gaire et al., 2019).

the insect cuticle (Sanchez-Arroyo et al., 2001). To further confirm the role of detoxification enzymes in deltamethrin resistance, we measured the activity of cytochrome P450s, esterases, and glutathione transferases using model substrates in both bed bug strains. The Knoxville strain showed significantly higher activity of all three enzymes when compared to the Harlan strain. Cytochrome P450 and esterase enzyme activities corroborated with the synergist bioassay data. However, the results for glutathione transferase were contrasting, wherein synergist bioassays with DEM did not indicate the role of glutathione transferase in resistance, but enzyme assays showed that CDNB-conjugation glutathione transferase activity was higher in the field-collected Knoxville strain. Since glutathione transferase enzymes are phase II enzymes that act on xenobiotics that are modified by Phase I enzymes (Yu, 2015), their contribution to the overall resistant phenotype could be minor and hence we did not see significant synergism or toxicity effects in synergist bioassays with DEM.

In addition to several other insect species, the role of three detoxification enzymes has been implicated in insecticide resistance in multiple strains of *C. lectularius* and *C. hemipterus* (Romero et al., 2009a; Lilly et al., 2016a; Karunaratne et al., 2007; Adelman et al., 2011; Romero and Anderson, 2016). However, in comparison to previous studies, the ECOD cytochrome P450 activity was higher (390% or 3.9-fold higher than the susceptible Harlan strain) in the Knoxville strain. Two previous studies with *C. lectularius* reported 20–40% increases in ECOD activity in resistant strains (Adelman et al., 2011; Romero and Anderson, 2016). In another deltamethrin resistant population of bed bugs, ECOD activity was not significantly elevated in comparison to the susceptible strain (Yoon et al., 2008). In a strain of resistant red flour beetles (*Tribolium casteneum* Herbst), a brain-specific cytochrome P450 (*CYP6BQ9*), which showed 200-fold higher gene expression in microarray experiments, was responsible for metabolizing deltamethrin into 4-hydroxy deltamethrin (Zhu et al., 2010b). The Knoxville bed bug strain may possess a similar cytochrome P450-based resistance mechanism that allows it to detoxify deltamethrin to a more polar and relatively less-toxic metabolite. Gene knockdown (RNA interference) experiments conducted with bed bugs or *C. lectularius* showed that four cytochrome P450s (CYP397A1, CYP398A1, CYP6DN1 and CYP4CM1), three cuticular proteins (c2, c10 and c13), and two ABC transporters (ABC8 and ABC9) were responsible for pyrethroid (beta-cyfluthrin) resistance (Zhu et al., 2013). Additionally, reduced cuticular penetration has also been linked to pyrethroid resistance in *C. hemipterus* (Lilly et al., 2016b).

Although metabolic enzymes and reduced penetration mechanisms may play important roles in deltamethrin resistance, mutations in the voltage-sensitive sodium channel α subunit gene are also known to impart resistance to pyrethroid insecticides, including deltamethrin (Yoon et al., 2008; Dang et al., 2015). The *kdr*-type mutations reduce or eliminate the ability of pyrethroid insecticides to disrupt the sodium channel function (Davies et al., 2007; Dong et al., 2014). In our study, we found that the Knoxville strain population is composed of mixed haplotype A and B bed bugs, where A refers to wild type homozygotes, and hence susceptible to pyrethroids, and B refers to bed bugs possessing the L925I mutation either in the homozygous or heterozygous state (Zhu et al., 2010a). No evidence for the presence of two other mutations

(V419L and I936F) was found in the Knoxville strain. Haplotype A individuals comprised 10% of the screened Knoxville strain samples and the remaining 90% samples were either heterozygous (30%) or homozygous (60%) for the L925I mutation and represented haplotype B. These results implicate the potential role of *kdr*-like mutations in deltamethrin resistance observed in the Knoxville strain. Previous studies have found the L925I mutation to be present in 78%–100% of *C. lectularius* and *C. hemipterus* infestations that were screened (Zhu et al., 2010a; Dang et al., 2015; Palenchar et al., 2015; Durand et al., 2012; Booth et al., 2015; Balvín and Booth, 2018). The presence of haplotype A and B bed bugs within the Knoxville population likely resulted from the absence of insecticide exposure in the laboratory-adapted colony for the past 6–7 years (> 30 generations). If *kdr*-like mutations impart a fitness cost, removal of this selective pressure may facilitate reappearance of susceptible genotypes within the population.

4.3. Susceptibility of the Knoxville strain to EOs and their active constituents

EOs contain complex mixtures of constituents and their composition may differ based on various factors, including the phenological state of the plant during sampling, plant part used for extraction, harvesting time, climatic and soil conditions, water level, and the presence of distinct chemotypic races of populations (Regnault-Roger et al., 2012). Therefore, we performed GC–MS analysis of EOs used in this study and confirmed the presence of major insecticidal constituents e.g., thymol, carvacrol, eugenol etc., at higher relative abundance (Table S2). With GC–MS analysis, it was confirmed that thyme, oregano, clove, coriander and geranium oils contained 45.34% thymol, 56.38% carvacrol, 89.87% eugenol, 66.26% linalool and 15.01% geraniol, respectively (Table S2). All these EO constituents were the most abundant compounds in each respective EOs except for geraniol, which was the second most abundant (Table S2). Our comparative bioassay findings revealed that the Knoxville strain, which possesses both metabolic and target site-based pyrethroid resistance mechanisms, was susceptible to all five EOs (thyme, oregano, clove, coriander, geranium), their insecticidal constituents (thymol, carvacrol, eugenol, linalool and geraniol) and a commercially available natural product formulation (EcoRaider®). These results for the Knoxville strain help explain the observed susceptibility of the field-collected bed bug population (Indy strain) to EcoRaider® and different EOs (Singh et al., 2014; Zha et al., 2018). The susceptibility of the field-collected and pyrethroid resistant Knoxville strain to an EO-based product, EcoRaider® also corroborates with the field efficacy data (> 90% reduction in bed bug count) reported for this product (Wang et al., 2014). In the future, additional deltamethrin-resistant *C. lectularius* strains as well as populations that exhibit resistance to other pyrethroid, class insecticides such as lambda cyhalothrin and alpha cyfluthrin, should be screened for their susceptibility to various EOs and their insecticidal constituents.

We did not investigate the mechanisms responsible for EO susceptibility in the deltamethrin-resistant Knoxville strain. However, we predict that differences in target sites of deltamethrin and major EO constituents (e.g., carvacrol, eugenol and thymol) are at least partially responsible for the susceptibility of the Knoxville strain, as observed in this study. In this regard, carvacrol, eugenol and thymol are known to act on the nicotinic acetylcholine, octopamine and gamma amino butyric acid receptors, respectively (Enan, 2001; Tong et al., 2013; Priestley et al., 2003). In recent years, EOs have been shown to synergize toxicity of pyrethroid insecticides in resistant mosquitoes and tobacco cutworm by inhibiting detoxification enzymes (Norris et al., 2018; O'Neal et al., 2019; Ruttanaphan et al., 2019). Thus, future studies should investigate the ability of EOs to synergize deltamethrin toxicity in the resistant Knoxville bed bugs.

5. Summary and conclusions

This study revealed a high-level of deltamethrin resistance in the Knoxville strain of *C. lectularius*. Synergist bioassays and enzyme assays support the involvement of detoxification enzymes in deltamethrin resistance. Additionally, DNA sequencing revealed the L925I *kdr*-type mutation in the voltage-sensitive sodium channel α subunit gene as another deltamethrin resistance mechanism in the Knoxville strain. However, the Knoxville strain was susceptible to EOs, their constituents, and an EO-based product. Overall, the findings of this study and evidence from previous studies (e.g., Singh et al., 2014; Wang et al., 2014; Zha et al., 2018) further confirm that EOs can be effective alternatives for the control of deltamethrin-resistant bed bugs. Nonetheless, other issues such as odor associated with the use of EOs and their short residual efficacy need to be addressed while formulating new natural product insecticides for bed bug management.

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Declaration of Competing Interest

The authors declare no competing interests.

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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.2020.104667.

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