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Insecticide Susceptibility Screening Against *Culex* and *Aedes* (Diptera: Culicidae) Mosquitoes From the United States

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Abstract

Mosquitoes exposed to sublethal doses of insecticides may be selected for resistance to insecticide active ingredients (AIs). Mosquitoes are exposed to AIs through agricultural, public/private mosquito control programs, homeowners, and other sources. Hence, mosquito control programs should routinely measure the resistance/susceptibility status of mosquito populations of public health concern.The objectives here were to determine resistance status for six AIs used in adult mosquito control in the United States to assess how resistance/susceptibility differs between AI, mosquito species (states where > 1 species collected), and between years (some populations sampled for 2 yr). Field-collected eggs from 21 mosquito populations of six different species or hybrid species (*Aedes albopictus* Skuse [Diptera: Culicidae], *Aedes aegypti* L. [Diptera: Culicidae], *Culex nigripalpus* Theobald, *Culex pipiens* L. [Diptera: Culicidae], *Culex quinquefasciatus* Say [Diptera: Culicidae], *Cx. pipiens/quinquefasciatus*) were obtained. Centers for Disease Control and Prevention bottle bioassays were used to assess the resistance/susceptibility status for six AIs (bifenthrin, deltamethrin, etofenprox, malathion, permethrin, and phenothrin). World Health Organization guidelines were used to classify mosquitoes as susceptible (98–100% mortality at diagnostic time [DT]), possibly resistant (80–97% mortality at DT), or resistant (<80% mortality at DT). Significant differences were observed in mosquito susceptibility/resistance between species and AIs. In states where both *Aedes* and *Culex* were collected, the odds of exhibiting resistance in *Culex* were 68–69 times higher than *Aedes* (Texas odds ratio: 69.30; 95% confidence interval: 5.86, 819.44; $P = 0.001$; North Carolina odds ratio: 67.99; 95% confidence interval: 15.21, 303.94; $P < 0.0001$). Some level of resistance was detected against all tested AIs in several mosquito populations and some varied between 2015 and 2016.

Key words: Chemical control, insecticide resistance, insecticide testing, mosquito control

The two most important genera of mosquitoes of public health concern in the United States are *Aedes* (e.g., *Aedes albopictus* Skuse [Diptera: Culicidae] and *Aedes aegypti* L. [Diptera: Culicidae]) (potential vectors of chikungunya virus, dengue virus [DENV], Zika virus [ZIKV]) and *Culex* (e.g., *Culex pipiens* L. [Diptera: Culicidae], *Culex quinquefasciatus* Say [Diptera: Culicidae]) (potential vectors of Saint Louis encephalitis virus and West Nile virus [WNV] (Centers for Disease Control and Prevention [CDC] 2015, 2016a). At present, the pathogens most frequently transmitted to humans within the United States by local populations of *Ae. albopictus* and *Culex* spp. are, respectively, La Crosse encephalitis virus (e.g., Westby et al. 2015) and WNV. *Ae. aegypti* has recently been implicated in focal transmission of chikungunya virus, DENV, and ZIKV in southern Florida (FL) and the lower Rio Grande valley of Texas (TX) (e.g., Brunkard et al. 2007, Adalja et al. 2012, Radke et al. 2012, Kendrick et al. 2014).

Others have shown pyrethroid resistance in *Ae. albopictus* and *Ae. aegypti* (review by Smith et al. 2016) and *Cx. pipiens* and *Cx. quinquefasciatus* (review by Scott et al. 2015) on a global scale, with limited studies on U.S. populations for adult *Aedes* (e.g., Marcombe et al. 2014; Richards et al. 2017) and *Culex* (e.g., McAbee et al. 2003; Richards et al. 2017). Most published studies on *Culex*

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assess resistance by exposing (male and female) larvae to insecticide solutions (e.g., Liu et al. 2004; Ayesa et al. 2005, Xu et al. 2005). Consequently, we evaluated insecticide resistance/susceptibility to two major classes of commonly used insecticides (organophosphates and pyrethroids) in adult females of mosquito species of public health concern. There is currently widespread reliance on pyrethroids in mosquito control, and organophosphates remain in use for public health emergencies (Likos et al. 2016; Smith et al. 2016).

Mosquito control programs (MCPs) are diverse in the United States and run the gamut from well-organized mosquito control districts, county Environmental Health programs, municipal public works, to private companies providing individual household level control (del Rosario et al. 2014, Hamer 2016). All MCPs are tasked with protecting public health from mosquito-borne disease, as well as controlling pests, and many use chemical insecticides to kill adult or larval mosquitoes. In order for a MCP to protect health and provide an effective community service, their mosquito control efforts must be regularly monitored and benchmarked through surveillance (e.g., American Mosquito Control Association 2017). Efficacy testing should be carried out prior to the use of a product and (at least) annually. If control benchmarks are not being met, determining the cause of failure is critical. A potential outcome of chemical control is the evolution of resistance, and this should be one of the causes of failure examined. This can be accomplished by seasonally and spatially monitoring the insecticide resistance/susceptibility status of target mosquito populations. This information informs management decisions about which insecticide products should be used and may vary from year to year, or within a single year, depending on variation in mosquito populations. Other reasons for control failure that should be considered may include equipment calibrations, weather conditions at the time of applications, and vegetation barriers preventing delivery of insecticides to the target population.

In spite of an increasing and comprehensive body of knowledge regarding the genetic basis of insecticide resistance, a sustained nationwide assessment of insecticide resistance for mosquitoes of public health importance in the United States is lacking and mapping of resistance may improve planning of mitigation measures. In 2016, CDC investigators at the Dengue Branch in Puerto Rico conducted resistance tests and mapped resistance to several active ingredients (AIs) (https://www.cdc.gov/zika/vector/testing-puertorico.html). This type of testing and mapping should be considered for other regions where mosquito control is needed. The CDC database called MosquitoNET has been set up, in part, to monitor resistance testing taking place in different states within the United States (CDC 2017). In addition, published diagnostic doses (DDs) for U.S. mosquitoes are lacking, especially for *Culex* species, for many of the AIs used here, hence this study builds baseline knowledge.

The evolution of insecticide resistance in mosquitoes is well known and can occur for commonly used AIs. There is currently widespread usage of pyrethroids and organophosphates in mosquito control, and these types of AIs are evaluated here. Mosquitoes can become resistant to AIs through different mechanisms, e.g., increases in cytochrome P450 monooxygenases that detoxify AIs, mutations in the voltage sensitive sodium channel (*kdr* genes), increases in metabolism, and other unknown mechanisms (Scott et al. 2015, Marriel et al. 2016, Smith et al. 2016). Some mosquitoes are not considered operationally susceptible to AIs, i.e., show decreased mortality after insecticide treatments but may experience decreases in fitness (e.g., reduced fecundity/fertility, shorter lifespan, etc.) due to AI exposure that may impact population dynamics (Scott et al. 2015). In some cases, insects can exhibit tolerance (rather than resistance) to sublethal insecticide doses where survival occurs, but resistance is not conferred to offspring (IRAC 2010). As with most biological processes, some individuals within mosquito populations (larvae and/or adults) may naturally exhibit a greater degree of tolerance to insecticides than others, and this may be difficult to distinguish from genetic resistance (inherited from one generation to the next so is present in some individuals before exposure to an insecticide), especially in areas with continual insecticide applications. Tolerance may be displayed in a generation of insects (such as mosquitoes) that is exposed to some type of stress (e.g., insecticide) and/or in subsequent generations after exposure to a similar type of stressor (Scott 1995) but is not an inherited trait, hence is not long-lasting.

Our objectives in the current study were to: 1) Determine the resistance status of several *Culex* and *Aedes* populations of public health importance against AIs commonly used in mosquito control, and 2) assess the extent to which resistance differs between AI, mosquito species, and (in some cases) between years.

Materials and Methods

Mosquitoes

Twenty-five mosquito abatement districts, control programs, universities, and government agencies were contacted within four regions (based on census regions in the United States): https://www2.census.gov/geo/pdfs/maps-data/maps/reference/us_regdiv.pdf. Programs from the following regions mailed eggs that were used in resistance/susceptibility assays: 1) West: California [CA], Utah [UT]; 2) South: FL, Georgia [GA], Louisiana [LA], North Carolina [NC], South Carolina [SC], TX; 3) Midwest: Minnesota [MN]; and 4) Northeast: Pennsylvania [PA]. Additional programs from these regions mailed us eggs, but we were unable to propagate enough mosquitoes for use in assays: Midwest (Nebraska), South (NC), West (Colorado).

Two susceptible colonies were used as controls in this study: 1) The CDC provided *Cx. quinquefasciatus* (Sebring strain) and 2) our existing *Ae. albopictus* colony originating from LA to establish AI doses and diagnostic times (DTs). We tested 21 field mosquito populations of six species from 17 participants (MCPs, universities, government agencies) including *Ae. albopictus* (N = 8), *Ae. aegypti* (N = 1), *Culex nigripalpus* Theobald (N = 1), *Cx. pipiens* (N = 3), *Cx. quinquefasciatus* (N = 6), and *Cx. pipiens/quinquefasciatus* (N = 2) (Table 1). Mosquitoes ($F_0 - F_5$) reared from field-collected eggs were processed as described previously (Richards et al. 2017). For mosquito populations that showed low hatch rate or where we wanted to increase sample size, mosquitoes were blood fed and additional generations propagated (Richards et al. 2017). The maximum number of generations used was five. For propagation, mosquito eggs were placed into 34 cm by 24 cm plastic pans (Bioquip, Rancho Dominguez, CA) with tap water and placed in incubators with a 14:10 light:dark cycle at 28°C and 85% humidity. Larvae were fed a 2:1 mixture of liver powder and yeast, and pupae were transferred to plastic cups containing tap water, placed into 30.5 cm by 30.5 cm by 30.5 cm square cages (BioQuip), and provided 20% sucrose ad libitum (Richards et al. 2017). Colonies were separated by mosquito collection location.

Active Ingredients

Concentrations of AIs used in bioassays were verified every 2 wk as described previously (Richards et al. 2017). Briefly, replicate samples of each stock solution were analyzed via capillary gas chromatograph with flame ionization detector (Agilent GC 6850 instrument, Agilent Technologies, Santa Clara, CA). Standard solutions of each AI were prepared by dissolving 0.01 g of each technical grade AI (Sigma Aldrich, St. Louis, MO) in acetone (40 ml) and were used

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to prepare the calibration curve for quantification. Analyses verified that AIs in stock solutions remained undegraded (data not shown).

Bioassays

Adult female mosquitoes were assayed for resistance to six AIs (bifenthrin, deltamethrin, etofenprox, malathion, permethrin, phenothrin) utilizing the CDC bottle bioassay method (CDC 2013). Stock solutions for bioassays were prepared by mixing each AI with acetone so that the final volume's concentration reflected the typical DD after a DT of 30–60 min (Table 2). Stock solutions were refrigerated at 4°C for the duration of the study (CDC 2013).

DDs used in this study were based on our previous study (Richards et al. 2017) and the current study using susceptible longstanding colonies of *Ae. albopictus* and *Cx. quinquefasciatus*. We used the same DDs for both *Aedes* and *Culex* populations in this study since we were interested in comparative susceptibility of species within these two genera. The DDs used were appropriate for lab colonies of both *Ae. albopictus* and *Cx. quinquefasciatus* and were optimized based on preliminary tests of both colonies using a variety of doses.

For deltamethrin, etofenprox, and malathion, two different DDs were used (Table 2). In these cases, during the initial determination of DD and DT, susceptible colonies exposed to the same AIs from a previous study carried out in 2015 (Richards et al. 2017) showed slightly different mortality rates than susceptible colonies exposed in the current study carried out in 2016. Consequently, for these three AIs, DDs from the previous study (Richards et al. 2017) were used in addition to doses determined in the current study, for comparison purposes between study years.

For each bioassay (21 mosquito populations × 6 AIs [9 doses]), CDC bottle bioassay procedures were followed (CDC 2013; Richards et al. 2017). Briefly, the interior of three to four 250-ml glass Wheaton bottles was coated with 1 ml of each AI stock solution or 1 ml of acetone (control) (CDC 2013). After coating, bottles were uncapped and placed on a bottle roller (Fisher Scientific, Kennesaw, GA) at 20 revolutions/min for 1–2 min until dry. This

Table 1. Mosquito collection locations from different states in the United States

State	Municipality	County/Parish	Mosquito species
Midwest			
Minnesota	St. Paul	Ramsey	*Cx. pipiens*
Northeast			
Pennsylvania	York	York	*Cx. pipiens*
South			
Florida	Vero Beach	Indian River	*Ae. albopictus*
Florida	Palmetto	Manatee	*Cx. nigripalpus*
Georgia	Savannah	Chatham	*Cx. quinquefasciatus*
Louisiana	Slidell	St. Tammany	*Cx. quinquefasciatus*
North Carolina	Winterville (Magnolia Ridge)	Pitt	*Cx. pipiens/quinquefasciatus*
North Carolina	Winterville (Magnolia Ridge)	Pitt	*Ae. albopictus*
North Carolina	Greenville (Curry Court)	Pitt	*Cx. pipiens/quinquefasciatus*
North Carolina	Greenville (Hop Tyson)	Pitt	*Ae. albopictus*
North Carolina	Jacksonville (Bayshore)	Onslow	*Ae. albopictus*
North Carolina	Bolivia (Research Station)	Brunswick	*Ae. albopictus*
North Carolina	Raleigh	Wake	*Ae. albopictus*
South Carolina	Columbia	Richland	*Cx. quinquefasciatus*
Texas	Beaumont	Jefferson	*Cx. quinquefasciatus*
Texas	Dallas (Balch Springs)	Dallas	*Cx. quinquefasciatus*
Texas	Dallas (Cockrell Hill)	Dallas	*Ae. aegypti*
Texas	Dallas (Highland Park)	Dallas	*Cx. quinquefasciatus*
Texas	Port Arthur	Jefferson	*Ae. albopictus*
West			
California	West Covina	Los Angeles	*Ae. albopictus*
Utah	Salt Lake City	Salt Lake	*Cx. pipiens*

Table 2. Diagnostic doses and times used for technical grade AIs

Insecticide	Classification	Final concentration per bottle (diagnostic dose[a])	Reference	Stock material (added to 1,000-ml acetone)	Diagnostic time[a]
Malathion	Organophosphate	100 µg/ml	2015 study	81.2 µl	60 min
Malathion	Organophosphate	250 µg/ml	Current 2016 study	203.2 µl	60 min
Etofenprox	Nonester pyrethroid	6 µg/ml	2015 study	5.1 µl	30 min
Etofenprox	Nonester pyrethroid	15 µg/ml	Current 2016 study	8.5 µl	30 min
Bifenthrin	Type I pyrethroid	12.6 µg/ml	2015 study	0.013 g	30 min
Permethrin	Type I pyrethroid	15 µg/ml	2015 study	0.015 g	30 min
Phenothrin	Type I pyrethroid	23 µg/ml	2015 study	19.6 µl	30 min
Deltamethrin	Type II pyrethroid	5 µg/ml	Current 2016 study	0.005 g	30 min
Deltamethrin	Type II pyrethroid	10 µg/ml	2015 study	0.01 g	30 min

[a]Diagnostic doses and times used here based on data from our susceptible F_{23} *Ae. albopictus* and $F_{>50}$ *Cx. quinquefasciatus* Sebring colonies.

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Table 3. Comparative susceptibility/resistance of *Aedes* mosquito populations to six active ingredients based on WHO guidelines

Source	Species	Malathion	Malathion	Etofenprox	Etofenprox	Bifenthrin	Permethrin	Phenothrin	Deltamethrin	Deltamethrin
		100 µg/ml	250 µg/ml	6 µg/ml	15 µg/ml	12.6 µg/ml	15 µg/ml	23 µg/ml	5 µg/ml	10 µg/ml
Vero Beach, FL	*Ae. albopictus* (F_2)	**Susceptible 100% *N*=13**	**Susceptible 100% *N*=13**	Resistant 7% *N* =14	Possible Resistance 92% *N* =12	Susceptible 100% *N* =14	Susceptible 100% *N* =15	**Susceptible 100% *N* =14**	Possible Resistance 87% *N* =14	Possible Resistance 93% *N* =15
Winterville, NC (Magnolia Ridge)	*Ae. albopictus* (F_3)	Not tested	**Susceptible 100% *N* =23**	Not tested	Not tested	**Susceptible 100% *N* =20**	**Susceptible 100% *N* =19**	Not tested	**Susceptible 100% *N* =22**	**Susceptible 100% *N* =17**
Jacksonville, NC (Bayshore)	*Ae. albopictus* (F_3)	**Susceptible 100% *N* =18**	**Susceptible 100% *N* =21**	Resistant 0% *N* =17	**Susceptible 100% *N* =16**	**Susceptible 100% *N* =22**	**Susceptible 100% *N* =16**	**Susceptible 100% *N* =17**	**Susceptible 100% *N* =18**	**Susceptible 100% *N* =24**
Bolivia, NC (Research Station)	*Ae. albopictus* (F_1)	Possible Resistance 88% *N* =16	Possible Resistance 82% *N* =16	Resistant 19% *N* =15	Susceptible 100% *N* =18	Possible Resistance 85% *N* =21	Possible Resistance 96% *N* =25	**Susceptible 100% *N* =26**	**Susceptible 100% *N* =28**	**Susceptible 100% *N* =22**
Raleigh, NC	*Ae. albopictus* (F_5)	**Susceptible 100% *N* =22**	**Susceptible 100% *N* =26**	Resistant 41% *N* =25	**Susceptible 100% *N* =23**	**Susceptible 100% *N* =21**	**Susceptible 100% *N* =24**	**Susceptible 100% *N* =22**	**Susceptible 100% *N* =25**	**Susceptible 100% *N* =22**
Greenville, NC (Hop Tyson)	*Ae. albopictus* (F_0)	Resistant 66% *N* =20	Resistant 79% *N* =19	Resistant 72% *N* =25	**Susceptible 100% *N* =19**	**Susceptible 100% *N* =17**	**Susceptible 100% *N* =18**	**Susceptible 100% *N* =17**	Possible Resistance 94% *N* =19	**Susceptible 100% *N* =18**
Dallas, TX	*Ae. aegypti* (F_0)	**Susceptible 100% *N* =36**	**Susceptible 100% *N* =40**	Resistant 6% *N* =35	Resistant 28% *N* =34	Resistant 69% *N* =40	Resistant 54% *N* =42	Resistant 40% *N* =30	Possible Resistance 85% *N* =41	Possible Resistance 96% *N* =32
Port Arthur, TX	*Ae. albopictus* (F_0)	Resistant 0% *N* =17	Resistant 77% *N* =17	Resistant 39% *N* =16	**Susceptible 100% *N* =15**	**Susceptible 100% *N* =19**	**Susceptible 100% *N* =16**	**Susceptible 100% *N* =18**	**Susceptible 100% *N* =17**	**Susceptible 100% *N* =16**
West Covina, CA	*Ae. albopictus* (F_1)	**Susceptible 100% *N* =17**	**Susceptible 100% *N* =18**	Possible Resistance 89% *N* =21	**Susceptible 100% *N* =15**	Resistant 67% *N* =19	**Susceptible 100% *N* =14**	**Susceptible 100% *N* =17**	**Susceptible 100% *N* =14**	**Susceptible 100% *N* =15**

N indicates the number of mosquitoes tested including all replicates for each AI, location, and mosquito population. Susceptible populations are given in bold.

process left AI residue in the treatment bottles and no AI in the control bottles. All bottles were stored uncapped in a drawer away from light for ≤24 h prior to bioassays.

Each mosquito population was assayed for six AIs (nine total doses) on the same day. At least three (population-specific) control bottles were used for each individual mosquito population and were also processed on the same day. Four- to 10-day old lab-reared adult female mosquitoes from field-collected eggs (Tables 3 and 4) were introduced into the bottles, i.e., 4–21 mosquitoes/bottle. Mosquito mortality was recorded at 10 time points (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min) following CDC bottle bioassay guidelines (CDC 2013). Mosquitoes that were knocked down (i.e., not able to stand up or fly) at these time points were classified as dead (CDC 2013).

Mortality rate was calculated as the number of dead mosquitoes divided by the total mosquitoes multiplied by 100 for each time point, AI and dose, and mosquito collection location. For each species, the mean proportion of dead mosquitoes at the DT was calculated and graphed (Figs. 1–3) (mortality curves are provided as Supplementary Data [Online only]). We used World Health Organization guidelines (as recommended by the CDC) (WHO 2013; CDC 2013) as follows: susceptible (98–100% mortality at DT), possible resistance (80–97% mortality at DT), or resistant (< 80% mortality at DT) (Tables 3 and 4). The proportions of tested mosquitoes dead at 30 min (bifenthrin, deltamethrin, etofenprox, permethrin, phenothrin) or 60 min (malathion) were used to determine resistance or susceptibility status.

Data Analyses

Ordinal logistic regression ($P < 0.05$) (PROC LOGISTIC, SAS Institute, Cary, NC) was used to determine differences, if any, in susceptibility, possible resistance, or resistance between 1) *Ae. albopictus* and *Culex* species (*pipiens*, *quinquefasciatus*, *pipiens/quinquefasciatus*) where species were collected from the same state (analyses conducted for mosquitoes collected from TX and NC) and 2) AIs (for all species and populations). Odds ratios were computed to illustrate the magnitude of differences in resistance observed between species and AIs.

Results

Resistance and Susceptibility Analyses

Mortality data are presented for each species (Figs. 1–3). DDs and times are shown in Table 2. Differences were observed between AIs in levels of resistance and susceptibility (range = 0–100% mortality at DT), and the degree of this variation differed between mosquito

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Table 4. Comparative susceptibility/resistance of *Culex* mosquito populations to six active ingredients based on WHO guidelines

Source	Species	Malathion 100 µg/ml	Malathion 250 µg/ml	Etofenprox 6 µg/ml	Etofenprox 15 µg/ml	Bifenthrin 12.6 µg/ml	Permethrin 15 µg/ml	Phenothrin 23 µg/ml	Deltamethrin 5 µg/ml	Deltamethrin 10 µg/ml
St. Paul, MN	*Cx. pipiens* (F_0)	Resistant 64% *N*=17	Possible Resistance 94% *N* =17	Resistant 0% *N* =17	Resistant 29% *N* =17	Possible Resistance 89% *N* =17	Resistant 48% *N* =21	Possible Resistance 90% *N* =28	Possible Resistance 89% *N* =16	Susceptible 100% *N* =17
York, PA	*Cx. pipiens* (F_0)	Resistant 61% *N* =28	Possible Resistance 93% *N* =27	Resistant 0% *N* =31	Resistant 30% *N* =17	Possible Resistance 81% *N* =25	Resistant 66% *N* =18	**Susceptible 100%** *N* =23	Possible Resistance 95% *N* =23	**Susceptible 100%** *N* =40
Palmetto, FL	*Cx. nigripalpus* (F_0)	Resistant 0% *N* =42	Resistant 13% *N* =33	Resistant 4% *N* =31	Resistant 31% *N* =39	Resistant 37% *N* =36	Resistant 61% *N* =38	Resistant 37% *N* =33	Resistant 59% *N* =37	Resistant 75% *N* =36
Savannah, GA	*Cx. quinquefasciatus* (F_0)	Resistant 11% *N* =19	Resistant 19% *N*=21	Resistant 5% *N*=22	Resistant 0% *N*=20	Resistant 0% *N*=29	Resistant 3% *N*=32	Resistant 14% *N*=26	Resistant 14% *N*=50	Resistant 35% *N*=44
Slidell, LA	*Cx. quinquefasciatus* (F_0)	Resistant 0% *N*=22	Resistant 0% *N*=22	Resistant 0% *N*=15	Resistant 0% *N*=20	Resistant 0% *N*=24	Resistant 4% *N*=20	Resistant 0% *N*=24	Resistant 0% *N*=24	Possible Resistance 90% *N*=22
Winterville, NC (Magnolia Ridge)	*Cx. pipiens/ quinquefasciatus* (F_0)	Resistant 40% *N*=15	Resistant 67% *N*=15	Resistant 53% *N*=16	Resistant 78% *N*=14	Resistant 72% *N*=14	Resistant 42% *N*=12	Resistant 72% *N*=15	Resistant 50% *N*=14	Possible Resistance 87% *N*=15
Greenville, NC (Curry Court)	*Cx. pipiens/ quinquefasciatus* (F_0)	Resistant 11% *N*=18	Possible Resistance 82% *N*=23	Resistant 21% *N*=18	Resistant 0% *N*=19	Resistant 0% *N*=18	Resistant 6% *N*=22	Resistant 16% *N*=19	Resistant 63% *N*=16	Resistant 58% *N*=16
Columbia, SC	*Cx. quinquefasciatus* (F_0)	Resistant 70% *N*=29	Resistant 58% *N*=24	Resistant 0% *N*=35	Resistant 3% *N*=33	Resistant 11% *N*=36	Resistant 0% *N*=34	Resistant 0% *N*=19	Resistant 19% *N*=38	Resistant 56% *N*=32
Beaumont, TX	*Cx. quinquefasciatus* (F_0)	Resistant 0% *N*=16	Resistant 0% *N*=18	Resistant 0% *N*=31	Resistant 0% *N*=24	Resistant 35% *N*=18	Resistant 26% *N*=26	Resistant 30% *N*=21	Possible Resistance 81% *N*=33	Resistant 63% *N*=16
Dallas, TX (Balch Springs)	*Cx. quinquefasciatus* (F_0)	Resistant 12% *N*=68	Resistant 78% *N*=44	Resistant 4% *N*=64	Resistant 40% *N*=43	Resistant 50% *N*=	Resistant 37% *N*=46	Resistant 39% *N*=42	Resistant 56% *N*=41	Resistant 62% *N*=42
Dallas, TX (Highland Park)	*Cx. quinquefasciatus* (F_0)	Resistant 53% *N*=60	Resistant 67% *N*=52	Resistant 0% *N*=36	Resistant 2% *N*=42	Resistant 6% *N*=46	Resistant 14% *N*=42	Resistant 12% *N*=48	Resistant 35% *N*=45	Resistant 72% *N*=60
Salt Lake City, UT	*Cx. pipiens* (F_0)	Resistant 59% *N*=47	Resistant 30% *N*=50	Resistant 0% *N*=32	Resistant 21% *N*=43	Resistant 16% *N*=33	Resistant 22% *N*=26	Resistant 23% *N*=30	Possible Resistance 87% *N*=39	Possible Resistance 97% *N*=38

N indicates the number of mosquitoes tested including all replicates for each AI, location, and species. Susceptible populations in bold.

populations (Tables 3 and 4). The number of generations propagated for each population is listed in Tables 3 and 4 (maximum number of generations was five; only three populations used were greater than two generations). All post hoc comparisons (except for the low dose of malathion) show the resistance status of mosquitoes exposed to the low dose of etofenprox was significantly higher than the other AIs in resistance status.

Malathion

Ae. albopictus from FL, CA, three different NC populations and *Ae. aegypti* from TX were susceptible to both doses (100 and 250 µg/µl) of malathion tested here (Table 3). Conversely, *Ae. albopictus* from NC (Greenville) and TX were resistant (range = 0–79% mortality at DT) to the same two malathion doses (Table 3). One NC population (Bolivia) exhibited possible resistance to both doses of malathion. All tested *Culex* populations from all locations were resistant (range = 0–64% mortality at DT) to the low dose of malathion (Table 4). For the high dose of malathion, 75% (*N* = 9 out of 12) or 25% (*N* = 3 out of 12) of *Culex* populations were respectively resistant (range = 0–78% mortality at DT) or possibly resistant (range = 82–94% mortality at DT) (Table 4).

Etofenprox

Most *Aedes* populations were resistant to the low dose of etofenprox (6 µg/µl) used here; however, *Ae. albopictus* from CA exhibited possible resistance (range = 0–100% mortality at DT) to this dose (Table 3). *Ae. aegypti* from TX were resistant to the high dose of etofenprox (15 µg/µl) used here and *Ae. albopictus* from FL exhibited possible resistance (Table 3). Six populations of *Ae.*

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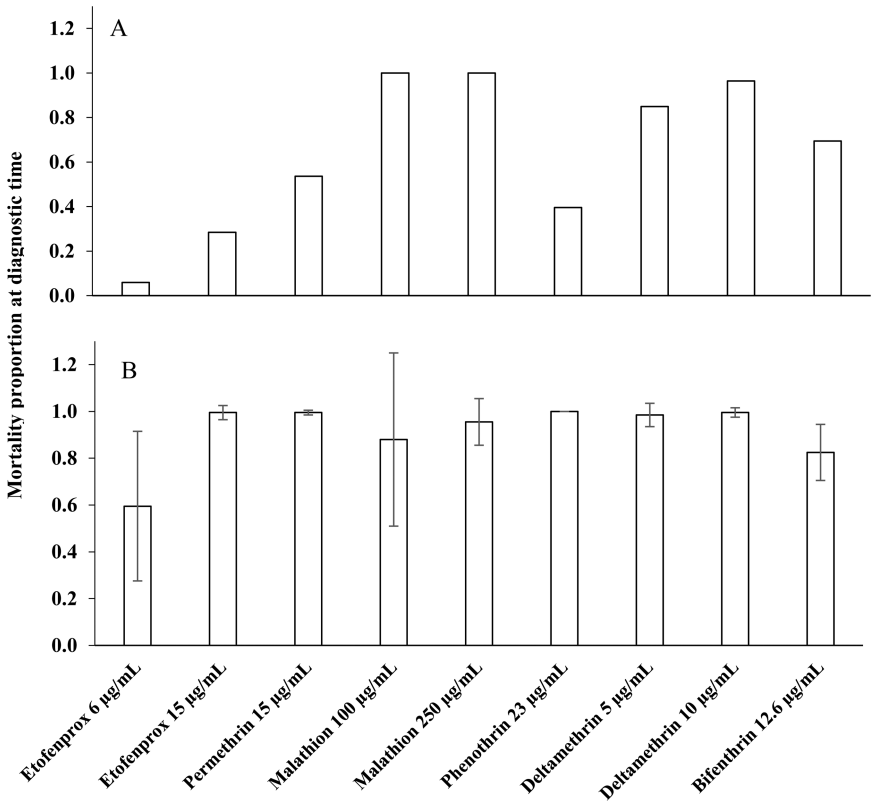

Fig. 1. Insecticide resistance test for (A) *Ae. aegypti* (one population tested) and (B) *Ae.albopictus* (eight populations tested). Standard deviation bars are shown when more than one population was tested.

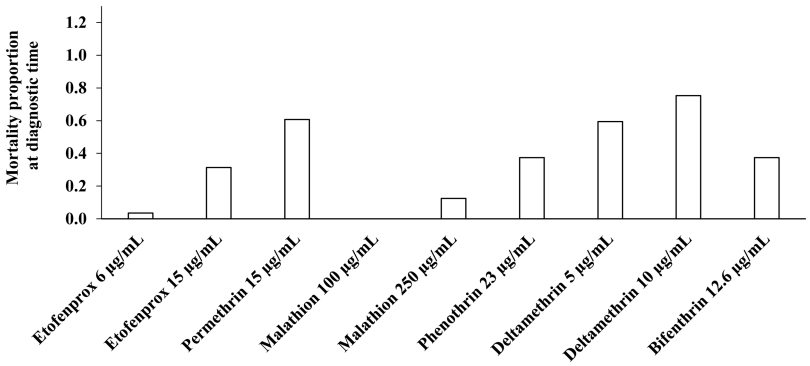

Fig. 2. Insecticide resistance test for *Cx. nigripalpus* (one population tested).

albopictus (NC, TX, CA) were susceptible to the high dose of etofenprox (Table 3). All *Culex* spp. (range = 0–78% mortality at DT) populations were resistant to etofenprox at the two doses (Table 4).

Bifenthrin

Six populations of *Ae. albopictus* (FL, NC, TX) were susceptible to bifenthrin (12.6 µg/µl) and two populations (*Ae. aegypti* from TX and *Ae. albopictus* from CA) were resistant (Table 3). Possible resistance was shown in one NC (Bolivia) *Ae. albopictus* population. Possible resistance was detected in two *Culex* (range = 81–89% mortality at DT) populations, and resistance was shown in the remaining 10 *Culex* populations (range = 0–72% mortality at DT) (Table 4).

Permethrin

Seven *Aedes* spp. populations were susceptible (100%) to permethrin (15 µg/µl) and one population (*Ae. aegypti* from TX) was resistant (Table 3). Possible resistance was shown in one NC (Bolivia) *Ae. albopictus* population. All *Culex* spp. were resistant to permethrin (range = 0–66% mortality at DT) (Table 4).

Phenothrin

All tested *Ae. albopictus* spp. populations were susceptible (100%) to phenothrin (23 µg/µl) and one population (*Ae. aegypti* from TX) was resistant (Table 3). Ten *Culex* populations were resistant (range = 0–72% mortality at DT) to permethrin, one population (*Cx. pipiens* from MN) was possibly resistant, and one population (*Cx. pipiens* from PA) was susceptible (Table 4).

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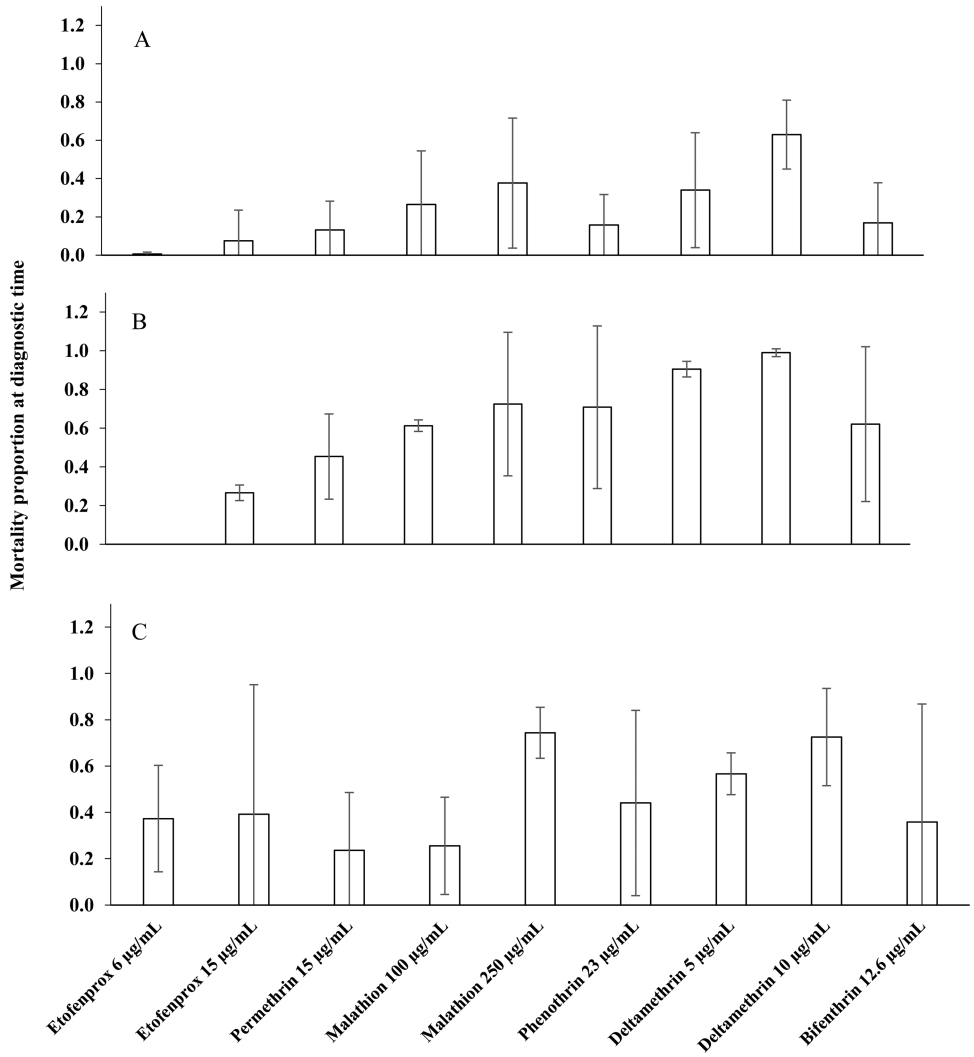

Fig. 3. Insecticide resistance test for (A) *Cx. quinquefasciatus* (six populations tested), (B) *Cx. pipiens* (three populations tested), and (C) *Cx. pipiens/quinquefasciatus* (two populations tested). Standard deviation bars are shown when more than one population was tested.

Deltamethrin

For the low dose (5 µg/µl) of deltamethrin used here, six *Ae. albopictus* populations were susceptible and the remaining three *Aedes* spp. populations exhibited possible resistance (range = 85–94% mortality at DT) (Table 3). At the high deltamethrin dose (10 µg/µl) used here, seven *Ae. albopictus* populations were susceptible and two *Aedes* spp. (*Ae. albopictus* from FL and *Ae. aegypti* from TX) were possibly resistant (range = 93–96% mortality at DT) (Table 3). Four *Culex* populations exhibited possible resistance (range = 81–95% mortality at DT) to the low deltamethrin dose, while the remaining eight populations were resistant (Table 4). For the high dose of deltamethrin, two *Culex* populations were susceptible (*Cx. pipiens* from MN and PA) and three populations showed possible resistance (range = 87–97% mortality at DT) (Table 4). The remaining seven *Culex* populations were resistant to the high dose of deltamethrin (Table 4).

Significant differences were observed in mosquitoes (categorized as susceptible, possibly resistant, or resistant) between species from TX (*Ae. albopictus, Cx. quinquefasciatus*) (df = 1, χ^2 = 11.31, P = 0.001) and NC (*Ae. albopictus, Cx. pipiens/quinquefasciatus*) (df = 1, χ^2 = 30.50, P < 0.0001) with *Culex* showing a higher degree of resistance than *Aedes*. The odds of *Culex* spp. mosquitoes being resistant were 69 (TX) and 68 (NC) times higher than the *Ae. albopictus* tested here.

Compared to bifenthrin, the odds of mosquitoes being resistant to the low dose of etofenprox were seven times greater (P = 0.022). Similarly, compared to the low and high doses of deltamethrin, the odds of mosquitoes being resistant to the low dose of etofenprox were 10 and 14 times higher (P = 0.006 and P = 0.002). Compared to the high dose of etofenprox, the odds of mosquitoes being resistant to the low dose of the same AI were six times greater (P = 0.028). Compared to phenothrin, the odds of resistance to the low dose of etofenprox was 11 times greater (P = 0.005). The same trend was true for the high dose of malathion (P = 0.013) and permethrin (P = 0.006) in that mosquitoes showed odds eight times higher of exhibiting resistance to the low dose of etofenprox.

Comparison Between 2015 and 2016

Two *Ae. albopictus* (FL and CA) and three *Culex* (*Cx. pipiens* from MN and UT; *Cx. pipiens/quinquefasciatus* from NC) populations were collected from the same counties in our study conducted in the 2015 (Richards et al. 2017) and the current study (conducted in 2016). For FL *Ae. albopictus*, the following differences were

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observed between years for low-dose malathion (2015: resistant, 2016: susceptible), bifenthrin (2015: possible resistance, 2016: susceptible), and deltamethrin (2015: susceptible, 2016: possible resistance). For the same *Ae. albopictus* in FL, no change was observed in susceptibility/resistance status for low-dose etofenprox (resistant), permethrin (susceptible), or phenothrin (susceptible). For the CA *Ae. albopictus*, yearly differences were observed for low-dose malathion (2015: resistant, 2016: susceptible), low-dose etofenprox (2015: resistant, 2016: possible resistance), bifenthrin (2015: possible resistance, 2016: resistant), and permethrin (2015: possible resistance, 2016: susceptible). In an *Ae. albopictus* population collected from the same area, no difference was observed between 2015 and 2016 for phenothrin or high-dose deltamethrin as mosquitoes in both years were susceptible to these AIs.

Cx. pipiens from MN only exhibited changes from year to year in response to permethrin (2015: possible resistance, 2016: resistance), while all other AIs showed no change between years (low-dose malathion: resistant, low-dose etofenprox: resistant, bifenthrin: possible resistance, phenothrin: possible resistance, high-dose deltamethrin: susceptible). For *Cx. pipiens/quinquefasciatus* from NC (Curry Court in Greenville), one change was noted in high-dose deltamethrin between 2015 (possible resistance) and 2016 (resistant). For this NC population, no changes were observed between the other AIs as all exhibited resistance for both 2015 and 2016. For *Cx. pipiens* from UT, their response to high-dose deltamethrin changed from 2015 (susceptible) to 2016 (possible resistance); however, mosquitoes exposed to all other AIs showed no yearly change as all remained resistant.

Discussion

We tested the hypothesis that mosquito populations have variable resistance to one organophosphate and several pyrethroid insecticides, depending on species and year. We found support for our hypothesis, as some tested populations have resistance to several pyrethroids. We also found differences between years, and that one AI, the low dose of etofenprox used here, was consistently less effective than the other tested AIs. This is practical information for mosquito control operators and may serve as a starting point for insecticide resistance/susceptibility analyses in their own regions.

The CDC recommends testing AIs that are currently being used or have recently been used, as well as others that are available for use (in case an alternative is needed) (CDC 2016b). It should be noted that resistance/susceptibility profiles found for technical grade AIs are not directly related to the efficacy of formulated products that contain additional ingredients (e.g., synergists that inhibit detoxification enzymes) that may improve efficacy. Thus, documented resistance in our assays does not mean a product will fail in a field application. Studies are underway to assess the relationships between selected AIs and associated formulated products via bottle bioassay. Although bottle bioassays are not a direct measure of field applications/conditions, the assays do evaluate resistance to AIs of the mosquito populations. Thus, the results should be interpreted with careful consideration for operational, genetic, and other relevant factors. We have used WHO classification for susceptibility/resistance here (as recommended by CDC guidelines; CDC 2013) so that some populations could be compared between years (WHO classifications used in previous study; Richards et al. 2017). Future studies will use CDC classifications of susceptibility/resistance that provide different measures.

We found differences in levels of resistance in collections sampled from the same place in consecutive years. We will expand these studies to include repeated testing of the same mosquito populations over the mosquito season in order to evaluate temporal changes in resistance/susceptibility in certain populations. Within season changes in resistance are known from agricultural settings, so the time of collection during the season may be important. The timing of population sampling may be even more important if chemical-based control practices are employed early in the season. We did not assess differences in control measures between years as this would be difficult to measure since there could be multiple sources of control, i.e., public/private pest control, agriculture, homeowner, and other unknown sources.

We expect resistance/susceptibility trends to change over time and with insecticide pressure. Furthermore, cross resistance (i.e., mosquitoes that become resistant to one pyrethroid may also become resistant to different pyrethroids in that class) may be an issue (Nauen 2007), especially where we observed widespread resistance in *Culex* species tested here. Cross resistance may occur when insecticides have similar (or the same) modes of action (Scott 1995).

Here, we have only tested two *Aedes* species (*Ae. albopictus* and *Ae. aegypti*) and four *Culex* species or hybrid species (*Cx. nigripalpus, Cx. pipiens, Cx. pipiens/quinquefasciatus, Cx. quinquefasciatus*). Three of the *Ae. albopictus* populations tested here were propagated for more than two generations (up to five generations) in the laboratory. Further testing of additional species and populations is warranted, depending on the regional needs of MCPs, as species diversity in resistance status may be important. Routine testing of multiple populations throughout mosquito control districts are certainly warranted due to (species, population, and seasonal) variability expected to occur.

In TX and NC where both *Culex* and *Aedes* species were sampled, we found that *Aedes* spp. showed greater susceptibility to AIs compared to the *Culex* spp. These results corroborate the findings of Richards et al. (2017) that conducted a similar study for mosquitoes collected in 2015. Others have also shown spatiotemporal differences in susceptibility of *Ae. aegypti* to deltamethrin (10 µg/bottle; tested here), bendiocarb (12.5 µg/bottle; not tested here), and chorpyrifos (50 µg/bottle; not tested here) (Deming et al. 2016). The same study showed a relationship between deltamethrin resistance and knock down resistance alleles (Ile1016 and Cys1534). Another study (using insecticide-treated papers following guidelines of a WHO tube assay for a 60-min exposure time, not the CDC bottle bioassay) assessed resistance/susceptibility of adult *Ae. albopictus* (New Jersey, PA, FL) to deltamethrin, phenothrin, and malathion (among others) (Marcombe et al. 2014). The same study showed susceptibility to deltamethrin and phenothrin; however, some mosquito populations from FL exhibited resistance to malathion. Since most published studies on *Culex* have exposed larvae (not adults) to insecticide solutions (e.g., Liu et al. 2004; Ayesa et al. 2005, Xu et al. 2005), results from those studies are not comparable to our study where we exposed adults to insecticides in bottle bioassays. Another study using the CDC bottle bioassay showed resistance in one population of *Cx. pipiens* from CA exposed to permethrin (60-min DT; 30 µg permethrin/bottle), resmethrin (*d*-phenothrin) (45-min DT; 10 µg phenothrin/bottle), and resistance to those exposed to malathion (DT not reported; 100 µg malathion/bottle) (McAbee et al. 2003). When considered with our findings, it is clear that variation in susceptibility varies among different mosquito populations and we reiterate that resistance/susceptibility testing (with technical grade and/or formulated products) should be carried out regularly.

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There may be different insecticide selection enacted on day-active mosquitoes, such as *Ae. albopictus* and *Ae. aegypti*, compared to *Culex* species that are active at dusk and dawn (Richards et al. 2017). Currently, the primary method for controlling *Ae. albopictus* and *Ae. aegypti* is reduction of oviposition sites (Smith et al. 2016) and barrier sprays that persist on vegetation where mosquitoes rest. Other methods, such as the sterile insect technique (the release of male genetically modified mosquitoes carrying a dominant lethal gene) and *Wolbachia*-infected mosquitoes are being used in some regions (e.g., Australia, South America, United States-CA, KY, NY) for controlling these day-active mosquitoes; however, insecticide are generally the preferred method for control in disease outbreak situations (Dobson et al. 2016, Smith et al. 2016, Schmidt et al. 2017).

In the current study, AI doses and DTs were based on susceptible *Aedes* and *Culex* laboratory colonies. MCPs interested in conducting insecticide resistance/susceptibility testing can use our (or the CDC's) AI-specific doses as a starting point for establishing baseline doses and DTs unique to their target mosquito population. However, as we have shown here, even laboratory colonies maintained in the same laboratory can show differences in susceptibility from year to year (as evidenced by some DD differences used in 2015 compared to 2016 here). It is important to monitor seasonal trends in resistance/susceptibility for field mosquito populations of interest to local programs. Control programs should also consider evaluating resistance/susceptibility to formulated insecticide products as these products are expected to have additional ingredients that may impact mortality rate (compared to technical grade AIs used here). Data from bottle bioassays using technical grade AIs are not necessarily directly correlated to field doses of formulated products, and these results should not be extrapolated to inform state-wide decisions on mosquito control applications. Furthermore, different products may be designed to persist on foliage for weeks (such as barrier spray products), while other products provide more short-term control (ultra-low volume [ULV] products). When designing the appropriate insecticide resistance/susceptibility monitoring plan, all of these components should be considered to assist in development of mosquito control policies.

Another factor to consider is the impact of insecticide resistance pressure from sources other than private or public MCPs. It is known that agricultural applications of insecticides can impact mosquito populations, even though mosquitoes are not the target of this application (e.g., Kibuthu et al. 2016; Luc et al. 2016). Mosquito larvae and adults may be exposed to insecticides used in agriculture. In addition, homeowners may apply insecticide products (for mosquitoes and other pests) to their properties, and this can influence insecticide resistance/susceptibility. For example, the *Cx. pipiens/quinquefasciatus* collected from Greenville, NC (Curry Court) was sampled from a retention pond in an area known to receive little to no insecticide treatment. However, Greenville, NC is located in a region of substantial agricultural production. Hence, runoff from nearby impervious surfaces (e.g., parking lots) may contain insecticide (or other) residue that could have contributed to this mosquito population's high degree of resistance to most of the AIs. Future studies are planned to evaluate the impact of agricultural runoff (containing AIs commonly used in adulticides) in relation to larval resistance/susceptibility and subsequent adult resistance. MCPs may also consider communicating with agricultural operations in order to determine the type and frequency of AIs being used. The runoff issue would not apply to container-inhabiting *Aedes* mosquitoes, hence this may be another reason contributing to differences in susceptibility/resistance we observed between *Culex* and *Aedes* mosquitoes. Others have shown high mortality rates in *Ae. aegypti* exposed to deltamethrin (Marriel et al. 2016). It is possible that droplets drifting from ULV or barrier insecticide sprays may enter containers with *Ae. aegypti* or *Ae. albopictus* larvae, hence causing mortality. This should be investigated further.

Insecticide resistance in mosquitoes is a growing issue of global concern (Smith et al. 2016). As pathogens such as DENV, WNV, ZIKV, malaria protozoans, and others continue to impact public health, MCPs should be vigilant to ensure the most efficacious control methods are being used. Resistance should be regularly monitored by more MCPs, and the types of AIs and dosages of products should be adjusted, based on these findings. In any case, targeted (to certain species and populations) surveillance-based mosquito control (rather than widespread calendar-based or complaint-based control) should be carried out to minimize the amount of insecticides applied. MCPs may consider rotating classes of products with different modes of action in order to minimize or delay the development of resistance (e.g., Nauen 2007) and/or using a product that contains more than one AI. Mosquito populations that are resistant to an insecticide with one mode of action may revert back to susceptible status over time after that insecticide is no longer used; however, the speed of this reversion depends on the degree of fitness costs incurred by the mosquito and other factors (IRMC 2010).

Insecticide resistance is a complex issue; however, the aforementioned practices have the potential to reduce the incidence of resistance in mosquito populations. More research is needed on fitness costs incurred by mosquitoes exposed to insecticides, but not killed, as this can inform risk assessments and models of epidemic and enzootic cycles. In addition to surveillance-based targeted control, it is increasingly important to develop new AIs with innovative modes of action and formulated products that target mosquitoes in different ways. As development of new AIs and products is expensive, maintaining the efficacy of current products is important and economically beneficial.

Supplementary Material

Supplementary data are available at *Journal of Medical Entomology* online.

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