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A Comparative Analysis of Resistance Testing Methods in Aedes albopictus (Diptera: Culicidae) from St. Johns County, Florida

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A comparative analysis of resistance testing methods in *Aedes albopictus* (Diptera: Culicidae) from St. Johns County, Florida

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Abstract

Aedes albopictus Skuse (Diptera: Culicidae) was tested for resistance to permethrin, bifenthrin, and malathion using Centers for Disease Control and Prevention (CDC) bottle bioassays and topical toxicology assays on adults and larval bioassays. Eggs were collected from 3 locations across St. Johns County, Florida, raised to the F3 generation and compared with an insecticide susceptible laboratory strain. Results from CDC bottle bioassays with permethrin indicate no significant differences between the 3 wild-type strains and the laboratory strain but suggest the possibility of resistance in 1 strain. Bottle bioassay results for malathion were inconclusive. Topical toxicological results for adults and bioassays for larvae showed a significant difference in permethrin resistance between the control strain and 1 of the wild-type strains. Results from this project indicate that insecticide susceptibility testing should be a regular part of mosquito surveillance programs. Upon detection of resistance, detailed dose response bioassays should be performed to quantify the resistance and mechanisms in local vector populations.

Key Words: Asian tiger mosquito; susceptibility; permethrin; bifenthrin; malathion

Resumen

Aedes albopictus Skuse (Diptera: Culicidae) fue sometida a pruebas de resistencia a permetrina, bifentrina y malatión utilizando bioensayos con botellas del CDC (Centro de Control de Enfermedades y Prevención) y ensayos de toxicología en larvas y adultos. Se recolectaron huevos de tres localidades del condado de St. Johns, Florida, los cuales fueron criados hasta la generación F3 y fueron probardos contra una cepa de laboratorio susceptible al. Los resultados de los bioensayos con botellas de CDC no indican diferencias significativas entre las tres cepas de tipo salvaje y la cepa de laboratorio, pero sugieren la posibilidad de resistencia a la permetrina en una cepa. Los resultados del bioensayo de botella para el malatión fueron inconclusivos. Los resultados toxicológicos para adultos y larvas mostraron diferencias significativas entre la cepa control y una de las cepas silvestres para la resistencia a la permetrina. Los resultados de este proyecto indican que las pruebas de susceptibilidad a los insecticidas deben formar parte de los programas de vigilancia de los mosquitos. En caso de detectar resistencia, deben realizarse bioensayos detallados de dosis-respuesta para cuantificar la resistencia y los mecanismos en las poblaciones locales de vectores.

Palabras Clave: mosquito tigre asiático; susceptibilidad; permetrina; bifentrina; malatión

Adulticide application, habitat source reduction, and larviciding are the most important tools for prevention and control of arthropod vector borne diseases. However, widespread and continued use of insecticides against mosquito populations has often led to the development of resistance to the chemicals used for their control (WHO 1998; Lima et al. 2003; Coleman & Hemingway 2007).

Simple bioassays to monitor and evaluate insecticide susceptibility are vital for effective vector control and resistance management. Although there are a variety of methods for testing the susceptibility of mosquito populations, the most widely used outside of the United States is the World Health Organization (WHO) diagnostic assay, which uses filter papers impregnated with insecticides and a carrier oil that test predetermined diagnostic dosages (WHO 1981). In the United States, a more common method of monitoring insecticide susceptibility is the Centers for Disease Control and Prevention (CDC) bottle bio-

assay, a method that involves aspirating mosquitoes into glass bottles treated with insecticide (Brogdon & McAllister 1998a, b).

Quantifying resistance and the underlying mechanisms requires more sensitive, time consuming tests such as direct topical application, biochemical screening, and molecular testing. Larval bioassays and topical application of insecticides to adults allow development of defined toxicological data for calculation of resistance ratios; a measure that WHO and CDC bioassays were not designed to produce. Biochemical testing can detect increased enzyme activity for systems involved in enhanced enzymatic detoxification and molecular methods use allele specific PCR assays and sequencing to test for genetic changes linked to resistance (Coleman & Hemingway 2007).

Many reports of insecticide resistance and regional or country-wide distributions of the vector are based on very limited datasets from a single location within a country and may be years, if not de-

cades old (Brogdon & McAllister 1998a, b). Insecticide resistance also can occur in patches throughout a region; this is most likely due to type of treatment and amount of exposure of the mosquito population to pesticides (Herath et al. 1987; Liu et al. 2004; Marcombe et al. 2014).

Insecticide resistance is well documented in vectors like *Aedes aegypti* (L.) and *Anopheles gambiae* Giles, but there have only been a few reports of limited insecticide resistance in the common invasive pest, *Aedes albopictus* Skuse (Liu et al. 2004; Coleman & Hemingway 2007; Vontas et al. 2012; Marcombe et al. 2014). Vontas et al. (2012) compiled studies from populations across a wide geographical area (i.e., India, Malaysia, Thailand, Cameroon, Greece, and Italy) and reported that the pyrethroids, deltamethrin and permethrin, were highly effective against *Ae. albopictus* adults. The data compiled from these regions indicated that *Ae. albopictus* has remained susceptible to pyrethroids as well as to the carbamate propoxur and the organophosphate malathion for over 20 years. There has been 1 report of knockdown (*kdr*) mutations in the sodium channel of *Ae. albopictus*, which reduces sensitivity of the sodium channel to pyrethroids and is the most common form of target site resistance found in numerous mosquito species (Kasai et al. 2011).

Aedes albopictus is an invasive species from Southeast Asia; it was first identified in Florida in Duval County in 1986 (Peacock et al. 1988; Benedict et al. 2007), and has spread throughout the entire state (Ali et al. 1995). It has displaced the yellow fever mosquito, *Ae. aegypti,* in many parts of Florida (O'Meara et al. 1995). *Aedes aegypti* and *Ae. albopictus* are potential threats to human health as they are capable vectors for dengue fever, chikungunya, and Zika viruses (Mitchell et al. 1987, Charrel et al. 2007). In addition, *Ae. albopictus* can transmit eastern equine encephalitis virus, La Crosse encephalitis virus, West Nile virus, and is also a likely vector of dog heartworm (Scott et al. 1990; Nayar & Knight 1999; Gerhardt et al. 2001, Turell et al. 2001; Liu et al. 2004). *Aedes albopictus* larval habitats are not limited to containers but also include sylvan ecosystems, tree holes, plants that hold water such as bamboo, bromeliads, and even grooves and pits in rocky surfaces (Washburn & Hartmann 1992; Johnson & Sukhdeo 2013). St. Johns County, Florida, encompasses a mix of urban, suburban, and agricultural habitats with established *Ae. albopictus* populations. The suburban northern region is bordered to the east by the Atlantic Ocean and to the west by the St. Johns River. The city of St. Augustine is a small densely populated urban region in the eastern portion of the county with large agricultural regions to the west that produce potatoes, cabbage, and silage. An earlier report of 2 *Ae. albopictus* colonies from this county indicated that some low levels of malathion resistance might be present in larvae (Marcombe et al. 2014). In this study we examined 3 field collected strains of *Ae. albopictus* from different habitats in St. Johns County, Florida (Fig. 1). We initially performed CDC bottle bioassays to develop time-series mortality curves to determine insecticide susceptibility in these geographically separate populations. We then compared the results of this initial bottle bioassay testing with 2 other methods of assessing resistance: the adult topical bioassay and larval bioassay, to determine the extent to which these methods are comparable to each other.

Materials and Methods

STUDY SITE

Three sites were chosen across St. Johns County, Florida (Fig. 1), which represented a mix of available habitats. The first collection site RAYS (29.877225°N, 81.324971°W) is a tire pile at a store centrally located in the collection area. The surrounding vegetation is oak (*Quer-*
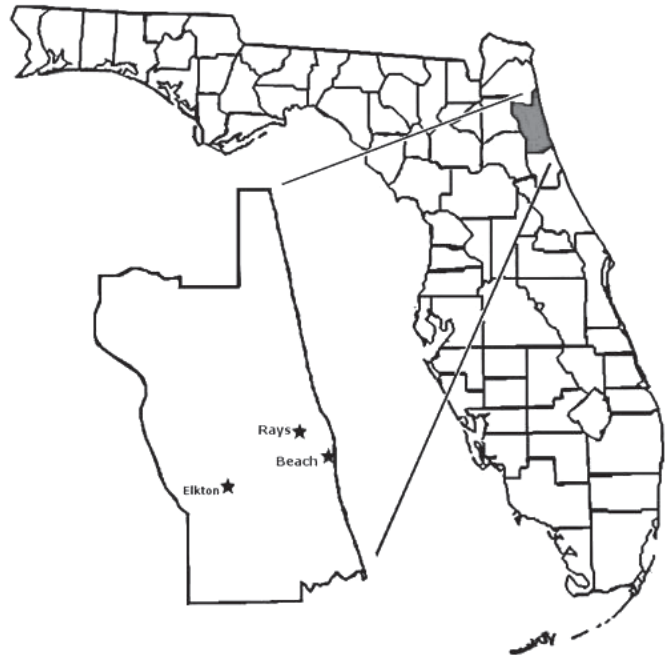
Fig. 1. Collection site locations in St. Johns County, Florida, for the 3 *Aedes albopictus* field strains tested for resistance in this study. RAYS is 14.7 km from ELKTON. RAYS is 6.6 km from BEACH. ELKTON and BEACH are 18.0 km apart. ELKTON and RAYS were the F1 and F2 sites in Marcombe et al. (2014).

cus spp.) with thick understory vegetation. The tires at this site are regularly treated with *Bacillus thuringiensis israelensis* (Bti) and the area is treated with permethrin products during the mosquito season. The second site, ELKTON (29.800105°N, 81.449266°W), is in the western rural part of the county with a large tire pile surrounded by farmland and ditching in an area regularly treated for agricultural pests and intermittently treated by the local mosquito control district with permethrin products during the mosquito season. Finally, the BEACH site (29.843097°N, 81.269832°W) is on the east side of the county and characterized by thick coastal oak and coastal understory vegetation in a 1980s-developed residential neighborhood occasionally treated with permethrin products by the local mosquito control district. The storm drains in the area are treated with methoprene slow release briquettes by the local mosquito control district.

EGG COLLECTION

Seed germination paper (30 × 10 cm) served as mosquito oviposition substrate (Anchor Paper Co., St. Paul, Minnesota). The containers that held the cards were 30.5 × 7.3 cm (height × width) green polypropylene cemetery vases with detachable spikes (Leggs Manufacturing Co., Fairfield, Illinois). Vases were allowed to season in the field for an average of 2 wk prior to placement of egg cards. The stock infusion water recipe was 3 parts oak leaves to 1 part grass clippings in a Rubbermaid 75 L black trash can filled with pond water. The mixture was allowed to ferment for an average of 2 wk depending on the ambient temperature. The infusion water was diluted 1:1 with tap water. Approximately 250 mL of the infusion water was placed in each vase after the egg cards were added. Vases were placed at least 0.25 m apart and near habitats preferred by container breeding mosquitoes. There were 5 to 10 vases at each site. Once a week, the egg cards and infusion water were replaced.

After collection, cards were brought back to the Anastasia Mosquito Control District (AMCD) laboratory (St. Augustine, Florida), covered

with paper towels, and allowed to dry for 3 days. After drying, the cards were placed in a 4.55 L (1 gallon) plastic storage bag with a cotton ball dampened with tap water.

MOSQUITO REARING

Eggs were delivered to the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), United States Department of Agriculture Agricultural Research Service (Gainesville, Florida). Eggs were hatched at room temperature (22.5 ± 1.5 °C), and larvae were reared following the standardized *Ae. aegypti* rearing methods described in Pridgeon et al. (2008).

Four strains of *Ae. albopictus* were tested: CMAVE, RAYS, ELKTON, and BEACH. The control strain (CMAVE) came from eggs from the Kline Laboratory at CMAVE originally collected in Gainesville, Florida, and has been in colony for 4 yr. Field collected eggs were limited in number; therefore, to ensure sufficient mosquito numbers for testing, field colonies were reared to the F2 and F3 generation using standardized methods (Pridgeon et al. 2008).

BOTTLE BIOASSAYS

CDC bottle bioassays were used to assess insecticide susceptibility in field collected *Aedes albopictus*. Bottle bioassays were conducted following Brogdon & McAllister (1998a). Technical grade permethrin, bifenthrin, and malathion (Chemservice, Westchester, Pennsylvania) were chosen to match the active ingredients used in local control measures. Stocks of 10 mg/mL and dilutions were prepared immediately before use. Permethrin and bifenthrin stocks were made in dimethyl sulfoxide (DMSO) and then diluted in acetone, and malathion, already in liquid form as technical grade material, was diluted directly in acetone. Glass Wheaton® bottles (250 mL) were treated with 1 mL of pesticide solution at 3 concentrations. Technical grade permethrin, bifenthrin, and malathion concentrations tested are specified in Table 1. Bottles treated with 1 mL acetone served as a control. Eight bottles were made for each trial and chemical; 2 bottles were acetone-only controls and 2 for each concentration of pesticide (= 2 + [2 × 3] = 8). Each replicate consisted of 3 chemical dilution bottles and 1 control bottle for the two strains to be tested, i.e. the CMAVE control strain and 1 of the wild type strains. Fifteen to twenty nonblood-fed females 5 to 7 days post-emergence were introduced into the glass bottles. Every 5 min, a mortality count was performed, this is different from the CDC protocol of a count every 15 min, and was done to generate a more detailed mortality time curve. This process was repeated for 2 h or until all mosquitoes were dead. Following CDC guidelines, the mortality criteria included mosquitoes with difficulty flying or standing on the bottle surface (Brogdon & McAllister 1998a). At the conclusion of the replicate, the bottles were placed at −20 °C to kill any remaining live mosquitoes and a second replicate was conducted with new mosquitoes from the control and test strains. Each trial consisted of 2 replicates, and a total of 3 independent repetitions were performed for each strain.

TOXICOLOGICAL ASSAYS

Direct topical application to adult females produces an LD_{50}, a quantitative measure, for a strain; the method has been used for extensive screening of natural products as well as laboratory derived compounds (Pridgeon et al. 2008). Permethrin adult topical assays and larval assays were conducted following protocols described previously (Akdag et al. 2014; Chang et al. 2014). The same technical grade permethrin was used to make a dilution series to provide an independent measure for comparison to the results observed in the CDC bottle bioassays.

The adult topical assay results are determined by the application of a known toxicant dose in 0.5 µL of acetone to the thorax of a cold anesthetized female. This allows precise plotting of a dose response curve to determine values of median lethal dose (LD_{50}) the dose required to achieve 50% mortality. Adult topical treatments were repeated at least 3 times for each strain on females 5 to 7 days post-emergence. The CMAVE *Ae. albopictus* strain was used as the susceptible control for comparison. The weight of the *Ae. albopictus* females used for these studies averaged 2.3 ± 0.3 mg (mean ± standard deviation) and organisms were cold anesthetized before application of dilutions of permethrin. Mortality was scored at 24 h after application. Permethrin was the only chemical used for these tests due to low mosquito numbers.

The larval bioassay used a similarly prepared dilution series to determine the effect on first instar larval mosquitoes. Due to limited numbers of test organisms, permethrin was tested in the larval assay. The protocol used the modified method described in Meepagala et al. (2015) to accommodate assays in 96 well plates. Each well contained 5 first instar larvae in 188 µL of water with an addition of food slurry (10 µL) and pesticide dilution (2 µL). The dilution series consists of the lowest concentration to cause 0% mortality to the highest concentration at which 100% mortality occurs.

STATISTICAL ANALYSES

Hypothesis testing was conducted on the bottle bioassay data at the 95% confidence level (CI; α = 0.05) to assess for significant differences in mortality and in time to 100% mortality among strains, among doses, and the strain × dose interaction. Preliminary goodness-of-fit testing using the Kolmogorov–Smirnov test for normality (Smirnov 1939) and the Bartlett test for homoscedasticity (homogeneity of variances) (Bartlett 1937a, 1937b) indicated that, even after 2 logarithmic data transformations to attempt to normalize the data, the data were non-normal and non-homoscedastic. Thus, the rank-based non-parametric Kruskal–Wallis (K–W) hypothesis test (α = 0.05) (Kruskal & Wallis 1952; Zar 1999) was used to assess for significant effects of strain and dose on mortality and on time to 100% mortality. Following the hypothesis test, an optimal post hoc multiple-comparison test (Tukey 1949, 1953) was conducted on the ranked data for each of the factors and interactions to identify the specific pairwise combinations of each factor and interaction to the overall variability (sources of variance). The statistical analysis was conducted using Intel Visual Fortran Compiler XE 2013 (Intel Corporation, Santa Clara, California).

Statistical analysis of adult topical bioassay and larval bioassay data were analyzed in a similar manner. In the adult topical bioassay, we calculated the 95% CI of the median lethal dose (LD_{50}), the dosage at which 50% mortality occurs. Similarly, the larval bioassay data provided the median lethal concentration (LC_{50}), the concentration required to achieve 50% mortality. Data was fit to a 4 parameter logistic equation

Table 1. Doses of toxicants used in Centers for Disease Control and Prevention (CDC) bottle bioassays for *Aedes albopictus*.

Chemical	0.5×DD (µg per bottle)	DD (µg per bottle)	2×DD (µg per bottle)
Permethrin	7.5	15.0	30.0
Bifenthrin	10.0	20.0	40.0
Malathion	50.0	100.0	200.0

Diagnostic doses (DD) are based on CDC recommendations for *Aedes aegypti*. CDC does not make recommendations for *Aedes albopictus* or for bifenthrin. 0.5×DD is half the diagnostic dose, DD is the diagnostic dose, and 2×DD is twice the diagnostic dose.

with the minimum and maximum specified as 0.00 and 1.00 respectively. Confidence intervals (95%) were calculated using the standard formula 95% CI = $LD_{50} \pm 1.96$ (SE_{LD50}). Where SE = standard error of the mean. In accordance with previous studies (Cumming et al. 2007; Marcombe et al. 2014), results for strains were considered to have significantly different LD_{50} values if the 95% CI did not overlap. Curve fitting and standard error calculation were performed with SigmaPlot v13 (Systat Software, Inc., San Jose, California).

Results

BOTTLE BIOASSAYS

Permethrin

The CDC bottle bioassay guide (Brogdon & Chan 2010) specifies 15 µg/bottle of permethrin as the diagnostic dose (DD) for *Aedes* species at the diagnostic time (DT) of 30 min. We used these guidelines to test permethrin-treated bottles against *Ae. albopictus* and found no insecticide resistance in the control CMAVE (87 ± 5% mortality, mean ± SE), Rays (95 ± 4%), and Elkton (90 ± 5%) strains, which all reached >80% mortality (Table 2). The CDC guidelines state that mortality between 80% and 97% indicates a potential for resistance, however, the dosage guidelines are set for genera and are not specifically defined to the species level, which could indicate different levels of susceptibility between species. The BEACH strain produced lower mortality to permethrin (68 ± 15%) at the 30-min DT. According to the bottle bioassay manual, the BEACH strain appears to show resistance (Table 2). However, the 30-min mortality among the 4 strains at 15 µg/bottle were not different (X^2 = 2.5500, X^2_{crit} = 7.8150, df = 3, 20; P = 0.4736). Specifically, post hoc analysis showed that the CMAVE control was not different from BEACH (Q = 0.0290, Q_{crit} = 2.7720, P = 0.8233), ELKTON (Q = 1.0455, Q_{crit} = 3.3140, P = 0.6947), or RAYS (Q = 1.8297, Q_{crit} = 3.6330, P = 0.5552).

Bifenthrin

Bifenthrin, another pyrethroid, was tested in the bottle bioassay. As there is no CDC recommendation for the DD of bifenthrin in the bioassay manual, a range of doses were used to determine the DD. Diagnostic dose is specified as the amount of insecticide that kills 100% of the test organisms within a given time (Brogdon and Chan 2010). We determined 20 µg/bottle for the DD. Three of the 4 strains had >90% mortality at 30 min exposure (DT) (CMAVE 98 ± 2%; RAYS 98 ± 2%, and ELKTON 97 ± 3%) (Table 2). The BEACH strain had 70% (70 ± 12%) mortality after 30 min exposure, but reached 100% mortality by 40 min. The 30-min mortality among the 4 strains at 20 µg/bottle were not different (X^2 = 6.6438, X^2_{crit} = 7.8150, df = 3, 20; P = 0.0874). Specifically, post hoc analysis showed that the CMAVE control was not different from ELKTON (Q = 0.0592, Q_{crit} = 2.7720, P = 0.8129), RAYS (Q =

0.1184, Q_{crit} = 2.7720, P = 0.7922), or BEACH (Q = 2.4277, Q_{crit} = 2.9180, P = 0.1090).

Malathion

An earlier study (Marcombe et al. 2014) indicated possible malathion resistance in *Ae. albopictus* larvae from 1 location that had a history of occasional use of malathion treatments for adulticiding operations. We assessed the effect of malathion exposure on the field-collected *Ae. albopictus* strains from our study. Initial bottle assays showed no difference in mortality at concentrations greater than 200 µg/bottle (data not shown), therefore, we used lower doses of 100 and 200 µg/bottle (Table 1), which are above the CDC recommendation of 50 µg/bottle for *Aedes* (Brogdon & Chan 2010). However, the CDC recommendation is suggested as a starting point, others have tested concentrations up to 474 µg/bottle (Sun et al. 2014). Complete (100%) mortality was not achieved for any of the 4 strains tested at these doses within the 2-h duration of the study, although it was most effective against the ELKTON strain (50 µg/bottle 98 ± 1.8%, 100 µg/bottle 98 ± 1.5%, and 200 µg/bottle 98 ± 1.7%), compared with the other strains. There was a significant difference between the ELKTON and the other 2 field strains as well as the susceptible control. Overall, 2-h mortalities at 200 µg/bottle among the 4 strains were different (X^2 = 9.5560, X^2_{crit} = 7.8150, df = 3, 20; P = 0.0234). Specifically, post hoc analysis showed that the ELKTON strain was different from RAYS (Q = 5.4073, Q_{crit} = 3.0496, P = 0.0003), BEACH (Q = 6.7591, Q_{crit} = 3.0496, P < 0.0001), and CMAVE (Q = 5.8579, Q_{crit} = 3.0496, P < 0.0001), whereas there were no differences between RAYS and CMAVE (Q = 0.4506, Q_{crit} = 3.0496, P = 0.6898), between BEACH and CMAVE (Q = 0.9012, Q_{crit} = 3.0496, P = 0.5702), or between BEACH and RAYS (Q = 1.3518, Q_{crit} = 3.0496, P = 0.4505).

ADULT TOPICAL ASSAYS

Topical bioassays were performed to confirm possible permethrin resistance in the BEACH strain. Three experiments were performed for each strain to develop LD_{50} 95% CI. The susceptible CMAVE strain resulted in a 95% CI of 0.10 to 0.15 ng/insect. Neither the RAYS nor BEACH strains were significantly different from the susceptible CMAVE strain (Table 3). Testing revealed the ELKTON strain was significantly more resistant at 0.25 to 0.40 ng/insect, with a minimal level of resistance of about 2-fold.

LARVAL BIOASSAYS

Larval bioassays with permethrin induced 50% mortality (LC_{50}) in the control (CMAVE) strain within the range of 29 to 45 pg/ml (95% CI). Larvae from both the RAYS and ELKTON strains had CIs that overlapped with the CMAVE strain thus indicating no significant difference. The BEACH strain with an LC_{50} range of 49 to 90 pg/ml (95% CI) was significantly different from the CMAVE strain (Table 4).

Table 2. Mortality (% ± standard deviation [SD]) of *Aedes albopictus* from St. Johns County, Florida, in the Centers for Disease Control and Prevention (CDC) bottle bioassay.

Chemical	DD/DT[1]	Mortality (%; mean ± SD) by strain			
		CMAVE	BEACH	ELKTON	RAYS
Permethrin	15 ug/30 min	87 ± 5	**68 ± 15**	90 ± 5	95 ± 4
Bifenthrin	20 ug/30 min	98 ± 2	**70 ± 12**	97 ± 3	98 ± 2
Malathion	200 ug/2 h	68 ± 3	**53 ± 15**	98 ± 2	**69 ± 10**

DD/DT, which is diagnostic dose/diagnostic time, are based on CDC recommendations for *Aedes aegypti*. CDC does not make recommendations for *Aedes albopictus* or for bifenthrin. Mortality readings below CDC specified parameters (<80%) that indicate the resistance cutoff are in **bold text**.

Table 3. Topical bioassay results of *Aedes albopictus* adult females from St. Johns County, Florida, with technical grade permethrin.

Strain	95% CI of LD_{50}[a] (ng/insect)	N	R^2
CMAVE (susceptible)	0.10–0.15	190	0.9714
BEACH	0.11–0.19	180	0.9731
RAYS	0.12–0.20	150	0.9332
ELKTON	**0.25–0.40**	**180**	**0.9750**

[a]lethal dose to 50% mortality (LD_{50}) are presented as the 95% confidence interval (CI) range. If ranges do not overlap, the LD_{50} values are considered statistically different at *P* <0.05 and are highlighted by **bold text** (Cumming et al. 2007)

Discussion

The purpose of our study was to evaluate a larger field sample of *Ae. albopictus* populations within St. Johns County, Florida, for insecticide resistance. Previous studies have shown that permethrin resistance in *Ae. albopictus* is relatively slow to develop compared with another container-inhabiting mosquito, *Ae. aegypti* (O'Meara et al. 1995; Vontas et al. 2012). An earlier study indicated a low level of larval resistance to malathion in a strain collected from the county (Marcombe et al. 2014). We used F2 and F3 *Ae. albopictus* to examine susceptibility to active ingredients in pesticides used in St. Johns County for mosquito control or in local agricultural operations. Although testing with later generations of mosquitoes from the field could increase susceptibility due to colonization effects, due to limited numbers of F1 generation mosquitoes we had to use F2 and F3 generations. A study done by Jirakanjanakit et al. (2007) also used F2 and F3 generations when they tested *Ae. albopictus* from a range of areas in Thailand. The study reported 1 area with some resistance to the organophosphate fenitrothion.

The first-line CDC bottle bioassay indicated some pyrethroid resistance in the BEACH strain. According to the CDC protocol (Brogdon and Chan 2010), if exposed mosquitoes do not reach greater than 80% mortality at the DD and DT, they are considered resistant. The BEACH strain did not reach this threshold after 30 min of exposure (the CDC DT), but it did achieve total (100%) mortality within 1 h of exposure. This result suggests low levels of permethrin resistance in the BEACH strain. With bifenthrin, another pyrethroid, the same result was observed, again indicating low levels of resistance in the BEACH strain by the CDC bottle bioassay. Doses of the 2 pyrethroids above or below the diagnostic dose gave differing results, indicating the importance of testing at the CDC determined dosage.

Malathion-treated bottles did not result in complete mortality within the 2 h exposure time for any of the strains and the results were much less clear. In the 200 µg bottles the ELKTON strain was the most sensitive with 98% mortality at the end of the 2 h exposure period, and the CMAVE, RAYS, and BEACH strains never reaching greater than 70%

Table 4. Larval bioassay results of *Aedes albopictus* first instars from St. Johns County, Florida, with technical grade permethrin.

Strain	LC_{50} (95% CI) (µg/ml)	N	R^2
CMAVE (susceptible)	2.86–4.45×10^{-5}	90	0.9808
BEACH	**4.85–9.04×10^{-5}**	**90**	**0.7892**
RAYS	2.13–4.07×10^{-5}	60	0.9695
ELKTON	4.25–6.12×10^{-5}	90	0.9964

[a]Lethal dose to 50% mortality (LC_{50}) are presented as the 95% confidence interval (CI) range. If the 95% CI ranges do not overlap, the LC_{50} values are considered statistically different at *P* <0.05 and are highlighted with **bold text** (Cumming et al. 2007).

mortality. Notably, mortality was lowest in the BEACH strain. These results are similar to the low level of malathion resistance noted by Marcombe et al. (2014). The reasons behind the increased sensitivity of the ELKTON strain to malathion are unclear and in fact seem at odds with what might be reasonably expected. The strain was collected from an agricultural area that would likely result in increased exposure to pesticides from agricultural operations, and it may be expected that they would possess an enhanced ability for detoxification, which would result in lower mortality (Mouchet 1988; Georghiou 1990).

Reduced mortality with malathion during the bottle bioassay could also be due to mode of action, as this organophosphate does not act as quickly as a pyrethroid and requires additional processing within the organism to the more toxic active form (Elliot et al. 1978). Several other studies have used a 24 h holding period before recording mortality (Juntarajumnong et al 2012; Marcombe et al. 2014), although Sun et al. (2014) described 100% mortality in only 40 min at a higher dose. The test dosage (200 µg/bottle) could be another reason for the lack of mortality we observed, but preliminary testing at a range of doses as high as 500 µg/bottle and 1,000 µg/bottle showed no increased mortality at dosages above 200 µg/bottle.

The CDC bottle bioassay is a field expedient method that gives some information about resistance in a population and can be altered to assess possible resistance mechanisms. It is easily accessible to mosquito control districts as it requires very little laboratory equipment and can be used with small numbers of organisms. As we found indications of permethrin resistance in the BEACH strain and wished to relate the bottle bioassay results to an actual dose or concentration, we performed both adult topical and larval bioassays with the same 4 strains using standard methods (Pridgeon et al. 2009; Ali et al 2013; Chang et al. 2014). These procedures are common in toxicological testing, but are infrequently compared with CDC bottle bioassays, which are mainly used to indicate resistance in field populations.

The larval assay confirmed the low level of pyrethroid resistance noted in the bottle bioassay for the BEACH strain. The levels of resistance noted was similar to the level noted by Marcombe et al. (2014) in St. Johns County *Ae. albopictus*. Larval exposure is an important element of resistance development that has been observed in *Anopheles gambiae* larvae that survive in puddles laced with residual toxicants from agricultural runoff (Yadouleton et al. 2011).

In the adult topical assay, we saw a significant difference in permethrin susceptibility in the ELKTON strain (compared with the CMAVE susceptible strain) that was not observed in the bottle bioassay or the larval assay. We did not detect the significant difference in the BEACH strain for permethrin that was indicated by the CDC bottle or larval bioassays (i.e., low levels of resistance were not observed in adult topical application). This discord between the bottle bioassay and the adult topical assay may point to possible differences in uptake rather than actual toxicity. In the bottle bioassay, the mosquitoes are only subjected to toxicant uptake when they remain in tarsal contact with the coated surface of the bottle. The actual dose received by any 1 mosquito is a function of contact time and is not precisely known. A toxicant that causes excitation or irritancy might reduce bottle resting time, thus reducing total uptake. Each individual organism can choose to fly or remain standing on the surface, resulting in a range of actual doses in the cohort. In contrast, the dose applied during the direct topical assay is known and the same dose is applied to all organisms. Although the acetone used evaporates quickly, the dose of toxicant remains on the cuticle and can continue to penetrate during the 24 h assay period, which may serve to enhance mortality. It is likely that both the adult topical and CDC bottle bioassay would agree in the case of stronger resistance.

In this study, we observed minor differences in *Ae. albopictus* resistance levels that were numerically significant but relatively small, and the biological significance of these small differences is not known. Two items do appear crucial; resistance testing should be a regular part of the surveillance program; and if resistance is detected, to follow up a first line indicator like a CDC bottle bioassay result with more detailed toxicology assays to more clearly identify the presence and types of resistance in local vector populations.

Acknowledgments

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