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## Insecticide Resistance Status of *Aedes aegypti* (Diptera: Culicidae) in California by Biochemical Assays

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### Abstract

Insecticide resistance in *Aedes aegypti* mosquitoes poses a major threat to public health worldwide. There are two primary biological mechanisms that can lead to insecticide resistance, target site and metabolic resistance, both of which confer resistance to specific classes of insecticides. Due to the limited number of chemical compounds available for mosquito control, it is important to determine current enzymatic profiles among mosquito populations. This study assessed resistance profiles for three metabolic pathways,  $\alpha$ -esterases,  $\beta$ -esterases, and mixed-function oxidases (MFOs), as well as insensitivity of the acetylcholinesterase (iAChE) enzyme in the presence of propoxur, among *Ae. aegypti* from the Central Valley and southern California. All field-collected *Ae. aegypti* demonstrated elevated MFOs and iAChE activity, indicating potential development of pyrethroid and organophosphate resistance, respectively. Although regional variations were found among  $\alpha$ -esterase and  $\beta$ -esterase activity, levels were generally elevated, further suggesting additional mechanisms for developing organophosphate resistance. Furthermore, mosquito samples from southern California exhibited a higher expression level to all three metabolic enzymes and iAChE activity in comparison to mosquitoes from the central region. These results could help guide future mosquito control efforts, directing the effective use of insecticides while limiting the spread of resistance.

**Key words:** *Aedes aegypti*, insecticide resistance, California

*Aedes aegypti* (Linnaeus), also known as the yellow fever mosquito, is the primary vector of chikungunya, dengue, yellow fever, and Zika viruses (Scott and Takken 2012, Smith et al. 2016). These arboviruses cause significant morbidity and mortality and incur billions of dollars in healthcare costs each year (Shepard et al. 2011). Half of the world's population live in dengue endemic areas with 50–100 million infections estimated annually worldwide (WHO 2009). *Aedes aegypti* and the viruses that they transmit have expanded into new geographic territories with the increase in global movement of people and goods. Zika and chikungunya viruses have spread throughout the Americas, while yellow fever virus was recently reported in China and resurged in Central Africa (Charrel et al. 2014, Staples et al. 2014, WHO 2014, Kraemer et al. 2015, Wasserman et al. 2016, Wilder-Smith et al. 2017).

Chemical control remains one of the primary means for combating mosquito populations worldwide (Marcombe et al. 2012). There are two major mechanisms that contribute to insecticide

resistance in mosquitoes: target site and metabolic resistance. Minor mechanisms of resistance include physical barriers, such as altered cuticle thickness preventing insecticide penetration, and behavioral resistance, such as altered mosquito behavior in the presence of insecticides (WHO 1998, Zalucki and Furlong 2017). Target site resistance is the failure of an insecticide to bind its target site due to alterations in the structure or inaccessibility of the target site. The primary protein associated with target site insensitivity to pyrethroids is the voltage-sensitive sodium channel (Vssc) protein, which is responsible for the initiation and propagation of action potentials in the nervous system (Du et al. 2013). Changes to the acetylcholinesterase enzyme, which is responsible for breaking down remnants of the neurotransmitter acetylcholine in the synaptic cleft, is associated with target site insensitivity to carbamates and organophosphates (Fukuto 1990). Alterations to the Vssc, known more commonly as knockdown resistance (*kdr*), and the acetylcholinesterase enzyme, commonly known as *Ace-1*, prevent insecticide binding;

therefore, mosquitoes may survive despite exposure to an insecticide. Metabolic insecticide resistance is associated with the mutation or elevated expression of specific enzymes that lead to the rapid detoxification or sequestration of insecticides (Hemingway et al. 2004). Metabolic resistance involves three enzyme families: cytochrome P450 monooxygenases (P450), glutathione-S-transferases (GST), and carboxy/cholinesterases (CCE) (Strode et al. 2008). Previous studies have indicated that the elevated activity of  $\alpha$ -esterase and  $\beta$ -esterase may confer resistance to organophosphates, while the elevated activity of mixed-function oxidases (MFOs) usually confer resistance to pyrethroids (Brogdon 1989).

In 2013, *Ae. aegypti* mosquitoes were discovered in three California counties: Madera, Fresno, and San Mateo. By the end of 2018, *Ae. aegypti* were detected within the jurisdictional boundaries of 247 cities and census-designated places in 13 counties of the Central Valley and southern region (Metzger et al. 2017, Pless et al. 2017). Genetic studies have indicated that there are at least two distinct populations of *Ae. aegypti* currently existing in California: the 'central' population comprising Fresno, Madera, and Tulare counties, and the 'southern' population including Imperial, Los Angeles, Orange, Riverside, San Bernardino, and San Diego counties (Pless et al. 2017). Another study suggests there may have been upwards of four separate introductions of *Ae. aegypti* into the state (Lee et al. 2019). These different populations may exhibit distinct resistance patterns requiring the use of unique chemical control efforts. For example, Liebman et al. (2019) indicated that from 2015 through 2017 the 'central' population displayed almost fixed resistance at the V1016I and F1534C Vssc alleles, potentially contributing to pyrethroid resistance, while the 'southern' population displayed a variation of both resistant and susceptible alleles.

There is little information regarding the enzymatic expression profiles of invasive *Ae. aegypti* populations in California. Due to a limited number of registered chemical products available for adult mosquito control in California, the development of pesticide resistance among *Ae. aegypti* populations throughout the state is of concern (Cornel et al. 2016, Liebman et al. 2019). Further investigation is required to ascertain whether introduced populations of *Ae. aegypti* are already partially or fully resistant to available insecticide products. Thus, biochemical assays were conducted by the California Department of Public Health (CDPH), Vector-Borne Disease Section, to measure the enzymatic activity of  $\alpha$ -esterase,  $\beta$ -esterase, and MFOs, along with insensitive acetylcholinesterase (iAChE) activity. Initially designed at the U.S. Centers for Disease Control and Prevention (CDC), these biochemical assays have been widely used to monitor *Ae. aegypti* populations for pesticide resistance (Brogdon 1989, Valle et al. 2006). Coupled with previously reported *kdr* results (Liebman et al. 2019), these assays provide important insecticide resistance information to local vector control agencies, supporting the design of efficient and effective chemical control strategies.

## Materials and Methods

### Mosquitoes

Local vector control agencies collected *Ae. aegypti* mosquitoes throughout the Central Valley and southern region of California for pesticide resistance testing. Adult mosquitoes were collected using traps (e.g., Biogents Sentinel [BGS]; carbon dioxide-baited), backpack aspirators, and opportunistic larval samples reared to adults prior to preservation and shipping to CDPH. In order to sustain a cold chain, freshly dead female *Ae. aegypti* were stored at  $-80^{\circ}\text{C}$ ,

transported on dry ice, and immediately stored at  $-80^{\circ}\text{C}$  upon receipt at the CDPH laboratory. Mosquito abdomens were removed on an ice block using flame-sterilized dissection tools. The head and thorax of each mosquito was used for biochemical assays and stored at  $-80^{\circ}\text{C}$  until ready for use. The abdomens of these mosquitoes were stored in 70% ethanol and utilized for *kdr* testing as summarized by Liebman et al. (2019). *Aedes aegypti* Rockefeller strain (ROCK) and *Ae. aegypti* Orlando strains were used as susceptible reference strains as available. ROCK (freshly frozen adult female mosquitoes) were obtained through BEI Resources, NIAID, NIH: *Aedes aegypti*, Strain ROCK, MRA-734, contributed by David W. Severson, Manassas, VA and Orlando strain was obtained from Benzon Research, Carlisle, PA.

### Biochemical Assays

Biochemical assay protocols provided by the CDC and the Brazilian Ministry of Health were followed with slight modifications in incubation time, reagent concentrations, and amount of homogenate (Valle et al. 2006, McAllister et al. 2012). Adult mosquito head and thoraces were homogenized in 100  $\mu\text{l}$  of potassium phosphate buffer ( $\text{KPO}_4$ , pH = 7.2) using  $3 \times 2.8$  mm beads in a Bead Ruptor 24 (OMNI, Kennesaw, GA) and then resuspended in 900  $\mu\text{l}$  of  $\text{KPO}_4$ . The resuspension was diluted further by mixing 700  $\mu\text{l}$  of the homogenate with 700  $\mu\text{l}$  of  $\text{KPO}_4$ . Large mosquito debris was excluded from testing during this step by allowing the homogenate to settle and pipetting only the supernatant. Samples were screened in triplicate in 96-well clear microplates (VWR, 62409 068, Visalia, CA) and plates were read using a Biotek Powerwave 340 plate reader (Winooski, VT). Susceptible (Orlando or ROCK susceptible strain) controls were included (in triplicate) with every run as quality control.

Protocols established by Brazil's Ministry of Health were modified and used to test for both  $\alpha$ -esterase and  $\beta$ -esterase activity (Valle et al. 2006). A substrate stock solution was prepared by dissolving 28 mg of  $\alpha$ - or  $\beta$ -naphthyl acetate (CAS # 830-81-9 and CAS # 1523-11-1, respectively) in 5 ml acetone and stored at  $4^{\circ}\text{C}$  in a light proof tube. A substrate working solution was prepared daily by diluting 250  $\mu\text{l}$  of the  $\alpha$ - or  $\beta$ -naphthyl acetate stock solution with 24.75 ml of  $\text{KPO}_4$ . A Fast Blue solution was prepared 5 min before use by dissolving 15 mg of *o*-dianisidine (CAS # 14263-94-6) in 15 ml of  $\text{ddH}_2\text{O}$  in a light proof tube. Fifty microliters of mosquito homogenate was added in triplicate to the 96-well plate, followed by 200  $\mu\text{l}$  of  $\alpha$ - or  $\beta$ -naphthyl acetate working solution. After a 15-min incubation, 50  $\mu\text{l}$  of the Fast Blue solution was added to the plate and incubated for another 2 min, which is a shorter incubation period comparing to CDC protocol to reduce acetone degrading on this 96-well clear plate. The plate was read at a 570-nm wavelength.

A modified CDC protocol was used to test for MFOs and iAChE activity, along with total protein concentration (McAllister et al. 2012). For the MFOs assay, 100  $\mu\text{l}$  of mosquito homogenate was added in triplicate to the 96-well plate, followed by 200  $\mu\text{l}$  of 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMBZ, CAS # 64285-73-0) and 25  $\mu\text{l}$  of 3% hydrogen peroxide with acetanilide (CAS # 7722-84-1) as stabilizer, which could provide a stable hydrogen peroxide in this reaction. The working solution for TMBZ was prepared by adding 50 mg of TMBZ into 25 ml absolute methanol (CAS # 67-65-1) and adding 75 ml of 0.25 M sodium acetate buffer (CAS # 64-19-7) (pH 5.0). Plates were incubated at room temperature for 10 min and read at a 620-nm wavelength. For the iAChE assay, 100  $\mu\text{l}$  of mosquito homogenate was added in triplicate to the 96-well plate, followed by 100  $\mu\text{l}$  of acetylthiocholine

iodide (ATCH, CAS # 1866-15-5) with propoxur (CAS # 114-26-1) inhibitor and 100 µl of dithio-bis-2-nitrobenzoic acid (DTNB, CAS # 69-78-3). The plate was read immediately (T0) and again at a 24-h time point (T24) at a 414-nm wavelength. For the protein concentration assay, a diluted dye reagent solution was prepared by adding 20 ml of concentrated protein dye reagent (Bio-Rad, Hercules, CA; 500-0006) to 80 ml of ddH<sub>2</sub>O. Next, only 20 µl of mosquito homogenate was added in triplicate to the 96-well plate, followed by the 200 µl of the protein dye solution. After a 5-min incubation, the plate was read at a 590-nm wavelength.

### Standard Curves

Standard curves were established by creating a serial dilution of a known standard. These serial dilutions were designed to include the possible range of optical density (OD) values for each assay. Both standards and samples were screened through the same procedure and measured by a spectrophotometer. By establishing a standard curve, measured OD values could be interpolated to enzymatic concentration/activity. The standards included bovine serum albumin (CAS # 9048-46-8), cytochrome c (CAS # 9007-43-6),  $\alpha$ - and  $\beta$ -naphthol (CAS # 90-15-3 and 135-19-3) for protein, MFO, and  $\alpha$ - and  $\beta$ -esterase assays to generate standard curves, respectively.

### Statistical Analysis

In order to compensate for variations in the size of mosquito samples, all enzymatic assay results were normalized by protein content for each mosquito by dividing the measured enzymatic activity by their corresponding protein concentration. All data were analyzed using R 3.5.1 (R Core Team 2018). Boxplots were used to visually compare field-caught samples to the reference ROCK strain. Unpaired Wilcoxon rank sum tests were conducted in order to establish statistically significant differences ( $\alpha \geq 0.05$ ) between field-collected mosquitoes and the reference ROCK strain. ArcGIS Desktop 10.5 was used to make maps of California.

### Results

In total, 12 and 17 agencies submitted *Ae. aegypti* to CDPH for biochemical testing in 2017 and 2018, respectively. Based on results of a previous study suggesting two separate introductions of *Ae. aegypti* into California (Pless et al. 2017), mosquito samples were divided according to regional collection sites (Table 1; Fig. 1). 'Central California' included seven vector control agencies from four counties (Fresno, Madera, Merced, and Tulare), and 'southern California' included eleven vector control agencies from six counties (Imperial, Los Angeles, Orange, Riverside, San Bernardino, and San Diego).

All tested *Ae. aegypti* exhibited significantly elevated iAChE and MFOs activity in comparison to the susceptible ROCK strain (Table 1; Fig. 2). Results for  $\alpha$ -esterases and  $\beta$ -esterases were more variable (Table 1; Fig. 2). For  $\alpha$ -esterase activity, 14 (78%) of 18 populations were significantly elevated ( $P < 0.05$ ); 5 (71%) of 7 were elevated in the central region and 9 (81%) of 11 were elevated in the southern region. For  $\beta$ -esterase activity, 8 (44%) of 18 populations exhibited significantly elevated activity ( $P < 0.05$ ); 2 (29%) of 7 from central region and 6 (55%) of 11 from southern region. In general, the southern *Ae. aegypti* populations expressed a higher level of  $\alpha$ -esterases (mean 34.65 vs 27.69 µg  $\alpha$ -naphthol/mg protein/15 min,  $P < 0.05$ ),  $\beta$ -esterases (mean 51.77 vs 38.75 µg  $\beta$ -naphthol/mg protein/15 min,  $P < 0.05$ ), MFOs (mean 1.85 vs 1.49 µg cytochrome c/mg protein,  $P < 0.05$ ), and iAChE (mean 2.03 vs 1.66,

$P < 0.05$ ) activity compared to the central populations (Table 2). Of note, samples from agencies in Fresno, Madera, and Tulare counties (DLTA population with 13 samples submitted) expressed significantly lower  $\beta$ -esterase activity in comparison to the reference strain.

### Discussion

Despite efforts to prevent the expansion of *Ae. aegypti*, these invasive mosquitoes are now present in many of the urbanized areas of the Central Valley and a large portion of urban southern California (Metzger et al. 2017). As *Ae. aegypti* continue to disperse and become well-established in California, utilizing effective chemical control methods is of utmost importance (Metzger et al. 2017). A recent publication indicated *Ae. aegypti* from the two regions displayed distinctly different resistance *kdr* profiles; the V1016I and F1534C resistance mutations of the *Vssc* gene were nearly fixed in mosquitoes tested from the Central Valley, whereas southern populations produced variable results with resistance frequencies ranging from 61 to 84% (Liebman et al. 2019). These results suggest that *Ae. aegypti* throughout California may be predisposed to survive pyrethroid treatment.

MFO are a class of enzymes that are also associated with pyrethroid resistance in insects (Hemingway and Ranson 2000). In this study, highly elevated MFOs activity was detected among field-caught populations from our study sites suggesting that, in addition to *kdr* resistance, a biochemical pathway may also be developing for the detoxification of pyrethrins/pyrethroids (Lewis et al. 1967, Brogdon and McAllister 1998). Although field-caught populations displayed both potential forms of resistance, it is unknown whether elevated MFO activity works separately or in parallel with *Vssc* gene mutations to confer resistance to pyrethroids, and how these two mechanisms affect the insecticide's mode of action.

Esterases are a class of enzymes that are associated with organophosphate, carbamate, and to a lesser extent pyrethroid resistance in mosquitoes (Hemingway and Ranson 2000, Sogorb and Vilanova 2002). The elevated expression of esterase enzymes can lead to resistance by either metabolizing insecticides through the hydrolysis of ester bonds, or through the sequestration of the insecticides (Poupardin et al. 2014). Esterase activity in this study was variable throughout the state with the highest expression exhibited in southern California *Ae. aegypti*. In general, field populations displayed a higher degree of both  $\alpha$ -esterase and  $\beta$ -esterase activity compared to susceptible strains, suggesting the development of resistance to organophosphates. Of note, the significantly low  $\beta$ -esterase activity found in Fresno and Madera counties corroborates previous studies indicating that *Ae. aegypti* populations from these two counties are genetically related relative to other regions of the state (Lee et al. 2019). Additional monitoring may help reveal trends in esterase activity.

In addition, iAChE activity was found to be highly elevated in all field populations, suggesting another potential mechanism for organophosphate resistance in California *Ae. aegypti*. Elevated iAChE activity in the presence of propoxur, a carbamate insecticide, was consistently reported throughout the central and southern regions of California. Mutations in the acetylcholinesterase enzyme can prevent binding of organophosphates to active sites, thereby decreasing or eliminating the insecticide's efficacy (Fukuto 1990). However, the *Ace-1* G119S mutation, the most common mutation conferring organophosphate resistance in mosquitoes, has yet to be described in California *Ae. aegypti* (Saavedra-Rodriguez et al.

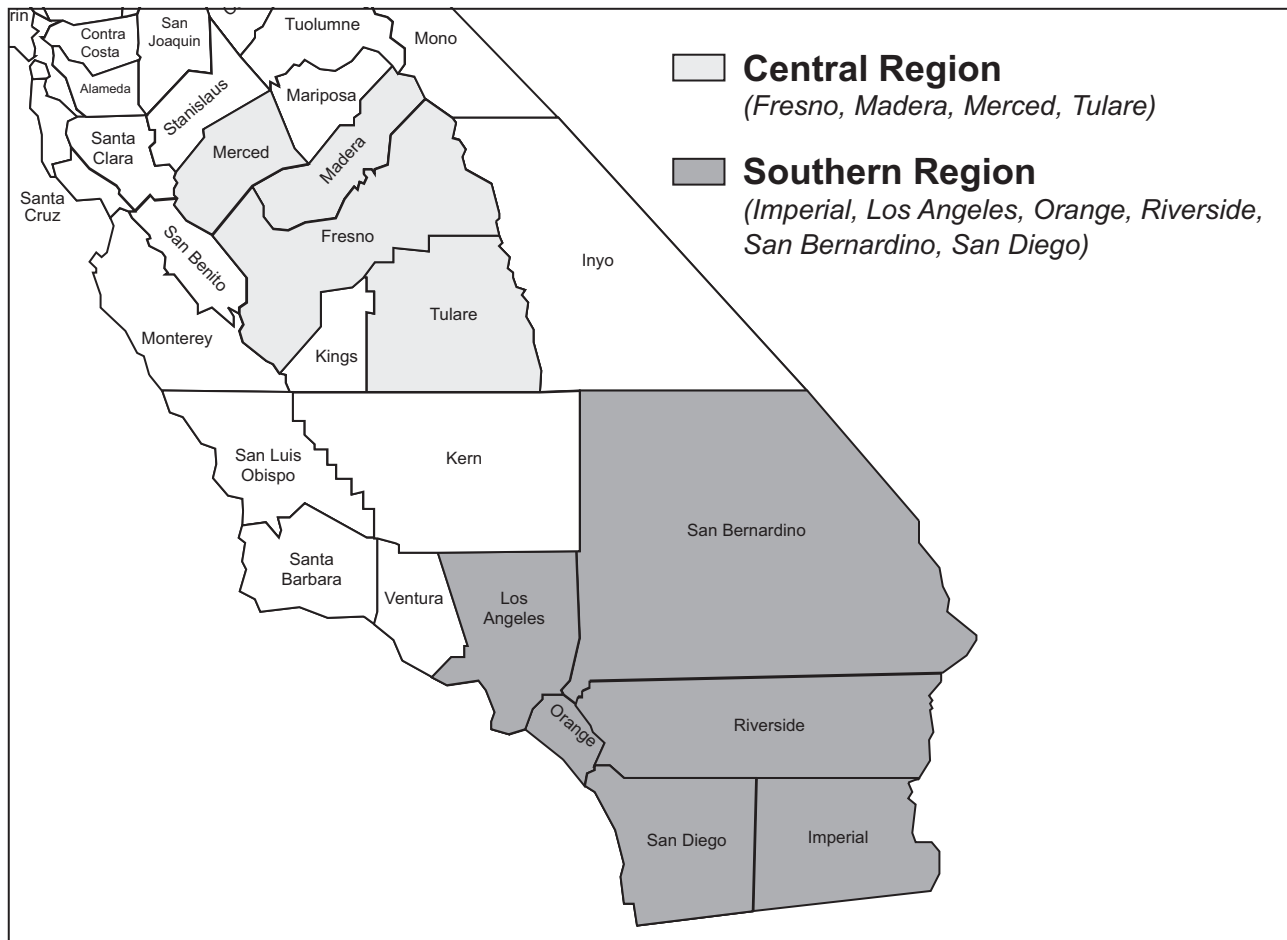
**Table 1.** Mean of  $\alpha$ -esterases,  $\beta$ -esterases, MFOs, and iAChE levels in *Aedes aegypti* in California from 2017 to 2018

| Strain<br>ROCK | Region <sup>a</sup> | County | Agency <sup>b</sup> | $\alpha$ -Esterases |     |          | $\beta$ -Esterases |     |          | MFOs            |     |          | iAChE           |     |          |
|----------------|---------------------|--------|---------------------|---------------------|-----|----------|--------------------|-----|----------|-----------------|-----|----------|-----------------|-----|----------|
|                |                     |        |                     | Mean $\pm$ SD       | N   | P-value  | Mean $\pm$ SD      | N   | P-value  | Mean $\pm$ SD   | N   | P-value  | Mean $\pm$ SD   | N   | P-value  |
|                |                     |        |                     | 23.36 $\pm$ 3.57    | 90  |          | 39.27 $\pm$ 6.33   | 90  |          | 0.94 $\pm$ 0.11 | 90  |          | 0.68 $\pm$ 0.12 | 90  |          |
|                |                     |        |                     | Mean $\pm$ SD       | N   |          | Mean $\pm$ SD      | N   |          | Mean $\pm$ SD   | N   |          | Mean $\pm$ SD   | N   |          |
| C              | Fresno              |        | GNSL                | 28.61 $\pm$ 10.55   | 259 | 6.52e-10 | 36.18 $\pm$ 15.08  | 259 | 1.26e-8  | 1.08 $\pm$ 0.32 | 259 | 0.0010   | 1.25 $\pm$ 0.67 | 259 | 2.2e-16  |
| C              | Fresno              |        | FRNO                | 25.07 $\pm$ 8.71    | 151 | 0.89     | 33.61 $\pm$ 11.9   | 151 | 1.52e-10 | 1.33 $\pm$ 0.93 | 151 | 3.59e-08 | 1.27 $\pm$ 0.75 | 151 | 2.2e-16  |
| C              | Fresno              |        | FRWS                | 28.7 $\pm$ 6.23     | 26  | 2.74e-05 | 35.54 $\pm$ 9.37   | 26  | 0.00084  | 1.3 $\pm$ 0.82  | 26  | 0.0014   | 1.17 $\pm$ 0.72 | 26  | 1.37e-06 |
| C              | Madera              |        | MADR                | 23.61 $\pm$ 4.41    | 142 | 0.95     | 37.76 $\pm$ 7.33   | 130 | 0.019    | 1.32 $\pm$ 1.39 | 286 | 0.0019   | 1.47 $\pm$ 2.81 | 280 | 1.56e-11 |
| C              | Merced              |        | MERC                | 31.32 $\pm$ 25.9    | 172 | 0.0089   | 45.41 $\pm$ 59.35  | 170 | 1.62e-06 | 2.07 $\pm$ 2.39 | 179 | 2.2e-16  | 2.35 $\pm$ 3.96 | 179 | 2.2e-16  |
| C              | Tulare              |        | DLTA                | 21.41 $\pm$ 6.91    | 10  | 0.39     | 28.37 $\pm$ 7.49   | 12  | 7.78e-06 | 1.91 $\pm$ 2.28 | 13  | 0.024    | 3.0 $\pm$ 4.18  | 13  | 3.59e-07 |
| C              | Tulare              |        | TLRE                | 37.46 $\pm$ 11.79   | 13  | 6.68e-06 | 46.73 $\pm$ 15.98  | 13  | 0.037    | 1.47 $\pm$ 0.7  | 13  | 1.47e-08 | 1.81 $\pm$ 0.71 | 13  | 1.38e-08 |
| S              | Imperial            |        | IMPR                | 29.16 $\pm$ 12.44   | 83  | 0.00054  | 48.16 $\pm$ 31.14  | 86  | 0.19     | 1.64 $\pm$ 1.6  | 87  | 1.09e-09 | 1.52 $\pm$ 1.93 | 87  | 1.13e-11 |
| S              | Los Angeles         |        | GRLA                | 33.52 $\pm$ 28.15   | 145 | 9.16e-13 | 55.0 $\pm$ 68.87   | 154 | 0.59     | 1.9 $\pm$ 2.61  | 123 | 6.63e-10 | 2.36 $\pm$ 3.84 | 154 | 2.2e-16  |
| S              | Los Angeles         |        | LACW                | 37.14 $\pm$ 21.47   | 110 | 2.2e-16  | 73.16 $\pm$ 63.56  | 110 | 2.2e-16  | 1.85 $\pm$ 2.23 | 110 | 2.08e-14 | 2.44 $\pm$ 3.81 | 110 | 4.69e-14 |
| S              | Los Angeles         |        | SGVA                | 26.11 $\pm$ 11.59   | 70  | 0.68     | 40.19 $\pm$ 16.97  | 71  | 0.083    | 1.4 $\pm$ 0.51  | 71  | 2.2e-16  | 1.66 $\pm$ 0.98 | 71  | 2.2e-16  |
| S              | Orange              |        | ORCO                | 29.87 $\pm$ 7.78    | 390 | 2.2e-16  | 42.65 $\pm$ 14.46  | 393 | 0.55     | 1.3 $\pm$ 0.62  | 375 | 9.30e-16 | 1.51 $\pm$ 1.11 | 393 | 2.2e-16  |
| S              | Riverside           |        | COAV                | 28.45 $\pm$ 13.04   | 25  | 0.053    | 55.27 $\pm$ 46.39  | 33  | 0.035    | 3.37 $\pm$ 2.96 | 34  | 2.98e-13 | 4.32 $\pm$ 4.35 | 34  | 2.35e-16 |
| S              | Riverside           |        | NWST                | 30.8 $\pm$ 6.96     | 63  | 6.37e-12 | 41.46 $\pm$ 13.12  | 63  | 0.66     | 1.53 $\pm$ 0.97 | 63  | 8.41e-11 | 1.21 $\pm$ 0.7  | 63  | 7.26e-11 |
| S              | Riverside           |        | RIVR                | 49.62 $\pm$ 64.84   | 94  | 2.2e-16  | 60.93 $\pm$ 70.93  | 95  | 0.00095  | 2.04 $\pm$ 2.11 | 95  | 2.2e-16  | 2.05 $\pm$ 2.12 | 95  | 2.2e-16  |
| S              | San Bernardino      |        | SANB                | 36.55 $\pm$ 18.85   | 332 | 2.2e-16  | 53.52 $\pm$ 34.37  | 345 | 1.08e-05 | 2.29 $\pm$ 3.04 | 388 | 2.2e-16  | 2.55 $\pm$ 3.42 | 379 | 2.2e-16  |
| S              | San Bernardino      |        | WVAL                | 42.13 $\pm$ 11.91   | 19  | 1.53e-11 | 60.55 $\pm$ 16.77  | 19  | 1.25e-09 | 1.47 $\pm$ 0.7  | 19  | 8.78e-10 | 1.48 $\pm$ 0.87 | 19  | 1.31e-09 |
| S              | San Diego           |        | SAND                | 51.6 $\pm$ 26.86    | 51  | 2.2e-16  | 68.96 $\pm$ 72.02  | 51  | 3.49e-10 | 3.53 $\pm$ 2.37 | 51  | 2.2e-16  | 2.95 $\pm$ 2.5  | 51  | 2.2e-16  |

P-values were calculated by unpaired Wilcoxon rank sum test and compared to ROCK strain.

<sup>a</sup>C = Central California, S = southern California.

<sup>b</sup>Central Region: Consolidated Mosquito Abatement District: CNSL, Delta Vector Control District: DLTA, Fresno Mosquito Vector Control District: FRNO, Fresno Westside Mosquito Abatement District: FRWS, Madera County Mosquito Vector Control District: MADR, Merced County Mosquito Abatement District: MERC, Tulare Mosquito Abatement District: TLRE; Southern Region: Coachella Valley Mosquito Vector Control District: COAV; Greater Los Angeles County Vector Control District: GRLA, Imperial County Vector Control Program: IMPR, Los Angeles County West Vector Control District: LACW; Northwest Mosquito Vector Control District: NWST; Orange County Mosquito Vector Control District: ORCO, Riverside County Vector Control Program: RIVR, San Bernardino County Vector Control Program: SANB, San Diego County Vector Control Program: SAND, San Gabriel Valley Mosquito and Vector Control District: SGVA, West Valley Mosquito Vector Control District: WVAL.



**Fig. 1.** Central and southern California counties. Mosquitoes from the highlighted counties were submitted for biochemical assay testing. Map generated using ArcGIS-ArcMap 10.5 (ESRI, Redlands, CA).

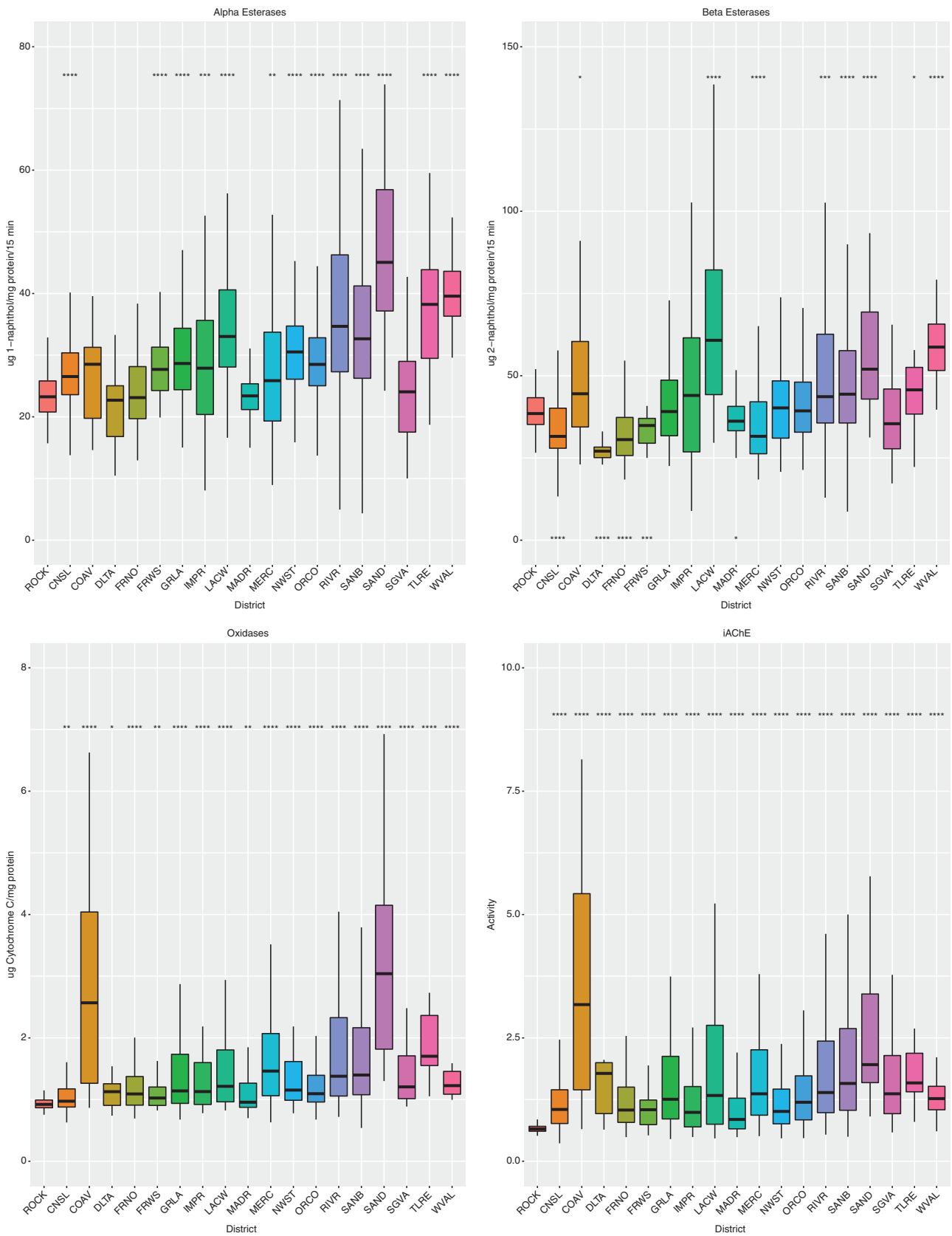
2014, Moyes et al. 2017). Since mosquito homogenates that we measured in this assay contain *Ace-1* and detoxification enzymes, the findings from this study and previous research indicate two possible reasons: there is an alternative organophosphate target site resistance in *Ae. aegypti* or upregulated detoxification enzyme expressions, like carboxylesterase, may cause this elevated iAChE activity in microplate results (Saavedra-Rodriguez et al. 2014, Grigoraki et al. 2016). Further sequencing of the *Ace-1* gene in California *Ae. aegypti* should be conducted to validate the development of known pesticide resistance-associated point mutations (Moyes et al. 2017).

Because the same specimens were tested by both *kdr* and biochemical assays, it was possible to compare and contrast the resistance patterns identified by both types of assays. As described above, *kdr* testing of central populations exhibited almost complete Vssc resistant mutations at both the V1016I and F1534C alleles, while southern populations showed a combination of resistant and susceptible alleles, with increasing resistant mutations from 2015 to 2017 (Liebman et al. 2019). In contrast, biochemical results from 2017 and 2018 indicated that southern *Ae. aegypti* exhibited higher  $\alpha$ -esterase and  $\beta$ -esterase, MFOs, and iAChE activity than central populations (Table 2). While these results do not show any clear resolution between *kdr* and biochemical testing, they do align with the concept that *Ae. aegypti* populations from the central and southern regions had distinct introductions (Pless et al. 2017). The integration

of *kdr* and biochemical results suggest that organophosphates may better control central *Ae. aegypti* populations, while properly formulated pyrethroid applications may better control southern populations (Saavedra-Rodriguez et al. 2014). To further confirm these suppositions, bioassays and field studies should be conducted on field populations.

Due to the variability in the quality of specimens submitted, size differences between specimens, as well as other factors, there were extreme outliers in all enzymatic assays. Samples were normalized by their protein concentrations; therefore, small protein values, along with high OD readings in subsequent assays, lead to extreme outliers. For this study, if OD readings were above or below the established detection limit, those samples were excluded from further analyses. Additionally, certain samples appeared desiccated upon arrival, indicating that mosquitoes may not have been freshly dead when frozen. For future studies, it is recommended that mosquito eggs or larvae collected from the field be reared under laboratory conditions to guarantee fresh specimens. Otherwise, greater care should be taken to standardize the quality of submitted specimens before testing to prevent possible biases in the data. Larger sample sizes within jurisdictional boundaries are also needed to increase the accuracy of testing.

To better understand the phenotypic resistance of *Ae. aegypti* in California, bottle bioassays and cage trials using field-collected adults or local colony mosquitoes are needed. This information is



**Fig. 2.** Boxplot of detoxification enzyme levels measured in *Aedes aegypti* from 2017 to 2018 (combined). \* On the top of graph indicates statistically significantly differences between *Ae. aegypti* sampled by vector control agency and the susceptible ROCK strain. ( $P$ -value < 0.05) higher; \* on the bottom of graph indicated significantly ( $P$ -value < 0.05) less. ROCK: *Aedes aegypti* Rockefeller susceptible strain. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001.

**Table 2.** Mean of  $\alpha$ -esterases,  $\beta$ -esterases, MFOs, and iAChE levels detected in *Aedes aegypti* from central and southern California, 2017–2018

|                     | Susceptible ROCK reference strain ( $N_a = 90$ ) | Central <i>Ae. aegypti</i>     | Southern <i>Ae. aegypti</i>      | <i>P</i> -value |
|---------------------|--|--------------------------------|----------------------------------|-----------------|
|                     | Mean $\pm$ SD                                    | Mean $\pm$ SD                  | Mean $\pm$ SD                    |                 |
| $\alpha$ -Esterases | 23.36 $\pm$ 3.57                                 | 27.69 $\pm$ 14.7 ( $N = 798$ ) | 34.65 $\pm$ 24.54 ( $N = 1357$ ) | 2.2e-16         |
| $\beta$ -Esterases  | 39.27 $\pm$ 6.33                                 | 38.75 $\pm$ 31.4 ( $N = 794$ ) | 51.77 $\pm$ 43.58 ( $N = 1387$ ) | 2.2e-16         |
| MFOs                | 0.94 $\pm$ 0.11                                  | 1.49 $\pm$ 1.56 ( $N = 961$ )  | 1.85 $\pm$ 2.16 ( $N = 1382$ )   | 2.2e-16         |
| iAChE               | 0.68 $\pm$ 0.12                                  | 1.66 $\pm$ 2.61 ( $N = 955$ )  | 2.03 $\pm$ 2.69 ( $N = 1422$ )   | 3.53e-15        |

Results demonstrate *Ae. aegypti* results combined by region and not by individual agency. *p*-values were calculated by unpaired Wilcoxon rank sum test between Central and Southern populations.

<sup>a</sup> $N$  = sample size.

critical to understand insecticide effects on field mosquito populations. Use of these testing methods will become increasingly important as *Ae. aegypti* spread further throughout the state and information regarding chemical control options becomes more imperative. The biochemical assays performed in this study, however, provide a baseline for future testing and expose a knowledge gap regarding potential resistance to pyrethroids and organophosphates in California *Ae. aegypti* populations.

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