

UC Irvine

UC Irvine Previously Published Works

Title

Emerging mosquito resistance to piperonyl butoxide-synergized pyrethroid insecticide and its mechanism

Permalink

<https://escholarship.org/uc/item/6t98q3zn>

Authors

Zhou, Guofa

Li, Yiji

Jeang, Brook

et al.

Publication Date

2021

DOI

10.1101/2021.09.29.462303



Vector Control, Pest Management, Resistance, Repellents

Emerging Mosquito Resistance to Piperonyl Butoxide-Synergized Pyrethroid Insecticide and Its Mechanism

Guofa Zhou,^{1,4} Yiji Li,^{1,3} Brook Jeang,¹ Xiaoming Wang,¹ Robert F. Cummings,² Daibin Zhong,¹ and Guiyun Yan¹

¹Program in Public Health, University of California, Irvine, CA, USA, ²Orange County Mosquito and Vector Control District, Garden Grove, CA, USA, ³Current address: Department of Pathogen Biology, Hainan Medical University, Haikou, Hainan, China, and ⁴Corresponding author, e-mail: zhoug@uci.edu

Subject Editor: Roberto Pereira

Received 20 October 2021; Editorial decision 15 December 2021

Abstract

Piperonyl butoxide (PBO)-synergized pyrethroid products are widely available for the control of pyrethroid-resistant mosquitoes. To date, no study has examined mosquito resistance after pre-exposure to PBO and subsequent enzymatic activity when exposed to PBO-synergized insecticides. We used *Culex quinquefasciatus* Say (Diptera: Culicidae), an important vector of arboviruses and lymphatic filariasis, as a model to examine the insecticide resistance mechanisms of mosquitoes to PBO-synergized pyrethroid using modified World Health Organization tube bioassays and biochemical analysis of metabolic enzyme expressions pre- and post-PBO exposure. Mosquito eggs and larvae were collected from three cities in Orange County in July 2020 and reared in insectary, and F₀ adults were used in this study. A JHB susceptible strain was used as a control. Mosquito mortalities and metabolic enzyme expressions were examined in mosquitoes with/without pre-exposure to different PBO concentrations and exposure durations. Except for malathion, wild strain *Cx quinquefasciatus* mosquitoes were resistant to all insecticides tested, including PBO-synergized pyrethroids (mortality range 3.7 ± 4.7% to 66.7 ± 7.7%). Wild strain mosquitoes had elevated levels of carboxylesterase (COE, 3.8-fold) and monooxygenase (P450, 2.1-fold) but not glutathione S-transferase (GST) compared to susceptible mosquitoes. When wild strain mosquitoes were pre-exposed to 4% PBO, the 50% lethal concentration of deltamethrin was reduced from 0.22% to 0.10%, compared to 0.02% for a susceptible strain. The knockdown resistance gene mutation (L1014F) rate was 62% in wild strain mosquitoes. PBO pre-exposure suppressed P450 enzyme expression levels by 25–34% and GST by 11%, but had no impact on COE enzyme expression. Even with an optimal PBO concentration (7%) and exposure duration (3h), wild strain mosquitoes had significantly higher P450 enzyme expression levels after PBO exposure compared to the susceptible laboratory strain. These results further demonstrate other studies that PBO alone may not be enough to control highly pyrethroid-resistant mosquitoes due to multiple resistance mechanisms. Mosquito resistance to PBO-synergized insecticide should be closely monitored through a routine resistance management program for effective control of mosquitoes and the pathogens they transmit.

Key words: *Culex quinquefasciatus*, insecticide resistance, piperonyl butoxide (PBO), PBO-synergized pyrethroid, metabolic enzyme expression

Mosquitoes are well-known vectors of many infectious pathogens affecting human health including malaria, dengue, West Nile, yellow fever, Zika, chikungunya, lymphatic filariasis, and others (David and Abraham 2016, Guzman et al. 2016, WHO 2020). Spray

applications of insecticides, especially pyrethroids, and pyrethroid-treated bed nets are the most commonly used adult mosquito control methods (Guzman et al. 2016, Pryce et al. 2018, Nasci and Mutebi 2019). Over time and following repeated use, mosquitoes have

developed very high resistance levels to multiple insecticides worldwide (Richards et al. 2018, WHO 2018, Nasci and Mutebi 2019). A number of studies have examined mosquito pyrethroid resistance mechanisms and demonstrated target site insensitivity due to mutations in the voltage-gated sodium channel (VGSC) gene, also known as knockdown resistance (*knr*; WHO 2018). The overproduction of metabolic detoxification enzymes, such as cytochrome P450 (CYP) monooxygenases, plays a critical role in insecticide resistance in mosquitoes (David et al. 2013, Liu 2015, WHO 2018).

To enhance the killing power of insecticides, synergized insecticides were developed by mixing insecticide synergists with pyrethroids (Brannon 1947, Goodwin-Bailey and Holborn 1952, Mallis et al. 1952). The insecticide synergist by itself does not harm insects at low concentrations; instead, it enhances insecticidal toxicity by inhibiting metabolic enzyme activities (Brannon 1947, Goodwin-Bailey and Holborn 1952, Mallis et al. 1952). Discovered in the 1940s, piperonyl butoxide (PBO) is one of the earliest insecticide synergists (Brannon 1947, Goodwin-Bailey and Holborn 1952, Mallis et al. 1952). PBO can synergize the effects of pyrethroid insecticides by reducing or nullifying the detoxifying capabilities of enzymes, primarily monooxygenases. PBO synergized pyrethroid ultra-low-volume (ULV) sprayings have been widely used for *Aedes* and *Culex* mosquito control (Osimitz et al. 2009, Suman et al. 2012, Farajollahi and Williams 2013, Xue et al. 2013). Field application of PBO-synergized insecticides performed far better than mono-pyrethroids (Goodwin-Bailey and Holborn 1952, Mallis et al. 1952). In addition to PBO, S,S,S-tributyl phosphorotrithioate (DEF), triphenyl phosphate (TPP), and diethyl maleate (DEM) are used as synergists for other classes of insecticides (Feyereisen 2015, Snoeck et al. 2017, Gonzalez-Morales and Romero 2019). However, PBO remains the most popularly used insecticide synergist (Osimitz et al. 2009, Snoeck et al. 2017). Today, more than 2,500 EPA-registered pesticide products in the United States contain PBO synergist (National Pest Information Center, <http://npic.orst.edu/factsheets/pbogen.pdf>). PBO-treated long-lasting insecticide-treated nets (PBO-LLITNs), which also use pyrethroids, have been tested for malaria control in many African countries (Gleave et al. 2021), with results showing that PBO-LLITNs outperformed regular LLITNs in reducing malaria infections and anopheline vector densities (Protopopoff et al. 2018, Gleave et al. 2021). PBO-LLITNs are soon to be rolled out on a large scale for malaria control in Africa with the support of the President's Malaria Initiatives (President's Malaria Initiative, <https://www.pmi.gov/annual-reports/>).

One important question regarding the use of synergized insecticides is whether they will select for vector population tolerance or even resistance to synergized insecticides; in other words, are PBO-synergized pyrethroids effective against mosquitoes that demonstrate high resistance levels to pyrethroids? Laboratory selection studies found that the addition of PBO slowed the development of pyrethroid insecticide resistance in *Aedes aegypti* L. and *Anopheles stephensi* Liston mosquitoes and agricultural insect pest white flies; however, insecticide-resistant mosquitoes quickly developed resistance to PBO-synergized insecticides (Kumar et al. 2002, 2004; Zimmer et al. 2017). A study in *Musca domestica* L. houseflies found that the flies developed resistance to PBO-synergized pyrethroids after just one year of continuous field applications of PBO-synergized pyrethroids (Davies et al. 1958). A recent study in Mozambique found that highly insecticide-resistant *Anopheles funestus* Giles also was insensitive to PBO-LLITNs (Riverson et al. 2019). A number of other studies also found that pretreatment of insecticide-resistant *Aedes*, *Anopheles*, and *Culex* mosquitoes with PBO did not fully restore the mosquitoes' susceptibility to insecticides and sometimes had a very limited effect on mosquito mortality

(Jones et al. 2012, Marcombe et al. 2019, Gunasekaran et al. 2020). There are several possible causes for mosquito tolerance to PBO-synergized insecticides, including A) the PBO concentration is too low or the exposure duration is too short; B) the resistance is so intense that PBO exposure cannot overcome the limit of the resistance, i.e., there is a maximum limit to the effect of PBO; C) mosquitoes develop tolerance to the PBO synergist, which is unlikely in places where PBO products have not been used; however, this is unknown in places such as the United States where PBO synergized insecticide products have been used for some time (Osimitz et al. 2009); and D) other resistance mechanisms are present, e.g., multiple mechanisms. However, little has been done to extensively examine mosquito resistance status to PBO-synergized pyrethroids concurrently with its enzymatic reactions after PBO exposure.

The aim of this study was to use *Cx. quinquefasciatus* as a model mosquito to examine levels of mosquito resistance to PBO-synergized pyrethroids and the underlying mechanisms in mosquitoes. We tested PBO exposure against both susceptible and field-collected mosquitoes and examined *knr* mutations and metabolic resistance mechanisms. The results will be useful for guiding large-scale use of PBO-synergized insecticide products, facilitating development of new methods of surveilling insecticide resistance, and informing the development of new vector and vector-borne disease control strategies.

Materials and Methods

Ethics Statement

No permits were required for the described field mosquito collections. Property owners at each location where mosquito larvae were collected consented orally to the collections. This study did not involve the collection of any human-related samples or personal information such as participants' names, addresses, and GPS location of their homes.

Culex quinquefasciatus Populations and Colonies

Wild strain *Cx. quinquefasciatus* (herein, OC-R strain) eggs and larvae were collected using standard dippers and pipettes in July 2020 from multiple places in the cities of Irvine, Garden Grove and Orange, Orange County, Southern California. This species is the most abundant mosquito in the county (Cummings et al. 2016), and breeds in a variety of urban/suburban water sources. It is the primary vector of West Nile virus (WNV) in southern California (Kwan et al. 2010). *Culex. quinquefasciatus* in Southern California are resistant to pyrethroids through elevated metabolic detoxification and knockdown resistance mechanisms (Ahmed et al. 2012, Richards et al. 2018, Yoshimizu et al. 2020). Since the mid-1980s, U.S. Environmental Protection Agency (EPA)-approved PBO-synergized pyrethrin/pyrethroid insecticides have been applied via ULV at approved label rates for adult mosquito control (adulticiding) at selected Orange County wetlands and underground stormwater drains in response to mosquito biting complaints from residents living near these locations. After two major WNV outbreaks in 2014 and 2015, pyrethroids have been applied in residential communities whenever WNV minimum infection rates in mosquitoes exceed 5 per 1,000 tested (Cummings et al. 2016, Orange County Mosquito and Vector Control District 2021, California Department of Pesticide Regulation 2021). However, these applications have been done infrequently and have always constituted less than 1% of total pesticide applications in the county (Orange County Mosquito and Vector Control District 2021, California Department of Pesticide Regulation 2021). In addition to the local governmental program,

mosquito larvae are exposed routinely to pyrethroid residuals from the control of other arthropod pests by homeowners, commercial pest control, and agricultural operations (Amweg et al. 2006, Wolfand et al. 2019, Budd et al. 2020, California Center Urban Runoff Research 2020, Sy et al. 2020).

Field-collected larvae from different habitats were mixed during collection, and the mix was divided into different trays (23 × 32 × 3.5 cm) in the insectary with about 150–200 larvae per tray. Field-collected eggs were reared in trays separate from field-collected larvae. Twenty-four-hours hatched larvae were put into different trays with about 150–200 larvae per tray. All larvae were fed with fish food plus yeast and the distilled rearing water was replaced once every 3–4 d. Pupations were observed, and pupae were picked twice a day, in the early morning (8:00–9:00 a.m.) and evening (6:00–7:00 p.m.). Pupae were put into 750 ml paper cups with about 100 pupae per cup and maintained inside the BugDorm-1 insect rearing cages (W30 × D30 × H30 cm). Emerged adults were maintained in the same BugDorm-1 cages with cotton balls saturated with 10% sugar solution before any experiments.

The eggs of an insecticide-susceptible *Cx. quinquefasciatus* strain, JHB F179, were obtained from BEI Resources (BEI Resources, NIAID, NIH, MRA No. NR-43025) and reared following the conditions and procedures described for the OC-R strain.

The OC-R and JHB strains of mosquitoes were reared in separate rooms of the insectaries in the Program in Public Health, University of California, Irvine. The conditions of mosquito rearing and resistance studies were conducted as follows: 26 ± 1°C, relative humidity 70 ± 10%, and 12:12 (L:D) h cycle. For both the OC-R and JHB strains, 3- to 5 d-old F₀ females were used for all tests.

Test of Mosquito Insecticide Resistance

The World Health Organization (WHO) standard tube bioassay was used to determine mortality rates of *Cx. quinquefasciatus* mosquitoes to four different insecticides (WHO 2016). Four insecticides belonging to these classes were tested: the pyrethroids, 1) deltamethrin (concentration 0.05%, 0.25% and 0.5%) and 2) permethrin (0.75%); 3) carbamate, bendiocarb (0.1%); and 4) organophosphate, malathion (5%). Silicone oil impregnated test paper was used as the control (Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia). In each test, a total of 20 female mosquitoes (3–5 d old) were exposed to insecticide-impregnated papers for 1 hour, then transferred to a holding tube and provided with cotton balls saturated with 10% sugar solution. Knockdown was recorded at 10-min intervals. Mortality was determined after 24 h. The field OC-R strain (F₀) was tested against all four insecticides; for the JHB strain (F₀), only the pyrethroids were tested. Four replicates of insecticide treatment for both OC-R and JHB mosquitoes, and two replicates of no insecticide control for each strain were performed concurrently for each insecticide according to standard WHO tube bioassay protocols (WHO 2016).

Mosquito Resistance to PBO-Synergized Pyrethroid

A U.S.-marketed PBO pyrethrin (ExciteR, 6% Pyrethrins, and 60% PBO, Central Garden & Pet Company, Schaumburg, Illinois 60173) was used as the reference insecticide. A mix of 6% pyrethrin and 60% PBO plus silicone oil was used to impregnate Whatman No. 1 (1 mm) paper. The solution formulation for indoor contact and surface spray was specified by the manufacturer, i.e., 4.25 fl oz/gallon of water and 1 gallon/750 square feet. Each test paper (12 cm × 15 cm) used approximately 0.947 ml of solution. A slightly modified WHO tube bioassay was used to examine the mortality of JHB

and OC-R strain females against the PBO-synergized pyrethrin described above, i.e., 20 females per test, 2-h exposure and mortality scored after 24 h, with four replicates for PBO-insecticide treatment and 2 for silicone oil test paper control concurrently. We used 2-h exposure here because we did not find any dead OC-R strain mosquitoes by the end of the 24-h holding period after 1-h insecticide exposure.

PBO Effect—Extended Exposure Time and Increased Concentration

For the first set of tests, a modified version of WHO-recommended PBO synergist–insecticide bioassays (WHO 2016), i.e., PBO 4% and deltamethrin 0.05%, was used. Twenty 3- to 5-d-old females were exposed to PBO-impregnated papers for 1 h, followed by 1-h exposure to insecticide-impregnated papers. Mosquitoes were then transferred to a holding tube and provided with cotton balls saturated with 10% sugar solution. Knockdown was recorded at 10-min intervals. Mortality was determined after 24 h. We additionally tested PBO exposure times of 2, 3, 4, and 6 h. Twenty females were used for each test, with four replicates for PBO + deltamethrin and two replicates for each control (PBO- and silicone oil-impregnated papers). The field OC-R strain (F₀) was tested for all treatments; for the JHB strain (F₀), only the exposure condition of PBO 1 h + deltamethrin 1 h was tested.

To determine the optimal PBO concentration and exposure time, deltamethrin 0.05% alone and with pre-exposure to PBO at concentrations of 4, 7, and 10% was tested, with exposure times of 1, 2, and 3 h using field OC-R strain mosquitoes. PBO controls, silicone oil controls, and PBO + insecticides tests were conducted concurrently.

Resistance Intensity: Dose–Response Relationship and Synergistic Effect

To determine the dose–response relationship between PBO synergist exposure and mosquito susceptibility to pyrethroids, WHO synergist-insecticide bioassays using different insecticide concentrations were conducted. The deltamethrin concentrations tested are listed in [Supp Table S1 \(online only\)](#). The PBO concentration was 4%, which is currently the WHO-recommended diagnostic concentration (WHO 2016). The test procedures were the same as described above, i.e., four replicates for treatment and two replicates for control, and duration of exposure was 1 h with PBO followed by 1 h with deltamethrin. Mosquito mortality was scored after 24 h and recorded for all tests.

Knockdown Resistance and Metabolic Enzyme Activity Measurement

Identification of L1014F *kdr* mutations was conducted on untreated mosquitoes using a TaqMan assay based on [Yoshimizu et al. \(2020\)](#). Individual mosquitoes were sequenced separately. Among the wild strain *Cx. quinquefasciatus*, only three mosquitoes were found dead 24 h after deltamethrin exposure. Therefore, *kdr* mutations were not examined separately for resistant and susceptible mosquitoes.

Cytochrome P450 monooxygenase enzyme activity was examined as follows: the susceptible JHB strain without exposure to deltamethrin insecticide or PBO, and the wild strain OC-R strain before and after 1-h exposure to 4% PBO (WHO standards), as well as after 3-h exposure to 7% PBO (which yielded robust mortality from this study). We also examined activities of glutathione S-transferases (GSTs) and carboxylesterases (COEs). We modified a previously published protocol to measure monooxygenase and

GST activities (Chang et al. 2014). Total protein was measured for each mosquito and corrected against a bovine serum albumin (BSA Fraction V; Sigma-Aldrich, Inc., St. Louis, MO) standard curve. All measurements were done in triplicate. Mean absorbance values for each tested mosquito and enzyme were converted into enzyme activity and standardized based on the total protein amount.

Data Analysis

WHO susceptibility test criteria are as follows: susceptible if mortality rate $\geq 98\%$; possible resistance if mortality is 90–97%; confirmed resistance if mortality rate $< 90\%$ or mortality rate $< 98\%$ with repeated tests using untested mosquitoes from the population exhibiting possible resistance as described; moderate to high-intensity resistance if mortality $< 98\%$ with 5 \times the diagnostic concentration; and high-intensity resistance if mortality $< 98\%$ with 10 \times the diagnostic concentration. We used χ^2 tests to determine the differences in mortalities (i.e., the total number of dead compared to the total number of mosquitoes tested for each experiment) between tests for each of the tested insecticides, different insecticide concentrations of the same insecticide, different PBO exposure durations, different PBO concentrations, and the same insecticide with/without PBO pre-exposure. A Student's *t*-test was used to determine if increase in PBO exposure duration (1 h vs 2 h and 2 h vs 3 h) significantly affected mosquito mortality; here, the mortalities were pooled among the 4, 7, and 10% PBO concentrations. A χ^2 test was also used to determine the difference in mortalities between wild strain and susceptible laboratory strain mosquitoes exposed to PBO-synergized pyrethrin after 2-h exposure. Lethal concentration was estimated using the Probit model. LC_{50} and LC_{90} , lethal concentrations that killed 50 and 90% of mosquitoes, respectively, were estimated for the susceptible and wild strain strains of mosquitoes with/without PBO pre-exposure. Resistance ratio, RR, was calculated as $(LC_{50} \text{ or } LC_{90} \text{ of field mosquitoes}) / (LC_{50} \text{ or } LC_{90} \text{ of susceptible mosquitoes})$. PBO synergistic ratios, SR, were calculated as $(LC_{50} \text{ or } LC_{90} \text{ of wild strain mosquitoes with PBO}) / (LC_{50} \text{ or } LC_{90} \text{ of PBO untreated wild strain mosquitoes})$.

The difference in *kdr* rates between susceptible and wild strain mosquitoes was not analyzed because susceptible mosquitoes did not show any *kdr* mutations and because the sample size of susceptible mosquitoes was too small. Analysis of variance (ANOVA) was used to compare P450, GST, and COE enzyme levels between

different mosquito strains without exposure to insecticide or PBO, and between wild strain mosquitoes before and after exposure to different concentrations of PBO. Reduction in enzyme activities due to PBO exposure was calculated by comparing enzyme levels before and after PBO exposure.

χ^2 tests were done using online freeware VassarStats: Website for Statistical Computation (<http://www.vassarstats.net/>; Vassar College, Poughkeepsie, NY). All other statistical analyses were done using R 4.0.1 (The R Project for Statistical Computing, Vienna, Austria).

Results

Intensity of Insecticide Resistance in

Cx. quinquefasciatus

WHO tube bioassays showed that using the diagnostic dosages, the mortality of wild strain *Cx. quinquefasciatus* was $< 10\%$ against both pyrethroids tested (deltamethrin 0.05% and permethrin 0.75%), 66.7 \pm 7.7% against bendiocarb 0.1%, and 96.3 \pm 4.7% against malathion 5%. The susceptible JHB strain mosquitoes were not fully susceptible to deltamethrin (97.5 \pm 1.7%; Fig. 1A). Even with 5 \times (0.25%) and 10 \times (5%) increased deltamethrin concentrations, wild strain *Cx. quinquefasciatus* mortalities were $< 60\%$ (Fig. 1A). For the wild strain mosquitoes, knockdown started late with the standard diagnostic insecticide dosages listed above, and the knockdown rate remained low by the end of 1-h exposure. The knockdown rate increased significantly with 5 \times and 10 \times deltamethrin (Fig. 1B).

Culex quinquefasciatus Against PBO-Synergized Pyrethroid

After 2-h exposure of *Cx. quinquefasciatus* to PBO-synergized pyrethrins (1:10 pyrethrins: PBO by volume), the mortality rate was 91.3% (95% CI: 84.0–98.6%) and the knockdown rate 98.8% (95% CI: 96.0–100%) for the susceptible JHB colony, whereas the wild strain had a mortality rate of only 1.6% (95% CI: 0–4.4%) and a knockdown rate of 13.3% (95% CI: 5.8–20.8%; Fig. 2). Both the mortality and knockdown rates were significantly higher among JHB strain mosquitoes compared to wild strain mosquitoes (χ^2 test, $P < 0.01$).

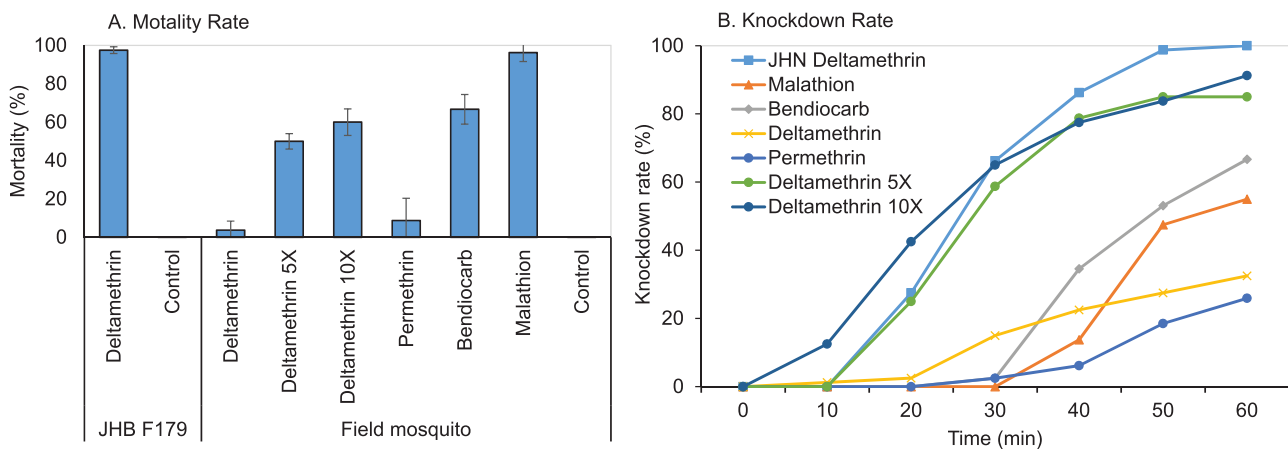


Fig. 1. Mortality of female *Culex quinquefasciatus* mosquitoes against different insecticides based on WHO tube test using standard diagnostic concentrations and 5 \times and 10 \times deltamethrin concentrations. (A) Mortality rate (%) and (B) Knockdown rate (%). JHN deltamethrin refers to JHB strain mosquitoes exposed to deltamethrin; all others curves represent field strain mosquitoes and the insecticides they were exposed to. As controls, field and JHB strain mosquitoes were separately exposed to silicone oil impregnated paper.

PBO Effect on Mosquito Mortality

For OC-R strain mosquitoes with pre-exposure to 4% PBO for 1 h, deltamethrin exposure led to more than doubled mosquito mortality compared to mortality observed without PBO pre-exposure; however, mortality rates remained <10% even after PBO pre-exposure (Fig. 3A). When the duration of 4% PBO exposure was extended to 2 h, increased mosquito mortality against deltamethrin was observed; however, mortality remained <30% (Fig. 3A). There was no significant difference in mortalities between exposure durations from 2 to 6 h ($\chi^2 = 0.74$, $df = 3$, $P = 0.8638$; Fig. 3A).

Bioassays with 0.05% deltamethrin without PBO pre-exposure resulted in mortality rates of <20% after 1–3 h of exposure, confirming high pyrethroid resistance in the OC-R strain *Cx. quinquefasciatus* mosquitoes (Fig. 3B). PBO pre-exposure led to higher mortality against deltamethrin for PBO concentrations up to 7%. Mortality rates at 7–10% PBO concentration were not significantly different (Fig. 3B). Increasing the PBO exposure duration from 1 h to 2 h increased mortality rates (three concentrations combined, $t = 7.74$, $df = 2$, $P = 0.0163$), but no significant difference in mortality was observed between 2- and 3-h PBO exposure ($t = 1.46$, $df = 2$, $P = 0.2809$; Fig. 3B).

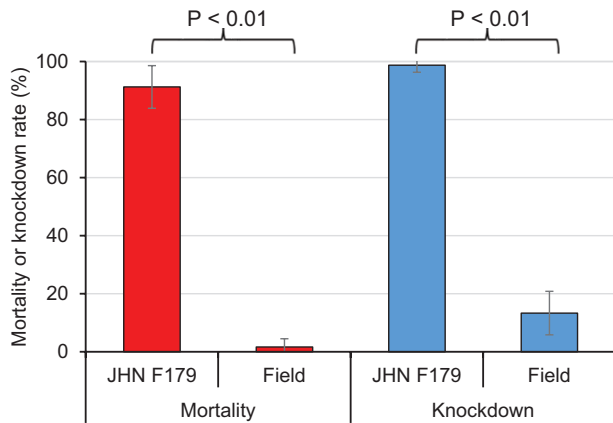


Fig. 2. Mortality and knockdown rates of female mosquitoes against PBO-synergized pyrethrin after 2-h exposure.

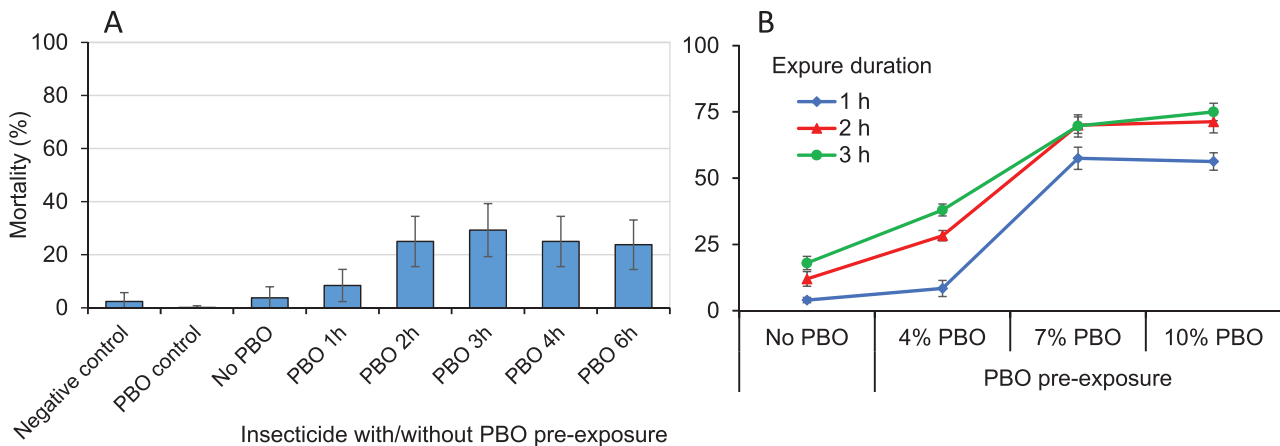


Fig. 3. Mortality (%) of wild strain *Culex quinquefasciatus* against different PBO concentrations and exposure durations. (A) No PBO and no insecticide (negative control), PBO control (no insecticide), and insecticide with 0–6 h pre-exposure to 4% PBO; and (B) 1–3 h pre-exposure to 4–10% PBO.

Dose–Response Relationship and PBO

Synergistic Ratio

Dose–response relationships for the JHB susceptible and OC-R wild strain mosquitoes against deltamethrin with/without PBO are shown in Fig. 4. The LC_{50} of the OC-R population was 0.22% without PBO pre-exposure, indicating a resistance ratio of 11.2 in relation to the susceptible JHB F₁₇₉ colony (Table 1). When the mosquitoes were pre-exposed to 4% PBO for 1 h, the LC_{50} was reduced to 0.10%, and the resistance ratio decreased to 4.9 with a synergistic ratio of 2.3 (Table 1). Similar resistance and synergistic ratios were observed using LC_{90} (Table 1).

Knockdown Resistance Gene Mutations and Metabolic Enzyme Expressions

The knockdown resistance mutation rate for L1014 was 61.8% in OC-R wild strain mosquitoes without exposure to pyrethroid insecticides and 0% in JHB strain mosquitoes.

Compared to the JHB strain susceptible mosquitoes, OC-R mosquitoes had significantly elevated enzyme activity for P450 (2.1-fold) and COE (3.8-fold) but not for GST (ANOVA, $P < 0.05$; Fig. 5, Table 2 and Supp Table S2 [online only]). For the field mosquitoes, pre-exposure to 4% PBO reduced P450 enzyme levels by about 25% but did not affect COE or GST enzyme activity. Pre-exposure to 7% PBO significantly suppressed enzyme activity for both P450 (33%) and GST (11%) but not for COE (ANOVA, $P < 0.05$; Fig. 5, Table 2 and Supp Table S2 [online only]). Even after 7% PBO exposure, P450 enzyme levels in field mosquitoes were still significantly higher than enzyme levels in JHB F179 mosquitoes (1.4-fold, ANOVA, $P < 0.05$).

Discussion

Mosquito resistance to insecticides is a major challenge for mosquito-borne disease prevention and control. PBO-synergized pyrethroid insecticides are one way to combat insecticide resistance. However, PBO-synergized insecticide products have their own limitations. Although PBO-synergized pyrethroids are widely used in the United States for *Aedes* and *Culex* control (Osimitz et al. 2009, Suman et al. 2012, Farajollahi and Williams 2013, Xue et al. 2013) and PBO-LLITNs have been tested and are being rolled out in many malaria endemic African countries (Prottopoff et al. 2018, Gleave

et al. 2021), potential mosquito tolerance to PBO-synergized pyrethroids and PBO-LLITN has not attracted attention from the scientific community and policy makers. Here, we used *Cx. quinquefasciatus* as a model mosquito to examine the interactions between highly insecticide-resistant field mosquitoes and PBO exposure. We found that wild strain *Cx. quinquefasciatus* from Southern California were highly resistant to pyrethroid and carbamate insecticides. Although PBO-synergized pyrethroids have been infrequently used for outdoor mosquito control at a small number of treatment sites since

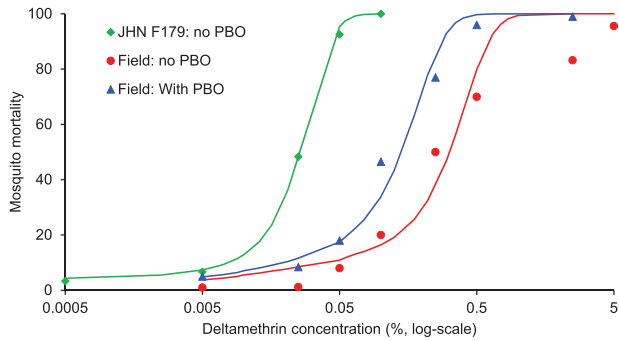


Fig. 4. Dose–response relationship against deltamethrin and PBO exposure. Dots/triangles/diamonds represent observed mortalities; curves represent model fitted results. Green (diamond), blue (triangle), and red (dot) colors respectively represent JHB strain without PBO pre-exposure, wild strain mosquitoes without PBO pre-exposure, and wild strain mosquitoes with PBO pre-exposure. Dash lines represent LC₅₀ and LC₉₀ levels.

Table 1. Estimation of 50 and 90% lethal concentrations of deltamethrin, resistance ratio (RR), and PBO synergistic ratio (SR)

LC ^a	Mosquito strain	Concentration (%)		RR ^b	SR ^c
		No PBO	With PBO		
LC ₅₀ ^a	JHB strain	0.0200	—	—	—
	OC-R strain	0.2246	0.0989	11.2	2.3
LC ₉₀ ^a	JHB strain	0.0248	—	—	—
	OC-R strain	0.3028	0.1351	12.2	2.2

^aLC: lethal concentration; LC₅₀: LC that kills 50% of mosquitoes; LC₉₀: LC that kills 90% of mosquitoes; ‘—’: not applicable. JHB strain: JHB F₁₇₉ susceptible strain; OC-R: field collected wild strain.

^bRR = (LC₅₀ or LC₉₀ of susceptible strain)/(LC₅₀ or LC₉₀ of wild strain).

^cSR = (LC₅₀ or LC₉₀ of wild strain with no PBO)/(LC₅₀ or LC₉₀ of wild strain with PBO).

1985 in Orange County, CA (California Department of Pesticide Regulation 2021), wild strain *Cx. quinquefasciatus* were highly resistant to the market formulation equivalent to PBO-synergized pyrethroids. In addition, even with optimal PBO concentrations and exposure duration, PBO pre-exposure paired with the diagnostic dose of deltamethrin killed only about 70% of wild strain *Cx. quinquefasciatus*, indicating the limitation of PBO exposure on mosquito mortality. Biochemical analysis showed that PBO could significantly reduce P450 enzyme activity. However, the reduced P450 activity in wild strain *Cx. quinquefasciatus* was still significantly higher than that observed in susceptible mosquitoes. This may partly explain why PBO pre-exposure and PBO-synergized pyrethroids could not fully restore mosquito susceptibility to insecticides. There was highly elevated COE enzyme activity in wild strain mosquitoes, and exposure to PBO had no impact on COE enzyme activity. In other words, the impact of PBO-synergized pyrethroids on mosquito mortality was limited because of multiple mechanisms. Other synergists such as DEF are essential to counteract the mosquito resistance to insecticides.

The phenomena of the insecticide-resistant mosquito insensitivity to PBO exposure or PBO-LLITNs have also been reported for *Aedes* and *Anopheles* mosquitoes from different countries (Bisset et al. 2013, Koou et al. 2014b, Ibrahim et al. 2019; Marcombe et al. 2019, Riveron et al. 2019, Keita et al. 2021, Zuharah and Sufian 2021). For example, Marcombe et al. (2019) found that in Laos, PBO exposure for highly permethrin-resistant *Ae. aegypti* increased mosquito mortalities from 2–11% to 10–25%. Although one may argue that mortalities increased 2- to 5-fold, the observed mortalities of 10–25% were too low for effective mosquito control (WHO 2006). In a study conducted by Cornet et al. in California, PBO + permethrin/pyrethrum was tested against *Ae. aegypti* using the Center for Disease Control and Prevention (CDC) bottle assay, and no mortality was observed in wild strain mosquitoes (Cornet et al. 2016). Similarly, during an insecticide resistance study with *An. gambiae* s.l. Giles in Mali, Keita et al. (2021) found that in several places, PBO exposure could only increase mosquito mortalities to pyrethroid insecticides from 24–41% to 34–45%. For *An. funestus* Giles in Mozambique, PBO exposure increased mosquito mortalities to pyrethroids from about 10 to <30%; in addition, mosquito mortalities increased from around 5% against regular LLITNs to about 15% against PBO-LLITNs even after 72 h of exposure (Riveron et al. 2019). We note that PBO-synergized insecticide products have not been used in any of the above-mentioned African study areas. These results suggest that highly insecticide-resistant mosquitoes may be tolerant of PBO exposure regardless of species. In nearly all cases, multiple resistance mechanisms have

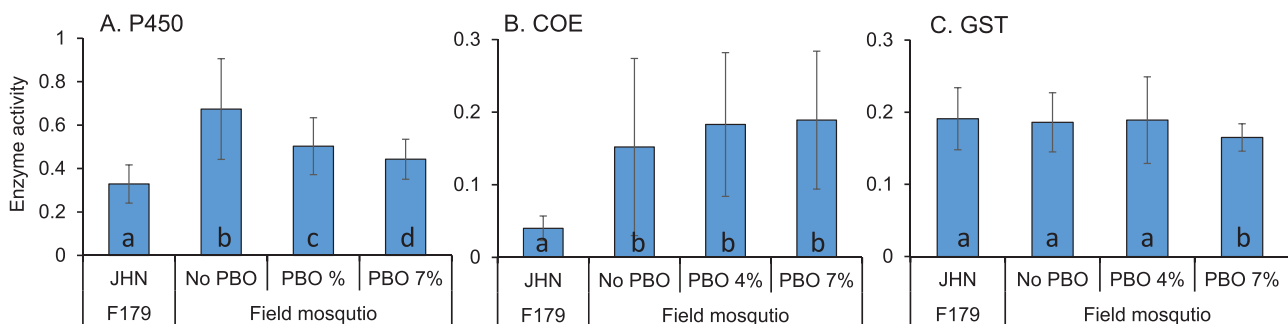


Fig. 5. Standardized enzyme activities based on the total protein amount for different mosquito strains and for field-collected wild strain mosquitoes at different PBO concentrations. No PBO exposure for JHB strain susceptible mosquito; No PBO—no PBO exposure; PBO 4% and PBO 7%—pre-exposed to 4 and 7% PBO, respectively.

Table 2. Ratio of enzyme levels between field-collected wild strain (OC-R) and susceptible mosquitoes (JHB F₁₇₉) and between different PBO treatments of field mosquitoes

Enzyme	OC-R control vs JHB	PBO 4% exposure vs OC-R control	PBO 7% exposure vs OC-R control
GST ^a	0.97	1.02	0.89**
COE ^a	3.80***	1.20	1.24
P450 ^a	2.05***	0.75***	0.66***

^aGST: glutathione S-transferase; COE: carboxylesterase; P450: monoxygenase.

Significantly different at level of 0.01; *significantly different at level of 0.0001; otherwise, not significantly different at level of <0.05.

been reported, including the presence of different detoxifying enzymes and different *kdr* mutations (Koou et al. 2014a, Ibrahim et al. 2019, Riveron et al. 2019, Lucas et al. 2020, Keita et al. 2021, Zuharah and Sufian 2021).

Although PBO-synergized pyrethrum and pyrethroid insecticides have been tested and used in the field since 1950 (Mallis et al. 1952, Davies et al. 1958, Plapp et al. 1963), insect resistance to PBO-synergized insecticides and insecticide products is seldom reported (Plapp et al. 1963, Riveron et al. 2019). For example, Davies et al. (1958) first reported housefly resistance to PBO pyrethroids in 1958, and Riveron et al. (2019) reported *An. funestus* resistance to PBO-pyrethroid treated LLITNs in 2019 years after these control products had been in use. Many studies found that pre-exposure of highly pyrethroid-resistant *Aedes*, *Anopheles*, and *Culex* mosquitoes to a synergist such as PBO, DEF, TPP, or DEM did not fully restore the mosquito mortality levels (Bisset et al. 2013, Mulamba et al. 2014, Yanola et al. 2015, Chang et al. 2017, Fagbohun et al. 2019, Leong et al. 2019, Lucas et al. 2020). However, most of these studies used the synergist to infer the mechanisms behind resistance without examining the mechanisms themselves, i.e., enzyme activity before and after synergist exposure. For example, most studies using PBO 4% + insecticide found that PBO pre-exposure increased the mortality of field-collected mosquitoes (Guntay et al. 2018, Fagbohun et al. 2019, Riveron et al. 2019, Lucas et al. 2020). Thus, researchers concluded that P450 enzyme was involved in resistance. To the best of our knowledge, our study is the first to examine in detail both enzyme activities and bioassay tests after mosquitoes were exposed to PBO. We examined how PBO exposure, at different concentrations and exposure durations, affected mosquito mortality as well as P450, COE, and GST enzyme activities.

Although increased enzyme activity and expression in P450 CYP genes have been well recognized as the major mechanisms for pyrethroid resistance (David et al. 2013, Riveron et al. 2015, Rakotondranaivo et al. 2018, Fagbohun et al. 2019, Lucas et al. 2020, Keita et al. 2021), it is unclear why the effect of PBO exposure is limited in reducing resistance levels. One possibility is the existence of multiple mechanisms of resistance. It is generally believed that PBO affects mainly P450 enzyme activity (Edi et al. 2014, Liu 2015, Silva Martins et al. 2019). Other studies found that both mixed-function oxidase and esterase activity were involved in *Ae. aegypti* and *Cx. quinquefasciatus* resistance to PBO-synergized insecticides, while resistant *Anopheles* also developed GST and A296S-RDL dieldrin resistance mechanisms (Koou et al. 2014a, Riveron et al. 2015, Cisse et al. 2017, Marcombe et al. 2019, Lucas et al. 2020). In this study, we found that both P450 and COE enzyme activities in wild strain *Cx. quinquefasciatus* were significantly elevated compared to those in susceptible strain

mosquitoes, whereas GST levels were similar between the susceptible and wild strain mosquitoes. This is similar to what has been observed in cases of extremely high and multiple insecticide resistance in the malaria mosquito *An. gambiae*, i.e., gene mutations in P450 enzymes and acetylcholinesterase ACE-1 duplication in COE enzymes (Edi et al. 2014). We found about 60% *kdr* gene mutations in wild strain *Cx. quinquefasciatus* mosquitoes, which is slightly higher compared to a previous study conducted in the same area (Yoshimizu et al. 2020). More interestingly, in addition to multiple resistance mechanisms, we found that even with robust PBO concentration and exposure duration, the reduction in P450 enzyme activity in field mosquitoes was limited, and this is a completely new finding. Rather than multiple mechanisms of resistance, we suspect that PBO has a maximum carrying capacity with regard to reducing P450 enzyme activity. When mosquito resistance exceeds a certain level, PBO exposure cannot overcome such a high level of enzyme activity. We hypothesize that the synergistic effect of PBO has a limit; however, more studies need to be conducted, as other unknown mechanisms may be involved.

This study has some limitations. First, WHO insecticide susceptibility test guidelines do not include standard diagnostic insecticide doses for *Culex* mosquitoes (WHO 2016). We used the standard diagnostic insecticide doses for *Anopheles* mosquitoes in this study (WHO 2016). We tested 5X and 10X diagnostic insecticide doses and observed that the mortalities were all <60% for wild strain *Cx. quinquefasciatus* mosquitoes. In addition, mortality of JHB F179 control strain *Culex* was 97% against standard 1X diagnostic insecticide doses. These results collectively suggest that field-collected *Culex* mosquitoes were highly resistant to pyrethroid insecticides. Second, we acknowledge that commonly applied control methods for *Culex* and *Aedes* mosquito management consist of aerial/ground ULV adulticiding with PBO-synergized pyrethrins/pyrethroids or organophosphates and larviciding with lightweight oils, insect growth regulators, and microbials (Cornel et al. 2016, Muhammad et al. 2017, Nasci and Mutebi 2019). ULV spraying of PBO-synergized pyrethrins/pyrethroids, such as Aqualuer 20-20 (Value Garden Supply, St. Joseph, MO), Evergreen EC 60-6 (McLaughlin Gormley King Company, Minneapolis, MN), and other formulations, have been extensively evaluated for lowering adult mosquito counts (Conover et al. 2015, Cornel et al. 2016, Muhammad et al. 2017) as well as reducing arboviral disease transmission (Elnaiem et al. 2008, Ruktanonchai et al. 2014, Holcomb et al. 2021). Although semifield (caged) experiments using direct spraying can be adapted to evaluate the efficacy of PBO-synergized pyrethroids (Conover et al. 2015, Cornel et al. 2016), the outcomes are affected by many factors including the specifications of sprayers; travel distance of droplets and droplet size; and environmental conditions (e.g., wind speed and direction; Conover et al. 2015, Fisher et al. 2015, Camelio et al. 2016, Cornel et al. 2016, Muhammad et al. 2017, Farooq et al. 2020), which likely contributed to inter-experimental variations in mosquito mortality (Muhammad et al. 2017). We did not conduct direct spraying experiments. Last but not least, results from laboratory-controlled tests likely differ from results following actual field applications (Muhammad et al. 2017). However, results are comparable under the same experimental conditions. Field applications of ULV spraying show large variations in reductions in mosquito densities (Cornel et al. 2016, Muhammad et al. 2017); it is unknown whether the low effectiveness is due to mosquito tolerance to insecticides, spraying methods, or the coverage of spraying, as it is infeasible for aerial/ground sprayings to cover every mosquito (Muhammad et al. 2017, Farooq et al. 2020). Furthermore, field environmental conditions and pesticide formulations can also affect spraying efficacy

(Muhammad et al. 2017, Dritz et al. 2020). Therefore, laboratory-controlled environment tests are a viable method for the evaluation of the efficacy of PBO-synergized insecticides. Indeed, we observed 92% mortality of JHB 179 control strain *Culex* mosquitoes against PBO-synergized pyrethrins compared to 2% mortality among our field mosquitoes. This indicates validity of our laboratory-controlled tests as well as field *Culex* tolerance of PBO-synergized pyrethrins.

Conclusion

Culex quinquefasciatus from Southern California were highly resistant to PBO-synergized pyrethroids. This raises serious concerns about the efficacy of currently available PBO synergized insecticide products against mosquitoes, including PBO-synergized pyrethroids for spraying and for LLITNs. With the increased use of insecticides with or without PBO, mosquitoes will develop increased resistance to insecticides, which will potentially compromise the efficacy of PBO-synergized insecticide products. Potential development of mosquito tolerance to PBO-synergized insecticide products should be closely monitored.

Supplementary Data

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

Acknowledgments

We wish to thank the Orange County Mosquito and Vector Control District, CA, for their support in collecting some of the field mosquitoes used in this study. We sincerely thank the three anonymous reviewers for their constructive suggestions, error corrections and comments. This study was partially funded by the National Institutes of Health (D43 TW01505).

References Cited

- Ahmed, M. A. I., A. Cornel, and B. Hammock. 2012. Monitoring of insecticide resistance of *Culex pipiens* (Diptera: Culicidae) colonies-collected from California. *Int. J. Environ. Sci. Devel.* 3: 346–349.
- Amweg, E. L., D. P. Weston, J. You, and M. J. Lydy. 2006. Pyrethroid insecticides and sediment toxicity in urban creeks from California and Tennessee. *Environ. Sci. Technol.* 40: 1700–1706.
- Bisset, J. A., R. Marín, M. M. Rodríguez, D. W. Severson, Y. Ricardo, L. French, M. Díaz, and O. Pérez. 2013. Insecticide resistance in two *Aedes aegypti* (Diptera: Culicidae) strains from Costa Rica. *J. Med. Entomol.* 50: 352–361.
- Brannon, L. 1947. Piperonyl cyclonene and piperonyl butoxide as synergists with rotenone. *J. Econ. Entomol.* 40: 933–934.
- Budd, R., D. Wang, M. Ensminger, and B. Phillips. 2020. An evaluation of temporal and spatial trends of pyrethroid concentrations in California surface waters. *Sci. Total Environ.* 718: 137402.
- California Center for Urban Horticulture. 2020. Urban run-of research. University of California, Davis. (<https://ccuh.ucdavis.edu/urban-runof-research>).
- California Department of Pesticide Regulation. 2021. Pesticide Use Reporting, 2014–2018. (<https://www.cdpr.ca.gov/docs/pur/purmain.htm>). Accessed November 20, 2021.
- Camelio, K., J. Gibson, M. L. Smith, L. L. Drake, and R. D. Xue. 2016. Evaluation of three commercial handheld ultra-low-volume foggers with Aqualure® 20-20 against adult *Aedes albopictus*. *J. Am. Mosq. Control Assoc.* 32: 163–166.
- Chang, X., D. Zhong, Q. Fang, J. Hartsel, G. Zhou, L. Shi, F. Fang, C. Zhu, and G. Yan. 2014. Multiple resistances and complex mechanisms of *Anopheles sinensis* mosquito: a major obstacle to mosquito-borne diseases control and elimination in China. *Plos Negl. Trop. Dis.* 8: e2889.
- Chang, K. S., H. C. Kim, T. A. Klein, and Y. R. Ju. 2017. Insecticide resistance and cytochrome-P450 activation in unfed and blood-fed laboratory and field populations of *Culex pipiens pallens*. *J. Pest Sci.* (2004). 90: 759–771.
- Cisse, M. B. M., D. Sangare, R. M. Oxborough, A. Dicko, D. Dengela, A. Sadou, J. Mihigo, K. George, L. Norris, and C. Fornadel. 2017. A village level cluster-randomized entomological evaluation of combination long-lasting insecticidal nets containing pyrethroid plus PBO synergist in Southern Mali. *Malar. J.* 16: 477.
- Conover, D., A. Fulcher, M. L. Smith, M. Farooq, M. K. Gaines, and R.-D. Xue. 2015. Evaluation of three commercial backpack sprayers with Aqualure® 20-20 against caged adult *Aedes Aegypti*. *J. Am. Mosq. Control Assoc.* 31: 190–192.
- Cornel, A. J., J. Holeman, C. C. Nieman, Y. Lee, C. Smith, M. Amorino, K. K. Brisco, R. Barrera, G. C. Lanzaro, and F. S. Mulligan III. 2016. Surveillance, insecticide resistance and control of an invasive *Aedes aegypti* (Diptera: Culicidae) population in California. *F1000Res.* 5: 194.
- Cummings, R., C. Fogarty, L. Krueger, L. Levy, T. Morgan, K. Nguyen, and A. Semro. 2016. Fighting an unpredictable foe: an overview of two consecutive years of record West Nile virus outbreaks, 2014–2015, in Orange County, California. *Proc. Mosq. Vector Control Assoc. CA.* 84: 116–123.
- David, S., and A. M. Abraham. 2016. Epidemiological and clinical aspects on West Nile virus, a globally emerging pathogen. *Infect. Dis. (Lond).* 48: 571–586.
- David, J. P., H. M. Ismail, A. Chandor-Proust, and M. J. Paine. 2013. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368: 20120429.
- Davies, M., J. Keiding, and C. G. Von Hosten. 1958. Resistance to pyrethrins and to pyrethrins-piperonyl butoxide in a wild strain of *Musca domestica* L. in Sweden. *Nature.* 182: 1816–1817.
- Dritz, D. A., S. Wheeler, and M. Ree. 2020. Resistance testing and mosquito management with pyrethroids. *Proc. Mosq. Vector Control Assoc. CA.* 88: 77–79.
- Edi, C. V., L. Djogbénou, A. M. Jenkins, K. Regna, M. A. Muskavitch, R. Poupardin, C. M. Jones, J. Essandoh, G. K. Kétoh, M. J. Paine, et al. 2014. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. *PLoS Genet.* 10: e1004236.
- Elnaïem, D. E., K. Kelley, S. Wright, R. Laffey, G. Yoshimura, M. Reed, G. Goodman, T. Thiemann, L. Reimer, W. K. Reisen, et al. 2008. Impact of aerial spraying of pyrethrin insecticide on *Culex pipiens* and *Culex tarsalis* (Diptera: Culicidae) abundance and West Nile virus infection rates in an urban/suburban area of Sacramento County, California. *J. Med. Entomol.* 45: 751–757.
- Fagbohun, I. K., T. A. Oyeniyi, T. E. Idowu, O. A. Otubanjo, and S. T. Awolola. 2019. Cytochrome P450 mono-oxygenase and resistance phenotype in DDT and deltamethrin-resistant *Anopheles gambiae* (Diptera: Culicidae) and *Culex quinquefasciatus* in Kosofe, Lagos, Nigeria. *J. Med. Entomol.* 56: 817–821.
- Farajollahi, A., and G. M. Williams. 2013. An open-field efficacy trial using AquaDuet® via an ultra-low volume cold aerosol sprayer against caged *Aedes albopictus*. *J. Am. Mosq. Control Assoc.* 29: 304–308.
- Farooq, M., J. E. Cilek, E. Summers, A. K. C. Briley, J. Weston, A. G. Richardson, and E. J. Lindroth. 2020. Potential of outdoor ultra-low-volume aerosol and thermal fog to suppress the Dengue vector, *Aedes aegypti*, inside wellings. *J. Am. Mosq. Control Assoc.* 36: 189–196.
- Feyerisen, R. 2015. Insect P450 inhibitors and insecticides: challenges and opportunities. *Pest Manag. Sci.* 71: 793–800.
- Fisher, M. L., D. F. Hoel, M. Farooq, and T. W. Walker. 2015. Deposition from ultra-low volume application of public health insecticides in a hot desert environment. *J. Am. Mosq. Control Assoc.* 31: 155–163.
- Gleave, K., N. Lissenden, M. Chaplin, L. Choi, and H. Ranson. 2021. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database Syst. Rev.* 5: CD012776.
- Gonzalez-Morales, M. A., and A. Romero. 2019. Effect of synergists on deltamethrin resistance in the common bed bug (Hemiptera: Cimicidae). *J. Econ. Entomol.* 112: 786–791.
- Goodwin-Bailey, K. F., and J. M. Holborn. 1952. Laboratory and field experiments with pyrethrins/piperonyl butoxide powders for the protection of grain. *Pyrethrum Post* 2: 7–17.
- Gunasekaran, K., S. S. Sahu, T. Vijayakumar, S. Subramanian, M. Rahi, and P. Jambulingam. 2020. Evaluation of DawaPlus 3.0 and DawaPlus 4.0, deltamethrin-PBO combination nets against pyrethroid-resistant *Anopheles culicifacies* in experimental huts in India. *Malar. J.* 19: 43.

- Guntay, O., M. S. Yikilmaz, H. Ozaydin, S. Izzetoglu, and A. Suner. 2018. Evaluation of pyrethroid susceptibility in *Culex pipiens* of Northern Izmir Province, Turkey. *J. Arthropod. Borne. Dis.* 12: 370–377.
- Guzman, M. G., D. J. Gubler, A. Izquierdo, E. Martinez, and S. B. Halstead. 2016. Dengue infection. *Nat. Rev. Dis. Primers.* 2: 16055.
- Holcomb, K. M., R. C. Reiner, and C. M. Barker. 2021. Spatio-temporal impacts of aerial adulticide applications on populations of West Nile virus vector mosquitoes. *Parasit. Vectors.* 14: 120.
- Ibrahim, S. S., M. M. Mukhtar, J. A. Datti, H. Irving, M. O. Kusimo, W. Tchappa, N. Lawal, F. I. Sambo, and C. S. Wondji. 2019. Temporal escalation of pyrethroid resistance in the major malaria vector *Anopheles coluzzii* from Sahelo-Sudanian Region of northern Nigeria. *Sci. Rep.* 9: 7395.
- Jones, C. M., C. Machin, K. Mohammed, S. Majambere, A. S. Ali, B. O. Khatib, J. McHa, H. Ranson, and L. A. Kelly-Hope. 2012. Insecticide resistance in *Culex quinquefasciatus* from Zanzibar: implications for vector control programmes. *Parasit. Vectors* 5: 78.
- Keita, M., N. Sogoba, F. Kané, B. Traoré, F. Zeukeng, B. Coulibaly, A. B. Sodio, S. F. Traoré, R. Djouaka, and S. Doumbia. 2021. Multiple resistance mechanisms to pyrethroids insecticides in *Anopheles gambiae* sensu lato population from Mali, West Africa. *J. Infect. Dis.* 223: S81–S90.
- Koou, S. Y., C. S. Chong, I. Vythilingam, C. Y. Lee, and L. C. Ng. 2014a. Insecticide resistance and its underlying mechanisms in field populations of *Aedes aegypti* adults (Diptera: Culicidae) in Singapore. *Parasit. Vectors.* 7: 471.
- Koou, S. Y., C. S. Chong, I. Vythilingam, L. C. Ng, and C. Y. Lee. 2014b. Pyrethroid resistance in *Aedes aegypti* larvae (Diptera: Culicidae) from Singapore. *J. Med. Entomol.* 51: 170–181.
- Kumar, S., A. Thomas, A. Sahgal, A. Verma, T. Samuel, and M. K. Pillai. 2002. Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Arch. Insect Biochem. Physiol.* 50: 1–8.
- Kumar, S., A. Thomas, A. Sahgal, A. Verma, T. Samuel, and M. K. K. Pillai. 2004. Variations in the insecticide-resistance spectrum of *Anopheles stephensi* after selection with deltamethrin or a deltamethrin-piperonyl-butoxide combination. *Ann. Trop. Med. Parasitol.* 98: 861–871.
- Kwan, J. L., S. Klueh, M. B. Madon, and W. K. Reisen. 2010. West Nile virus emergence and persistence in Los Angeles, California, 2003–2008. *Am. J. Trop. Med. Hyg.* 83: 400–412.
- Leong, C. S., I. Vythilingam, J. W. Liew, M. L. Wong, W. S. Wan-Yusoff, and Y. L. Lau. 2019. Enzymatic and molecular characterization of insecticide resistance mechanisms in field populations of *Aedes aegypti* from Selangor, Malaysia. *Parasit. Vectors* 12: 236.
- Liu, N. 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annu. Rev. Entomol.* 60: 537–559.
- Lucas, K. J., R. B. Bales, K. McCoy, and C. Weldon. 2020. Oxidase, esterase, and KDR-associated pyrethroid resistance in *Culex quinquefasciatus* field collections of Collier County, Florida. *J. Am. Mosq. Control Assoc.* 36: 22–32.
- Mallis, A., A. G. Miller, and R. V. Sharpless. 1952. Effectiveness against house flies of six pyrethrum synergists alone and in combination with piperonyl butoxide. *J. Econ. Entomol.* 45: 341–343.
- Marcombe, S., B. Fustec, J. Cattel, S. Chonephetsarath, P. Thammavong, N. Phommavanh, J. P. David, V. Corbel, I. W. Sutherland, J. C. Hertz, et al. 2019. Distribution of insecticide resistance and mechanisms involved in the arbovirus vector *Aedes aegypti* in Laos and implication for vector control. *PLoS Negl. Trop. Dis.* 13: e0007852.
- Muhammad, F., F. Ali, X. Rui-De, L. S. Michael, L. A. Jeremy, R. R. Alex, and A. K. Jennifer. 2017. Effect of nozzle orientation on dispersion of Aqualuer 20-20@ sprayed by a truck-mounted ultra-low volume sprayer against caged *Aedes aegypti*. *J. Am. Mosq. Control Assoc.* 33: 209–214.
- Mulamba, C., J. M. Riveron, S. S. Ibrahim, H. Irving, K. G. Barnes, L. G. Mukwaya, J. Birungi, and C. S. Wondji. 2014. Widespread pyrethroid and DDT resistance in the major malaria vector *Anopheles funestus* in East Africa is driven by metabolic resistance mechanisms. *PLoS One* 9: e110058.
- Nasci, R. S., and J. P. Mutebi. 2019. Reducing West Nile virus risk through vector management. *J. Med. Entomol.* 56: 1516–1521.
- Orange County Mosquito and Vector Control District. 2021. Mosquito Control. Orange County, CA. (<https://www.ocvector.org/mosquito-control>).
- Osimitz, T. G., N. Sommers, and R. Kingston. 2009. Human exposure to insecticide products containing pyrethrins and piperonyl butoxide (2001–2003). *Food Chem. Toxicol.* 47: 1406–1415.
- Plapp, F. W., Jr., W. S. Bigley, G. A. Chapmen, and G. W. Eddy. 1963. Synergism of malathion against resistant house flies and mosquitoes. *J. Econ. Entomol.* 56: 643–649.
- Protopopoff, N., J. F. Mosha, E. Lukole, J. D. Charlwood, A. Wright, C. D. Mwalimu, A. Manjurano, F. W. Mosha, W. Kisinza, I. Kleinschmidt, et al. 2018. Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two factorial design trial. *Lancet* 391: 1577–1588.
- Pryce, J., M. Richardson, and C. Lengeler. 2018. Insecticide-treated nets for preventing malaria. *Cochrane Database Syst. Rev.* 11: CD000363.
- Rakotonranaivo, T., S. F. Randriamanarivo, M. R. Tanjona, I. Vigan-Womas, M. Randrianarivojosia, and M. O. Ndiath. 2018. Evidence of insecticide resistance to pyrethroids and bendiocarb in *Anopheles funestus* from Tsararano, Marovoay District, Madagascar. *Biomed Res. Int.* 2018: 5806179.
- Richards, S. L., J. A. G. Balanay, A. V. White, J. Hope, K. Vandock, B. D. Byrd, and M. H. Reiskind. 2018. Insecticide susceptibility screening against *Culex* and *Aedes* (Diptera: Culicidae) mosquitoes from the United States. *J. Med. Entomol.* 55: 398–407.
- Riveron, J. M., M. Chiumia, B. D. Menze, K. G. Barnes, H. Irving, S. S. Ibrahim, G. D. Weedall, T. Mzilahowa, and C. S. Wondji. 2015. Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: a major concern for malaria vector control. *Malar. J.* 14: 344.
- Riveron, J. M., S. Huijben, W. Tchappa, M. Tchouakui, M. J. Wondji, M. Tchoupo, H. Irving, N. Cuamba, M. Maquina, K. Paaijmans, et al. 2019. Escalation of pyrethroid resistance in the malaria vector *Anopheles funestus* induces a loss of efficacy of piperonyl butoxide-based insecticide-treated nets in Mozambique. *J. Infect. Dis.* 220: 467–475.
- Ruktanonchai, D. J., S. Stonecipher, N. Lindsey, J. McAllister, S. K. Pillai, K. Horiuchi, M. Delorey, B. J. Biggerstaff, T. Sidwa, J. Zoretic, et al. 2014. Effect of aerial insecticide spraying on West Nile virus disease—north-central Texas, 2012. *Am. J. Trop. Med. Hyg.* 91: 240–245.
- Silva Martins, W. F., C. S. Wilding, A. T. Isaacs, E. J. Rippon, K. Megy, and M. J. Donnelly. 2019. Transcriptomic analysis of insecticide resistance in the lymphatic filariasis vector *Culex quinquefasciatus*. *Sci. Rep.* 9: 11406.
- Snoeck, S., R. Greenhalgh, L. Tirry, R. M. Clark, T. Van Leeuwen, and W. Dermauw. 2017. The effect of insecticide synergist treatment on genome-wide gene expression in a polyphagous pest. *Sci. Rep.* 7: 13440.
- Suman, D. S., S. P. Healy, A. Farajollahi, S. C. Crans, and R. Gaugler. 2012. Efficacy of Duet dual-action adulticide against caged *Aedes albopictus* with the use of an ultra-low volume cold aerosol sprayer. *J. Am. Mosq. Control Assoc.* 28: 338–340.
- Sy, N., S. S. Wheeler, S. Su, M. Reed, K. K. Brisco, A. J. Cornel, and J. Gan. 2020. Evaluation of pyrethroid residues in California urban catch basins and associated *Culex pipiens* permethrin resistance. *Proc. Mosq. Vector Control* 88: 24–26.
- WHO. 2006. Indoor residual spraying. Use of indoor residual spraying for scaling up global malaria control and elimination. WHO/HTM/MAL/2006.1112. WHO, Geneva, Switzerland. (https://apps.who.int/iris/bitstream/handle/10665/69386/WHO_HTM_MAL_2006.1112_eng.pdf;sequence=1).
- WHO. 2016. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2nd ed. WHO, Geneva, Switzerland. (<http://www.who.int/iris/handle/10665/250677>).
- WHO. 2018. Global report on insecticide resistance in malaria vectors: 2010–2016. WHO, Geneva, Switzerland. (<https://apps.who.int/iris/handle/10665/272533>).
- WHO. 2020. Mosquito-borne diseases. WHO, Geneva, Switzerland. (<https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases>).

- Wolfand, J. M., C. Seller, C. D. Bell, Y. M. Cho, K. Oetjen, T. S. Hogue, and R. G. Luthy. 2019. Occurrence of urban-use pesticides and management with enhanced stormwater control measures at the watershed scale. *Environ. Sci. Technol.* 53: 3634–3644.
- Xue, R. D., M. L. Smith, and W. A. Qualls. 2013. Field evaluation of truck-mounted thermal fog of DUET™ and Aqualuer using different solvents against *Aedes albopictus*. *J. Am. Mosq. Control Assoc.* 29: 301–303.
- Yanola, J., S. Chamnanya, N. Lumjuan, and P. Somboon. 2015. Insecticides resistance in the *Culex quinquefasciatus* populations from northern Thailand and possible resistance mechanisms. *Acta Trop.* 149: 232–238.
- Yoshimizu, M. H., K. Padgett, and V. Kramer. 2020. Surveillance of a *kdr* resistance mutation in *Culex pipiens* (Diptera: Culicidae) and *Culex quinquefasciatus* in California. *J. Med. Entomol.* 57: 645–648.
- Zimmer, C. T., M. Panini, K. S. Singh, E. L. Randall, L. M. Field, E. Roditakis, E. Mazzoni, and C. Bass. 2017. Use of the synergist piperonyl butoxide can slow the development of alpha-cypermethrin resistance in the whitefly *Bemisia tabaci*. *Insect Mol. Biol.* 26: 152–163.
- Zuharah, W. F., and M. Sufian. 2021. The discovery of a novel knockdown resistance (*kdr*) mutation A1007G on *Aedes aegypti* (Diptera: Culicidae) from Malaysia. *Sci. Rep.* 11: 5180.