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Pyrethroid tolerance in *Culex pipiens pipiens* var *molestus* from Marin County, California†

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Abstract: In May 2001 a sample of *Culex pipiens pipiens* variety *molestus* Forskål from Marin County, California, collected as larvae and reared to adults, was found to show reduced resmethrin and permethrin knock-down responses in bottle bioassays relative to a standard susceptible *Cx pipiens quinquefasciatus* Say colony (CQ1). Larval susceptibility tests, using CQ1 as standard susceptible, indicated that the Marin mosquitoes had LC_{50} resistance ratios of 18.3 for permethrin, 12 for deltamethrin and 3.3 for pyrethrum. A colony of Marin was established and rapidly developed higher levels of resistance in a few generations after exposure to permethrin as larvae. These selected larvae were shown to cross-resist to lambda-cyhalothrin as well as to DDT. However, adult knock-down time in the presence of permethrin, resmethrin and pyrethrum was not increased after increase in tolerance to pyrethroids as larvae. Partial and almost complete reversion to susceptibility as larvae was achieved with *S*, *S*, *S*-tributylphosphorotrithioate and piperonyl butoxide (PBO), respectively, suggesting the presence of carboxylesterase and P450 monooxygenase mediated resistance. Insensitive target site resistance (*kdr*) was also detected in some Marin mosquitoes by use of an existing PCR-based diagnostic assay designed for *Cx p pipiens* L mosquitoes. Carboxylesterase mediated resistance was supported by use of newly synthesized novel pyrethroid-selective substrates in activity assays. Bottle bioassays gave underestimates of the levels of tolerance to pyrethroids of Marin mosquitoes when compared with mortality rates in field trials using registered pyrethroid adulticides with and without PBO. This study represents the first report of resistance to pyrethroids in a feral population of a mosquito species in the USA.

Keywords: *Culex pipiens* complex; pyrethroid resistance

1 INTRODUCTION

Since the 1950s California mosquito abatement districts have persistently maintained a chemical control program to reduce mosquito populations. Some emphasis has been placed on controlling members of the *Culex pipiens* complex because of their propensity to become a nuisance. In addition to being major pest mosquitoes, *Cx pipiens* complex members are vectors of *Wuchereria bancrofti* (Cobbald), which causes filariasis in humans in the tropics.[1] They also vector West Nile[2] and St Louis encephalitis viruses in Eastern USA,[3] and Rift Valley Fever virus in Egypt.[4]

In California, *Cx p pipiens* L and *Cx p quinquefasciatus* Say interbreed, but *Cx p pipiens* predominates in northern California, while *Cx p quinquefasciatus* is localized in Southern California. Throughout California there are varying levels of hybridization between the sub-species and this is most apparent in central California; for simplicity, we refer to them as populations of *Cx pipiens* complex.[5–7]

California populations of *Cx pipiens* complex mosquitoes developed resistance to DDT and other organochlorines not long after these chemicals were introduced.[8] Organophosphates then

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replaced organochlorines and shortly thereafter, in the early 1960s, Isaak[9] detected the first evidence of organophosphate resistance in California *Cx p quinquefasciatus*. By the 1970s organophosphate resistance was widespread;[10] but despite this early evidence of resistance, organophosphates are still used today, although there is now a strong propensity to use pyrethroids.

Pyrethroid tolerance in members of the *Cx pipiens* mosquito complex has been documented in countries such as Tunisia,[11] Cuba,[12,13] Ivory Coast and Burkina Faso,[14] Saudi Arabia,[15] French Polynesia[16] and China.[17] There has not, however, been a published report of pyrethroid tolerance in North America in any member of the *Cx pipiens* complex or, in fact, in any mosquito species.

For surveillance purposes, in 2000 we conducted time-knock-down adulticide bottle bioassays on *Cx pipiens* complex populations from various locations in California in order to test for tolerance levels to organophosphate and pyrethroid chemicals.[18] All of the populations evaluated, except for one, showed no tolerance to the three pyrethroids tested (pyrethrum, permethrin and resmethrin). This single population that showed pyrethroid tolerance as adults and larvae was collected in 2001 from San Rafael (Marin County). In this study we have focused on further elucidating, by susceptibility and biochemical assays, the adult and larval pyrethroid tolerance characteristics of the Marin mosquitoes.

2 EXPERIMENTAL METHODS

2.1 Colony strains and maintenance

Culex pipiens complex mosquitoes collected as larvae from a flooded basement of an apartment complex in San Rafael (Marin County, CA) in May 2001 were reared to adults and called the Marin colony. Before colonization the Marin mosquitoes had been exposed to treatments two or more times a year for 6 years with Scourge^{TM} (12% resmethrin + 4% PBO active ingredient). Based on morphology of male genitalia (DV/D ratios), 100% of the Marin mosquitoes sampled fell within the range typical for *Cx p pipiens*.[19] Because of their DV/D ratio measurements, the habitat they originated from, and because all females lay autogenous eggs, we refer to them as *Cx p pipiens* var *molestus* Forskål. From F4 generation the colony has been maintained solely from autogenous eggs requiring no blood feeding. Starting with the F2 generation, they were selected with permethrin six times over 26 generations in order to increase their resistance to levels where the possible pyrethroid resistance mechanism(s) could be more easily discernable. Selection comprised of exposing the 4th-stage larvae to a permethrin solution for 24 h, increasing the strength of the solution to maintain approximately 50% larval mortality.

For comparative purposes, a susceptible *Cx p quinquefasciatus* (CQ1) colony was used as an organophosphate- and pyrethroid-susceptible strain. The CQ1 colony originated from adults collected from Merced, CA in the 1950s, have male genitalia DV/D ratios typical for *Cx p quinquefasciatus*, and the colony is non-autogenous. Susceptibility profiles of CQ1 as larvae and adults closely matched those of S-Lab (standard susceptible *Cx p quinquefasciatus* colony used in other laboratories).[20]

2.2 Adult bottle bioassays

The time-knock-down adulticide bottle bioassay was conducted by treating the insides of 250-ml Wheaton bottles (Fisher #06-404B) with technical grade insecticide purchased from Chem Service (West Chester, PA). The insecticides were diluted in acetone and evenly applied to the inside of each bottle, following the procedure described by Brogdon and McAllister.[18] For each insecticide, four replicates of 25 three-day-old mosquitoes were used to determine percentage mortality (organophosphates) or percentage knock-down (pyrethroids). Controls consisted of bottles coated with acetone only, or acetone and synergist. A count was taken of dead or knocked down mosquitoes every 15 min for up to 3 h in both treatment and control bottles. A mosquito was considered dead or knocked down if it could not right itself when the bottle was slowly rotated. Mosquitoes were exposed to a predetermined dosage of insecticide, which represented the amount of insecticide that resulted in 100% mortality or knock-down in the CQ1 colony within 1 h. All bioassays conducted on Marin mosquitoes were tested concurrently with CQ1 mosquitoes to confirm consistency of method. The concentrations used for the insecticides were as follows: malathion, 100 μg per bottle; naled (dibrom), 10 μg per bottle; fenthion, 200 μg per bottle; chlorpyrifos, 50 μg per bottle; permethrin, 30 μg per bottle; pyrethrum, 15.6 μg per bottle; resmethrin, 10 μg per bottle; deltamethrin, 20 μg per bottle; and piperonyl butoxide, 63, 150 and 400 μg per bottle. The 400 μg PBO per bottle dose used with permethrin and pyrethrum was the maximum amount that did not cause mortality when used alone. The lower two amounts corresponded to the same proportion of active ingredient (AI) of pesticide to PBO provided on the labels for the formulations used in the ULV field application tests. These were 63 μg per bottle for permethrin and 150 μg per bottle for pyrethrum. Time-mortality and time-knock-down data were plotted on a probability versus time scale using SigmaPlot (Jandel Corporation, San Rafael, CA).

2.3 Larval bioassays

Larvicide bioassays were conducted on 15 4th-stage larvae placed in waxed cups (Sweetheart #S-304, Owings Mills, MD) with four replicates tested per concentration ($n = 60$). Each cup contained 100 ml of tap water, 5 mg of ground rodent food, and varying concentrations of technical grade pyrethrum, permethrin, deltamethrin, lambda cyhalothrin or p,p'-DDT diluted in acetone. Controls containing

only acetone, or only acetone and synergist as appropriate, were run concurrently with each test. Bioassays were run with the synergists *S, S, S*-tributylphosphorotrithioate (DEF, at 1 mg liter^{-1}) and piperonyl butoxide (PBO, at 5 mg liter^{-1}), and 3-octylthiol-1,1,1-trifluoro-2-propanone (OTFP, at 2 mg liter^{-1})[21] where larvae were exposed to the synergist for 4 h prior to pyrethroid addition. Each synergist was diluted in acetone before application to the test solution. Following the addition of pyrethroid or synergist, the test solution was stirred briefly to ensure uniform mixture. The larval cups were then placed in an incubator at 28 °C, and percentage mortality was recorded after 24 h. LD_{50} and LD_{90} data based on larval mortality was determined using POLO PC.[22]

2.4 Ultra-low-volume field study

Commercially available synergized and unsynergized ULV permethrin and pyrethrum formulations were applied to caged Marin and CQ1 mosquitoes in a fallow field next to the property of the Merced Mosquito Abatement District. These formulations were Permanone® RTU (39.8 g liter^{-1} permethrin *trans* ≤ 65%, *cis* ≥ 35% + 84.8 g liter^{-1} PBO technical) (Aventis Environmental Science USA LP, Montvale, NJ) applied at 0.0078 kg AI ha^{-1}; Permanone EC (100 g liter^{-1} permethrin *trans* ≤ 65%, *cis* ≥ 35%) (Aventis Environmental Science USA LP, Montvale, NJ) mixed 1:1.32 by volume with Citgo Duoprime Oil 90 (Citgo Petroleum Corp, Tulsa, OK) applied at 0.0078 kg AI ha;$^{-1}$ Pyganid® (50 g liter^{-1} pyrethrins, <20% mineral oil) (McLaughlin Gormley King Co, Minneapolis, MN) mixed 1:2.33 by volume with Citgo Duoprime Oil 90 (Citgo Petroleum Corp., Tulsa, OK) applied at 0.0028 kg AI ha^{-1}; and Pyrocide® 12–60 (120 g liter^{-1} pyrethrins, 600 g liter^{-1} PBO technical) (McLaughlin Gormley King Co, Minneapolis, MN) applied at 0.0028 kg AI ha^{-1}. The pesticide formulations were applied by truck-mounted Leco 800 (Clark Mosquito Control, Roselle, IL) cold-foggers at a speed of 8 km h^{-1} along an east–west aligned spray path at sunset after formation of a temperature inversion. Not all the tests were conducted the same day and the conditions for each test are provided in Table 1. Droplet size was measured using the slide waving method of Carroll and Bourg,[23] with Teflon-coated slides (BioQuip Products, Gardena, CA) and flow rate calibrations for treatments were conducted on the afternoon of each test shortly before the treatment.

Approximately 30 three-day-old mosquitoes from each colony were mouth aspirated into screened cages (16.5 cm diameter × 5.08 cm deep; Clark Mosquito Control, Roselle, IL). The cages were hung from 1-m high stakes by means of adhesive-backed Velcro screened sides perpendicular to wind direction. The stakes were arranged in a 3 × 3 pattern, with a cage from each colony tested on each stake. These stakes were situated at 30.5, 61.0 and 91.4 m downwind from the path of the truck, in rows 15.2 m apart. At each distance there were three replicates for each colony, giving a sample size of approximately 90 for each population at each distance. Controls were placed in an area away from the spray site. Cages were left on the stakes for 15 min post-treatment, then transported back to the laboratory and held at room temperature.

Table 1. Field knock-down and mortality of CQ1 and Marin mosquitoes[a]

Treatment	Colony	Distance from application (m)			Mean value[b]
		31	61	91	
Knock-down % (1 h)					
Permethrin	Marin F25	3.8 (±6.5)	1.5 (±1.4)	0.6 (±1.1)	2.0 (±3.6) A
	CQ1	70.6 (±36.1)	68.8 (±50.7)	53.3 (±43.4)	64.2 (±38.8) B
Permethrin + PBO	Marin F25	40.3 (±28.4)	52.0 (±28.5)	21.0 (±11.2)	37.7 (±24.9) B
	CQ1	98.3 (±2.9)	97.3 (±4.6)	98.4 (±2.7)	98.0 (±3.1) C
Mortality % (12 h)					
Permethrin	Marin F25	35.5 (±32.0)	21.3 (±33.7)	12.3 (±14.7)	23.0 (±26.4) A
	CQ1	97.2 (±4.8)	82.6 (±26.8)	81.9 (±27.7)	87.2 (±20.8) BC
Permethrin + PBO	Marin F25	91.1 (±8.4)	88.9 (±10.6)	55.5 (±19.1)	78.5 (±20.9) B
	CQ1	100	100	100	100C
Knock-down % (1 h)					
Pyrethrum	Marin F25	0	0	0.9 (±1.5)	0.3 (±0.9) A
	CQ1	64.1 (±7.1)	31.8 (±1.4)	32.2 (±18.0)	42.7 (±18.8) B
Pyrethrum + PBO	Marin F25	81.7 (±8.3)	68.9 (±17.5)	44.5 (±38.5)	65.1 (±27.1) B
	CQ1	97.9 (±1.9)	100	97.9 (±3.6)	98.6 (±2.3) C
Mortality % (12 h)					
Pyrethrum	Marin F25	2.9 (±2.4)	1.2 (±1.5)	2.3 (±3.9)	2.1 (±2.5) A
	CQ1	79.6 (±3.5)	44.0 (±12.2)	21.0 (±12.6)	48.2 (±27.1) B
Pyrethrum + PBO	Marin F25	80.0 (±9.8)	76.0 (±23.4)	44.5 (±36.5)	66.8 (±27.9) B
	CQ1	100	100	100	100 C

[a] Average temperature was 17.3 °C (15.4–21.0 °C) with a 0.3–1 °C inversion between 1.8 m and 9.1 m.
[b] Means followed by the same letter are not significantly different within each treatment (ANOVA least significance difference; $P \leq 0.05$).

Mosquitoes were left in the cage for 1 h after treatment, then transferred into a clean holding cup (1/2-pint paper food cans, Neptune Paper Products, Newark, NJ) with a screened top, and supplied with a cotton ball soaked with a 100 g liter^{-1} sucrose solution. For mosquito transfer, each test cage was emptied into a larger cage from which the mosquitoes were collected by mouth aspiration for placement in the holding cups. Knock-down was assessed at 1 h and mortality at 12 h post-treatment.

2.5 Non-specific esterase allozyme electrophoresis

Non-specific esterase allozyme phenotype was determined by polyacrylamide gel electrophoresis (PAGE). Wild Marin and CQ1 colony mosquitoes tested were ground in 30 µl of a Tris-citrate buffer (pH 7.1) containing sucrose (100 g liter^{-1}) Triton X-100 (5 g liter^{-1}) and bromophenol blue (0.1 g liter^{-1}). The homogenate was then electrophoresed through a Tris-boric acid–EDTA (60 g liter^{-1}; pH 8.9) gel in a Hoefer SE 600 series electrophoresis unit for about 5 h. The gel was stained for esterase activity according to the recipe provided by Steiner and Joslyn.[24] Enzyme action was halted after 10 min with a weak 1% acetic acid solution. Bands were characterized based on mobility and their affinity to hydrolyze either α or β-naphthyl acetate. Bands that preferentially hydrolyzed α-naphthyl acetate stained black, while bands that had a higher activity on β-naphthyl acetate stained red. Colony mosquitoes that had been selected for the common $\alpha 2 \beta 2$ pattern (A2B2) were run alongside each gel for comparison, along with susceptible (CQ1) colony mosquitoes.

2.6 Detection of insensitive target site

DNA was extracted from wild Marin and CQ1 colony adult mosquitoes by means of the method described by Collins *et al*.[25] Polymerase chain reactions (PCR) were carried out for each sample according to the methods outlined by Martinez-Torres *et al*[26] in order to detect the presence of a leucine to phenylalanine mutation at position 1014 of the voltage-dependent sodium channel (*kdr*-type resistance). PCR products were run through on a 1.5% agarose gel, and banding was visualized by staining with ethidium bromide under ultraviolet light (302 nm).

2.7 Carboxylesterase activity assays

Assays with all substrates were performed on whole mosquito homogenates of individual mosquitoes homogenized in sodium phosphate buffer (pH 7.8, 100 mM; 200 µl) at 4 °C.[27]

The esterase activities between male and female adult CQ1 and Marin mosquitoes were measured using a standard surrogate esterase substrate *p*-nitrophenyl acetate (PNPA), a general esterase fluorescent substrate ((*R*/*S*)-acetic acid cyano(6-methoxynaphthalen-2-yl)methyl ester; compound 1) and a compound 2 structurally similar to Type II pyrethroids known as *trans*/*cis*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylic acid cyano(6-methoxynaphthalen-2-yl)methyl ester.[27,28] PNPA assays were performed in 96-well microtiter styrene flat bottom plates (Dynex Technologies, Inc, Chantilly, VA). The total assay volume was 200 µl, consisting of 150 µl of sodium phosphate buffer (pH 7.8, 100 mM) and 10 µl of mosquito homogenate. The substrate was added to produce a final concentration of 0.5 mM.[29] Activity was monitored for 2 min at 405 nm at room temperature with a Uvmax™ (Molecular Devices Corporation, Palo Alto, CA). Assays with the general fluorescent substrate (compound 1) were performed in black 96-well polystyrene flat clear bottom microtiter plates (Corning Inc, New York, NY) and hydrolysis activity was measured at 30 °C in a SPECTRAFlour Plus (Tecan, Research Triangle Park, NC). The reaction mixture consisted of 150 µl of sodium phosphate buffer (pH 7.8; 0.1 mM) and 10 µl of mosquito homogenate. Activity monitoring was begun immediately upon addition of 40 µl of substrate solution to each well (final concentration 4.4 µM). The fluorescence was read for 5 min with excitation at 330 nm (bp 35) and emission at 465 nm (bp 35). Assays with the cypermethrin-selective substrate (compound 2) were performed in individual 4-ml quartz cuvettes with a Fluoromax-2 fluorospectrometer (Instruments SA, Inc Edison, NJ). In 3 ml of the buffer, 10 µl of mosquito homogenate was first added, and the reaction was initiated by the addition of 3 µl of substrate solution (final concentration 10 µM).

3 RESULTS

3.1 Adulticide bottle bioassay

No mortality was observed in control bottles coated with acetone only, or acetone and synergist only, in any of the assays. Based on the time-knock-down bottle bioassay, wild Marin County *Cx p pipiens* var *molestus* (F0 generation) showed more tolerance to resmethrin (Fig 1, A), pyrethrum (B) or permethrin (C) than the susceptible CQ1 mosquitoes. The Marin mosquitoes responded similarly to permethrin and resmethrin, but were much less tolerant to pyrethrum. At the threshold time (α, the time necessary for 100% knock-down of the CQ1 colony) of 60 min for permethrin and pyrethrum and 45 min for resmethrin, 50%, 5% and 59% of the Marin mosquitoes, respectively, were not knocked down. All of the Marin mosquitoes were knocked down after 90 min of exposure to pyrethrum, while approximately 10% of the Marin mosquitoes were still not knocked down after 3 h of exposure to resmethrin and permethrin. There was no difference between Marin and CQ1 time mortality for any of the organophosphates tested, which included naled, malathion, fenthion and chlorpyrifos (data not shown).

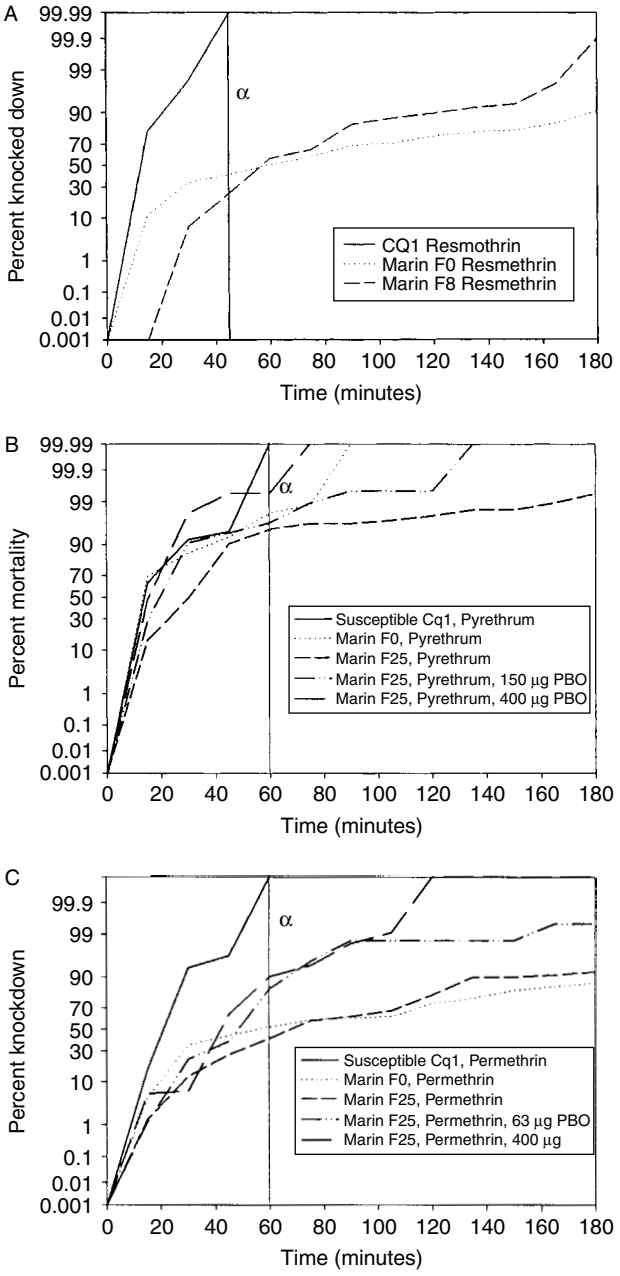

Figure 1. Marin County *Cx pipiens molestus* and susceptible *Cx quinquefasciatus* bottle bioassay percentage knockdown plotted over time for (A) resmethrin, (B) pyrethrum (C) permethrin.

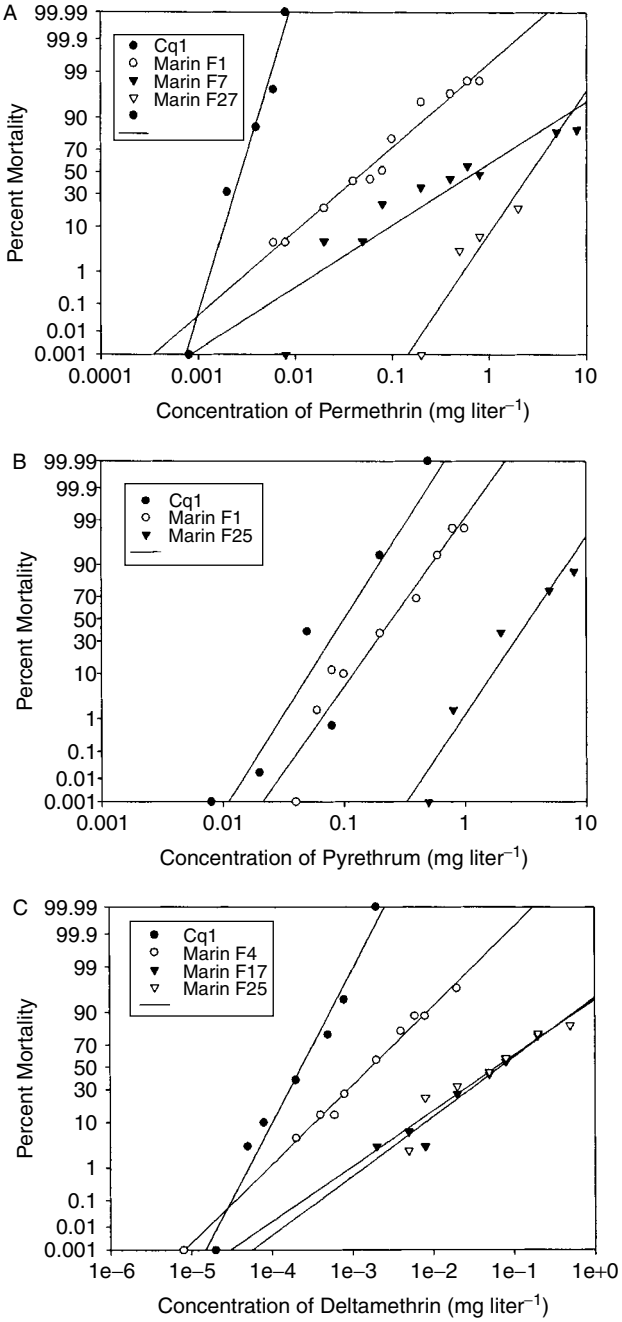

Figure 2. Fourth-instar CQ1 and Marin colony susceptibility profiles to (A) permethrin, (B) pyrethrum and (C) deltamethrin.

3.2 Larvicide bioassay

To reflect as much as possible responses of wild Marin mosquitoes, larval susceptibility assays were done at as low a filial generation as dictated by availability of sufficient numbers of larvae and before selection for higher levels of resistance had proceeded too far. No mortality was observed in any of the control (acetone with and without synergist, and water only) cups in any of the assays. First-generation Marin larvae were resistant to permethrin, slightly tolerant to pyrethrum and fourth-generation Marin were resistant to deltamethrin (Fig 2) with resistance ratios at LC_{50} (defined as $LC_{50\ \text{resistant(Marin)}}/LC_{50\ \text{susceptible(CQ1)}} = RR_{50}$) of 18.3 for permethrin, 3.3 for pyrethrum and 12 for deltamethrin. All larval insecticide susceptibility assays were subjected to probit analysis, and, in all instances, the index of significance for potency estimation was below 0.1 at a confidence limit of 95%, with exception to the assays using F25 and F17 on permethrin and DDT respectively because of low mortality at saturated concentrations. Because of the high tolerance to permethrin of the FI Marin mosquitoes it was decided to select for higher levels of resistance with further exposure to permethrin. Exposure to permethrin began at a dosage of 0.05 mg liter^{-1}, and progressed from 0.08 mg liter^{-1} in F2, 0.2 mg liter^{-1} in F3, 0.4 mg liter^{-1} in F5, 0.5 in F11 and 0.5 mg liter^{-1} in F18. After the 18th generation, no further exposures to permethrin were made. At

a high dose of 0.5 mg liter^{-1}, saturation point was reached and the permethrin solution appeared murky. During this succession of exposures to permethrin, RR_{50} ratios to pyrethrum and deltamethrin also increased to 50 and 274 respectively. With all three chemicals increased levels of tolerance produced evolving responses close to straight lines with low slopes, until, as is strongly indicated with permethrin, resistance suddenly increased sharply with a steep slope, an indication of polyfactorial resistance.

The wild Marin mosquitoes showed high to partial reversion to susceptibility in the presence of the synergist PBO (Fig 3), slightly to DEF (Fig 3) and no reversion in the presence of OTFP (data not shown).

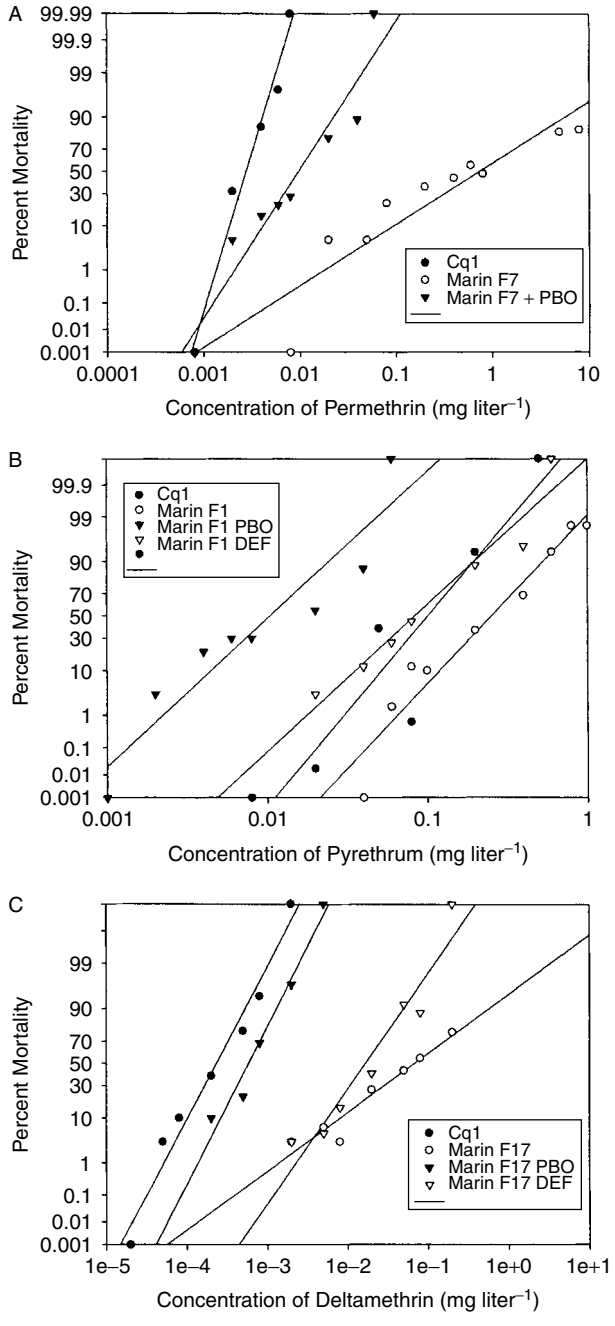

Figure 3. Fourth-instar CQ1 and Marin colony susceptibility profiles to (A) permethrin, (B) pyrethrum and (C) deltamethrin in the presence of synergists PBO and DEF.

After several generations of permethrin selection the Marin larvae showed extremely high tolerance to DDT, with an LC_{50} of 170 and an LC_{90} of an immeasurable amount due to solubility issues at high DDT concentrations (Fig 4A). Cross-resistance to lambda-cyhalothrin (Marin F13 RR_{50} 60.7) was also apparent (Fig 4B).

Bottle bioassay time-knock-down responses of adults of higher filial generation Marin mosquitoes indicated that, while permethrin selection as larvae increased or maintained resistance to permethrin and resmethrin as adults, resistance to pyrethrum was not increased. The synergist PBO increased knock-down rates in the presence of pyrethrum and permethrin on Marin F25 adults with knock-down rates higher at higher ratios of PBO to insecticide (Fig 1B and C).

3.3 Field study

In most instances there was no mortality in 1 h and 12 h in CQ1 and Marin controls. When mortality did occur in the controls it never exceeded 4.5% and percentage mortality was corrected for natural mortality by Abbott's formula.[30] Since the bottle bioassays are designed to provide an estimate of knock-down responses to pyrethroids, mortality of selected Marin mosquitoes was determined by exposure in the field to high label doses of registered ULV adulticide pyrethroid formulations. Pyrethrum when used alone was significantly ($P < 0.05$) less effective,

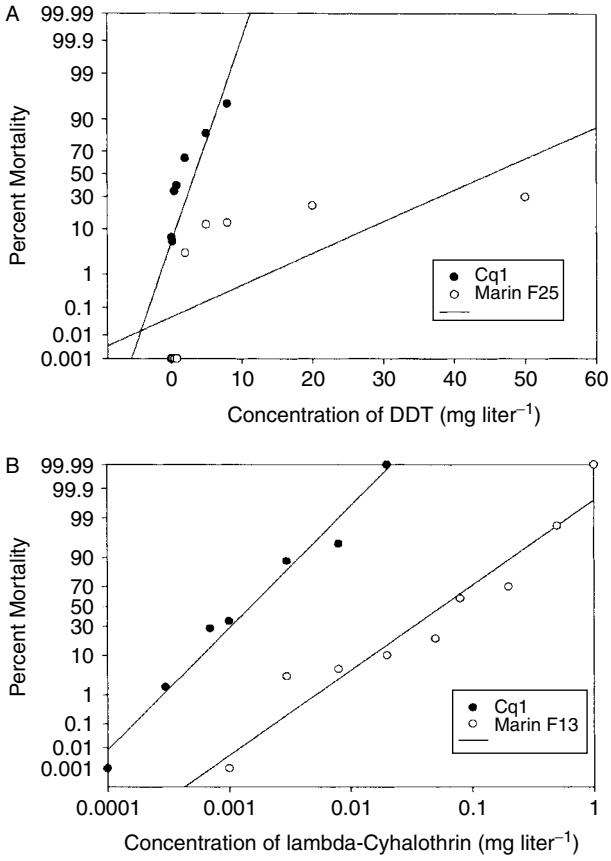

Figure 4. Fourth-instar CQ1 and Marin colony susceptibility profiles to (A) *p*, *p*′-DDT and (B) lambda-cyhalothrin.

producing only 48% mortality in the CQ1 and 2% in Marin mosquitoes (Table 1). The extent of mortality in the CQ1 mosquitoes was also significantly affected by distance from source, with least control at 91.4 m. Permethrin when used alone produced much higher mortality than pyrethrum at all three distances from spray source in the CQ1 mosquitoes (87%) and slightly higher mortality than pyrethrum alone in the Marin mosquitoes (23%). For either pyrethroid the CQ1 mosquitoes were killed at a significantly ($P < 0.05$) higher rate than Marin. The addition of the synergist PBO to permethrin and pyrethrum markedly reversed any degree of tolerance in the CQ1 mosquitoes, producing 100% mortality at all distances. Permethrin and pyrethrum with PBO provided partial, but still significant ($P < 0.05$), synergism to the Marin mosquitoes. Beyond 30 meters from the spray source, however, mortality was still at an operational unacceptable low level. Percentage knock-down of both mosquito colonies after 1 h was always lower than mortality at 12 h, with CQ1 significantly ($P < 0.05$) more than Marin. This indicates that those knocked down after 1 h never recovered, and that some not knocked down within 1 h still had received a lethal dose leading to mortality 11 h later. The addition of PBO to both pyrethrum and permethrin produced a significant ($P < 0.05$) increase in knock-down in both the Marin and CQ1 mosquitoes.

3.4 Non-specific esterase frequencies

Most wild Marin mosquitoes showed weak staining similar to the CQ1, indicating lack of elevated esterase enzymes (Fig 5). Fifty-two Marin of both sexes were tested, with three showing highly elevated levels of B1 esterase, and four individuals showing weakly elevated B1 esterase. The two that had strong B1 esterase banding were visually as strong as the banding seen typically in members of the *Cx pipiens* complex in other locations in California where they are organophosphate resistant, such as Reedley. Two examples of those that had weak B1 banding are shown in Fig 5. There was no correlation between sex and intensity of staining.

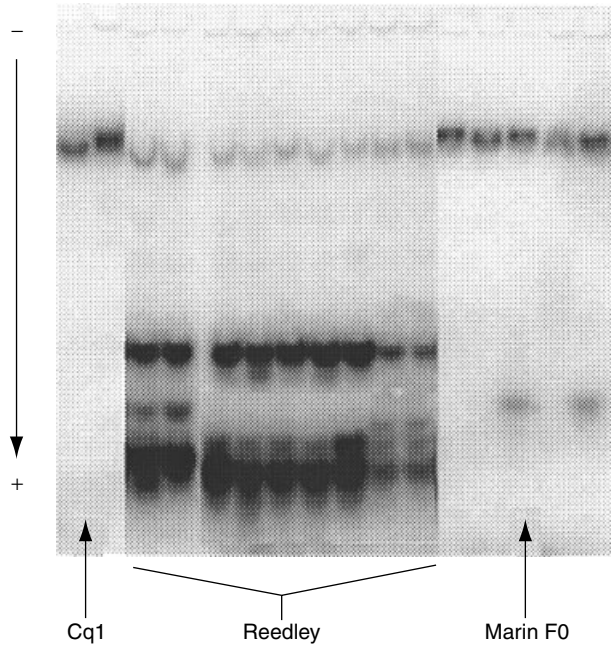

Figure 5. Polyacrylamide electrophoresis gels stained with α and β naphthyl acetate of adult colony CQ1, wild Marin and wild Reedley *Cx pipiens* complex member mosquitoes.

3.5 Detection of insensitive target site

Fifty wild Marin individuals were tested along with several CQ1 individuals for presence of insensitive target site using a PCR-based diagnostic assay designed for detection of *kdr*-type resistance in *Cx pipiens*.[26] Replacement of the amino acid leucine with phenylalanine at position 1014 in the voltage-dependent sodium channel protein renders the protein less sensitive to binding of DDT and pyrethroids. Of the 50 Marin individuals tested, six were homozygous for leucine, 26 were homozygous for phenylalanine and 18 were heterozygotes. None of the CQ1 mosquitoes showed any presence of phenylalanine at position 1014, thus were absent for the *kdr*-type mutation. The frequencies of each allele of the sodium channel gene in the wild Marin mosquitoes did not differ significantly (chi-square at $P = 0.05$) from frequencies predicted by Hardy–Weinberg equilibrium (Genepop version 1.2).[31]

3.6 Carboxylesterase activity

Carboxylesterase activity levels as measured by all three substrates (Table 2) produced highly variable levels in both males and females (high standard errors), indicating that levels of carboxylesterases expression were highly heterogeneous amongst individuals in each colony. The carboxylesterase assays (enzyme and substrate concentrations) were designed to produce a linear response (up to 20 min) with <1% variability

Table 2. Carboxylesterase activity in individual mosquito homogenates: PNPA in nmol $min^{-1}mg^{-1}$ protein and compounds 1 and 2 in pmol min^{-1} mg^{-1} protein

Substrate (colony)	Male (SE)	Male R/S[a]	Female (SE)	Female R/S[a]	Male female R/S[a]
PNPA (CQ1)	91.2 (8.1)		88.8 (10.2)		
PNPA (Marin)	98.5 (14.4)	1.07	58.4 (2.24)	0.65	0.87
Compound 1 (CQ1)	515.1 (35.6)		749.3 (124.7)		
Compound 1 (Marin)	728.9 (353.7)	1.41	459 (132.2)	0.61	0.93
Compound 2 (CQ1)	216.7 (19)		155.9 (16)		
Compound 2 (Marin)	411.1 (70)	1.9	129.5 (12)	0.83	1.4

[a] Ratio = activity of Marin (R)/activity of CQ1 (S).

between replicates,[29] and therefore the observed variability was due to differences amongst individual mosquitoes and not due to inter-assay variability. Large gender differences in carboxylesterase activity levels were regularly observed. Both carboxylesterase fluorescent substrates produced consistently lower levels of activity in Marin females than males, but the differences were less obvious with PNPA. Marin male carboxylesterase activities were consistently higher than CQ1 males, the most evident occurring with the cypermethrin-specific substrate, where Marin males were almost double that of CQ1 males. In contrast, Marin female carboxylesterase activity levels were consistently almost half of those of CQ1 females with PNPA and the general fluorescent substrates.

4 DISCUSSION AND CONCLUSIONS

Priester and Georghiou[20] were able to show that resistance to pyrethroids due to a single heritable factor could evolve in a California population of *Cx p quinquefasciatus* under continued exposure to pyrethroids in the laboratory. It was inevitable that tolerance to pyrethroids would eventually arise in wild *Cx pipiens* complex mosquitoes in California because of the high pyrethroid usage against agricultural pests and that resistance to pyrethroids has arisen in Africa[14,32] and Asia[15,17] in *Cx pipiens sensu latu*.

The Marin mosquitoes originated from a population that bred in pools of water from underneath an apartment complex. Mosquito control personnel did not have sufficient access to be able to use larvicides, and so resorted to treating the adults. Environmental concerns over organophosphorus insecticides prompted the local abatement agency to discontinue their use, leaving pyrethroids as the only option for adult control. Years of treatment with pyrethroids most likely created the ideal situation for the development of resistance in this population. The propensity for selection of pyrethroid resistance may have arisen from pre-existence of the *kdr* mutant allele of the *para* gene from past use of DDT. However, we also cannot rule out the possibility that mosquitoes carrying pyrethroid resistant gene(s) may have been introduced from outside populations via sea or air transport.

At the time of colonization Marin mosquitoes were tolerant to resmethrin and permethrin and slightly tolerant to pyrethrum. After several rounds of selection with permethrin as larvae they were also highly tolerant to DDT and lambda-cyhalothrin, suggesting that at the time of colonization they were probably cross-resistant to DDT and lambda-cyhalothrin as well. The larvicide susceptibility synergist assays suggest that the tolerance to pyrethroids observed in the Marin population of *Cx p pipiens* var *molestus* is due, in large part, to oxidative metabolism based upon the strong reversal action of PBO and slight action of DEF. PBO, which is a widely recognized suicide substrate for some P450 enzymes (but is known to inhibit other enzymes such as esterases), shows almost complete reversion to susceptibility, whereas DEF, which primarily inhibits esterases and some oxidases, showed partial reversion to susceptibility. The synergist OTFP, which so far is only known to act on esterases, had no significant synergistic effect on the Marin mosquitoes when exposed to permethrin or deltamethrin. The low general esterase activity of Marin mosquitoes that was similar to CQ1 levels was corroborated by the low PAGE naphthyl acetate staining intensities and similar PNPA and general flourescent substrate activity levels. For comparative purposes mosquitoes that had intense naphthyl acetate PAGE staining propensities (Reedley mosquitoes in Fig 5) had mean male and female PNPA activity levels 2.5 times, and general fluorescent substrate activity levels four times higher than Marin and CQ1 mosquitoes. It is interesting that male Marin mosquitoes possessed almost double the cypermethrin-metabolizing activity of CQ1 mosquitoes, which suggests the presence of male-linked carboxylesterase pyrethroid-metabolizing properties that are not inhibited by DEF or OTFP.

The almost complete reversion to susceptibility of Marin larvae in the presence of the synergist PBO does not, however, corroborate with the detection of *kdr*-type mutation and remains an enigma. The presence of *kdr*-type mutation had little effect on pyrethroid resistance on Marin larvae despite the fact that they are highly resistant to DDT. Electrophysiological studies on pyrethroid resistant *Cx p quinquefasciatus* from Saudi Arabia[15] did indicate that, even though there was no *kdr*-type mechanism conferring resistance to pyrethroids, they were cross-resistant to DDT. The frequencies of the mutant voltage-gated sodium channel allele in wild Marin were also in Hardy–Weinberg equilibrium, which would suggest that the mutant allele is not under selective pressure in the field. However, field ULV spray data on adults does suggest a non-oxidative metabolizing component to resistance as adults. Even in the presence of PBO many Marin survived a dose lethal to susceptible mosquitoes and had low permethrin and pyrethrum knock-down activity after one hour. Knock-down activity with all three pyrethroids with and without PBO was always much higher in the bottle bioassays after 1 h than with the registered formulations in the field. These data indicate that the bottle bioassays were underestimating the extent of operational control failure that would occur in the field in the case of Marin mosquitoes. This inability to extrapolate and predict the extent of resistance in the field from bottle bioassay data was most likely due to differences in doses, and that multiple mechanisms of resistance to pyrethroids are present in the Marin colony, including the *kdr*-mutant allele, high oxidase and low esterase mediated resistance. Multiple resistance mechanisms in the form of *kdr*-type and cytochrome P450 oxidase metabolism have been proposed to occur in several *Cx p quinquefasciatus* populations in West Africa.[14,32] Whether the same P450 oxidases are responsible

for pyrethroid resistance in the West African, Saudi Arabian[33] and Marin mosquitoes remains to be determined. Biochemically, the natural pyrethrins have many more sites susceptible to P450 oxidation than compounds such as deltamethrin and permethrin, while, based on steric properties, the natural pyrethrins should be more resistant to esterase action.

We do not know the extent of genetic exchange that takes place between the below-ground *Cx p pipiens* var *molestus* and above-ground Marin *Cx p pipiens* populations. However, the possibility exists that genetic exchange does occur[5–7] and that pyrethroid resistant genes could be expected to spread. Marin larvae display considerable cross-tolerance to lambda-cyhalothrin, and this chemical is currently used extensively in rice cultivation in California. Since 1998 lambda-cyhalothrin use has increased from 0 to 10 787 kg per year on rice and various vegetable crops (See www.cdpr.ca.gov/docs/pur/purmain.htm). Rice plantations are a favored breeding habitat for *Cx pipiens* complex members, and the use of this chemical alone would provide sufficient selective pressures for maintenance and spread of pyrethroid resistance.

Unlike all populations of *Cx pipiens* complex members thus far evaluated in California, the Marin population showed high susceptibility to organophosphates, raising the possibility that resistance to pyrethroids occurs at the cost of resistance to organophosphates. This phenomenon, also reported in China[17] may be exploited in the future to mitigate and reverse resistance to pyrethroids by using rotations of pyrethroids and organophosphates.

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