High level methoprene resistance in the mosquito *Ochlerotatus nigromaculis* (Ludlow) in Central California

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Abstract: In the summer of 1998, failures of methoprene field applications to control the mosquito *Ochlerotatus nigromaculis* (Ludlow) were noticed in several pastures in the outskirts of Fresno, California, USA. Effective control with methoprene had been achieved for over 20 years prior to this discovery. Susceptibility tests indicated that the Fresno *Oc nigromaculis* populations had developed several thousand-fold higher LC_{50} and LC_{90} tolerance levels to methoprene compared with methoprene-naïve populations. The synergists piperonyl butoxide (PBO), *S,S,S*-tributyl phosphorotrithioate and 3-octylthio-1,1,1-trifluoro-2-propanone had little synergistic effect, suggesting that the mechanism of methoprene tolerance was not mediated by P450 monooxygenase or carboxylesterase enzyme degradation. As part of initiating a resistance management strategy, partial reversion back to methoprene susceptibility was achieved in a resistant population after six consecutive applications of *Bacillus thuringiensis israelensis* Goldberg & Marga coupled with two oil and two pyrethrum + PBO applications.
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1 INTRODUCTION

The first signs of methoprene control failures of the common pasture mosquito *Ochlerotatus nigromaculis* (Ludlow) were observed during September of 1998 in two pastures in Fresno County, California, USA. Methoprene (Altosid® liquid formulation [ALL]) had been used to control *Oc nigromaculis* in these pastures since 1974 and was shown to be effective since then by observations of lack of flying adults and intermittent sampling to confirm 95–100% pupal death. By September 1999 the lack of control by ALL had spread approximately nine miles due east and southeast to a further nine pastures in Fresno County. Additional methoprene failures were found 34 miles south in one pasture in Kings County.[1] A field study conducted in 1999 indicated that in some of these pastures low level to no control was achieved with ALL or Altosid XR-G. A range of control from 52 to 99% was reported with Altosid pellets.[1]

Oc nigromaculis, first collected in California in 1937, was suspected of being introduced into California from east of the Sierra Nevadas on alfalfa plants.[2] Thereafter, *Oc nigromaculis* spread throughout the San Joaquin Valley. It avidly blood-feeds on man and domestic animals and represents a pest species. The short (less than 5–7 days from egg to adult) and multivoltine life cycle from April to October compound to facilitate rapid increase in numbers, requiring constant and costly abatement. Laboratory virus vector competence studies have proven that *Oc nigromaculis* is capable of transmitting western equine encephalomyelitis and St Louis encephalitis, although no isolations of either virus have been made from wild specimens in California.[3]

This study was undertaken to confirm that the lack of control by applications of methoprene on *Oc nigromaculis* in the field was due to resistance by comparing methoprene susceptibility profiles on individuals from pastures where methoprene failures had been noted and from pastures where methoprene had never been used. Susceptibility tests were also conducted with synergists to ascertain whether the methoprene resistance was mediated by carboxylesterase and oxidase metabolic detoxification. In response to the methoprene failures, the use of *Bacillus thuringiensis israelensis* Goldberg & Marga (*Bti*) was evaluated as an alternative control agent in a pasture populated with resistant *Oc nigromaculis*. At the same time, metho-

prene susceptibility assays were performed on larvae of *Oc nigromaculis* collected from this pasture after several *Bti* treatments to determine whether there was notable reversion to methoprene susceptibility.

2 EXPERIMENTAL METHODS

2.1 Field collection and rearing

Resting *Oc nigromaculis* were collected with a sweep net and blood-seeking females attracted to us were collected during the day in pastures. The mosquitoes were kept in 30.5-cm collapsible cages (BioQuip® Products, Gardena, CA, 90248-0620) and maintained at a constant 26 °C, 85% RH, and cycles of 12:10 h light:dark separated by 1-h periods of red light simulating dusk and dawn. The mosquitoes were blood-fed on mice and thereafter supplied with a 100 g liter^{-1} sucrose solution. After trying numerous substrates, moistened dark purple and green O-Cel-O™ sponges (3M, St. Paul, MN 55133) appeared to be the most efficient artificial oviposition substrate. After sufficient eggs had been oviposited, the sponges were held in humid chambers at 23 °C until flooded. Eggs remained viable for at least 8 months in this manner. Portions of sponges, according to the numbers of larvae needed for bio-assay testing, were cut, soaked in tap water, deoxygenated under a vacuum and held for 2–3 h at room temperature (21 °C). All larvae that had hatched within that time period were transferred to rearing pans containing tap water (2 liter), liver concentrate diluted in water (100 g liter^{-1}; 5 ml) and a handful of dried grass. Larvae were provided with constant aeration and incubated at 28 °C (14:10 h light:dark) and given daily (from day 2 onwards) 500 mg of crushed rodent LabDiet® (PMI® Nutrition International Inc, Brentwood, Missouri) pellets. In these conditions, with no more than 150 larvae per pan, it typically took five days for *Oc nigromaculis* to synchronously reach early fourth stage. In instances where *Oc nigromaculis* larvae were collected from the field for methoprene tolerance testing, they were collected before *Bti* applications as first- and second-stage larvae. These larvae were reared following the same procedures as described above until they had reached early fourth stage.

2.2 Methoprene susceptibility assay

Details for the methodologies of the assays are provided as this species of mosquito is particularly difficult to rear. Due to the possibility of methoprene adhering to surfaces, all glass bottles and glass microsyringes used to prepare and hold methoprene stock solutions were presoaked for at least 24 h in the concentration of methoprene for which they were destined. Bio-assay glass jars (50 ml), were silanized with SigmaCote® (Sigma Chemical Co, St Louis, MO 63178) and between bio-assays all the glass jars were washed with detergent (Liqui-Nox, Alconox Inc, New York, NY 10003), rinsed five times with tap water and distilled water each and twice with acetone and then

baked for at least 5 h at 80 °C. When obvious beading of water was no longer evident on the bio-assay jars, they were re-silanized and thoroughly cleaned to remove silane residue. All methoprene bio-assays were performed in tap water, as higher levels of natural mortality consistently occurred when distilled water was used. The tap water originated from an underground well at the laboratory premises. A report on metals, cations, anions, heavy metals and organic chemicals in the tap water is available upon request from the first author.

Early fourth-stage larvae were transferred to preconditioning pans containing equal quantities of rearing water and tap water and kept for 30 min at room temperature. While the larvae were preconditioning, silanized bio-assay bottle jars were filled with tap water (47 ml) and rearing water (2.5 ml) to which a solution (0.5 ml) of the appropriate concentration of methoprene (technical *s*-methoprene; Wellmark International, Schaumberg, IL) in acetone + water was subsequently added (see below). In preparing methoprene dilutions the technical s-methoprene was assumed to have a purity of 97% and a density of 1. Whenever numbers of mosquitoes allowed, each assay test series consisted of seven replicates of 15 larvae at each methoprene concentration. Controls consisted of seven replicates each of water only, and acetone in water. Methoprene stock solutions were prepared in HPLC grade acetone and kept refrigerated. Test solutions diluted in water of appropriate concentration of methoprene (following procedures recommended by Dame D, 1999, pers comm.) were only prepared on the day of the test. After the larvae had been transferred with a wire mesh to each bio-assay jar, methoprene test solution (0.5 ml) and finely crushed rodent LabDiet (4 mg) was added and stirred with the glass pipette used to add the methoprene. Each test jar was covered with a waxed cup with the bottom cut out to fit snuggly over the test jar. Each waxed cup was capped with a plastic lid to prevent evaporation. Bio-assay test jars were held at constant 28 °C and 14:10 h light:dark). Every 12 h dead larvae were removed, counted and discarded, and the pupae were transferred to 50 ml of tap water in waxed holding cups covered with netting from which a pad of cotton-wool soaked in 100 g liter^{-1} sugar water was suspended. Pupal holding cups were also kept at 28 °C and every 12 h counts of dead pupae, partially emerged adults and live adults were made. Percentage mortality was calculated on the basis of the combined total numbers of dead pupae, dead larvae, partially emerged adults and adults unable to fly from the surface of the water. LC$_{50}$ and LC$_{90}$ levels were calculated by probit analysis (PriProbit, PriProbitNM (C) 1998–2000 Masayuki Sakuma, Kyoto University, Kyoto, Japan) after Abbott's correction for natural mortality.[4]

2.3 Methoprene/synergist susceptibility assays

The effects of three synergists, piperonyl butoxide (PBO; Chem Service Inc, West Chester, PA), an

inhibitor of oxidases, *S,S,S*-tributyl phosphorotrithio-ate (DEF; Chem Service Inc, West Chester, PA) and 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) inhibitors of carboxylesterases, were tested on methoprene-resistant *Oc nigromaculis*. The synergist OTFP (provided by Bruce Hammock, UC Davis) was included as it is a known inhibitor of juvenile hormone esterase (JHE) which hydrolyzes juvenile hormone for normal insect development.[5] JHE is an atypical carboxylesterase that may not be inhibited by DEF, and we wished to examine if JHE could be responsible for hydrolytic degradation of the juvenoid methoprene before it reaches its target site. Concentrations of synergists (diluted in acetone + water) were predetermined on the basis of the highest concentration that did not result in larval or pupal mortality, 1 mg liter^{-1} (ppm) for OTFP and PBO and 0.05 mg liter^{-1} for DEF. Larvae were exposed to these concentrations of synergist for 6 h prior to addition of methoprene.

2.4 *Bacillus thuringiensis israelensis* applications
In June 2000 pasture 11, populated with methoprene-resistant *Oc nigromaculis*, was selected for conducting *Bti* efficacy trials. The pasture, covering 8 ha, was divided into four sections, NE, NW, SE and SW. Location and extent of irrigation flooding was done at the owner's discretion, resulting in some parts of the pasture receiving more water than others. In a typical situation, each section was flooded and dried up within a day, except in areas of slight depression where larvae were drawn into pools of water that remained long enough for *Oc nigromaculis* to complete their immature cycle. In only three out of the six occasions when the SW section was flooded were first and second stage *Oc nigromaculis* larvae found. In one of three occasions the water had dried up by the following day, hence preventing post-*Bti* sampling. In the other two occasions *Bti* was applied in the SW section. In most instances sampling and evaluation of *Bti* control was accomplished in the other three sections (see Fig 2). No data were available after certain irrigation cycles because of insufficient flooding and rapid evaporation.

The study lasted for 108 days, within which the owner flooded the entire pasture six times, and portions of it on an additional two occasions. This provided an opportunity to apply and evaluate the efficacy of *Bti* (VectoBac® TP on 16-mesh sand) across several consecutive treatments. All VectoBac TP treatments were applied with a Herd® seeder mounted on an all-terrain vehicle at a rate of *c* 11.18 kg ha^{-1}. *Bti* was applied when *Oc nigromaculis* larvae were in their first through third stages. Numbers of larvae per dip based on 40 dips were taken just before and 48 h after *Bti* application. Due to the sensitivity of spread of methoprene-resistant *Oc nigromaculis* and complaints by the public of biting adults, a control site with no application of *Bti* could not be justified. Based on results from a previous trial conducted in the same pasture a year before, in areas that received no larviciding, adults emerged from 89–94% of the pupae

that were collected.[1] For the purpose of this trial we assumed that untreated areas would have produced 89–100% adult emergence. In addition, to circumvent possible spread of methoprene-resistant individuals on occasions when low numbers of fourth-stage larvae, pupae and adults were still observed after *Bti* treatments, the pasture was treated with larvicide oil (GB-1111®, Golden Bear Oil Specialties Inc, Oildale, California) or pyrethrins (Pyrocide®, McGlaughlan Gormley King Co, Minneapolis, MN or Pyrenone® 25-5, AgrEvo Environmental Health, Montvale, NJ) on days shown in Fig 2. GB-1111 was applied with a hand-held pump-pressurized sprayer at a rate of 28 liters ha^{-1}. Pyrocide (5% pyrethrins + 25% PBO) was applied at 0.0022 kg ha^{-1} with a Leco® ULV fog generator (Model HD-D) mounted on a truck. Larval counts per 40 dips were analyzed by one-way analyses of variance followed by *post hoc* Scheffé *F* test to determine significance of differences on larval counts before and after *Bti* application and also to compare the pre-*Bti* larval levels between days 1, 15, 31, 61, 77 and 107. (StatView®, SAS institute Inc, Cary, NC).

Just before the 1st, 3rd, 5th and 6th applications of *Bti*, samples of larvae were taken from pools from all sections of the pasture for bioassay evaluation of methoprene insecticidal activity. Larvae were brought back to the laboratory and reared in the same manner as those originating from eggs as described above. Methoprene susceptibility assays were performed as described in Section 2.2.

3 RESULTS
3.1 Methoprene susceptibility profiles
A summary of probit analyses of methoprene susceptibility profiles of *Oc nigromaculis* populations from Merced (susceptible), pasture 11 (resistant) and Fresno[6] is provided in Table 1. Probit calculations of methoprene insecticidal data on populations from 1972, more than 25 years ago with a methoprene numbered compound known as ZR515, are included to compare the sensitivities of the assays and to confirm that the Merced population were indeed sensitive to methoprene. Methoprene was first registered for use in the mid-1970s, thus the 1972 Fresno *Oc nigromaculis* population was guaranteed to be methoprene naïve. Merced and Fresno susceptible *Oc nigromaculis* produced similar LC$_{50}$ levels, but the LC$_{90}$ levels of the Merced mosquitoes were slightly higher than the 1972 Fresno population, indicating more heterogeneity in methoprene response in the Merced population (more gradual slope of regression line). In contrast, *Oc nigromaculis* from pasture 11, where failed field methoprene applications were first observed in 1999,[1] had 3100-fold higher LC$_{50}$ and 1533-fold higher LC$_{90}$ levels than the susceptible Merced population. *Oc nigromaculis* originating from pastures of recorded methoprene application failures in 1999 (all within 15 miles of pasture 11) produced similar levels of methoprene resistance (data not

Table 1. Summary of probit analysis of the effect of methoprene on mortality of Californian populations of *Ochlerotatus nigromaculis* exposed as early fourth-stage larvae. (95% confidence limits and standard error in parentheses)

	Sample size					LC_{50}		LC_{90}
	Test	Controls	Slope	Index[a]	LC_{50} (μg $liter^{-1}$)	ratio	LC_{90} (μg $liter^{-1}$)	ratio
Fresno[b]	2050	Unknown	0.989 (0.054)	0.117	0.01 (0.0–0.01)		0.13 (0.07–0.37)	
Merced (susceptible.)	1170	165	0.667 (0.038)	0.037	0.01 (0.01–0.03)		1.2 (0.48–4.5)	
Pasture 11 (resistant 2000)	1350	165	0.72 (0.037)	0.218	31[c] (8–167)	3100	1840 (280–167 000)	1533
Pasture 11 (June 2001)	735	105	0.476 (0.035)	0.123	1.65 (0.04–5.5)	165	818 (140–22 000)	681

[a] Index of significance for potency estimation.
[b] Reference 6.
[c] Rejection of linearity of log dose-probit mortality response ($P < 0.05$).

shown). The shape of the susceptibility profiles between the methoprene-susceptible and -resistant populations differed markedly. The susceptible mosquitoes followed closely a linear response to increasing concentration, whereas the resistant population followed more of a sigmoidal response with an extensive plateau falling below the 20% mortality level and extending from 0.00001 to 0.01 mg liter^{-1} methoprene. Pre-exposure of methoprene-resistant *Oc nigromaculis* (pasture 11) to PBO, DEF and OTFP showed no significant synergistic effect towards methoprene (based on RR$_{50}$ log probit regression line at $P < 0.05$), suggesting very minor to no role for carboxylesterase- or oxidase-mediated metabolism in causing resistance to methoprene (Fig 1).

Synchronous development and rapidity of mosquito life cycle (5–7 days) proved critical factors for producing valid and conclusive methoprene bio-assays on *Oc Nigromaculis*, with mortality less than 15% in controls. Overcrowding (>150 larvae per 2 liter) and rearing at temperatures less than 27 °C lengthened the life cycle and caused asynchronous development which became exacerbated when the fourth-stage larvae were transferred to the confines of methoprene and control test jars. Larvae often lingered on in the fourth stage for 4–5 days and most eventually died before pupating, and those that did pupate also died. Ideal methoprene bio-assay tests were achieved when most larvae that were transferred to test jars pupated within 48 h.

3.2 *Bacillus thuringiensis israelensis* applications and reversal of methoprene resistance

In the three sections where *Bti* control could be evaluated (Fig 2), *Bti* resulted in significant ($P < 0.05$) decline in *Oc nigromaculis* larvae 48 h after *Bti* application on all occasions except for once in the northeast section (day 30). *Bti* applications were augmented with application of oil (larvicide and pupicide) three times where low numbers of larvae and pupae were found in isolated pools, and once with pyrethrins to kill biting adults. The owner partially flooded the pasture on day 48 in the northeast section, which resulted in a few isolated pools with larvae in them. These pools were treated with methoprene as an Altosand$^®$ (methoprene liquid larvicide formulation

on 16-mesh sand; RMC Lonestar$^®$, Pleasanton, CA) and a drying agent (HiSil 233 Harwick$^®$, Pico Rivera, CA) at an application rate of 11.21 kg AI ha^{-1}. High survival rates of pupae (89%) confirmed the expected failure of Altosand, based on the high levels of methoprene resistance observed in the methoprene bio-assay conducted on larvae collected approximately four weeks earlier (Post *Bti* collection 1, Fig 3). Pupal mortality of 11% was expected due to natural mortality caused by handling of the mosquitoes similar to that observed in earlier field studies.[1] After pupae were collected for laboratory evaluation, the remaining fourth-stage larvae and pupae were treated with larvicide oil to ensure that no methoprene-tolerant mosquitoes were left to re-infest the pasture.

Although the density of larvae was not consistent across the pasture, larvae collected in each section before the first two *Bti* treatments were generally significantly higher than numbers prior to the four later *Bti* treatments. Hence, *Bti* and applications of other chemicals most likely caused significant reduction in adult emergence and gravid females that would normally have contributed to subsequent generations. Most floodings, however, still produced some larvae and these likely originated from eggs deposited by the few surviving females after *Bti* treatment, females migrating into the pasture from surrounding areas and from eggs that were dormant from previous generations.

Methoprene susceptibility assays were conducted on larvae collected from pools dispersed throughout the pasture on four occasions to monitor for possible progressive decline in tolerance to methoprene due to insecticides other than methoprene. These collections were denoted as post-*Bti* collection 1, 2, 3 and 4, and represent collections before the 2nd, 5th and 6th *Bti* applications and after the first flooding the following year in June 2001 respectively (Fig 3). Insufficient larvae were collected for testing at all methoprene concentrations for collections 1 to 3, which precluded probit analysis calculations. However, by visual inspection of Fig 3, appreciable decline in tolerance to methoprene was observed after the 5th *Bti* application and other chemical treatments. A large enough sample size was collected in the fourth collection (June 2001) to allow for probit analysis (summarized in

Table 1), and this showed a marked reduction (18-fold) in the LD_{50} level relative to the original pre-*Bti*-treatment population. Avoidance of methoprene applications in pasture 11 also resulted in a more linear bioassay response, with only a slight inflection at 60% mortality that extended across 0.05–0.1 mg liter^{-1} of methoprene.
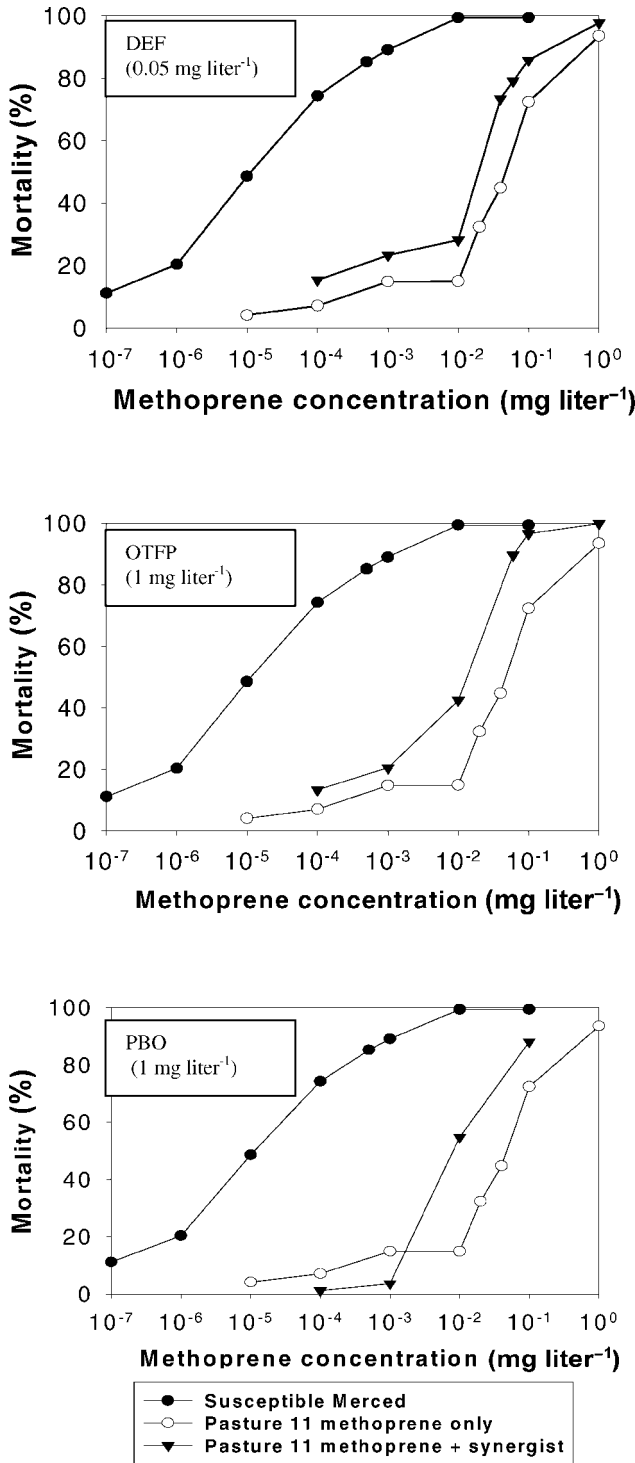

Figure 1. Effects of synergists on methoprene resistant *Ochleatus nigromaculis*. Based on seven replicates of 15 larvae at each methoprene concentration.
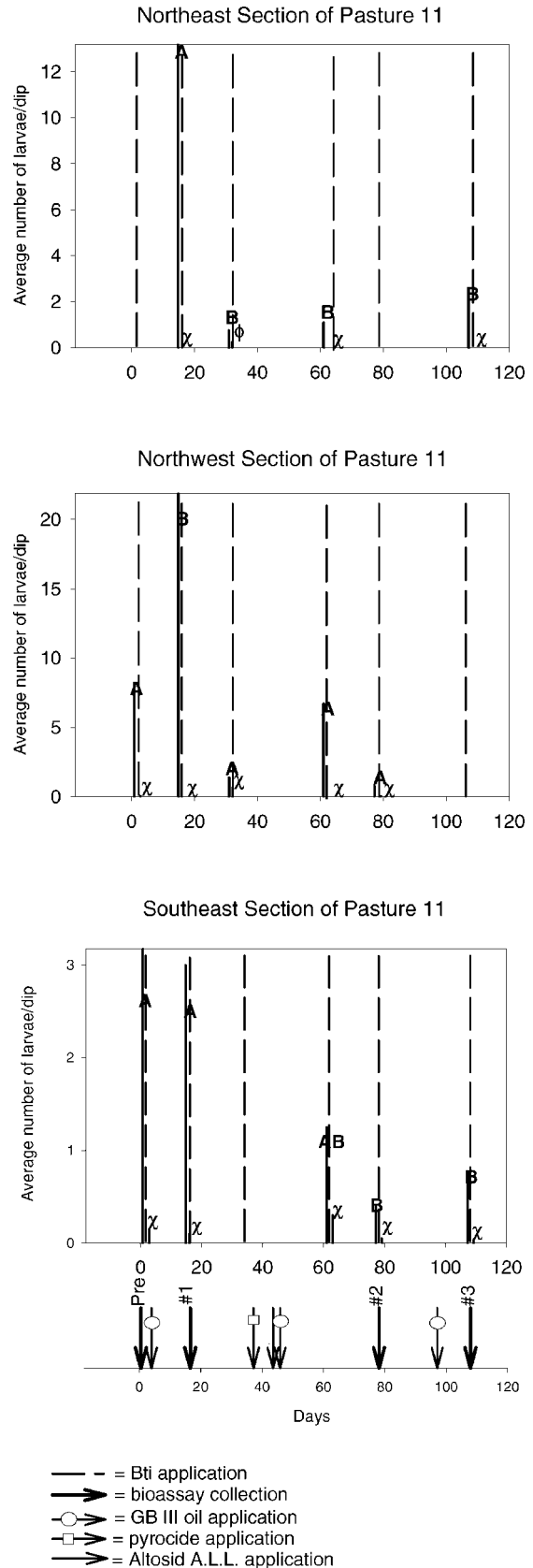

Figure 2. Schematic presentation of days pasture 11 was treated with *Bacillus thuringiensis israelensis (Bti)*, pyrethrins and oils, and control of *Ochlerotatus nigromaculis* after *Bti* application. Different letters indicate significant differences in numbers of larvae collected per dip (based on an average of 40 dips) per *Bti* application by Scheffe test *F* test at 0.05 significance level. χ = significant difference ($P < 0.05$) between pre- and 48-h-post-*Bti* applications. ϕ = no significant difference ($P < 0.05$) between pre- and 48-h-post-*Bti* applications.
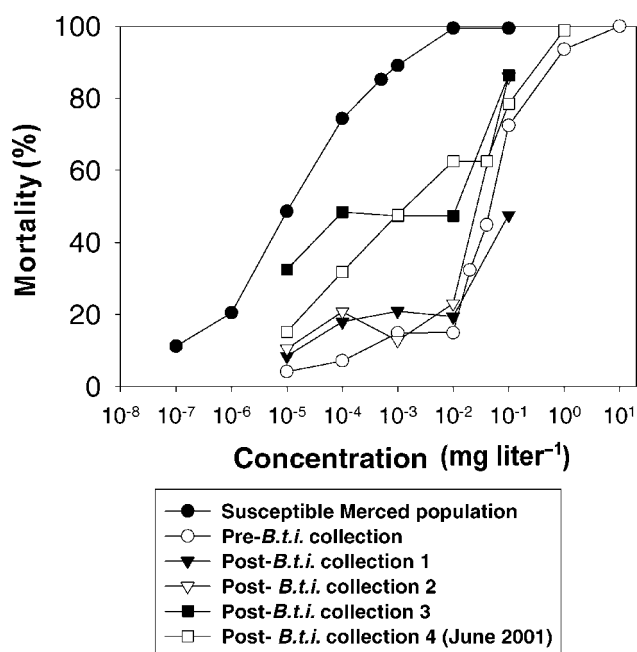
Figure 3. Series of methoprene bio-assays conducted on methoprene-resistant *Ochlerotatus nigromaculis* larvae collected after consecutive *Bacillus thuringiensis israelensis* applications.

4 DISCUSSION AND CONCLUSIONS

The methoprene-naïve population of *Oc nigromaculis*, originating from eastern Merced County along the Sierra Nevada foothills, displayed methoprene susceptibility profiles similar to the Fresno mosquitoes of 1972. This indicates that the susceptibility assay protocol that we designed produced similar degrees of sensitivity to earlier tests on the same mosquito species,[6] and that the Merced *Oc nigromaculis* populations were highly susceptible to methoprene.

Mosquito abatement districts world-wide have enjoyed the luxury of methoprene usage for over 20 years to control immature mosquitoes, as there have been no reports of tolerance to methoprene except on two occasions which have both appeared only in the past 5 years. This is despite the fact that significant tolerance to methoprene has been shown to be possible in less than twenty generations by laboratory selection of *Culex* mosquitoes[7–10] and in other insects such as house flies[11] and flour beetles.[12]

The mosquito *Ochlerotatus taeniorhynchus* (Weid) (formerly *Aedes taeniorhynchus*) was the first mosquito to have spontaneously developed resistance to methoprene in the field in isolated barrier islands off the coast of Florida. Methoprene assays demonstrated that the island populations had developed a 14.9-fold higher LC_{50} than susceptible mainland populations with which there should have been very little gene flow.[13] The selection for much higher levels of methoprene tolerance proven by susceptibility assays in this study to be present in large non-isolated populations of *Oc nigromaculis* provides an interesting, contrasting situation. An ideal situation for selection of methoprene-resistant genotypes was obviously created in Fresno as

methoprene had been applied for 20 years with 10 applications or more each summer, depending on the numbers of times the pastures were flood-irrigated. A single plateau below the 20% mortality level in the methoprene-resistant *Oc nigromaculis* suggests that over 80% of the mosquitoes possessed and expressed a methoprene-resistant factor by the time methoprene resistance was suspected in the field. Hence, this factor either was very rapidly selected for in the field within 2 years from 1998 to 2000 in certain pastures, or methoprene tolerance and the methoprene resistance factor had been present in the Fresno area a long time before resistance to methoprene was suspected. Mark-release-recapture studies have shown that *Oc nigromaculis* are capable of dispersing at least up to 11 km from their breeding source.[14] However, amongst mosquito-abatement personnel there is debate concerning the extent of, and the conditions under which, dispersal occurs (personal communication). In general, most believe that the incentive to disperse remains low when domestic animals, moisture and breeding sites are all available within a pasture. This would create an almost island-like situation of rapid intense selection for methoprene resistance genotypes in isolated pastures, and hence explain the patchy distribution of methoprene tolerance observed in Fresno.[1] If dispersal were more frequent than believed, the existence of un-detected methoprene resistance genotypes occurring long before 1998 would seem a more likely scenario.

Induction of methoprene-resistant *Culex pipiens* L was associated with reduction in reproductive success for 20 generations of selection.[10] Continued selection resulted in sudden further increase of methoprene resistance and return of normal reproductive success. Before the 20th generation, when methoprene toler-ance was moderate, reversion to methoprene sus-ceptibility was rapidly attained in the absence of methoprene exposure. However, after the 20th gen-eration and high levels of methoprene resistance, there was no reversion to susceptibility in the absence of methoprene. A similar phenomenon may have oc-curred in field populations of *Oc nigromaculis* where moderate levels of resistance to methoprene existed for several years without its presence being noticed because of compromised reproductive success and hence few nuisance adults. Continued use of metho-prene may then have resulted in sudden selection of high tolerance to methoprene and return of normal reproductive success, leading to noticeable adult accu-mulations and complaints.

Few studies have been done to elucidate the mechanisms of methoprene resistance in insects induced for resistance by selection. By *in vitro* and *in vivo* metabolic assays increased oxidative degradation of methoprene and, to lesser extent, increased ability to excrete methoprene and lower penetration of methoprene, accounted for the higher tolerance to methoprene in one strain of resistant housefly.[11] Based on the results from that housefly study, and the prediction of an increase in juvenoid degradation due

to oxidative metabolism in mosquitoes,[7–9] we had anticipated that the oxidase inhibitory action of PBO would have shown synergistic effect in methoprene resistant *Oc nigromaculis*. This was not the case. In fact a general esterase inhibitor such as DEF or a known juvenile hormone esterase inhibitor, OTFP, also had no significant synergistic effect. This does not, however, rule out the possibility that there may still be degradation of methoprene in *Oc nigromaculis* by enzymes not inhibited by these synergists, or that perhaps our concentrations of the synergists were not high enough to cause synergism. Other resistance mechanisms in *Oc nigromaculis* may also be likely, such as reduced penetration of methoprene, increased excretion or an insensitive target site. The identification of the target site of methoprene and the proteins that bind to methoprene still eludes insect physiologists. However, evidence has been found of a sex-linked gene, known as the *Met*[+] gene that codes for a receptor or a protein that is stoichometrically involved in JH or methoprene reception in *Drosophila melanogaster* Meig.[15] A methoprene-resistant strain of *D melanogaster* was induced by mutagenesis (known as the *Met* strain) and this putative receptor protein in the *Met* strain possessed a low affinity for binding to JH III and possibly to methoprene.[16] In more recent work, by transposon tagging and sequencing, the gene coding for the putative JH receptor showed homology to the bHLH-PAS family of transcriptional regulators that are involved in JH regulation of gene expression[17] and other genes which respond to external cell signals.[15] No difference in numbers of males and females surviving methoprene exposure was observed in our assays, but comparisons of the homolog *Met* gene between methoprene-susceptible and -resistant populations of *Oc nigromaculis* could still prove useful.

Changes in accumulation and distribution of ^{14}C-methoprene between susceptible and methoprene resistant *Cx pipiens* larvae have been found.[18] Resistant individuals had three-quarters of the radioactivity and eliminated the radioactivity at faster rates once placed in clean water.[18] Methoprene-resistant *Cx pipiens* had similar radioactive levels in the alimentary canals but much less in the rest of the body. A behavioral response of reduced feeding and hence less oral uptake of ^{14}C-methoprene was discounted and that lower cuticular penetration was found to be the major cause of less accumulation of methoprene in the body. We observed no difference in rates of pupation between the susceptible methoprene Merced populations and methoprene-resistant *Oc nigromaculis* from both the control (no methoprene) and treated jars. This suggests that both the methoprene-susceptible and -resistant larvae fed at similar rates whether they were exposed to methoprene or not. In addition, the *Oc nogromaculis* larvae were continuously exposed to methoprene until they had pupated and hence they could not avoided being exposed to the methoprene. Behaviorial avoidance is an unlikely mechanism of methoprene resistance in *Oc nigromaculis*. Reduced cuticular penetration of methoprene and increased elimination of methoprene, however, cannot be discounted as a possible mechanism of resistance in *Oc nigromaculis*.

Unfortunately studies to determine the mechanisms of methoprene resistance in *Oc nigromaculis* will prove challenging, as attempts to colonize methoprene-resistant and -susceptible colonies using induced mating techniques have so far failed.[19] Swarms of mating *Oc nigromaculis* above prominent objects such as bushes and fence poles have been observed at sunset in pastures (by AJC), and this eurygamous mating behavior precludes our abilities to establish spontaneously mating colonies.

These findings of considerable resistance to methoprene indicate the need for strategies to mitigate methoprene resistance that should apply to *Oc nigromaculis* and other mosquito species. Since this study has shown the effectiveness of *Bti* as a larvicide and as an agent that does not cross-resist with methoprene, strategies could include mixtures of methoprene with other larvicides such as *Bti* (commonly known as Duplex) or rotations. Some mosquito-abatement personnel have suggested using temephos. However, we caution this approach, as extensive OP resistance has been reported in Californian *Oc nigromaculis* in the past,[20] and the Fresno county *Oc nigromaculis* were found still to possess high levels of non-specific esterase activity when run in 6% polyacrylamide gel electrophoresis stained with equal quantities of α- and β-naphthyl acetate (data not shown).

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