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METHOPRENE TOLERANCE IN *AEDES NIGROMACULIS* IN FRESNO COUNTY, CALIFORNIA

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ABSTRACT. Methoprene, a juvenile hormone analogue, has been used for at least 20 years as the primary insecticide to control the pasture mosquito *Aedes nigromaculis* in Fresno County, California. First reports of apparent methoprene control failures were noted in a pasture west of Fresno in September 1998. Insufficient control was noted in 12 different pastures the following season from April to September 1999. In September of 1999, field trials were conducted to better ascertain the level of control. Results based on pupal counts from different methoprene formulations and rates of application indicated that in some pastures low levels of control were achieved with Altosid® (Liquid Larvicide) and Altosid XR-G. Control with Altosid Pellets was reported at 52–99%.

KEY WORDS *Aedes nigromaculis,* methoprene tolerance, California, insecticide resistance, mosquito control

INTRODUCTION

Aedes nigromaculis (Ludlow) is a floodwater, multivoltine mosquito species with a range from southern Canada, through central and western United States, to Mexico (Carpenter and LaCasse 1955). The 1st collections of *Ae. nigromaculis* were made by Aitken in 1937 (Bohart and Washino 1978). Since then, collection records of this mosquito have demonstrated its association with irrigated pastures, alfalfa fields, almond orchards, and wetlands. In Fresno County, *Ae. nigromaculis* numbers peak in summer from July to September (average maximum temperatures of 32–40°C and minimum of 20°C), and by mid-October, they produce overwintering eggs. During this period of peak activity, completion of the aquatic cycle takes between 5 and 7 days, but reports of 4 days have been noted in hot conditions (Bohart and Washino 1978).

Aedes nigromaculis, an opportunistic bloodfeeder, will bite humans readily. If not controlled, their numbers can rapidly reach nuisance levels in areas neighboring breeding sites. In Fresno and surrounding counties, pastures are generally flooded in 10–14-day cycles, resulting in new hatches every flooding cycle. Because of its nuisance status, *Ae. nigromaculis* control constitutes an important and costly component of several mosquito control agencies in California. Additionally, laboratory studies have demonstrated that *Ae. nigromaculis* is capable of transmitting western equine encephalomyelitis and St. Louis encephalitis viruses, although no isolations of either have been made from collections in California (Hardy and Reeves 1990).

Aedes nigromaculis has a notorious reputation for rapidly developing resistance to insecticides. Resistance was detected only 3 years after the introduction of DDT (Bohart and Murray 1950). By 1952, some populations had developed resistance to other chlorinated hydrocarbons such as lindane, aldrin, and toxaphene (Schaefer and Wilder 1970). In the 1950s, organophosphates became the main pesticides used, and the 1st reports of resistance to parathion were recorded in *Ae. nigromaculis* in 1958 in Kings and Tulare counties (Lewallen and Nicholson 1959). Because of cross reactivity with other organophosphates and carbamates by the 1970s, there were few chemicals available to control *Ae. nigromaculis,* and some attempts to control it in the Central Valley had stopped. Methoprene proved to be a highly effective control agent of *Ae. nigromaculis* (Schaefer and Dupras 1973, Schaefer et al. 1975) and is still in extensive use today.

Three formulations containing methoprene are used to control *Ae. nigromaculis* in California. They are Altosid® Pellets (Pellets), Altosid XR-G (XR-G), and Altosid liquid formulation (ALL). The formulation used is essentially driven by the type of breeding site and the cost of application. After initial field trial evaluations of methoprene showed 100% control in Central Valley pastures (Schaefer and Wilder 1973), records show that ALL has been used effectively in pastures since 1974 in parts of Fresno County (particularly west of Fresno).

In the last few floodings of a west-Fresno pasture in September of 1998, the 1st signs of ALL control failures appeared. High numbers of flying and host-seeking adult *Ae. nigromaculis* remained after the field was sprayed. The following year, more careful observation and attention were given to applications of ALL in these and other pastures during the *Ae. nigromaculis* season. High numbers of adults remained after treatment, and control failures were noticed in 12 additional pastures. This provided opportunities to examine other factors that could be

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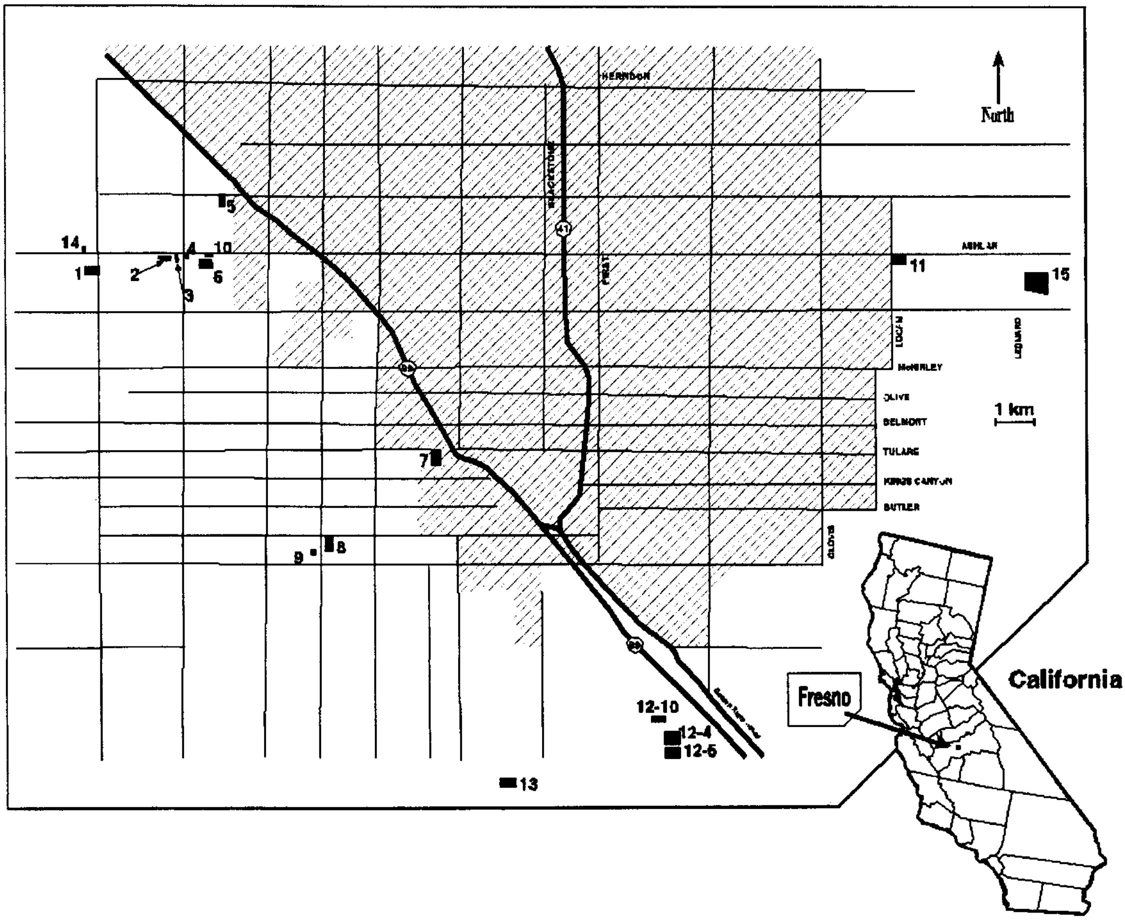

Fig. 1. Location of field site. Numbers refer to pasture or site number. Hatched areas represent urban and residential areas of Fresno.

responsible for control failures such as incorrect pesticide application, flooding regime anomalies, and methoprene formulation problems. All of these other factors failed to explain the unusually high numbers of adults remaining after treatment, and the possibility of resistance to methoprene was considered for the 1st time.

To avoid reliance on anecdotal information and to ascertain more specifically the percent emergence that was occurring in these and other pastures, a field evaluation was done in the final month of the 1999 *Ae. nigromaculis* season in Fresno and Kings counties.

MATERIALS AND METHODS

Study site and methoprene application: Small pasture sizes, lack of control of flooding and drying regimes, time constraints, sensitivity associated with methoprene failures, and spread of methoprene resistant adults limited the design of the field study. The study site (7,702.4 ha) is presented in Fig. 1; it shows all but 1 pasture. Each pasture was designated with a site number. The site not included in the figure was 38.4 km southwest of downtown Fresno in Kings County and is referred to as Kings 4004. Only 1 pasture where previous methoprene failures were noted covered a large enough area in a single flooding for simultaneous application and evaluation of all 3 methoprene formulations (site 11). This site was divided into 4 areas. To one, ALL (Zoecon division of Wellmark International, Dallas, TX; 5% active ingredient (AI), *S*-methoprene) was applied, XR-G (Zoecon; 1.5% AI, *S*-methoprene) in a 2nd, Pellets (Zoecon; 4.25% AI, *S*-methoprene) in a 3rd, and a 4th area was left untreated as a control. For comparative purposes, ALL was applied in 6 other pastures, 3 of which were pastures of previous methoprene failures (sites 2, 10, and 14). The 3 other pastures had shown no evidence of failure (sites 12, 13 and 15). In addition to pasture 11, XR-G was applied in 3 pastures (sites 1, 6, and Kings 4004), and Pellets were applied in 3 pastures (sites 2, 3, and 4). Simultaneous application of ALL and Pellets was accomplished at site 2. In addition to a portion of site 11, site 9 was used as a control to determine natural mortality. All methoprene formulations were applied either by hand with a granule bag or Whirly Bird® seeder or applied with a Herd® seeder mounted on an all-terrain vehicle. Altosid Liquid Larvicide sand was prepared by using a formulation of 118.3 ml of ALL adhered to 4.536 kg of 16-mesh sand (RMC Lonestar®, Pleasanton, CA) and a drying agent (Hi-Sil 233 Harwick®, Pico Rivera, CA). The larvicide was applied at a rate of 623 g of methoprene to 90 kg of sand granule mixture when larvae were in late 3rd and early 4th stages. XR-G and Pellets were applied 1 day prior to pasture flooding to maximize effectiveness. Actual methoprene application rates were determined for each pasture using the following formula:

$$\frac{W_1 - W_2}{A} = \text{Application Rate}$$

The weight of material before application is W_1, W_2 is the weight after application, and A is the area of application. Actual application rates are provided in Tables 1 and 2.

Methoprene failures had been observed in 3 additional sites (5, 7, and 8), but these were not included because they were not flooded at the time of this study.

Sampling: Aedes nigromaculis immatures develop synchronously, allowing for simultaneous collections of pupae. Pupae were only collected from treated and control pastures, ensuring that the larvae received sufficient exposure to methoprene. Whenever possible, 2 or more pupal collections, 24 h apart, were made from each pasture so that mortality rates could be calculated based on more than 1 day's collections. Additionally, pupal samples were taken from several remaining pools of water from each pasture to avoid bias caused by uneven methoprene application across pastures. Samples were transported in 5-liter buckets to the laboratory where the pupae and the water were transferred to BioQuip® mosquito breeders or Pyrex® storage dishes. A maximum of 100 pupae was placed in each breeding container and maintained at room temperature (~21°C). A pad of cotton wool soaked in 10% sucrose solution was placed on the top of each adult container. Pupae were allowed to complete development (24 h after field collection), and counts of dead pupae, partially emerged adults, dead adults, and pupal cases were made. Percent mortality was then calculated based on the following formula: percentage mortality = (dead pupae + dead adults ÷ total mosquitoes collected) × 100. Corrected percentage of mortality rates were calculated using Abbott's (1925) formula.

In all pastures that were used in this study, remaining pupae were treated with larvicidal oil. Adults were killed with adulticide a day after emergence to prevent the spread of resistant mosquitoes and reduce nuisance problems.

Climatic conditions were recorded at a weather station located in Fresno.

RESULTS

Results based on field-collected pupal death and adult emergence after applications of 3 Altosid methoprene formulations revealed unexpectedly high emergence rates from various dispersed pastures and expected control in others.

Sites 2 and 11: During the study period (1 September–2 October 1999), daily maximum and minimum temperatures ranged from 27.79 to 35.57°C (mean, 32.2°C) and 11.11 to 19.45°C (mean, 14.5°C), respectively. Precipitation (0.254 mm) occurred on 1 day.

Percent control obtained for each of the methoprene formulations at sites 2 and 11 is presented in Table 1. Percent mortality was not corrected for natural death, as more pupae died in the control plots than in the ALL and XR-G treatment plots at site 11, and the water dried up before pupae could develop in site 2. Essentially, no control of *Ae. nigromaculis* was achieved with either ALL or XR-G at sites 2 and 11. Some control, albeit at unacceptably low levels, was obtained with Pellets at site 11 (53% and 41%) and 2 (85.12%).

Interestingly, higher mortalities were consistently observed in early collections of pupae from site 11. This could be due to a bias in the early collections—the sick and lethargic pupae were more easily collected on the 1st day, whereas the 2nd and 3rd day favored the collection of healthy and more resistant pupae. Another explanation could be that not all of the eggs hatched at once, and those that hatched 1st, developing into the early pupae, were exposed to slightly higher concentrations of methoprene than those hatching and developing later. In mosquitoes, males often develop more quickly and pupate earlier than females, and a sex difference in susceptibility to methoprene may be possible. Methoprene has been noted to slow down larval and pupal development, and thus most pupae were present a day later in the plots treated with Pellets. Pellets are designed to provide 30 days of control, and a 2nd collection of pupae was obtained from a subsequent flooding (17 days later) in the portion of site 11 that had been originally treated with Pellets. An 11.9% drop in mortality was recorded in *Ae. nigromaculis* from the 2nd flooding when compared with mortality from the 1st flooding.

Other pastures: Pupal mortality rates obtained with methoprene treatments in all sites are summarized in Table 2. Site 9 was used as a control, recording 98.25% emergence in the 571 pupae collected. This control was used in Abbott's (1925) formula to calculate the corrected mortalities for the other treated pastures.

Prior to the study, increased ALL failure was noticed in a pasture in Kings County, and XR-G as a more potent formulation was then applied. In the

Table 1. Pupal mortality evaluations of *Aedes nigromaculis* treated with various Altosid® methoprene formulations simultaneously in 2 pastures in Fresno County, California.

Treatment[1] (g active ingredient/ha)	Area treated (ha)	Date pupae collected	Percent mortality (*n*)	Average mortality
Control (no treatment)	na	15 Sep	10.85 (258)	
		16 Sep	11.35 (141)	
				11.03
ALL[2] (12.33)	1.54	15 Sep	10.28 (253)	
		16 Sep	3.36 (149)	
				7.71
ALL[3] (11.21)	0.26	17 Sep	2.98 (168)	
XR-G[2] (290.85)	0.89	15 Sep	23.26 (86)	
		16 Sep	2 (150)	
		17 Sep	1.69 (59)	
				8.14
Pellets[2] (395.37)	0.77	15 Sep	60 (5)	
		16 Sep	84.56 (149)	
		17 Sep	20.55 (146)	
				53
		1 Oct	41.1 (146)	
Pellets[3] (333.44)		17 Sep	85.12 (121)	

[1] ALL, Altosid liquid formulation; XR-G, Altosid XR-G; Pellets, Altosid Pellets.
[2] Site 11 data.
[3] Site 2 data.

1st day's collection of 160 pupae, 96.22% of pupae and adults either drowned or died in another way. A day later the pasture was revisited, and a sample of 25 pupae was brought back from which only 44% died. The Kings pasture was treated a few days before the trial in Fresno County began, and it was from our experience in this pasture that we attempted to continue evaluating methoprene control from more than 1 day's pupal collection whenever possible. Unfortunately, all of the ALL-treated pastures other than pasture 11 dried up too rapidly for more than 1 day of pupal collection.

As in site 11, earlier pupal collections showed higher mortality rates. For example, from Pellet treatments at site 3, the percentage of control of 1st-day pupae was 90.64% (n = 203), whereas 2nd-day control was only 68.5% (n = 254). This difference of 22.14% was double that observed at site 11. Higher mortality rates for earlier collections also occurred in XR-G–treated plots. Control at site 1 of 1st-day pupal collections was 37.17% (n = 374) and 16.76% (n = 149) for 2nd-day collections. This 20% difference is similar to that seen at the site 11 XR-G–treated area.

Table 2. Pupal mortality evaluations of *Aedes nigromaculis* treated with various Altosid® formulations in several pastures in Central California, September, 1999.

Pasture	Treatment (g active ingredient/ha)[1]	Area treated (ha)	Percent mortality (*n*)	Corrected mortality (%)
11	12.33 ALL	1.54	7.71 (402)	6.07
2	11.21 ALL	0.26	2.98 (168)	1.25
10	10.65 ALL	0.52	0 (6)	Na
14	11.21 ALL	0.4	1.81 (386)	0.06
12[2]	11.21 ALL	3.04	87.04 (54)	86.81
13[2]	11.21 ALL	0.81	97.37 (76)	97.32
15[2]	13.67 ALL	2.03	99.06 (841)	99.04
11	2.90.85 XR-G	0.89	8.14 (295)	6.5
1	312.71 XR-G	2.9	31.33 (553)	30.11
6	403.5 XR-G	1.03	8.34 (51)	6.7
Kings 4004	168.12 XR-G	3.24	90 (185)	89.82
11	396.37 Pellets	1.46	53 (300)	52.16
3	571.62 Pellets	0.7	79.57 (457)	79.21
4	314.39 Pellets	1.08	99.18 (488)	99.17
2	333.44 Pellets	0.26	85.12 (121)	84.85

[1] ALL, Altosid liquid formulation; XR-G, Altosid XR-G; Pellets, Altosid Pellets.
[2] Pastures noted to still have effective ALL control prior to study.

Generally, very low control was achieved with ALL and XR-G methoprene applications in pastures in which previous failures had occurred. An unintentional application of XR-G at a rate higher than the recommended rate (26.9 kg/ha) still did not produce acceptable control levels at site 6. Much higher control with ALL was achieved in the 3 pastures where methoprene still appeared to kill *Ae. nigromaculis* prior to the study (sites 12, 13, and 15). At site 15 (<1 mile from site 11), the same ALL sand and drying agent mixture that was used at site 11 was used 16 days after application at site 11. This fell within the recommended period of 20 days of shelf life of methoprene (ALL) sand mixture. All other ALL treatments were with material that had not necessarily come from the same mixture but that had originated from the same liquid methoprene batch.

DISCUSSION

In the early 1970s, the question of why insecticide resistance always appears to start in alkaline pastures was raised. Schaefer (1972) suggested that it had nothing to do with the affect of alkaline soil and water on the insecticides. Rather, it was because the pastures in question were on soils with poor drainage and could not be used for crops. Constant irrigation to create a sufficient grazing area created a mosquito problem. These pastures were thus targeted for mosquito control. If one assumes a very conservative estimate of flooding every 18 days, each pasture has to be treated 5 times during the peak *Ae. nigromaculis* season. The repetitive chemical treatment that has been conducted since 1974 (25 years) created a strong selection pressure on mosquito populations that favored chemical-tolerant individuals. In addition, those pastures that are closest to urban areas are treated more extensively than pastures further away because of their proximity to more people. In the sites in this study, it appears that the highest levels of methoprene failures occurred in pastures closest to residential areas (sites 1, 3, and 11). All the resistant and susceptible pastures occur in similar sandy loam soil types. Hence, different soil substrates affecting methoprene silica binding properties between pastures is unlikely.

The mechanisms of resistance and spread of tolerance to methoprene in *Ae. nigromaculis* needs additional study. Resistance to methoprene has arisen in a mosquito population in isolated barrier islands (Captiva and Lover's Key Islands) off the west coast of Florida. In Florida, the salt marsh mosquito *Aedes taeniorhynchus* (Wied.) on Captiva island and Lover's Key island was found to be 14.9- and 14.8-fold more tolerant of methoprene, respectively, than was a mainland (Flamingo strain) population (Dame et al. 1998). *Ae. taeniorhynchus* appears to have developed increased tolerance in isolated island habitats where little to no gene flow occurs between the islands and the mainland. However, in California, the pastures are not isolated habitats and form a continuum across the Central Valley. Hence, one would expect the *Ae. nigromaculis* to be a single panmictic population. In California, methoprene tolerance appears to have been selected in a large population by repetitive treatment. Dispersal studies have shown that *Ae. nigromaculis* mosquitoes are capable of flying at least 11 km (Husbands and Rosay 1952), and genetic exchange should occur from one pasture to the next. Interestingly, the present study shows that pastures with susceptible populations are dispersed among pastures with treatment-resistant populations. This may suggest that development and spread of methoprene resistance is still at its early stages. Methoprene failures were originally noticed at site 1 during the past summer. Numerous pastures southwest of this pasture, essentially downwind, developed resistance. However, that some levels of tolerance were noticed more than 30 km south in Kings 4004 may indicate that methoprene resistance is developing independently in several locations.

Selection for increased tolerance to methoprene has been achieved in the laboratory after many generations with *Culex tarsalis* Coq. (Georghiou 1974) and *Culex pipiens pipiens* L. (Brown and Brown 1974). The mechanisms of resistance in these mosquitoes were not investigated, but cytochrome P450 or carboxylesterase mediated detoxification mechanisms (Brown and Brown 1974, Feyereisen 1998) have been postulated. Additionally, methoprene resistant strains of *Drosophila melanogaster* Meigen have been selected in the laboratory by *P*-element mutagenesis (Turner and Wilson 1995), and recent molecular genetic studies on these strains have provided considerable insights into a possible insensitive target site resistance mechanism involving the *Met* gene (Ashok et al. 1998, Wilson and Ashok 1998). The *Met* gene appears to be a transcription factor gene coding for a family of bHLH-PAS proteins, and the role that this protein has in juvenile hormone and juvenile hormone mimic metabolism is presently under investigation. Comparisons of the homolog *Met* gene between methoprene susceptible and resistant populations of *Ae. nigromaculis* could also prove insightful.

The high methoprene failure rates observed in this study and the lack of evidence of alternative explanations suggest that *Ae. nigromaculis* has developed increased tolerance to methoprene. It had been hoped that an endogenous compound similar in molecular structure or physiological action would be less likely to induce resistance. To determine quantitatively what the levels of methoprene resistance or tolerance levels are, bioassays comparing methoprene mortality dose responses of susceptible, resistant, and if possible, methoprene-naïve populations are currently being conducted. Offspring from host-seeking *Ae. nigromaculis* females that were collected this past season are being used for these bioassays.

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