ISOLATION AND CHARACTERIZATION OF TWO NOVEL ORGANOPHOSPHATE RESISTANCE MECHANISMS IN *CULEX PIPIENS* FROM CYPRUS

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ABSTRACT. Two novel mechanisms of organophosphate resistance were isolated and characterized from a population of *Culex pipiens* L. from Cyprus. Two strains, one expressing the novel, highly active esterases A5 and B5 (strain A5B5-R), and one expressing insensitive acetylcholinesterase (strain Ace-R), were developed by single pair crosses and selection with temephos and propoxur, respectively. The A5B5-R strain demonstrated resistance toward organophosphate insecticides that could be suppressed by the esterase inhibitor *S,S,S*-tributyl phosphorotrithioate (DEF). No cross-resistance to carbamates occurred. The Ace-R strain demonstrated resistance to organophosphate as well as to carbamate insecticides. Propoxur and temephos resistance was not affected by the monooxygenase inhibitor piperonyl butoxide or by DEF. The Ace-R strain possessed a novel toxicologic profile as well as a unique acetylcholinesterase inhibition pattern. Inheritance of temephos or propoxur resistance was codominant in F_1 offspring. Backcrosses to a susceptible strain in both cases failed to fit a single gene model, suggesting that multiple loci may be involved. Combining the A5B5-R and the Ace-R strains resulted in high levels of temephos resistance, similar to that of the parents.

KEY WORDS   *Culex pipiens*, resistance, esterase, insensitive acetylcholinesterase

INTRODUCTION

Cyprus maintains an extensive mosquito abatement program for the control of pest mosquitoes and to prevent the reestablishment of eradicated malaria vectors. Since 1971 the program has relied primarily on organophosphate (OP) insecticides, particularly temephos, dichlorvos, and pirimiphos methyl. We recently reported the presence of resistance to OP and carbamate insecticides in collections of *Culex pipiens* L. from Cyprus (Wirth and Georghiou 1996). The mechanisms of OP resistance in these collections were 5 different highly active esterases (esterases A1, A2, A5, B2, and B5) detected by starch gel electrophoresis, and insensitive acetylcholinesterase detected by microtiter enzyme inhibition tests. Five of these mechanisms (esterases A2, A5, B2, and B5 and insensitive acetylcholinesterase) were present at varying frequencies in every site sampled, whereas highly active esterase A1 was found in only one site at extremely low frequency.

Highly active esterases and insensitive acetylcholinesterase are the 2 most common mechanisms of OP resistance in *Culex* mosquitoes (Georghiou 1994, Pasteur and Raymond 1996) and these mechanisms have been well studied in field and laboratory populations. Highly active esterases are classified as either type A or B based on their preferential hydrolysis of α- or β-naphthyl acetate, respectively, using starch gel electrophoresis. To date, 10 different highly active esterases have been identified in *Culex* (esterases A1, A2, A3, A4, A5, B1, B2, B3, B4, and B5). A and B esterases are encoded by 2 distinct, closely linked loci (Pasteur and Raymond 1996). As a result, some highly active A and B esterases such as A2 and B2 in *Culex quinquefasciatus* Say (Wirth et al. 1990), A3 and B3 in

Culex tarsalis Coquillett (Prabhaker et al. 1987), A4 and B4 in *Cx. pipiens* (Poirié et al. 1992), and A5 and B5 in *Cx. pipiens* from Cyprus (Poirié et al. 1992) are found in linkage disequilibrium.

Overproduction of B esterases results from amplification of the esterase B structural gene (Mouchès et al. 1986) producing 120- to 500-fold greater esterase activity and temephos resistance levels of 590- to 800-fold in mosquitoes with esterase B1 (Mouchès et al. 1987, Ferrari and Georghiou 1990). Overproduction of esterase B5 in mosquitoes from our Cyprus collections also results from gene amplification (Poirié et al. 1992). Recently, highly active esterase A2 was shown to result from gene amplification (Vaughn and Hemingway 1995) due to coamplification of both the A and B loci (Rooker et al. 1996) and other highly active A esterases that are linked with amplified B esterases in *Culex* are likely to be the product of amplified genes. In contrast, esterase A1 is not linked with a highly active B esterase and elevated activity of this esterase appears to result from a regulatory process (Rooker et al. 1996). Highly active A and B esterases are believed to confer OP resistance by sequestering and detoxifying insecticides (Ketterman et al. 1992, Cuany et al. 1993).

The enzyme acetylcholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter acetylcholine, terminating transmission of the impulse at the cholinergic receptor. Acetylcholinesterase is the target of both OP and carbamate insecticides. Modified AChEs that are less sensitive to insecticide inhibition have been reported in *Culex* (Raymond et al. 1986; Takahashi and Yasutomi 1987; Bourguet et al. 1996a, 1996b; Mamiya et al. 1997) and reports of *Culex* populations that contain both highly active esterases and insensitive AChE are in-

creasing, especially in populations under intensive mosquito control (Raymond et al. 1986, Bisset et al. 1990, Bonning et al. 1991, Rivet et al. 1994, Raymond and Marquine 1994, Wirth and Georghiou 1996, and others).

Among the OP resistance mechanisms identified in the Cyprus collections, highly active esterases A1 and A2 plus B2 have been characterized from mosquitoes collected in France and the United States (Raymond et al. 1986, Wirth et al. 1990). However, esterases A5 plus B5 were originally reported only in mosquitoes from our collections from Cyprus, and more recently Italy (Severini et al. 1997), and thus represent novel alleles whose spectrum of resistance has not been characterized. Further, inhibition studies on the AChE of Cyprus mosquitoes suggested that this insensitive AChE had a unique pattern of sensitivity to insecticide inhibition (Wirth and Georghiou 1996). Because of the high levels of resistance to temephos observed in our field collections we have investigated the possible contributions of these 2 novel resistance mechanisms, either singly or in combination, to the observed high resistance. Here we report on the isolation and selection of these 2 resistance mechanisms, their toxicologic profiles singly and in combination, and their pattern of resistance inheritance.

MATERIALS AND METHODS

Strains

Three different mosquito strains were used in this study. Strain S-Lab (Georghiou et al. 1965) is a susceptible laboratory reference strain of *Cx. quinquefasciatus* originating from California. Strains A5B5-R and Ace-R were derived from the 1987 Mitsero, Cyprus, collection of *Cx. pipiens* (Wirth and Georghiou 1996) by single pair crosses and selection with temephos or propoxur, respectively, to reach high resistance. The A5B5-R strain possesses only the highly active esterases A5 plus B5 and lacks insensitive AChE, whereas the Ace-R strain possesses only the insensitive AChE and lacks highly active esterases.

Isolation of resistance mechanisms

Virgin male and female mosquitoes were obtained from the Mitsero colony by isolating individual pupae in glass scintillation vials. Two hundred pairs were held in cups for 3 days to permit mating. Males were removed and frozen at $-70°C$ until they were analyzed for elevated esterase activity and insensitive AChE by microtiter plate enzyme assays. Females, identified as having mated to a male expressing only elevated esterase or only insensitive AChE, were released into separate cages and bloodfed. Bloodfed females were removed daily and isolated in oviposition cups. After oviposition, females were frozen for biochemical analysis.

Egg rafts, identified as the offspring of parents with insensitive AChE activity, were hatched separately and a sample of 10 individuals from each raft was used to confirm the presence of insensitive AChE activity and the absence of elevated esterase activity, by enzyme assay. The offspring of parents identified with elevated esterase activity were pooled to form a colony and their offspring were used for additional screening as described above, to eliminate undesired esterases and produce a strain expressing only esterases A5 plus B5. A total of 9 and 4 rafts, respectively, were used to establish the A5B5-R and Ace-R strains.

Bioassay and selection procedure

Larvae were bioassayed by standard methods (Georghiou et al. 1965). Briefly, groups of 20 early 4th-instar larvae were tested in 100 ml of tap water in 6-oz. (237-ml) waxed paper cups. At least 5 (but usually 6–10) concentrations of insecticide giving mortality between 2 and 98% were used. Mortality was determined after 24 h and tests were replicated on 5 different days. Insecticides were dissolved in acetone, and test cups and controls received equal volumes of this solvent. Data were analyzed by probit analysis (Finney 1971, Raymond et al. 1993). Dose–response values with overlapping fiducial limits were not considered significantly different. Resistance ratios (RRs) with fiducial limits that included the integer 1 were not considered to be significantly different from the reference strain.

Tests with the synergists *S,S,S*-tributyl phosphorotrithioate (DEF), an esterase inhibitor, and piperonyl butoxide (PB), an inhibitor of monooxygenases, utilized the maximum sublethal dose of each synergist determined for each strain. These doses were 0.5 and 0.05 mg/liter DEF and 1 and 5 mg/liter PB, for A5B5-R and Ace-R, respectively.

Larvae from the Ace-R and A5B5-R strains were mass selected with propoxur or temephos, respectively, in each generation until a high level of resistance was obtained. The selection procedure consisted of placing groups of 1,000 early 4th-instar larvae in a liter of tap water in an enameled pan, and exposing the larvae to the desired concentration of insecticide for 24 h. Survivors were recovered into clean water, fed, and used to continue the colony. Generations were initially maintained separately, then permitted to overlap after 13 generations of selection pressure. Mortality in the A5B5-R colony averaged 68.6% up to generation 13. Thereafter, selection pressure was maintained at concentrations yielding mortality of about 50%. Mortality of the Ace-R colony averaged 47.6% up to generation 11, after which increasing concentrations of propoxur (up to 50 mg/liter) produced mortalities between 0 and 20%. The strain was subsequently maintained under propoxur selection with concentrations of 10–20 mg/liter. Toxicology and synergism tests were done at approximately generation

50, estimated on the basis of 12 generations per year.

Insecticides

Eight insecticides of technical grade dissolved in acetone were tested: temephos (91%, American Cyanamid, Princeton, NJ), chlorpyrifos (98%, Dow Chemical Co., Midland, MI), malathion (92.8%, American Cyanamid), fenthion (95%, Mobay, Kansas City, KS), ethyl parathion (98.5%, Monsanto, St. Louis, MO), propoxur (99%, Mobay), bendiocarb (100%, Nor-Am Chemical Co., Wilmington, DE), and carbaryl (99.9%, Union Carbide, Research Triangle Park, NC). The synergists DEF (99%) and PB (100%), were obtained from Mobay and MGK (Minneapolis, MN), respectively.

Biochemical tests

Three different biochemical tests were used to screen the adults of the single pair crosses and to characterize the Ace-R and A5B5-R strains. These tests have been described in detail elsewhere; therefore, only a brief description of each test is provided.

Microtiter plate esterase test: The esterase assay was as described by Dary et al (1990) with minor modifications (Wirth and Georghiou 1996). Total esterase activity in individual mosquitoes was determined relative to a standard curve of α-naphthol (2–20 nmoles/well). The mean value for the susceptible reference strain, S-Lab, was 8.654 nmoles/min/mosquito (SD = 1.249). Individual mosquito homogenates with values greater than the mean esterase activity of S-Lab plus 3 standard deviation units (13 nmoles/min/mosquito) were considered to possess elevated esterase activity.

Microtiter plate AChE assay: The AChE assay was as described by ffrench-Constant and Bonning (1989) with minor modifications (Wirth and Georghiou 1996). The percentage of residual uninhibited AChE activity in individual mosquitoes was determined by dividing the AChE activity in the presence of propoxur by the AChE activity present in the control (ethanol). In the S-Lab strain, the average residual activity in the presence of propoxur was 0.06% (SD = 0.142). An insect was considered to have insensitive AChE if the residual activity exceeded the mean residual AChE activity of S-Lab plus 3 standard deviation units (2%).

Starch gel electrophoresis: Esterase phenotypes were identified by starch gel electrophoresis using the method of Pasteur et al. (1981). Individual adult mosquitoes were homogenized and esterase allozymes were separated by electrophoresis and stained with Fast Garnet GBC salt in the presence of equal amounts of α- and β-naphthyl acetate. Identification of esterase allozymes was based on the mobility of highly active esterases from the different colonies relative to laboratory reference strains with known esterase composition, including esterase B1 (Georghiou et al. 1980), esterases A2 plus B2 (Wirth et al. 1990), and esterases A5 plus B5 (Poirié et al. 1992).

Genetic crosses

Reciprocal crosses and backcrosses were prepared between the respective resistant strains and S-Lab. Virgin males and females were obtained from isolated pupae as described above. A minimum of 200 males and females were used for each mass cross. The following crosses were made, with the female parent listed first: I, Ace-R × S-Lab; II, S-Lab × Ace-R; III, A5B5-R × S-Lab; IV, S-Lab × A5B5-R; V, A5B5-R × Ace-R; VI, Ace-R × A5B5-R; VII, (S-Lab × Ace-R) F_1 × S-Lab; and VIII, (S-Lab × A5B5-R) F_1 × S-Lab.

The combined effect of esterases A5 plus B5 and the insensitive AChE was evaluated by crosses between the respective resistant strains (crosses V and VI). The levels of temephos resistance in the offspring of these crosses were contrasted with resistance levels in the F_1 offspring from crosses between S-Lab and Ace-R (crosses I and II) or A5B5-R (crosses III and IV). Temephos resistance levels in A5B5-R and Ace-R, as well as those reported earlier in Cyprus field collections (Wirth and Georghiou 1996) were also compared. These tests were performed at approximately generation 70, whereas the toxicologic analysis utilized generation 50; therefore, the susceptibility values for A5B5-R and Ace-R in Table 4 differ from those presented in Tables 1 and 2.

RESULTS

Evolution of temephos resistance and resistance characteristics of A5B5-R

All individual adult mosquitoes of strain A5B5-R that were examined by starch gel electrophoresis expressed highly active esterases A5 and B5 (48 h old, n = 200). Resistance levels at the median lethal concentration (LC_{50}) increased under temephos selection pressure from 14.2-fold in generation 2 to 33.3-fold in generation 50 (Fig. 1). Bioassays at generation 50 showed high levels of resistance at the 95% lethal concentration (LC_{95}) to temephos (RR, 50.8), chlorpyrifos (RR, 46.1), and fenthion (RR, 50.6) and a moderate level of resistance to malathion (RR, 11.6) (Table 1). Resistance was insignificant or extremely low to propoxur and bendiocarb (RR, 2.7 and 4.2, respectively) (Table 2). In the presence of the synergist DEF, temephos resistance decreased to 5.4-fold at the LC_{95} (Table 3). No synergism of propoxur by PB was observed.

Evolution of propoxur resistance and resistance characteristics of Ace-R

All individual adult mosquitoes of the Ace-R strain examined by microtiter enzyme inhibition as-
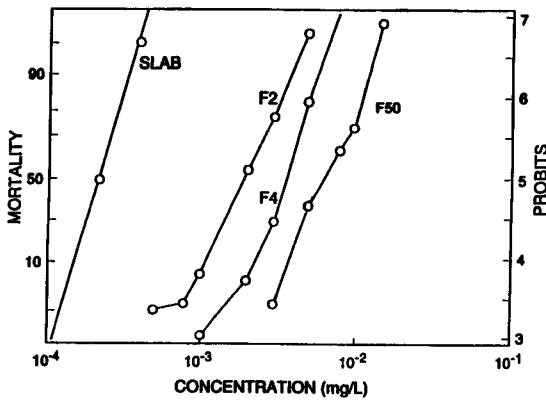
Fig. 1. Evolution of temephos resistance in the A5B5-R strain in response to artificial selection pressure. S-Lab is the laboratory reference strain. F2 represents the offspring of the first selected generation.

say possessed insensitive AChE (n = 32). Between generations 3 and 50, propoxur resistance increased from 2- to 85-fold at the LC$_{50}$ (Fig. 2) with a concomitant increase in the average residual AChE activity in the presence of propoxur from 28.2% (n = 32, SD = 11.2) to 42.3% (n = 48, SD = 12.5).

No highly active esterases were observed in Ace-R mosquitoes examined by starch gel electrophoresis (n = 200).

After 50 generations of selection the Ace-R strain showed high levels of resistance at the LC$_{95}$ to propoxur (RR, 93.1), bendiocarb (RR, 48.3), and carbaryl (RR, 45.2) (Table 2), as well as temephos (RR, 56.5) (Table 1). Only moderate resistance to fenthion (RR, 10.3) and malathion (RR, 11.6) was detected, and extremely low resistance to chlorpyrifos (RR, 4.7) and parathion (RR, 2.0) was noted. No reduction in resistance was observed when temephos was bioassayed with DEF or when propoxur was bioassayed with PB (Table 3).

Inheritance of resistance

No significant difference was found in the bioassay results of the reciprocal crosses of S-Lab to each resistant strain (cross I, II; III, IV), suggesting absence of maternal effects. The results of only one set of crosses for each strain are shown (Figs. 3 and 4 and Table 4). The temephos dose–response line for A5B5-R did not overlap with that of S-Lab, indicating that A5B5-R was homogeneous for temephos resistance (Fig. 3). The F$_1$ offspring of the cross between the A5B5-R strain and S-Lab

Table 1. Organophosphate dose–response values and resistance ratios for larvae of mosquito strains of S-Lab, A5B5-R, and Ace-R after 50 generations of selection pressure.[1]

Insecticide	Strain	Slope (±SE)	LC$_{50}$	LC$_{95}$	RR at LC$_{50}$	RR at LC$_{95}$
Temephos	S-Lab	6.9 (0.577)	0.00180 (0.00171–0.00190)	0.00312 (0.00285–0.00351)		
	A5B5-R	4.4 (0.306)	0.0524 (0.0486–0.0562)	0.123 (0.109–0.141)	33.3 (14.2–78.4)	50.8 (8.3–310)
	Ace-R	3.1 (0.212)	0.0398 (0.0362–0.0436)	0.136 (0.116–0.167)	25.3 (11.2–56.9)	56.5 (38.9–94.5)
Chlorpyrifos	S-Lab	7.1 (0.613)	0.00091 (0.00087–0.00092)	0.00156 (0.00141–0.00175)		
	A5B5-R	3.3 (0.294)	0.0225 (0.0206–0.0247)	0.0719 (0.0590–0.0944)	24.7 (20.2–30.1)	46.1 (29.3–72.5)
	Ace-R	5.4 (0.401)	0.00362 (0.00339–0.00388)	0.00731 (0.00654–0.00844)	4.0 (3.2–4.9)	4.7 (3.0–7.2)
Malathion	S-Lab	6.6 (1.03)	0.0350 (0.0288–0.0426)	0.0620 (0.0410–0.0991)		
	A5B5-R	4.6 (0.776)	0.315 (0.241–0.412)	0.718 (0.425–1.23)	9.0 (5.2–15.5)	11.6 (3.8–35.5)
	Ace-R	3.4 (0.253)	0.400 (0.365–0.436)	1.22 (1.05–1.49)	11.4 (8.0–16.2)	19.7 (9.2–42.3)
Fenthion	S-Lab	5.6 (0.481)	0.00346 (0.00325–0.00367)	0.00677 (0.00612–0.00776)		
	A5B5-R	2.9 (0.511)	0.0958 (0.0693–0.132)	0.342 (0.169–0.726)	27.6 (19.2–39.8)	50.6 (22.9–111.8)
	Ace-R	5.0 (0.385)	0.0325 (0.0300–0.0349)	0.0695 (0.0624–0.0797)	9.4 (7.57–11.7)	10.3 (6.98–15.1)
Parathion	S-Lab	6.4 (0.668)	0.00281 (0.00263–0.00300)	0.00508 (0.00455–0.00596)		
	Ace-R	10.1 (0.905)	0.00688 (0.00654–0.00719)	0.0100 (0.00941–0.0108)	2.4 (1.86–3.2)	2.0 (1.23–3.16)

[1] LC$_{50}$, median lethal concentration (mg/liter); LC$_{95}$, 95% lethal concentration (mg/liter); RR, resistance ratio. The 95% confidence intervals are given in parentheses.

Table 2. Carbamate dose–response values and resistance ratios for larvae of mosquito strains of S-Lab, A5B5-R, and Ace-R after 50 generations of selection.[1]

Insecticide	Strain	Slope (±SE)	LC_{50}	LC_{95}	RR at LC_{50}	RR at LC_{95}
Propoxur	S-Lab	4.9 (0.437)	0.183 (0.169–0.197)	0.395 (0.357–0.453)		
	A5B5-R	4.5 (0.287)	0.457 (0.426–0.491)	1.07 (0.951–1.23)	2.5 (2.0–3.0)	2.7 (1.9–3.8)
	Ace-R	4.3 (0.319)	10.7 (9.99–11.6)	25.7 (22.5–30.5)	85.6 (54.5–134)	93.1 (39.1–221)
Bendiocarb	S-Lab	5.7 (0.456)	0.185 (0.173–0.198)	0.360 (0.322–0.417)		
	A5B5-R	3.7 (0.315)	0.539 (0.495–0.587)	1.51 (1.28–1.88)	2.9 (2.3–3.6)	4.2 (2.7–6.5)
	Ace-R	5.1 (0.977)	8.28 (6.17–11.1)	17.4 (10.3–30.1)	44.7 (25.4–78.8)	48.3 (17.3–135)
Carbaryl	S-Lab	4.9 (0.432)	0.701 (0.651–0.752)	1.52 (1.35–1.81)		
	Ace-R	5.1 (0.426)	32.8 (30.6–35.2)	68.9 (61.2–80.7)	46.8 (37.7–58.1)	45.2 (29.5–69.2)

[1] LC_{50}, median lethal concentration (mg/liter); LC_{95}, 95% lethal concentration (mg/liter); RR, resistance ratio. The 95% confidence intervals are given in parentheses.

showed an intermediate level of temephos resistance. The data on the backcross offspring ([S-Lab × A5B5-R] F_1 × S-Lab) revealed a slight inflexion at the intermediate doses between the susceptible and the hybrid individuals; however, the line deviated significantly from that expected under a single gene model, particularly at lower temephos concentrations ($\chi^2 = 78.05$, $n = 10$, $P < 0.05$).

The propoxur dose–response line for Ace-R did not overlap with that for S-Lab (Fig. 4), and F_1 offspring of the crosses between Ace-R and S-Lab were intermediate in expression of resistance to propoxur (Table 4). Bioassay of the backcross offspring was statistically consistent with a straight line ($\chi^2 = 13.6$, df 11, $P > 0.05$). Therefore inheritance of propoxur resistance did not fit a single gene model ($\chi^2 = 65.4$, $n = 12$, $P < 0.05$).

Resistance characteristics of artificially recombined esterases A5 and B5 and insensitive AChE

Temephos bioassays on the F_1 offspring of cross V showed a resistance level of 157.9, considerably higher than the level of resistance (RRs at LC_{95}, 14.7 and 23.9, respectively) present in the F_1 offspring of crosses A5B5-R and Ace-R to the S-Lab strain (Table 4), but lower than the level of resistance in the A5B5-R parent (RR at LC_{95}, 281). However, temephos resistance in that cross was en-

Table 3. Effect of the enzyme inhibitors S, S, S-tributyl phosphorotrithioate (DEF) and piperonyl butoxide (PB) on the toxicity of temephos and propoxur, respectively.[1]

Strain	Slope (±SE)	LC_{50}	LC_{95}	RR at LC_{50}	RR at LC_{95}
		Temephos + DEF			
S-Lab	4.0 (0.281)	0.00035 (0.00032–0.00037)	0.00089 (0.00079–0.00103)		
A5B5-R	2.5 (0.187)	0.00106 (0.00094–0.00118)	0.00466 (0.00382–0.00602)	2.9 (2.50–3.56)	5.4 (3.80–7.61)
Ace-R	4.1 (0.258)	0.0252 (0.0235–0.0271)	0.0633 (0.0561–0.0736)	71.3 (59.6–85.3)	73.1 (51.9–102.9)
		Propoxur + PB			
S-Lab	6.6 (0.903)	0.0270 (0.0252–0.0281)	0.0478 (0.0375–0.0467)		
A5B5-R	3.5 (0.347)	0.0819 (0.0734–0.0898)	0.242 (0.205–0.306)	3.1 (2.40–3.95)	5.9 (3.59–9.69)
Ace-R	3.2 (0.221)	1.65 (1.51–1.81)	5.43 (4.64–6.61)	65.2 (51.3–82.8)	131.9 (82.6–210)

[1] LC_{50}, median lethal concentration (mg/liter); LC_{95}, 95% lethal concentration (mg/liter); RR, resistance ratio. The 95% confidence intervals are given in parentheses.
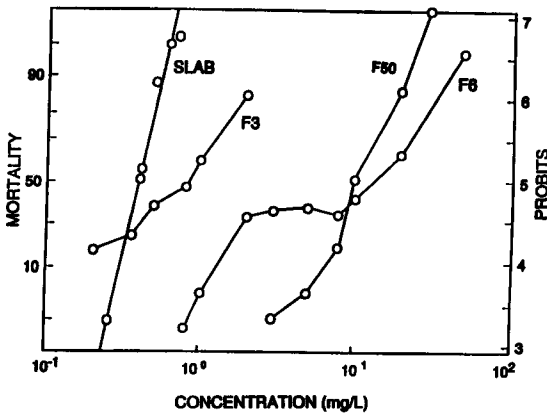
Fig. 2. Evolution of propoxur resistance in the Ace-R strain in response to artificial selection pressure. S-Lab is the laboratory reference strain. F3 represents the offspring of the first selected generation.

Fig. 3. Temephos dose–response lines observed in parental S-Lab (S) and A5B5-R (R) strains, their F_1 offspring (S-Lab × A5B5-R), and backcross (BC) ([S-Lab × A5B5-R] × S-Lab). The expected line is the ld-p line calculated under monofactorial inheritance of resistance.

hanced relative to that of the Ace-R parent (RR at LC_{95}, 81.1). In contrast, propoxur resistance in cross VI (RR at LC_{95}, 14.4) did not differ greatly from that observed in the F_1 offspring of crosses of Ace-R to S-Lab (RR at LC_{95}, 15.3) but was significantly higher than those of the A5B5-R parent and the A5B5-R cross with S-Lab (RR at LC_{95}, 2.3 and 2.2, respectively), indicating that esterases A5 plus B5 make no contribution to propoxur resistance (Table 4).

DISCUSSION

Novel OP resistance mechanisms, esterases A5 plus B5 and an insensitive AChE, in addition to esterases A2 plus B2, contribute to high levels of OP resistance in populations of Cx. pipiens from Cyprus. Esterases A2 plus B2 have a cosmopolitan distribution (Raymond et al. 1991) and evidence is accumulating that these esterases have expanded their range through migration, possibly associated with commercial traffic (for a review see Pasteur and Raymond 1996). In contrast to the widespread distribution of A2 plus B2, esterases A5 plus B5 have a narrower distribution, having been initially detected in Cyprus and recently in Italy (Severini et al. 1997).

Multiple mechanisms of OP resistance are reported in many populations of the Cx. pipiens complex under insecticide control. Insecticide selection pressure can lead to high frequencies of multiple highly active esterases (Raymond et al. 1987, Ben Cheik and Pasteur 1993, Yébakima et al. 1995) or highly active esterases and insensitive AChE (Raymond et al. 1986, Bisset et al. 1990, Bonning et al. 1991, Severini et al. 1993, Raymond and Marquine 1994, Rivet et al. 1994). In only a few cases has the relative importance of the different mechanisms present in the resistant population been determined.
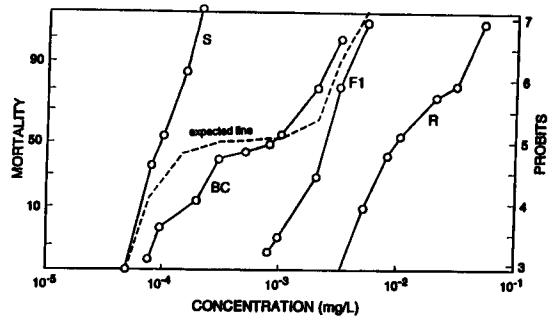
In OP-resistant Cx. pipiens from France, 2 resistance mechanisms, esterase A1 and insensitive AChE, were detected (Raymond et al. 1986). The insensitive AChE was associated with much higher chlorpyrifos resistance than was esterase A1, so that the relative contribution of esterase A1 to chlorpyrifos resistance in the presence of the insensitive AChE was insignificant. Multiple OP resistance genes were also identified in Corsican populations of Cx. pipiens (Raymond and Marquine 1994). The identified resistance genes (insensitive AChE and esterases A1, A4, and B4) conferred only low temephos resistance, despite 17 years of insecticide treatment. In contrast, the results from the Cyprus mosquitoes suggest that each of the 3 major resistance mechanisms can potentially provide high levels of temephos resistance, either alone or in combination.

Although the direct association of temephos resistance and esterases A5 plus B5 has not been demonstrated, considerable circumstantial evidence supports this association. All mosquitoes in the
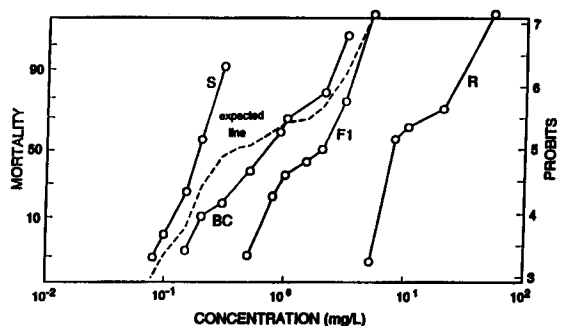

Fig. 4. Propoxur dose–response lines observed in parental S-Lab (S) and Ace-R (R) strains, their F_1 offspring (S-Lab × Ace-R), and backcross (BC) ([S-Lab × Ace-R] × S-Lab). The expected line is the ld-p line calculated under monofactorial inheritance of resistance.

Table 4. Dose–response values and resistance ratios for temephos and propoxur tested on larvae of mosquito strains S-Lab, Ace-R, and A5B5-R and F_1 offspring of crosses between these strains.[1]

Insecticide	Strain and crosses	Slope (±SE)	LC_{50}	LC_{95}	RR at LC_{50}	RR at LC_{95}
Temephos	S-Lab	6.4 (0.477)	0.00096 (0.00091–0.00101)	0.00174 (0.00159–0.00194)		
	Ace-R	2.4 (0.165)	0.0288 (0.0256–0.0322)	0.141 (0.117–0.179)	30.1 (25.0–36.2)	81.1 (57.3–114)
	A5B5-R	2.6 (0.196)	0.113 (0.102–0.125)	0.488 (0.400–0.631)	118.3 (98.2–142)	281 (194–406)
	Ace-R × S-Lab	4.0 (0.290)	0.00994 (0.00924–0.0106)	0.0256 (0.0226–0.0299)	10.4 (8.58–12.5)	14.7 (10.2–21.2)
	S-Lab × A5B5-R	6.6 (0.758)	0.0235 (0.0146–0.0329)	0.0416 (0.0228–0.103)	24.5 (19.4–31.1)	23.9 (15.3–37.6)
	Ace-R × A5B5-R	2.9 (0.213)	0.0741 (0.0675–0.0818)	0.274 (0.225–0.335)	77.4 (64.2–93.3)	157.9 (106.8–233.6)
Propoxur	S-Lab	5.2 (0.411)	0.184 (0.173–0.196)	0.381 (0.337–0.447)		
	Ace-R	5.2 (0.876)	9.37 (6.62–13.4)	19.4 (9.90–41.3)	50.9 (30.9–83.8)	51.1 (18.6–140.4)
	A5B5-R	5.3 (0.373)	0.421 (0.388–0.457)	0.858 (0.763–0.994)	2.3 (1.81–2.88)	2.3 (1.46–3.48)
	Ace-R × S-Lab	3.8 (0.304)	2.16 (1.99–2.34)	5.83 (4.99–7.16)	11.7 (9.69–14.2)	15.3 (9.98–23.5)
	S-Lab × A5B5-R	6.0 (0.421)	0.447 (0.420–0.474)	0.835 (0.762–0.935)	2.4 (1.96–2.99)	2.2 (1.45–3.3)
	Ace-R × A5B5-R	2.9 (0.436)	1.53 (1.13–2.07)	5.49 (2.66–12.2)	8.4 (5.89–11.6)	14.4 (6.27–33.2)

[1] LC_{50}, median lethal concentration (mg/liter); LC_{95}, 95% lethal concentration (mg/liter); RR, resistance ratio. The 95% confidence intervals are given in parentheses.

A5B5-R strain expressed the 2 esterases, and demonstrated a significant level of temephos resistance, which was suppressed in the presence of the esterase inhibitor DEF. Further, the esterase B5 gene from Cyprus mosquitoes has been shown to be amplified, and to be hybridized by the esterase B1 cDNA clone. The B5 protein is recognized by the esterase B1 antibody. These characteristics are in common with all other highly active B esterases associated with OP resistance (Poirié et al. 1992).

The insensitive AChE isolated from the Cyprus mosquitoes has unique characteristics relative to another well-studied insensitive AChE found in Cx. pipiens from the French MSE strain (Raymond et al. 1985). First, the Ace-R mosquitoes revealed extremely low chlorpyrifos resistance, whereas the MSE strain expressed high levels of resistance to chlorpyrifos (Raymond et al. 1986). Second, the percent residual AChE activity in the presence of propoxur differed. Acetylcholinesterase from the MSE strain retained virtually all of its activity (Raymond et al. 1985), whereas AChE from Ace-R mosquitoes lost 50–60% of its activity in the presence of the identical concentration of propoxur. These differences were confirmed in a kinetic study that showed the unique catalytic properties of the Cyprus enzyme (Bourguet et al. 1996b). Insensitive AChEs in mosquito collections from Europe and Africa had similar sensitivity to propoxur inhibition, except the enzyme from Cyprus mosquitoes. The kinetic characteristics and toxicologic spectrum

suggest that the insensitive AChE from the Ace-R strain represents (a) unique mutation(s). This can only be confirmed by molecular analysis of the different modified AChEs.

The pattern of inheritance of temephos resistance in the A5B5-R strain was not consistent with those reported for other highly active esterases, including esterase B1 (Georghiou et al. 1980), esterases A2 plus B2 (Wirth et al. 1990), and esterases A3 plus B3 in Cx. tarsalis (Prabhaker et al. 1987). In these 3 studies, temephos resistance was inherited as a monofactorial character. In the case of the insensitive AChE, the intermediate level of resistance in the F_1 offspring is consistent with that reported for Cx. pipiens from France (Raymond et al. 1987), but not for Anopheles albimanus Wiedemann (Ayad and Georghiou 1975) or Culex tritaeniorhynchus Giles (Takahashi and Yasutomi 1987), in which resistance was almost fully dominant. Because the backcross dose–response line showed no evidence of any plateau, multiple loci may be involved. However, the backcross method has limited ability to discriminate among modes of inheritance (Tabashnik 1991). In particular, the variation resulting from crossing strains of 2 closely related species with different genetic backgrounds, as well as the variation inherent in the bioassay technique, limit the sensitivity of this approach. These results should be considered preliminary, pending confirmation by a more sensitive test method.

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