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ORGANOPHOSPHATE RESISTANCE IN *CULEX PIPIENS* FROM CYPRUS

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ABSTRACT. Populations of *Culex pipiens* were sampled from 8 locations in Cyprus between 1987 and 1993. All population samples generally revealed organophosphate resistance to malathion, temephos, chlorpyrifos, fenthion, dichlorvos, and pirimiphos methyl, in decreasing order of magnitude. Of 7 populations assessed with the carbamate propoxur, all proved to be resistant to different degrees. Of the 6 populations tested with permethrin, 2 were resistant to permethrin. Resistance was associated with the presence of 5 different overproduced esterases (esterases A1, A2, A5, B2, and B5) as well as an insensitive form of acetylcholinesterase. These results are discussed in relation to the ongoing mosquito abatement program in Cyprus and to similar programs in other parts of the world.

INTRODUCTION

In 1949, Cyprus became one of the first countries in the eastern Mediterranean to eradicate malaria and the primary vector *Anopheles sacharovi* Favre. Since then, an extensive program of mosquito surveillance and abatement has been in effect to prevent the reintroduction of the disease and to control biting pests such as *Culex pipiens* Linnaeus. Despite the successful eradication of malaria, there continues to be concern over the possible reintroduction of the disease because of the arrival of people from neighboring areas where malaria is endemic and where the vector *An. sacharovi* is resistant to most classes of insecticides (Clarke 1985, Hemingway et al. 1992). Another potential source of reintroduction is the importation of temporary laborers from Asian countries in which *Plasmodium falciparum* is resistant to antimalarial drugs.

The mosquito control program in effect on the island is based primarily on larviciding. Between March and October, potential breeding sites are identified and treated at 10–30-day intervals. Typical breeding sites include sewage treatment plants and standing pools of water near rivers and streams and on farms. The program originally relied on DDT, which was phased out in 1971 and replaced by temephos. Pirimiphos methyl, dichlorvos, pyrethroids, and fuel oil are used to a lesser extent. Adulticiding is limited to mosquito shelters such as caves. *Gambusia affinis* is established in rivers and irrigation dams.

In 1987, a population of *Cx. pipiens* was collected from a farm near the village of Mitsero. A subsequent collection was made near Athienou in 1992. Both collections were colonized and tested in our laboratory at the University of California, Riverside, and revealed a broad spectrum of resistance to organophosphate (OP) insecticides. At the request of the Cyprus Ministry of Health, a more extensive study was undertaken in 1993 to determine the distribution of insecticide resistance and to characterize the mechanisms of resistance involved.

MATERIALS AND METHODS

Strains: Between 1987 and 1993, 10 breeding sites were sampled (Fig. 1). Eight of these yielded *Cx. pipiens.* Two sites (Pyrga and Paramytha) yielded significant numbers of *Anopheles claviger* (Meigen), and one site (Ayias Trias) contained *Aedes mariae* (Sergent and Sergent). The non-*Culex* larvae produced no eggs under laboratory conditions and were not studied further. The *Culex* larvae were colonized in our laboratory, and the F_1 offspring were tested. A susceptible strain of *Culex quinquefasciatus* Say, S-Lab (Georghiou et al. 1966), was used as a reference population for bioassays and biochemical tests. The S-Lab strain has frequently been used as a reference for comparisons to *Cx. pipiens* (Raymond et al. 1986, Poirie et al. 1992, Failloux et al. 1994). Its LC_{50} values do not show a significant difference from those published for *Cx. pipiens* (Curtis and Pasteur 1981, Bonning et al. 1991, Khayrandish and Wood 1993, Severini et al. 1993).

Bioassay method: The F_1 offspring of the field-collected larvae were bioassayed according to the procedure of Georghiou et al. (1966). Tests were conducted on groups of 20 early 4th-instar larvae in 100 ml of tap water in 6-oz. waxed paper cups. At least 5 concentrations of insecticide giving 24-h mortality between 2 and 98% were utilized. Tests were replicated on 5 different days. The concentration of acetone in control and test cups was adjusted to 1%. Tests with the bacterial insecticide *Bacillus thuringiensis israelensis* (*B.t.i.*) were similar to those

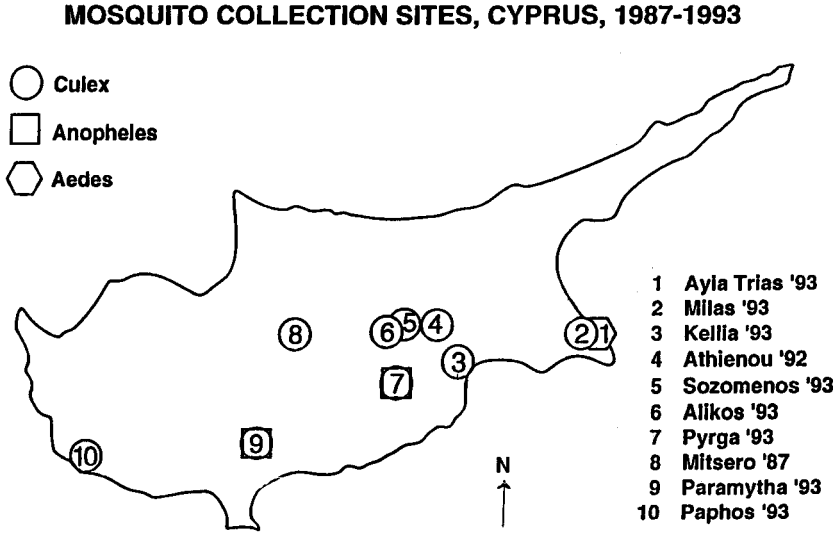

Fig. 1. Map of Cyprus indicating the mosquito collection sites from 1987 to 1993.

used with conventional insecticides except they were conducted in 8-oz. plastic cups in distilled water without acetone. Data were subjected to probit analysis (Finney 1971) using the program of Raymond (1985).

Insecticides used: Six OP insecticides of technical grade were used: temephos (91%; American Cyanamid, Princeton, NJ), chlorpyrifos (98%; Dow Chemical Co., Midland, MI), malathion (92.8%; American Cyanamid), fenthion (95%; Mobay, Kansas City, KS), pirimiphos methyl (91.7%; ICI Americas, Inc., Richmond, CA), and dichlorvos (98%; Shell Development Co., Modesto, CA). The carbamate propoxur (99%; Mobay), the pyrethroid permethrin (94.6%; ICI Americas), and the bacterial insecticide *B.t.i.* (preparation IPS 80; Pasteur Institute, Paris) were also tested. All insecticides were dissolved in acetone except for *B.t.i.*, which was solubilized in distilled water and agitated using glass beads to create a homogeneous solution.

Starch gel electrophoresis: Samples of 50 adult mosquitoes from each collection (except that from Mitsero, of which 45 were tested) were analyzed for elevated esterases by starch gel electrophoresis, according to the method of Pasteur et al. (1981). Individual adult insects were homogenized, electrophoresed, and stained with Fast Garnet GBC salt in the presence of equal amounts of α- and β-naphthyl acetate. Esterase phenotypes were evaluated relative to the mobility of known esterases in OP-resistant reference strains of the *Cx. pipiens* complex, including esterase B1 from Tem-R (Georghiou and Pasteur 1978) and esterases A2 and B2 from Selax (Wirth et al. 1990).

Enzyme assays: A sample of 105 adult mosquitoes from the F_2 generation of the Mitsero collection was analyzed by microtiter enzyme assay to determine the presence of elevated esterase activity and insensitive acetylcholinesterase (AChE) in individual insects.

Esterase activity tests: Total esterase activity was determined on individual adult mosquitoes of either sex in 96-well, flat-bottomed microtiter plates (Dynatech, Chantilly, VA) according to the method of Dary et al. (1990). Briefly, mosquitoes were homogenized in 100 μl of 100 mM sodium phosphate buffer (pH 7.5) with 0.5% Triton X-100. After centrifugation to remove debris, 20 μl of supernatant was diluted with 180 μl of 100 mM sodium phosphate (pH 6.5) containing 0.5% Triton X-100. Fifty microliters of the diluted homogenate was aliquoted into each of 2 wells in the microtiter plate, and 100 μl of substrate solution (50 mM sodium phosphate [pH 6.5], 0.25% Triton X-100, 2 mM α-naphthyl acetate, 0.003 mM BW284C5 [a specific AChE inhibitor], 3.5% ethanol) was added to each well. After a 10-min incubation at room temperature, 100 μl of 0.8 mg/ml Fast Garnet GBC salt was added and the plate was incubated for an additional 10 min. Absorbance was determined at 550 nm on a microtiter plate reader (Model 2550; BioRad Laboratory, Richmond, CA). Total esterase activity was calculated based on a standard curve of α-naphthol (2–20 nmoles/well). Values greater than 12 nmoles/min/mosquito were considered as signifying elevated es-

terase activity. The mean value for the susceptible reference strain S-Lab was 8.654 nmoles/min/mosquito (SD = 1.249).

Acetylcholinesterase activity: Inhibition of AChE was as described by ffrench-Constant and Bonning (1989) with minor modifications. Seventy microliters of the original homogenate prepared for the esterase test was diluted 10-fold with 100 mM sodium phosphate buffer (pH 7.5) containing 0.5% Triton X-100. One hundred microliters of homogenate was aliquoted to each of 4 wells in a microtiter plate. Each well received a different 5-µl treatment, i.e., 1) ethanol, 2) 0.08 mM BW284C5, 3) 2 mM propoxur, or 4) 0.08 mM paraoxon. The plates were equilibrated for 10 min, then 100 µl of developing solution (9.4 ml distilled water, 0.2 ml 100 mM acetylthiocholine iodide, 0.4 ml 12 mM DTNB [5,5-dithio bis[2-nitrobenzoic acid]) was added to each well. Absorbance was read at 414 nm at 0, 15, and 30 min after addition of the developing solution. The absorbance values in the wells containing insecticide were corrected by the absorbance in the presence of the specific AChE inhibitor. The percentage of residual AChE activity in individual mosquitoes was calculated by dividing the activity in the presence of insecticide by the activity in the control (ethanol). An insect was considered to possess insensitive AChE if its residual activity exceeded the mean value (+3 SD) of the S-Lab strain, i.e., 2% for propoxur or 15% for paraoxon. The average residual value for the S-Lab strain in the presence of propoxur was 0.06 ± 0.14% and in the presence of paraoxon, 8.0 ± 1.2%.

RESULTS

Bioassays: The bioassay results for each insecticide and mosquito population are indicated by means of dose–response lines in Figs. 2–4. All populations tested possessed high resistance to organophosphates and carbamates but lower resistance to pyrethroids. The highest levels of resistance observed are presented in Table 1. For organophosphates, resistance was highest to malathion, for which resistance ratios ranged from 8.0- to 97.2-fold at the LC_{50} and from 83.0- to 320-fold at the LC_{95}. Resistance was also high for temephos and chlorpyrifos (Fig. 2) but lower for pirimiphos methyl, fenthion, and dichlorvos (Fig. 3). Propoxur resistance was high in most populations, especially at the LC_{95} (Table 1 and Fig. 4). Tests with the bacterial insecticide *B.t.i.* revealed significant differences in susceptibility among the 6 collections examined (Fig. 4). Resistance ratios at the LC_{50} ranged from 1.7- to 6-fold, whereas resistance ratios at the LC_{95} were 3.6–11-fold. Three collections had resistance ratios greater than 5-fold at the LC_{95}. These differences remain unexplained because this product is not included in the mosquito control program of Cyprus and agricultural *Bacillus thuringiensis* products are nontoxic to mosquito larvae.

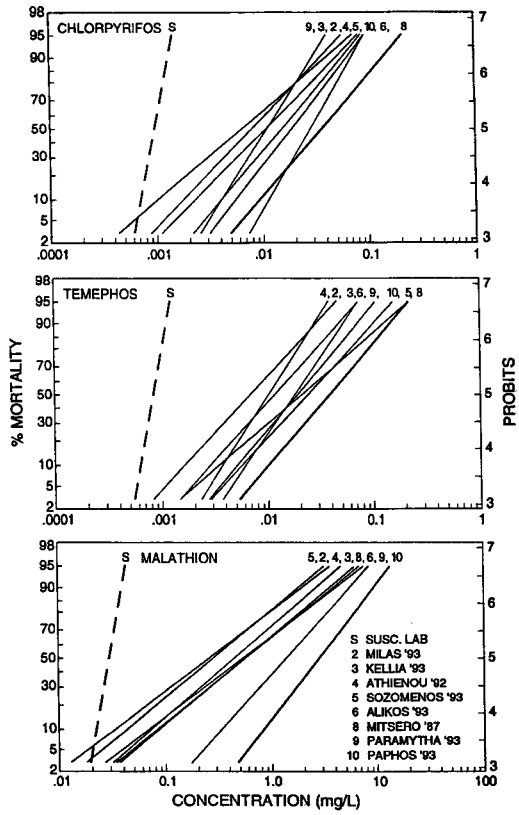

Fig. 2. Dose–response lines for chlorpyrifos, temephos, and malathion for Cyprus collections of *Culex quinquefasciatus.*

Starch gel electrophoresis: A variety of amplified esterases were present in each population (Table 2). The most common esterases found were a pair designated A5 and B5 (Poirie et al. 1992), which so far have been reported only from Cyprus. A second pair of esterases, A2 and B2 (Raymond et al. 1987, Wirth et al. 1990), were also present. Esterase A1 (Pasteur et al. 1981) was detected at an extremely low frequency in the 1987 Mitsero collection (2.2%), and its presence in Cyprus was not confirmed in any of the subsequent collections. The frequency of esterases A2 and B2 ranged from 14 to 72%, whereas that of esterases A5 and B5 ranged from 8 to 98% in the collections examined.

Biochemical tests: The Mitsero population contained both elevated esterase activity and insensitive AChE. Thirty-five insects were identi-

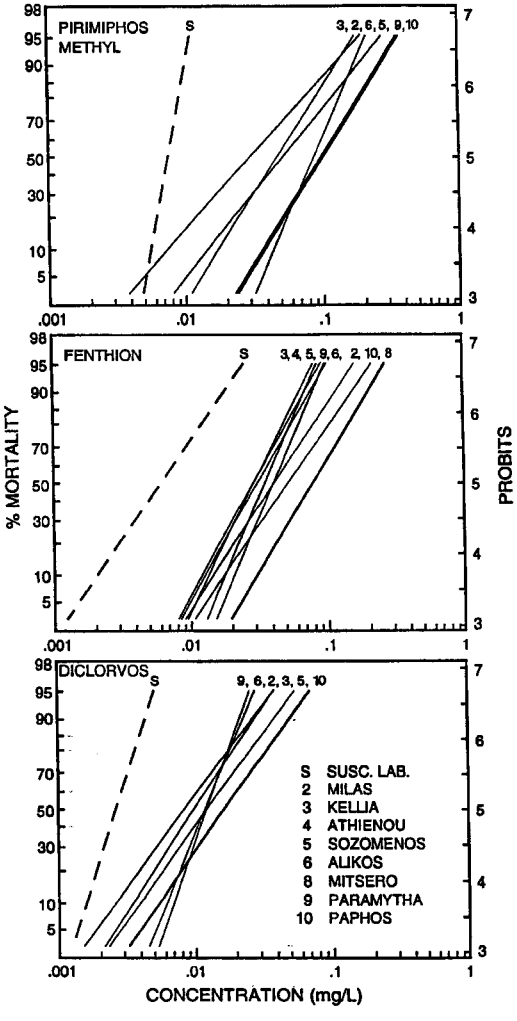

Fig. 3. Dose–response lines for pirimiphos methyl, fenthion, and dichlorvos for Cyprus collections of *Culex quinquefasciatus.*

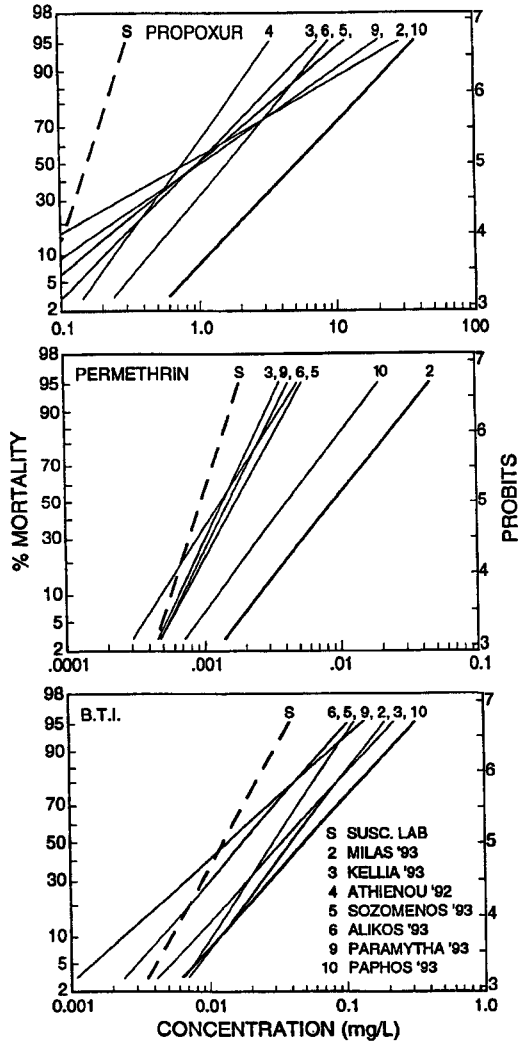

Fig. 4. Dose–response lines for propoxur, permethrin, and *B.t.i.* for Cyprus collections of *Culex quinquefasciatus.*

fied as possessing only elevated esterase activity (33.3%), which could be the result of the presence of either esterases A5 and B5, esterases A2 and B2, or all 4. Sixteen individuals (15.2%) possessed only the insensitive AChE. Thirty (28.6%) contained both elevated esterase activity and the insensitive acetylcholinesterase, whereas 24 mosquitoes (23%) possessed neither mechanism. These frequencies are not in strict agreement with the electrophoresis results, probably because of the different generations sampled.

DISCUSSION

Culex pipiens mosquitoes from Cyprus possess high levels of resistance to several OP insecticides as a consequence of the prolonged and general use of this class of chemicals in mosquito control. Populations sampled from a variety of geographic locations during 1987, 1992, and 1993 showed a consistent pattern of resistance to both OP and carbamate insecticides. Examination of the mechanisms of resistance present in these populations revealed 2 major types: elevated activity of detoxifying esterases and an altered acetylcholinesterase.

Five different elevated esterases were present. Esterases A2 and B2 have been widely reported in the *Cx. pipiens* complex (Raymond et al. 1987, Pieris and Hemingway 1990, Bonning et al. 1991, Ben Cheikh and Pasteur 1993, Khayr-

Table 1. Dose–response values for Cyprus collections with the highest level(s) of insecticide resistance to each of the chemicals tested.

Insecticide	Strain	LC_{50} (mg/liter)	LC_{95} (mg/liter)	Resistance ratios	
				At LC_{50}	At LC_{95}
Temephos	S-Lab	0.0009	0.0013	—	—
	Mitsero	0.0411	0.2391	18.1	62.9
Chlorpyrifos	S-Lab	0.0010	0.0015	—	—
	Mitsero	0.0393	0.2302	18.5	80.5
Malathion	S-Lab	0.0295	0.0434	—	—
	Paphos	2.82	13.76	97.2	320.0
Pirimiphos methyl	S-Lab	0.0076	0.0115	—	—
	Paramytha	0.0955	0.3462	12.5	28.8
	Paphos	0.0970	0.3389	12.8	28.3
Fenthion	S-Lab	0.0063	0.0259	—	—
	Mitsero	0.0770	0.2588	12.2	28.5
Dichlorvos	S-Lab	0.0268	0.0489	—	—
	Paphos	0.1550	0.6049	5.7	12.3
Propoxur	S-Lab	0.1604	0.3240	—	—
	Paphos	5.474	122.0	34.2	376.0
Permethrin	S-Lab	0.00088	0.0019	—	—
	Paphos	0.00414	0.0202	4.7	10.6
	Milas	0.00865	0.0446	9.8	23.5
B.t.i.	S-Lab	0.0082	0.0295	—	—
	Paphos	0.0500	0.3254	6.1	11.0

andish and Wood 1993, Failloux et al. 1994, Rivet et al. 1994). Esterase A1, reported only from Europe (Pasteur et al. 1981), was observed in one Cyprus collection made in 1987 but not in subsequent collections. Esterases A5 and B5 were recently identified by Poirie et al. (1992) only in mosquitoes from Cyprus that were provided from our collections. Originally thought to be identical to esterases A4 and B4 reported in France, they were found to be associated with higher OP resistance and to be unique, based on the restriction fragment length polymorphism (RFLP) pattern revealed in Southern blot experiments (Poirie et al. 1992) using the B1 esterase gene as a probe. To date, all of the B esterases have been shown to be overproduced because of gene amplification (Mouches et al. 1986, Raymond et al. 1989, Poirie et al. 1992). Recently, gene amplification was also shown to be the basis for overproduction of the esterase A2 (Vaughan and Hemingway 1995). It is probable, therefore, that the other known A esterases (A1, A3, A4, and A5) will also be found to be overproduced because of this mechanism.

Table 2. Frequency (in percent) of the different overproduced esterases, singly and in combination, in *Culex pipiens* from Cyprus, as revealed by starch gel electrophoresis. Samples of 50 adult insects were tested with the exception of Mitsero, where 45 adults were tested.

Strain	Esterase				
	None	A5B5	A2B2	A5B5/ A2B2	A1
Mitsero	15.6	53.3	17.8	11.1	2.2
Athienou	60.0	26.0	12.0	2.0	0
Sozomenos	10.0	68.0	0.0	22.0	0
Alikos	60.0	16.0	10.0	14.0	0
Milas	40.0	4.0	52.0	4.0	0
Kellia	24.0	44.0	20.0	12.0	0
Paramytha	10.0	62.0	0.0	28.0	0
Paphos	2.0	26.0	0.0	72.0	0

In addition to OP resistance, all populations of *Cx. pipiens* from Cyprus were found to possess propoxur resistance. Because no carbamates have been used in mosquito control, this observation led to the assumption that an insensitive acetylcholinesterase selected by OPs was present. This was subsequently confirmed by biochemical tests on the Mitsero population. Therefore, OP resistance in these populations is probably a consequence of the presence of a variety of amplified esterases as well as an insensitive AChE. Propoxur resistance results from cross-

resistance caused by the insensitive AChE. A similar pattern of OP resistance associated with multiple mechanisms (i.e., overproduced esterases and insensitive AChE) has been reported in a number of countries, such as France (Rivet et al. 1994), Italy (Bonning et al. 1991), and Cuba (Bisset et al. 1991), that conduct intensive mosquito abatement programs.

Resistance to permethrin was noted in 2 of the 6 collections tested. This could be the result of cross-resistance from previous DDT use because of the Kdr resistance mechanism or to agricultural application of pyrethroids.

One of the potential alternative materials for mosquito control is *B.t.i.* This bacterial insecticide is highly specific, being toxic only to mosquitoes and blackflies (de Barjac 1990). Our tests on 6 Cyprus collections showed greater variation in LC values (*ca.* 11-fold) than were observed in a field survey of *Cx. pipiens* and *Cx. quinquefasciatus* in California during 1990 and 1991 (Georghiou et al. 1991). However, long-term selection of *Cx. quinquefasciatus* in our laboratory with *B.t.i.* (preparation IPS 80) has produced only limited resistance (Georghiou 1990).

Slope values for the field collections were generally lower than those observed in the susceptible reference strain. The slope values indicate that the Cyprus collections are highly variable in response to insecticides and may be able to respond further to selection pressure leading to increased resistance.

The observed spectrum of multiple resistance to OP and carbamate insecticides in Cyprus based on both metabolic esterases and insensitive acetylcholinesterase serves to emphasize the need for incorporating alternative approaches in the existing mosquito control programs. Greater emphasis on the elimination of breeding sites, reduction in the frequency of treatments with conventional insecticides, and the incorporation of environmentally benign insecticides, such as the bacterial insecticides and highly refined oils, is indicated. Such approaches should reduce the need for routine chemical treatments with OPs and thus delay the further evolution of OP resistance.

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