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SELECTION AND CHARACTERIZATION OF TEMEPHOS RESISTANCE IN A POPULATION OF *AEDES AEGYPTI* FROM TORTOLA, BRITISH VIRGIN ISLANDS

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ABSTRACT. A collection of *Aedes aegypti* from Tortola, British Virgin Islands, with a high level of temephos resistance (46.8-fold at the 95% lethal concentration [LC_{95}]) was selected to higher resistance with temephos in the laboratory. After 13 generations of pressure, the temephos resistance ratio increased to 180.6 (LC_{95}), whereas in the absence of selection pressure the resistance ratio declined to 8.5. Relatively low levels of resistance or cross-resistance to other organophosphate and carbamate insecticides, and a high level of resistance to the pyrethroid permethrin were also observed. Synergism tests implicated detoxifying esterases in temephos resistance and the presence of elevated esterase activity was confirmed by biochemical tests; however, no evidence was found of insensitive acetylcholinesterase. Mendelian crosses indicated that temephos resistance was inherited as a monofactorial trait. The presence of high levels of temephos and permethrin resistance in *Ae. aegypti* has important implications for *Aedes* control programs.

KEY WORDS *Aedes aegypti,* resistance, organophosphate, pyrethroid, esterase, genetics

INTRODUCTION

Aedes aegypti (L.), the principle vector of yellow fever, dengue, and dengue hemorrhagic fever, represents a significant public health threat in Central and South America, Africa, and Southeast Asia. In the urban tropics, 2 billion persons are considered to be at risk for dengue virus infection (Tabachnik 1991). Because *Ae. aegypti* is a highly domesticated mosquito, this species thrives in crowded urban areas where reliable water supplies and modern sanitation facilities may be lacking. Consequently, containers that are used to collect rainwater and store domestic water supplies provide important breeding sites (Nathan 1993, Gubler and Clark 1994). Source reduction and community-based sanitation programs are important components of *Aedes* control strategies, however community action alone may not be sufficient to control larval populations. Of 25 Caribbean countries and territories surveyed in 1990, 22 relied upon residual insecticides as focal treatments for larval habitats (Nathan 1993). Organophosphate (OP) insecticides, including temephos sand granules for domestic water containers, and malathion, fenthion, and fenitrothion for residual or space sprays, are used to reduce vector densities (Georghiou et al. 1987, Gratz and Jany 1994).

Because of the importance of insecticides in *Aedes* control programs, and the declining development and registration of new insecticides, maintaining the effectiveness of current materials is an important goal. Caribbean and South American populations of *Ae. aegypti* have generally revealed low to moderate levels of OP resistance (5- to 10-fold) (Georghiou et al. 1987, Rawlins and Ragoonansingh 1990, Mekuria et al. 1991, Rawlins and Hing Wan 1995, Mazzarri and Georghiou 1995, Rawlins 1998). Occasionally, higher levels of resistance (>10-fold) are found, which provides evidence of potential control problems in this species (Georghiou et al. 1987, Rawlins and Ragoonansingh 1990, Rawlins and Hing Wan 1995, Rawlins 1998).

During a field survey for OP resistance in *Ae. aegypti,* Georghiou et al. (1987) reported a high level of temephos resistance, 46.8-fold at the 95% lethal concentration (LC_{95}), in a population from Tortola, British Virgin Islands. This collection provided the opportunity to study field-developed temephos resistance in *Ae. aegypti* in more detail. Here we report the capacity of this population to respond to further temephos selection pressure, the resistance spectrum, the primary resistance mechanism, and the pattern of inheritance of temephos resistance.

MATERIALS AND METHODS

Mosquito strains: Three strains of mosquitoes were used in this study. The Tortola colony was derived from a collection of *Ae. aegypti* eggs from Sea Cows Bay, Tortola, British Virgin Islands, which was received in January 1985 (Georghiou et al. 1987). After 3 generations in the laboratory, this colony was divided into 2 strains; 1 was subjected to temephos selection and identified as Tortola-Sel and the other was maintained without selection and identified as Tortola. A long-established laboratory reference strain, Rock (originally obtained from the late G. B. Craig, University of Notre Dame, Notre Dame, IN) was used for comparisons.

Insecticides: Seven technical-grade insecticides were used for selection and bioassay: the organophosphates temephos (91%; American Cyanamid, Princeton, NJ), fenthion (95%; Mobay, Kansas City, KS), malathion (92.8%; American Cyanamid), chlorpyrifos (98%; Dow Chemical Co., Midlands, MI), and ethyl-parathion (98.5%; Monsanto,

St. Louis, MO), the pyrethroid permethrin (94.6%, ICI Americas, Inc., Richmond, CA), and the carbamate propoxur (99%; Mobay). Two synergists were used to help detect detoxification enzymes involved in resistance: *S,S,S*-tributyl phosphorothioate (DEF), an esterase inhibitor, and piperonyl butoxide (pb), an inhibitor of mixed function oxidases.

Selection and bioassay procedures: The selection procedure for the Tortola-Sel strain involved exposing groups of 50 early 4th-stage larvae to temephos in 100 ml of tap water in 177-ml waxed paper cups for 24 h. Concentrations used for selection ranged from 0.5 μg/ml in generation 4 to 1 μg/ml in generation 16. Mortality levels were maintained between 60 and 90%. Survivors were transferred to clean water, fed, and used to continue the colony. One thousand to 5,000 larvae were selected in each generation. Generations were nonoverlapping. The Tortola colony was reared under similar conditions but without insecticide selection pressure.

Bioassay tests utilized standard methods (Georghiou et al. 1966, 1987), which are briefly described here. Groups of 20 early 4th instars were placed in 177-ml waxed cups in 99 ml of tap water and 1 ml of insecticide solution in acetone. Control cups received acetone without insecticide. Five or more concentrations of insecticide, providing mortality between 2 and 98% after 24 h, were replicated on 5 different days. Data were subjected to probit analysis (Finney 1971) using a BASIC program (Raymond 1985). Resistance ratios were calculated at the median lethal concentration (LC_{50}) and LC_{95} by comparing the estimated lethal concentration values of the 2 Tortola strains with those of the susceptible Rock strain. Lethal concentration values with overlapping fiducial limits were not considered to be significantly different.

Synergism tests were similar to the bioassay tests except that 0.5 ml of the desired concentration of synergist was added to each cup, followed by the concentration of insecticide. The sublethal concentrations of synergists against all 3 mosquito strains were 1.0 μg/ml DEF and 3.0 μg/ml pb.

Microtiter plate esterase assay: Total esterase activity in individual, frozen adult mosquitoes (3 days postemergence) from Tortola-Sel, Tortola, and Rock strains was determined according to the method of Dary et al. (1990). Thirty-two mosquitoes (16 males and 16 females) from each colony were examined. Mosquitoes were homogenized in 100 μl of 100 mM sodium phosphate buffer (pH 6.5) with 0.5% Triton X-100. Debris was removed by centrifugation. Supernatant (20 μl) was diluted with 180 μl of homogenization buffer. Diluted homogenates (50 μl) were then aliquoted into each of 2 wells in a microtiter plate, and 100 μl of substrate solution (50 mM sodium phosphate, pH 6.5; 0.25% Triton X-100; 2 mM α-naphthol acetate; 0.003 mM BW284C5, a specific acetylcholinesterase [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. After an additional 10-min incubation, absorbance was read at 550 nm on a microtiter plate reader (model 2550; BioRad Laboratory, Richmond, CA). Total esterase activity was calculated relative to a standard curve of α-naphthol (2–20 nmoles/well). Specific esterase activity was calculated relative to the total protein present in each mosquito to compensate for size differences (Dary et al. 1990).

Microtiter plate AChE assay: Inhibition of AChE was as described by ffrench-Constant and Bonning (1989) with minor modifications. The original homogenate (70 μl) from the samples that were prepared for the esterase test was diluted 10-fold with 100 mM sodium phosphate buffer (pH 7.5) containing 0.5% Triton X-100. Homogenate (100 μl) was aliquoted into each of 4 wells in a microtiter plate. Each well received a different 5-μl treatment, either ethanol, 0.08 mM BW284C5, 2 mM propoxur, or 8 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 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 propoxur or paraoxon by the activity in the control (ethanol).

Inheritance of temephos resistance: Reciprocal mass crosses using adult mosquitoes were prepared between the Rock and Tortola-Sel strains. Virgin males and females were obtained from pupae that were isolated in glass scintillation vials. Approximately 300 males and 300 females were used in each cross. Both parental colonies and the F_1 offspring of the reciprocal crosses were bioassayed concurrently. The backcross was prepared between the F_1 (Tortola-Sel × Rock) females and Rock males. The backcross offspring were bioassayed with temephos.

RESULTS

The dose–response values and resistance ratios for the Rock, Tortola-Sel, and Tortola strains toward a spectrum of insecticides are reported in Table 1. After 13 generations of selection, temephos resistance in the Tortola-Sel strain increased almost 4-fold, from an initial resistance ratio of 46.8 to 180.6 at the LC_{95}. Significant, but much lower, levels of resistance were detected to fenthion (13.8-fold), parathion (8.8-fold), malathion (6.0-fold), and chlorpyrifos (5.8-fold). Propoxur resistance

Table 1. Resistance and cross-resistance in *Aedes aegypti* from Tortola, British Virgin Islands, after 13 generations in the presence or absence of temephos selection.[1]

Insecticide Strain	LC_{50} (μg/ml) (fiducial limits)	LC_{95} (μg/ml) (fiducial limits)	Slope	Resistance ratio	
				LC_{50}	LC_{95}
Temephos					
Rock	0.0124 (0.0118–0.0131)	0.0222 (0.0205–0.0245)	6.6	1.0	1.0
Tortola-Sel	0.953 (0.697–1.30)	4.01 (1.92–8.53)	2.6	76.9	180.6
Tortola	0.0603 (0.0510–0.0712)	0.189 (0.136–0.266)	3.3	4.9	8.5
Fenthion					
Rock	0.0142 (0.0114–0.0177)	0.0274 (0.0181–0.0418)	5.8	1.0	1.0
Tortola-Sel	0.169 (0.159–0.180)	0.378 (0.338–0.435)	4.7	11.9	13.8
Tortola	0.0530 (0.0493–0.0575	0.158 (0.133–0.201)	3.5	3.7	5.8
Malathion					
Rock	0.112 (0.0910–0.137)	0.126 (0.143–0.331)	5.7	1.0	1.0
Tortola-Sel	0.337 (0.313–0.359)	0.758 (0.687–0.854)	4.7	3.0	6.0
Tortola	0.282 (0.263–0.302)	0.591 (0.523–0.700)	5.1	2.5	4.7
Chlorphyrifos					
Rock	0.0111 (0.00928–0.0133)	0.0203 (0.0140–0.0306)	6.3	1.0	1.0
Tortola-Sel	0.0489 (0.0456–0.0523)	0.116 (0.104–0.132)	4.4	4.5	5.8
Parathion					
Rock	0.0125 (0.00970–0.0161)	0.0239 (0.0138–0.0419)	5.8	1.0	1.0
Tortola-Sel	0.119 (0.113–0.126)	0.211 (0.194–0.236)	6.6	9.5	8.8
Propoxur					
Rock	0.883 (0.826–0.939)	1.87 (1.69–2.11)	5.1	1.0	1.0
Tortola-Sel	2.42 (1.99–2.93)	8.90 (6.05–13.3)	2.9	2.7	4.8
Tortola	2.62 (1.96–3.49)	10.4 (5.98–18.4)	2.7	3.0	5.6
Permethrin					
Rock	0.00101 (0.00078–0.00131)	0.00430 (0.00258–0.00731)	2.6	1.0	1.0
Tortola-Sel	0.0511 (0.0470–0.0555)	0.185 (0.160–0.219)	2.9	50.6	43.0
Tortola	0.0246 (0.0201–0.0301)	0.122 (0.0835–0.183)	2.4	24.3	28.4

[1] LC_{50}, median lethal concentration; LC_{95}, 95% lethal concentration.

was 4.8-fold at the LC_{95}. A surprisingly high level of permethrin resistance, 43.0-fold at the LC_{95}, was also detected.

In the absence of selection pressure, the temephos and fenthion resistance ratios in the Tortola strain declined to 8.5 and 5.8, respectively, at the LC_{95}. However, the malathion and propoxur dose–response values were not significantly different between the selected and unselected colonies. The resistance ratio to permethrin was 28.4 at the LC_{95}.

The temephos resistance ratio in the Tortola-Sel strain was reduced from 180.6 to 3.1 at the LC_{95} in the presence of the esterase inhibitor DEF (Table 2). The oxidase inhibitor pb had no effect on the

Table 2. Effect of the synergists *S,S,S*-tributyl phosphorothioate (DEF) and piperonyl butoxide (pb) on resistance levels to temephos and propoxur on the susceptible Rock strain and the temephos-resistant Tortola-Sel strain of *Aedes aegypti*.[1]

Insecticide and synergist Strain	LC_{50} (μm/ml) (fiducial limits)	LC_{95} (μg/ml) (fiducial limits)	Slope	Resistance ratio LC_{50}	Resistance ratio LC_{95}
Temephos + DEF					
Rock	0.00485 (0.00300–0.00780)	0.0113 (0.00471–0.0278)	4.4	1.0	1.0
Tortola-Sel	0.0159 (0.0150–0.0169)	0.0349 (0.0341–0.0398)	4.8	3.3	3.1
Propoxur + pb					
Rock	0.219 (0.157–0.311)	0.474 (0.201–1.29)	4.9	1.0	1.0
Tortola-Sel	0.816 (0.666–0.994)	3.13 (2.27–4.37)	2.8	3.7	6.6

[1] LC_{50}, median lethal concentration; LC_{95}, 95% lethal concentration.

activity of propoxur and the resistance ratio for this combination was 6.6 at the LC_{95}.

Biochemical testing revealed the presence of elevated esterase activity in both the Tortola-Sel and the unselected Tortola strains (Table 3). The average nonspecific esterase activity in the Tortola-Sel and Tortola strains was 9.8- and 2.6-fold higher, respectively, than the mean esterase activity of the Rock strain. No evidence was found of insensitive AChE in the Tortola-Sel strain. The percent residual AChE activity in the presence of propoxur was not significantly different for the Rock (0.049 ± 0.19% [SD], n = 32) and Tortola-Sel (0.063 ± 0.11, n = 32) strains, nor was any significant difference observed using paraoxon as the inhibitor (Rock, 10.9 ± 4.6%; Tortola-Sel, 12.1 ± 4.3%).

The F_1 offspring of the reciprocal crosses between the Rock and Tortola-Sel strains showed intermediate levels of temephos resistance. Resistance was not significantly different between the reciprocal F_1 families (Fig. 1). Analysis of the temephos dose–response line of the backcross offspring revealed a distribution with a distinct plateau over a range of concentrations resulting in 50–70% mortality. However, chi-square analysis indicated that the observed mortality was greater than expected under a monofactorial model, because of high mortality at the 3 lowest temephos concentrations (χ^2 = 56.7, df = 11, $P < 0.05$). If these 3 dose–mortality values were excluded, the observed and expected mortalities of the remaining concentrations were not significantly different from each other (χ^2 = 5.89, df = 8, $P > 0.05$).

Table 3. Average esterase activities in the susceptible Rock strain, the temephos-resistant Tortola-Sel strain, and the unselected Tortola strain of *Aedes aegypti*.

Strain	Number	Mean esterase activity (nmoles/min/mosquito)	SD
Rock	32	2.327	1.383
Tortola-Sel	32	22.785	6.654
Tortola	32	6.025	2.238

DISCUSSION

In this study we have shown that *Ae. aegypti* has the potential to develop a high level of temephos resistance in the field and that such resistance can increase under intensive selection pressure. This resistance declined in the absence of selection pressure, was associated with elevated esterase activity, and was most likely inherited as a monofactorial trait.

Despite the potential for high resistance, the levels observed at most field sites have been considerably lower. Recent analysis of *Ae. aegypti* susceptibility from the same location (Sea Cows Bay, Tortola, British Virgin Islands) demonstrated declining temephos resistance levels from the 46.8-fold level detected in 1985 (Georghiou et al. 1987) to 12.1-fold in 1992–93 (Rawlins and Hing Wan 1995) and to 6.3-fold in 1995–96 (Rawlins 1998). Larvicidal control for *Ae. aegypti* has not been recommended for the past 2–3 years in this region (Rawlins 1998), which may account for the observed decline. These data, in conjunction with the decline in temephos resistance in the unselected Tortola colony, suggest that temephos resistance is unstable in the absence of selection pressure. Thus, changing to a new insecticide without temephos cross-resistance, or rotating among several such insecticides, may be advantageous in managing such resistance.

The high level of temephos resistance in our selected colony was associated with low to moderate levels of resistance and/or cross-resistance to the other OP insecticides tested. The highest level of resistance noted was to fenthion, which is consis-

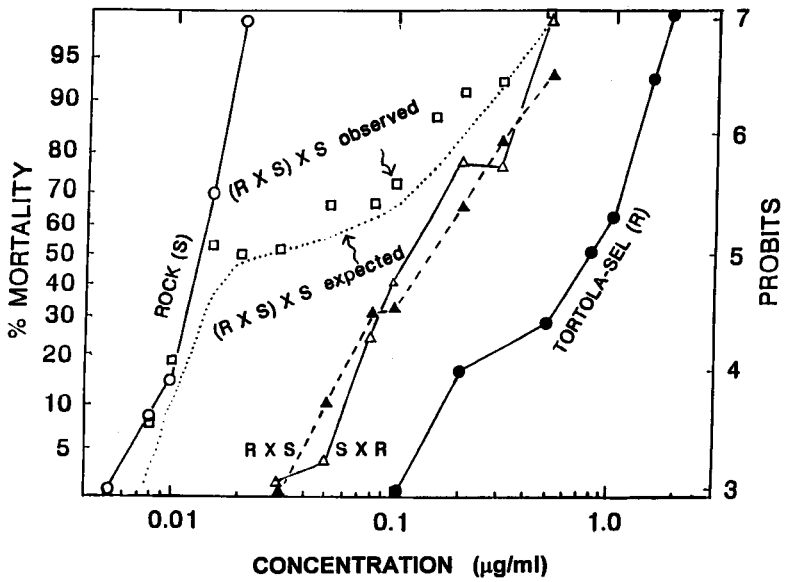

Fig. 1. Temephos dose–response regression lines on susceptible Rock strain (S), temephos-resistant Tortola-Sel strain (R), their F_1 offspring (R × S, Tortola × Rock; S × R, Rock × Tortola), and the backcross [(R × S) × S, (Tortola × Rock)F_1 × Rock]. The dotted line represents the line expected under a monofactorial model of inheritance.

tent with resistance patterns in field surveys of the Caribbean region, as is the low level of malathion resistance (Georghiou et al. 1987, Rawlins and Ragoonansingh 1990, Rawlins and Hing Wan 1995, Rawlins 1998). Malathion resistance in the Tortola strain failed to increase significantly under additional temephos selection pressure, which, together with the low malathion resistance levels reported from this area, suggests this material will continue to be effective.

Toxicologic and biochemical tests established increased esterase activity as the primary mechanism of temephos resistance in the Tortola strains. Elevated esterase activity associated with chlorpyrifos and temephos resistance, respectively, has been reported in *Ae. aegypti* from Venezuela (Mazzarri and Georghiou 1995) and Trinidad (Vaughan et al. 1998), and was linked with malathion resistance in *Ae. aegypti* from Puerto Rico (Field et al. 1984). The linkage between increased esterase activity and OP resistance is well established in the *Culex pipiens* complex (Georghiou et al. 1980, Prabhaker et al. 1987, Wirth et al. 1990, Poirié et al. 1992, Wirth 1998) and in other insects (Devonshire and Sawicki 1979, Devonshire and Field 1991). Although overproduction of esterases associated with OP resistance in *Culex* results from amplification of the esterase genes and/or increased transcription (Mouches et al. 1986, Raymond et al. 1989, Poirié et al. 1992, Vaughan and Hemingway 1995), the underlying mechanism of increased esterase activity in *Ae. aegypti* is not yet known.

Simple crossing experiments showed that temephos resistance was intermediate in dominance in the F_1 generation and the backcross regression line suggested that a single major gene is involved, despite the deviation from the monofactorial model at the lowest dose–response concentrations (Fig. 1). Variation in susceptibility between the 2 parental strains was considered the source of a similar deviation in *Cx. pipiens* that were resistant to *Bacillus sphaericus* (Nielsen-LeRoux et al. 1997) and is likely in this case as well.

The focus of this work was the characterization of temephos resistance and the discovery of significant permethrin resistance in the Tortola and Tortola-Sel strains in the later stages of this study was unanticipated. Pyrethroid resistance has been reported in *Ae. aegypti* from Puerto Rico (Hemingway et al. 1989), the Dominican Republic (Mekuria et al. 1991), and Venezuela (Mazzari and Georghiou 1995). Such resistance could be cross-resistance from DDT resistance in the Tortola-Sel strain, which demonstrates 100% survival to the DDT World Health Organization diagnostic dose (0.012 mg/liter) (data not shown), or it may have been independently selected. Further research is needed to determine the precise mechanism(s) involved, because both metabolic-based resistance and nerve insensitivity have been demonstrated in DDT- and pyrethroid-resistant *Ae. aegypti* (McDonald and Wood 1979, Hemingway et al. 1989).

High levels of temephos and permethrin resistance in the Tortola population of *Ae. aegypti* have serious implications for the continued effectiveness of these materials. Alternative materials, such as *Bacillus thuringiensis* var. *israelensis* and methoprene should be seriously considered for incorporation into larval

control programs. Continued monitoring of insecticide susceptibility in *Aedes* populations is critical for informed decisions on insecticide use; however, the emphasis on source reduction and environmental sanitation must continue to decrease reliance on insecticides and reduce selection pressure on resistant populations.

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