Journal of Medical Entomology

Articles

DR. JUAN BISSET LAZCANO

INSTITUTE OF TROPICAL MEDICINE “PEDRO KOURÍ”.

Autopista Novia del Mediodia K m 6 1/2. A. Postal

601.”Marianao 13. Havana City. Cuba.

Telephone: 53-7-220650

E-mail: bisset@ipk.sld.cu

Running head: Insensitive *ace*tylcholinesterase in *Ae.aegypti*

SELECTION OF INSENSITIVE *ACE*TYLCHOLINESTERASE AS A RESISTANCE MECHANISM IN *AEDES AEGYPTI* FROM *SANTIAGO DE CUBA*

JUAN BISSET, MARÍA M. RODRÍGUEZ, AND DITTER FERNÁNDEZ

Vector Control Department

Institute of Tropical Medicine ¨Pedro Kourí¨

Autopista Novia del Mediodía Km 61/2, entre Autopista Nacional y Carretera Central

A. Postal 601, Marianao 13, Ciudad Habana, Cuba.

ABSTRACT

A sample of *Aedes aegypti* (Diptera: Culicidae) from SANTIAGO DE CUBA, Cuba, with a high level of propoxur resistance compared to the reference susceptible ROCKEFELLER strain (12.60x at the 50 % lethal concentration [LC_{50}] and 18.08 at the 90 % lethal concentration [LC_{90}]), with a 4.3 per cent frequency of insensitive AChE frequency was subjected to propoxur selection for 13 successive generations to increase the frequency of this resistance mechanisms in *Aedes aegypti*. High resistance to propoxur was developed during this selection (41.73 fold) and the frequency of insensitive *ace*tylcholinesterase mechanism was increased 13.25 fold. Other mechanisms (overproduced esterases, glutathione transferases or monooxygenases) were not detected in the propoxur selected strain. The selection of an insensitive *ace*tylcholinesterase resistance mechanism in *Aedes aegypti.* has important implications and will be a valuable resource for genetic studies and molecular characterization of the *ace* gene mutation(s) associated with insecticide resistance in *Ae. aegypti*.

KEY WORDS: *Aedes aegypti*, insensitive *ace*tylcholinesterase, cross resistance, mechanisms, Cuba

Insecticide resistance in vector mosquito populations remains a major problem in the control of parasitic diseases. It results from three main types of mechanism: reduction in the insecticide penetration; increased metabolism of the insecticide by esterases, monooxygenase, or glutathione transferases; and modification of the insecticide target (Mutero *et al.* 1994). *Ace*tylcholinesterase (*AChE*: *ace*tylcholine *ace*tylhydrolase) is the major target for *organophosphate* and carbamate insecticides which inhibit enzyme activity by phosphorylating or carbamylating the serine residue within the active site gorge (Anthony *et al.* 1995). *AChE* catalyses the hydrolysis of the neurotransmitter *ace*tylcholine and is essential for cholinergic transmission in the insect nervous system (Toutant, 1989).

AChE insensitivity is a frequent resistance mechanism in insects and responsible mutations in the *ace* gene associated with resistance were first described in two Diptera*: D. melanogaster* (Mutero *et al.* 1994) and *M. domestica* (Kozaki *et al.* 2001 and Walsh *et al.* 2001). More recently a single mutation in the ace-1 gene associated with insecticide resistance was reported in *Cx. pipiens* and *An. gambiae* (Weilll *et al.* 2004a). However involvement of *ace* gene in insecticide resistance had not been described in some species such as *Aedes aegypti*. Indeed, it has been shown, based on a silent base in single codon of the *Aedes aegypti* ace-1 gene, that the mutation leading to AChE insensitivity is unlikely to occur (Weilll *et al.* 2004b)

Here we report an *Aedes aegypti* strain with a high frequency of an insensitive *AChE* resistance mechanism which will allow investigation of gene mutations responsible for insecticide resistance in this species.

MATERIALS AND METHODS

Insects: (1) ROCKEFELLER: A reference susceptible laboratory strain of Caribbean origin, colonized in the early 1930's, provided by the CDC laboratory in San Juan, Puerto Rico, (2) SANTIAGO DE CUBA (STC), collected during the epidemic of dengue occurred in 1997 from the locality of the same name, colonized and maintained in the Institute of Tropical Medicine "Pedro Kourí" (IPK). This strain was selected for 13 generations by exposing 4th instars to propoxur at the 90% mortality level, (3) Each generation from the propoxur selection procedure was labeled as FxP where x corresponds to the number of each generation of selection from F1P to F13 P.

Larval bioassays followed Georghiou *et al.* (1987). Twenty early 4^{th} instar larvae of uniform size were pl*ace*d in plastic cups containing 99 ml of tap water and 1 ml of insecticide solution of the desired concentration in *ace*tone. Five or more concentrations of each insecticide, prepared in standard (weight/volume) *ace*tone solution, were used in at least 5 replicates on different days. The control cup was treated with 1 ml of *ace*tone alone. Mortality was determined 24 h after insecticide treatment. Each bioassay was replicated at least twice. Results were subjected to probit analysis by the method of Finney (1971) using a basic program (Raymond 1985). Resistance ratios were calculated at the LC_{50} by comparison to the susceptible reference strain.

Resistance mechanisms

The action of two synergists S,S,S, tributyl phosphorotrithioate (DEF) and piperonyl butoxide (PB) were determined by exposing 4^{th} instar larvae to 0.008 mg/l DEF or 5.0 mg/l PB for 4h prior to the addition of insecticide solution (Ranasinghe & Georghiou 1979). At these concentrations there was no mortality with the synergist alone.

Biochemical tests

The normal activity and propoxur inhibited ***AChE*** was measured following the method of Hemingway *et al.* 1986. Standard solutions of $1x10^{-2}$ M solutions of 100 % pure propoxur were prepared in acetone. These were diluted 1:3 with phosphate buffer (0.02 M, pH 7.5) immediately before use. Solutions of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), (1, 36 mg/ml) and acetylthiocholine iodide (1.08 mg/ml) in phosphate buffer were prepared fresh on the day of testing. A quarter of the mosquito homogenate was used to assay normal uninhibited *AChE* activity; a further 0.25 ml was used for the propoxur inhibition studies. Two replicate aliquots of homogenate from a single mosquito were placed in a microtitre plate, and 10 μl of propoxur solution were added to one replicate. After 2 min, 25 μl of the acetylthiocholine iodide solution plus 20 μl of the DTNB solution were added to all replicates. The enzyme reaction was then allowed to run for 30 min, and the absorbance was read at 420 nm in Labsystems iMS Reader MF.

The esterase activity was determined in fourth instar larvae according to modified methods for *Aedes aegypti* (Rodríguez *et al.* 2001). 20 μl of each homogenized larvae were added to microtitre plates of 96 wells, and mixed with 200 μl of 0.7 mM β-naphthyl *ace*tate substrate. After 10 minutes of reaction, 40 μl of Fast-blue were added and the O. D. was read at 570 nm in a Labsystems iMS Reader MF. Values of O.D above 1.227 were considered as high esterase activity.

The glutathione- S-transferase (GST) activity was determined according to the method of Booth *et al.* (1961), and modified for *Ae. aegypti (*Rodríguez *et al.* 2001). Each larvae homogenate (20 μl) was added to a reaction mixture of 250 μl of 50 mM 1-chloro-2, 4 dinitrobenzene and 5 ml of 20 mM reduced glutathione. After 3 min, the O.D. was read at 340

nm in a Labsystems iMS Reader MF. . Each value of enzymatic GST activity was determined in the homogenate and activity was expressed in μmol/ mg.ml. Values above 0.6694 μmol/ mg.ml were considered significantly different from the susceptible (Rodríguez *et al.* 2001).

RESULTS

The SANTIAGO DE CUBA (STC) strain was 12.60 fold more resistant than the susceptible reference ROCKEFELLER strain at the 50 % lethal concentration (LC_{50}) and 18.08 at the 90 % lethal concentration (LC_{90}). After 13 generations of propoxur selection the resistance ratio at the LC_{50} increased to 41.73 fold and 86.19 fold respectively (table 1). The level of propoxur resistance was increased approximately 10.66 fold during propoxur selection, from a Resistance Ratio (RR_{90}) value of 8.08 in STC to 86.19 in F13 strain (Fig 1). The insensitive *AChE* frequency was increased in each generation of selection, from 4.3 % in STC to 57 % in F13P strain (Fig 1). A highly significant correlation (r= 0.92) between the variation in propoxur resistance and the insensitive *AChE* frequency ($p<0.05$) was observed in each generation from the propoxur selection (F1P-F5P, F7P, F9P, F13P).

The effect of the synergists DEF and PB on resistance to propoxur was evaluated in STC and in the last generation of selection F13P. Results indicated that neither esterase nor monooxygenase based mechanisms are involved in propoxur resistance in STC or F13 P because the Synergism values (SR) value in both were less than five and very close to the susceptible reference strain ROCKEFELLER (Table 2). This observation correlates well with the observed frequencies of esterase and GST activity during propoxur selection (Table 3). The frequencies of overproduced GST and esterase did not show appreciate variation after 13 generations of selection.

Resistance levels to the organophosphates: temephos, malathion, chlorpyrifos, fenthion, fenitrothion and pirimiphos-methyl are shown in Figure 2. The Resistance Ratio calculated from 50 % lethal concentration (RR_{50}) declined for temephos, but all the RR_{50} values were less than 10 fold to this chemical. The RR_{50} values for malathion and pirimiphos-methyl remained stable but it showed a tendency to increase for chlorpyrifos. Similar results were observed for

fenitrothion and fenthion, in both the RR_{50} did not exhibit remarkable change from STC to F3P, but it was increased from F3 to F4 and declined from F5P to F13P, showing an negative cross resistance effect at thirteen generations of propoxur selection.

DISCUSSION

The toxicity of OP and carbamate insecticides is due to the inhibition of *AChE* activity in cholinergic synapses and resistance to these compounds is the result of the reduced inhibition of cholinergic *AChE*, a phenomenon that has developed following extensive use of these pesticides for pest management in agriculture and Public Health.

The insensitive *AChE* resistance mechanism has been reported in *Culex pipiens* from Italy (Villani & Hemingway, 1987); Bourguet *et al.* (1996) showed that this mechanism was solely responsible for resistance to propoxur in the PRAIAS *Culex pipiens* strain, from Portugal and insenstivie AChE has also been reported in *Culex quinquefasciatus* from Cuba (Bisset *et al.* 1991, Díaz *et al.* 1993).

Propoxur selection pressure increased resistance in SANTIAGO DE CUBA strain of *Ae aegypti* to about 41.72 fold, when compared the 13^{th} generation of propoxur selection ($F_{13}P$) with the susceptible reference strain. The increase in levels of propoxur resistance during the selection process was well correlated with an increase in the frequency of insensitive AChE mechanism. Significant changes were not detected in the other resistance mechanisms evaluated. The effect of DEF and PB synergists on propoxur resistance was low or absent, indicating that increased detoxification by esterase or monooxygenase-based mechanisms of resistance can only play a minor role in propoxur resistance. These results point towards the insensitive *AChE* as the mechanism responsible for resistance to propoxur. In *Aedes aegypti* from Cuba, collected during the last epidemic of dengue which occurred in 2002 focusing in Havana City, Playa Municipality showed the highest *Aedes aegypti* infestation. In this municipality the *AChE* insensitive mechanism was observed in larvae to a high frequency, and propoxur resistance was high too, the synergism study with DEF and PB did not support a role for the metabolic enzymes (Bisset *et al.* 2004) in conferring resistance.

This is the first report of such resistance mechanism in *Aedes aegypti*. Although the *ace-1* gene has been cloned from this species, no mutations have been detected in this gene associated with an insensitive *AChE* (Weill *et al.* 2002). This contrasts with the report of a single mutation associated with insecticide resistance in *Cx. pipiens* and *An. gambiae* (Weill *et al.* 2004a). It has been established that a silent base of a single codon represents a major constraint to the evolution of an insensitive AChE-based insecticide resistance mechanism in *Aedes aegypti* (Weill *et al*. 2004b).

Little or not cross resistance was observed to the Ops insecticides evaluated during propoxur selection process, however a negative cross resistance was observed to fenitrothion and fenthion at the thirteen generation of selection (F13P). This contrasts with observations on insensitive AChE-based resistance in *Culex* species. For example, in *Culex quinquefasciatus* from Cuba, where a strain was selected with malathion for 22 generations, a high frequency of an insensitive *AChE* mechanism was responsible for the cross resistance to propoxur (Díaz *et al.* 1993) and this *AChE* mechanisms, together with an overproduced esterase-based mechanism in *Culex quinquefasciatus* from Cuba generated more broad spectrum to organophosphate and carbamate resistance than each mechanism separately (Bisset *et al.* 1990, 1991, Rodriguez *et al*. 1996). In *Culex pipiens* from Tunisia the chlorpirifos resistance was associated with an insensitive *AChE* mechanisms and not metabolic enzyme-based resistance mechanisms (Pasteur *et al.* 1999).

The diagnosis and selection of an insensitive AChE-based mechanism of propoxur insecticide resistance in a strain of *Aedes aegypti* from Cuba reported here has important implications in relation to the evolution of insecticide resistance in this important vector species. The strain will be an important tool in further molecular and biochemical studies to elucidate the mutation(s) associated with the insensitive AChE mechanism.

ACKNOWLEDGMENTS

We thank Dr. Graham Small and Dr. Hilary Ranson for their advice. This work was supported by a World Health Organization/TDR special program for research and training.

REFERENCES

Anthony, N., T. Rocheleau, G. Mocelin, H.J. Lee, and R. Ffrench-Constant. 1995. Cloning, sequencing and functional expression of an *ace*tylcholinesterase gene from the yellow fever mosquito *Aedes aegypti* . FEBS Lett. 368: 461-465.

Bisset, J.A., M.M. Rodríguez, C. Díaz, E. Ortiz, M.C. Marquetti, and J. Hemingway. 1990. The mechanism of organophosphate and carbamate resistance in *Culex quinquefasciatus* (Diptera: Culicidae) from Cuba. Bull. Entomol. Res. 80: 245-250.

Bisset, J.A., M.M. Rodríguez, J. Hemingway, C. Díaz, G.J. Small, and E. Ortiz. 1991. Malathion and pyrethroid resistance in *Culex quinquefasciatus* from Cuba : efficacy of pirimiphos methyl in the presence of at least three resistance mechanisms. Med. Vet. Entomol. 5: 223-228.

Bisset, J.A., M.M. Rodríguez, D. Fernández, O. Pérez. 2004. Estado de la resistencia a insecticidas y mecanismos de resistencia en larvas del Municipio Playa, colectadas durante la etapa intensiva contra el *Aedes aegypti* en Ciudad de la Habana, 2001-2002. Rev. Cub. Med. Trop. 56: 61-6.

Booth, J. E., E. Boyland, and P. Sims. 1961. An enzyme from the rat liver catalyzing conjugation with glutathione. Biochem. J. 79 : 516-523.

Bourguet, D., R. Capela, and M. Raymond. 1996. An insesitive *ace*tylcholinesterase in *Culex pipiens* (Diptera: Culicidae) from Portugal. J. Econ. Entomol. 89: 1060-66.

Díaz, C., J.A. Bisset, M.M. Rodríguez. 1993. Selección de una cepa de Culex quinquefasciatus homocigótica para la resistencia al insecticida organofosforado malation. Rev. Cub. Med.Trop. 45:193-196.

Finney, D. J., 1971. Probit analysis. Cambridge University Press. Cambridge. pp.333.

Georghiou, G.P., M. Wirth, H. Tran, F. Saume, and A. B. Knudsen. 1987. Potential for organophosphate resistance in Aedes aegypti in the Caribbean area and neighboring countries.

J. Med. Entomol. 24: 290-294.

Hemingway, J., C. Smith, K.G.I. Jayawardena, P.R.J. Hearth. 1986. Field and laboratory detection of the altered *AChE* resistance genes which confer organophosphate and carbamate resistance in mosquitoes (Diptera: Culicidae) Bull. Ent. Res. 76: 359-66.

Kozaki, T., T. Shono, and Y. Kono. 2001. Fenitroxon insensitive *ace*tylcholinesterases of the housefly, *Musca domestica* associated with point mutations. Insect. Biochem. Mol. Biol. 31: 991-97.

Mutero, A., M. Pralavorio, J.M. Bride, and F. Fournier. 1994. Resistance-associated point mutations in insecticide-insensitive *ace*tylcholinesterase. Proc. Natl. Acad. Sci. USA. 91: 5922-26.

Pasteur, N., M. Marquine, H. Ben Cheikh, C. Bernard, D. Bourguet. 1999. A new mechanism conferring unprecedented high resistance to chlorpyrifos in *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. 36:794-802.

Ranasinghe, L.E., and G. P. Georghiou. 1979. Comparative modification of insecticide resistance spectrum in Culex pipiens fatigans Wied by selection with temephos and temephos synergist combinations. Pest. Sci. 10: 510-518.

Raymond, M. 1985. Presentation d'une programme d'analyse log-probit pour microordinateur cahiers Orstrom Sér. Ent. Méd. et. Parasitol. 23: 117-21.

Rodríguez, M.M., J.A. Bisset, , L. Mastrapa, C. Díaz. 1996. Asociación de la resistencia a insecticidas organofosforados, carbamatos y piretroides con los mecanismos de resistencia observados en cepas de *Culex quinquefasciatus* de Ciudad de la Habana. Rev. Cub. Med.Trop. 47 : 154–59.

Rodríguez, M.M., J.A. Bisset, D. Molina, L. Lauzan, and A. Soca. 2001. Detection of resistance mechanisms in Aedes aegypti from Cuba and Venezuela. J. Med. Entomol .38: 623-628.

Severson, D. W., N. M, Anthony, O. Andreev, and R. Ffrench-Constant. 1997. Molecular

mapping of insecticide resistance genes in the yellow fever mosquito (*Aedes aegypti*). The Journal of Heredity. 88: 520-24.

Toutant, J.P. 1989. Insect *ace*tylcholinesterase : catalytic properties, tissue distribution and molecular forms. Progress in Neurobiology. 32: 423-46.

Villani, F. and J. Hemingway. 1987. The dection and interaction of multiple organophosphorous and carbamate insecticide resistance genes in field populations of *Culex pipiens* from Italy. Pestic. Biochem. Phisiol. 27: 218-28.

Walsh, S. B., T. A. Dolden, G. D. Moores, M. Kristensen, T. Lewis, A.L. Devonsshire, and M. S. Williamson. 2001. Identification and Characterization of mutations in housefly (*Musca domestica*) *ace*tylcholinesterase involved in insecticide resistance. Biochem. J. 359: 175-81.

Weilll, M., P. H. Fort., A. Berthomieu, M. Pierre Dubois, N. Pasteur, and M. Raymond. 2002. A novel *ace*tylcholinesterase gene in mosquitoes codes for the insecticide target an dis non-homologous to the *ace* gene in Drosophila. The Royal Society. 2007-16.

Weilll, M. C. Malcolm, F. Chandre, K. Mongensen, A. Berthomieu, M. Marquine, M. Raymond. 2004a. The unique mutation in *ace*-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol. Biol. 13: 1-7.

Weilll, M et al. 2004b. Insecticide resistance: a silent base prediction.. Curr Biol. 14: R552-3

ANNEXES

Table 1. Resistance ratio to propoxur at the 50 % (LC_{50}) and 90 % (LC_{90}) lethal concentration (RR_{50} and RR_{90}) in SANTIAGO DE CUBA (STC), the last generation of selection (F13P) and the susceptible reference strain (ROCKEFELLER).

Strains	LC_{50} [1] (fiducial limits)	RR_{50} [3]	LC_{90} [1] (fiducial limits)[2]	RR_{90} [3]	Slope (± SD) [2]
STC	4.7 (4.35-5.20)	12.6	9.7 (8.48-2.02)	18.08	4.1 (+/-0.50)
F13P	15.8 (4.26-7.35)	41.7	46.4 (33.56-76.98)	86.19	5.4 (+/-0.18)
ROCKEFELLER	0.4 (0.35-0.41)	-	0.5 (0.49-0.62)	-	8.4 +/-1.08)

[1] LC in mg/liter; 95% FL in parenthesis

[2] Standard deviation

[3] Resistance Ratio ($RR_{50 \text{ or } 90}$): $LC_{50 \text{ or } 90}$ resistant strain/ $LC_{50 \text{ or } 90}$ ROCKEFELLER strain

Fig. 1. Resistance Ratio to propoxur at the 90 % lethal concentration (RR_{90}) . Frequencies of altered acetylcholinesterase mechanism in *Aedes aegypti* from SANTIAGO DE CUBA and the successive generation of selection with propoxur F1, F5, F7, F9, F13 P.

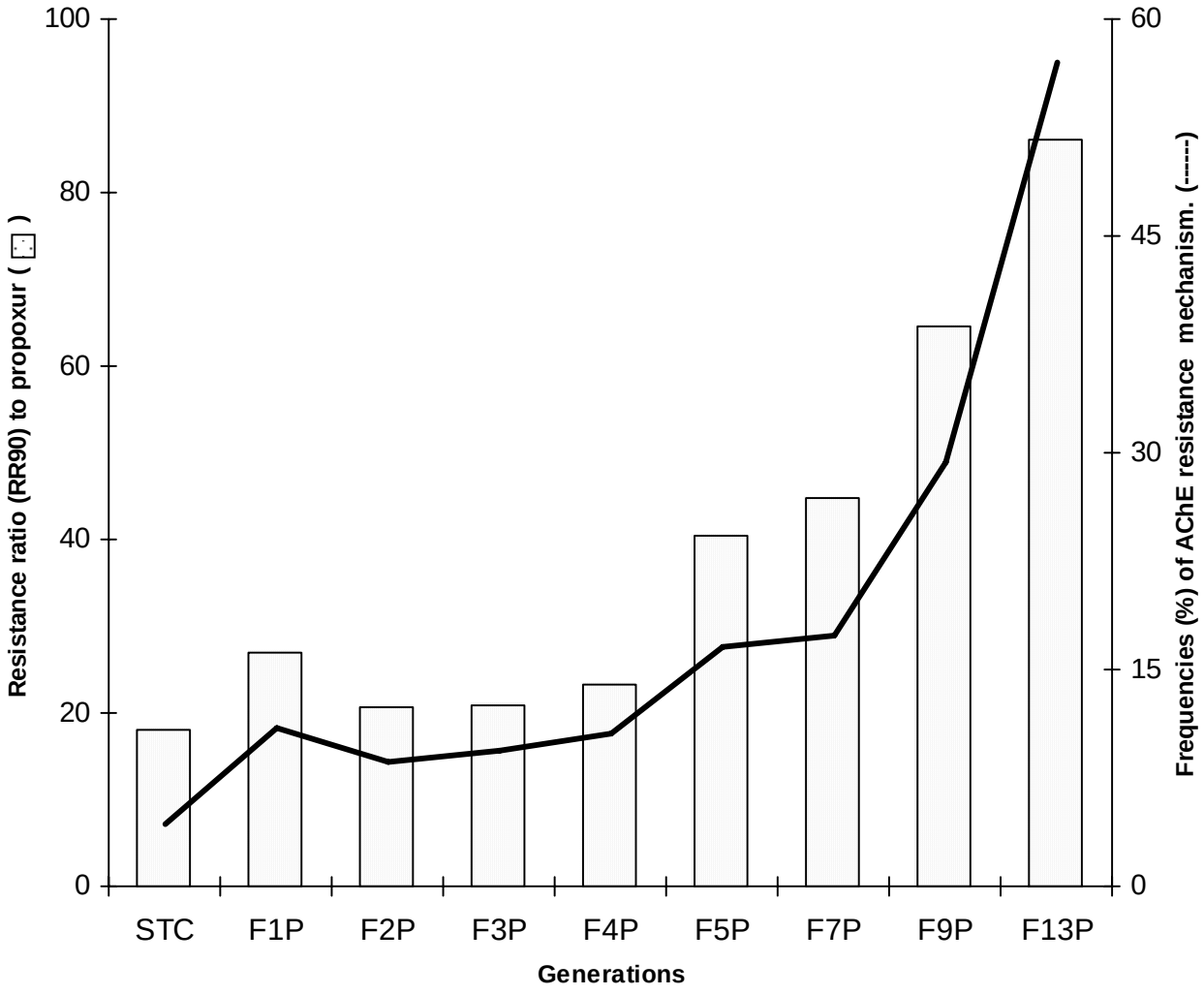

Table 2. Toxicity of propoxur with the synergists DEF and PB to the original strain SANTIAGO DE CUBA, and the last generation of selection with propoxur (F13P) and the *Ae. aegypti* susceptible strain (ROCKEFELLER).

Strains	SR[1] DEF + Propoxur	SR[1] PB + Propoxur
ROCKEFELLER	0.5	0.59
SANTIAGO DE CUBA	1.2	1.81
F13P	1.04	0.99

[1]Synergism ratio (SR)= LC_{50} insecticide alone/ LC_{50} insecticide + synergist DEF or PB.

Table 3. Frequency values (%) of the esterases and GST mechanisms observed in *Aedes aegypti* from SANTIAGO DE CUBA (STC) and the thirteen generation of selection with propoxur (F13P).

Strain	Esterase (%)[1] (n)	GST (%)[2] (n)
STC	12.0 (350)	13.0 (350)
F13P	11.2 (186)	12.5 (186)

[1] Esterase activity was measured as optical density.

[2] Glutathione transferase was measured as enzymatic activity

(n) The total of individual tested is in breakers

Fig. 2. Resistance Ratio to the organophosphates temephos, malathion, chlorpiriphos, fenitrothion, fenthion and p. methyl at the 50 % lethal concentration LC_{50} in Santiago de Cuba (STC) and the successive generation of selection F1-F5 and F13P.