Research Article

Effect of phenobarbital on inducing insecticide tolerance and esterase changes in *Aedes aegypti* (Diptera: Culicidae)

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Abstract

The effect of phenobarbital (PB) on the induction of tolerance to the organophosphorous insecticide temephos (TE) was investigated in *Aedes aegypti* L4 larvae submitted to two different PB-treatments:(1) continuous treatment from the egg to the larval L4 stage and (2) discontinuous treatment in which L4 larvae were exposed for 30 h. Mosquitoes from two Brazilian cities were studied: São José do Rio Preto (SJ) in São Paulo State and Goiânia (GO) in Goiás State. According to criterions established by World Health Organization (WHO) mosquitoes from SJ are organophosphate-susceptible while mosquitoes from GO are organophosphate-resistant. For both SJ and GO larvae the two different PB-treatments resulted in significantly increased tolerance (measured by reduced mortality) to 0.01mg/L TE while for larvae exposed to 0.02 mg/L TE only continuous PB-treatment resulted in significantly increased TE-tolerance. The reduction of mortality rate was greater in SJ larvae than in GO larvae, confirming data from other organisms indicating that the effect of PB is more pronounced in susceptible strains. To test if oxidase enzymes were involved in PB-induced tolerance we treated PB-pretreated SJ and GO larvae with the oxidase inhibitor piperonyl butoxide (PBO) before exposure to TE and observed increased (rather than decreased) tolerance, suggesting that oxidases are not involved in the tolerance process and that PB and PBO can act in concert or synergistically. Esterase patterns of PB-pretreated larvae indicated that the cholinesterases EST-13 and EST-14 are involved in the PB-induced TE- tolerance, reinforcing a previous study carried out in our laboratory which suggested that increased esterase synthesis is the mechanism responsible for the development of insecticide resistance in *Aedes aegypti*.

Key words: *Aedes aegypti*, insecticide resistance, phenobarbital, tolerance, pyperonyl butoxide, esterases.

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Introduction

In mammals and insects, organophosphorous insecticides are detoxified by several enzymes such as cholinesterases, carboxylesterases, oxidases and glutathione-S-transferases (GSTs), which act by reducing the amount of free organophosphate (OP) molecules (Terriere, 1984; Kaliste-Korhonen *et al.,* 1998). The detoxification system may be enhanced by chemical stimuli, leading to the production of additional enzymes and causing insecticide tolerance (Scott, 1995). A wide variety of chemicals have been shown to elicit this inductive response. Among them is the barbiturate phenobarbital (PB) which has been used in research into OP insecticide toxicity in organisms such as flies (Scott and Lee, 1993; Fuchs *et al.*, 1994) and rodents (Clement, 1983; Kaliste-Korhonen *et al.*, 1990). Data in the literature has shown that PB moderately decreases the toxic response to some OPs and increases the insecticide LD_{50} values. For the OP soman, this increase was two to twelvefold in rats and two to threefold in mice (Dubois and Knoshita, 1968; Clement, 1983).

Tolerance caused by treatment with PB has been mainly attributed to the induction of carboxylesterases, cholinesterases and cytochrome P-450 isozymes (Ali *et al.*, 1985; Carino *et al.*, 1994; Fuchs *et al.*, 1994; Kaliste-Korhonen *et al.* 1998). In rat liver, PB increased the activity of three distinct carboxylesterases by three to eightfold (Ali *et al.*, 1985), while in *Drosophila melanogaster* PB-treated, the activity of cytochrome P450 enzymes increased by up to 2.5 times, being the induction dose-dependent (Fuchs *et al.*, 1994).

The mechanism or mechanisms by which PB increases enzyme activity in eukaryotes remains unknown, although the hypothesis that insecticide resistance and PB induction involve the same genetic mechanisms is frequently mentioned in the literature (Plapp, 1984; Terriere 1984; Scott and Lee, 1993; Liu and Scott, 1997).

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In recent years our research group has been involved in the study of the development of resistance to insecticides and the mechanisms of such resistance in some Brazilian populations of the mosquito *Aedes aegypti*, which is currently one of the most widespread disease vectors in the world. Dengue, dengue hemorrhagic fever and yellow fever are diseases caused by viruses maintained in a cycle that involves humans and *A. aegypti*. The results of one of our previous studies in *A. aegypti* from the city of São José do Rio Preto in the State of São Paulo suggested that changes detected in the esterase synthesis could be related to the 20-year struggle by sanitary authorities to control these mosquitoes with intensive use of insecticides (Lima-Catelani *et al.*, 2004).

In the research reported in the current paper, we describe the effect of PB-treatment on the induction of tolerance to the organophosphorous insecticide temephos (TE), in two Brazilian populations of *A. aegypti* of different OP-susceptibility. One of these populations is from the city of Goiânia in the State of Goiás (GO) and the other is the already mentioned population from São José do Rio Preto in the State of São Paulo (SJ). According to the criterions established by the World Health Organization (WHO) GO is OP-resistant (Macoris *et al.*, 1995) while SJ, although showing a small decrease of insecticide-susceptibility is still considered OP-susceptible (Macoris *et al*., 1999).

Materials and Methods

Mosquitoes

Four stages characterize the development of *A. aegypti,* which is a holometabolous insect: egg, larva (with subphases L1, L2, L3, L4), pupa and adult.

Larvae and pupae of *A. aegypti* were collected in São José do Rio Preto (SJ) by people who work in the Superintendency for the Control of Endemic Diseases (*Superintendência de Controle de Endemias do Estado de São Paulo*: SUCEN) from tires, cans, bottles and other *A. aegypti* usual breeding sites and brought to be raised in the Laboratory of Vectors at IBILCE-UNESP. Eggs of the mosquitoes from Goiânia (GO) were supplied by Dr. Ionizete Garcia da Silva from the Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia (*Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia*) and also raised in the Laboratory of Vectors. The present work was carried out in 2000 and 2001.

Phenobarbital treatment

Mosquitoes were exposed to phenobarbital (PB) (Gardenal®, Rhodia Farma, Brazil) using two different treatments: (1) continuous treatment, in which *A. aegypti* eggs were allowed to develop in a 0.2 mg/mL PB aqueous solution until L4 larval stage was reached (which took about one week) and (2) discontinuous treatment, in which

L4 larvae were treated during 30 h in 0.26 mg/mL PB aqueous solution. Eggs and larvae were kept in Petri dishes containing 40 mL of the solution, under suitable physical conditions for normal development. Simultaneously to the treatments, controls were made using water without the addition of PB.

Exposure to the organophosphate temephos

PB-pretreated and control (untreated with PB) L4 larvae from SJ and GO were exposed to three concentrations (0.005, 0.01 and 0.02 mg/L) of the organophosphorous insecticide temephos (TE) (Abate®, Cyanamid, Brazil) in aqueous solutions. In each test, groups of 10 larvae were exposed to every insecticide concentration in Petri dishes containing 40 mL of the solution. Two other types of parallel controls were made, some groups of 10 larvae being placed in PB solution in the same concentration as that to which PB-pretreated larvae had been exposed, while other groups were placed in water only. Larval mortality was evaluated after 24 h.

Piperonyl butoxide treatment

The action of the oxidase inhibitor piperonyl butoxide (PBO) (Acros Organics, U.S.A.) was investigated by subjecting different groups of 10 larvae to different combinations of treatments as follows: (a) PB-untreated L4 larvae were placed into 6.0 µL/L PBO aqueous solution for 24 h and then into 0.01 mg/L TE aqueous solution for a further 24 h; (b) PB-discontinuously-pretreated L4 larvae were placed into a 6.0 µL/L PBO plus 0.01 mg/L TE aqueous solution for 24 h; (c) PB-discontinuously-pretreated larvae were placed into a 10 µL/L PBO aqueous solution for 4 h and then exposed to 0.01 mg/L TE aqueous solution for 24 h. In every case, controls were performed omitting the PBO in the sequence of treatments.

Esterase patterns

The esterase patterns of L4 larvae, pupae and male and female adult *A. aegypti* taken from continuous and discontinuous PB treatments along with untreated controls were examined. Esterase analysis was carried out using (8%) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The mosquitoes were individually squashed in 25 µL of buffer (1.5 M Tris-HCl, pH 8.8, plus 10% glycerol) and the mixture allowed to stand until the supernatant separated, 10 µL of the supernatant being used in each sample dropped onto the gel. Electrophoresis was performed for two hours at ~25 °C using a constant voltage of 200 V and 0.1 M Tris-glycine (pH 8.3) solution as the running buffer. After electrophoresis, esterases were identified in the gels as described by Johnson *et al*. (1966) and Steiner and Johnson (1973), by pre-incubating the gels for 45 min at ~25°C in 50 mL 0.1 M aqueous sodium phosphate (pH 6.2) followed by staining the gels in the dark for one hour with a solution containing 30 mg of -naphthyl acetate and

15 mg of β-naphthyl acetate as substrates, 60 mg of fast blue and 5 mL of N-propanol in 50 mL of 0.1 M aqueous sodium phosphate solution. The gels were dried at room temperature using gelatin and cellophane wound slab gels in an embroidering hoop (Ceron *et al.*, 1992). The esterase bands in the gels were numbered according to the system of Lima-Catelani *et al.*, 2004.

Esterase Activity

The activity of esterases in polyacrylamide gels of pupae submitted to continuous treatment with PB was evaluated by using the Global Lab Image Program (SP0550, Data Translation, Inc., MA, USA), a Windows®-based monochrome image processing and analysis package. In this method the intensity analysis is displayed in linear profiles and histograms. The profile tool graphs the gray values (in the present study equating to the intensity of band staining) along a line segment in an image (in this study being the horizontal sequence of bands in the gel). The horizontal axis of the resultant profile graph represents the points along the line segment while the vertical axis represents the pixel value at each point. The full range of grayscale values is from 0 (completely black) to 255 (the absence of color), between which lie intermediate shades of gray. In the present study, the grayscale values given to the bands by the program were used for comparison between treatments and controls.

Statistics

Treatments were compared using the chi-squared (χ^2) test for homogeneity (Mood *et al.*, 1974).

Results

Susceptibility to TE of PB-pretreated and non-pretreated L4 larvae

As shown in Table 1, no mortality of L4 larvae occurred in either water (control) or PB aqueous solution, while in TE aqueous solution mortality of both SJ and GO larvae increased with increasing TE concentration. In the tests involving discontinuous and continuous PB pretreatment the percentage mortality also increased with increasing TE concentration.

In comparisons between PB-pretreated and non-pretreated SJ and GO larvae the mortality of larvae exposed to TE decreased in every test involving pretreated larvae, although the decrease in mortality was only significant for every comparison when TE concentration was 0.01 mg/L (Table 1). In the discontinuous pretreatment, decreased mortality of the SJ larvae was also significant at 0.005 mg/L TE, while in the continuous pretreatment decreased mortality was significant for both the SJ and OP populations at 0.02 mg/L TE.

Comparisons between the SJ and GO populations regarding percentage mortality of L4 larvae in tests without

Table 1 - Percentage mortality of *Aedes aegypti* larvae from São José do Rio Preto (SJ) and Goiânia (GO) in control tests without phenobarbital (PB) pretreatment and in experimental tests after discontinuous and continuous PB-pretreatment. Mortality was measured after the larvae had been exposed to the different concentrations of the organophosphate temephos (TE) for 24 h.

					Control tests							Experimental tests					
					TE (mg/L) (without PB-pretreatment)							TE (mg/L) (after PB pretreatment)					
	Water		PB		0.005		0.01		0.02		PB-Pretreatment	0.005		0.01		0.02	
Population	N	M	N	M	N	M	N	M	N	M		N	M	N	M	N	M
SJ	150	0	150	0	65	63.0	40	80.0	55	94.5	Discontinuous	65	46.1*	40	47.5**	55	87.2
GO	320	0	320	0	135	25.2	110	56.4	90	82.2		135	18.5	110	37.3**	90	71.1
SJ	50	0	50	0	60	45.0	50	86.0	60	100.0	Continuous	60	38.3	50	54.0**	50	86.0**
GO	50	0	50	0	70	20.0	60	46.6	70	81.4		50	12.0	60	23.3**	60	65.0*

N = number of larvae used; M = percentage mortality. Asterisks indicate significant decrease in mortality in tests with TE after PB-pretreatment in comparison with control tests. * = p ≤ 0.05; ** = p ≤ 0.01.

pretreatment or with discontinuous or continuous PB pretreatment, followed by exposure to TE showed significantly higher mortality values for SJ larvae for both types of pretreatment in every comparison except for discontinuous PB pretreatment in which larvae were exposed to 0.01 mg/L TE (Table 2). This data was computed from Table 1, calculating the mortality difference between SJ and GO populations in PB-pretreated and non-pretreated mosquitoes.

Effect of piperonyl butoxide (PBO) on mortality of L4 larvae exposed to TE

As shown in Table 3, a reduction in mortality occurred in experiments in which PB-pretreated larvae were exposed to PBO and 0.01 mg/mL TE simultaneously or in which exposure to TE occurred after PBO treatment. In both cases mortality was significantly lower than in the experiments in which larvae were not exposed to PBO. No mortality was observed in larvae exposed to PBO alone, in the concentrations used for 4- or 24-h treatments, before submitting them to TE.

In the experiments in which the mosquitoes were not PB-pretreated, the mortality differences between the experiments in which larvae were exposed to TE only and those in which the mosquitoes had been pretreated with PBO and then exposed to TE were not significant.

Esterase patterns of PB-pretreated mosquitoes from SJ population: changes in esterase frequency and activity

Esterase band patterns in polyacrylamide gels of mosquitoes from SJ population, in different developmental stages (L4 larvae, pupae and adult males and females), submitted to continuous or discontinuous PB treatments were compared to control mosquitoes (which had not been treated with PB) (Table 4). Significant expression variation, indicated by changes in the frequency of mosquitoes giving band in gels, was observed for PB-treated larvae in respect to bands EST-2 and EST-20 ($p < 0.05$), which showed decreased frequency in the discontinuous treatment. In the continuous treatment, the frequencies of the same two bands and also band EST-4 were significantly reduced in larvae ($p < 0.01$), but the frequency of the band

Table 3 - Comparative mortality (%) of *A. aegypti* L4 larvae from São José do Rio Preto in tests using temephos (TE) only or piperonyl butoxide (PBO) or phenobarbital (PB) before exposure to temephos (PBO → TE or PB → TE), or using the sequence of treatments (PB → PBO → TE or PB → PBO + TE).

PB-pretreatment	Test	N	M (%)
not PB pretreated	TE	14	50.0
	PBO → TE	14	57.5
PB pretreated	PB → TE	30	40.0**
	PB → PBO + TE	30	6.7
PB pretreated	PB → TE	40	52.5*
	PB → PBO → TE	50	32.0

N = number of larvae used; M = mortality percentage. Asteriscs indicate a significant difference in mortality in the comparison of the two tests for each PB-pretreatment (* = $p \leq 0.05$; ** = $p \leq 0.01$).

EST-14 was significantly increased in larvae ($p < 0.01$) and in pupae ($p < 0.05$).

Changes in esterase activity evaluated on the basis of the degree of staining and thickness of the bands in the gels were observed for the continuous PB treatment. In the discontinuous tests the changes were not as evident as in the continuous ones. Bands EST-1, EST-13 and EST-14 showed the greatest changes in esterase activity. Band EST-1 showed decreased activity in larvae, pupae and adults but bands EST-13 and EST-14 showed an increased activity in the larval and pupal stages. Means of pixel numbers and standard deviations obtained for the three bands from treated and control pupae are set out in Table 5. Pupae were chosen for measurements because they showed the greatest activity increase in bands EST-13 and EST-14. Differences in pixel numbers between treated pupae and controls were significant for esterases EST-1 and EST-13. A polyacrylamide gel showing the esterase patterns, and the profile graphs of the three esterase bands are given in Figures 1 and 2, respectively.

Discussion

Insect resistance to insecticides is one of the central subjects as far as disease vectors or agricultural pests are

Table 2 - Comparison between the percentage mortality of São José do Rio Preto (SJ) and Goiânia (GO) *A. aegypti* exposed to the organophosphate temephos (TE) in tests with L4 larvae which had not been pretreated with phenobarbital (PB) and L4 larvae submitted to discontinuous and continuous pretreatments with PB.

Comparisons	PB-pretreatment	Test	Mortality differences (%) in temephos (mg/L) tests		
			0.005	0.01	0.02
SJ x GO	Not pretreated	TE	37.8**	23.6**	12.3*
	Discontinuous	PB → TE	27.6**	10.2	16.1*
	Not pretreated	TE	25.0**	39.4**	18.6**
	Continuous	PB → TE	26.3**	30.7**	21.0*

Asteriscs indicate significant differences. * = $p \leq 0.05$; ** = $p \leq 0.01$.

Table 4 - Effect of PB-treatment on esterase patterns. Percentage of *A. aegypti* mosquitoes from São José do Rio Preto submitted to continuous and discontinuous treatments (T) with phenobarbital (PB) and controls (C) bearing the esterase bands during different developmental stages.

	C (not PB-treated)				(T) Discontinuous				C (not PB-treated)				(T) Continuous			
	L4	P	F	M	L4	P	F	M	L4	P	F	M	L4	P	F	M
N	60	50	28	32	60	50	28	32	63	67	39	39	73	67	41	41
Bands																
EST-21	3.3	-	-	-	1.7	-	-	-	-	-	-	-	-	-	-	-
EST-20	80.0	-	-	-	58.3*	-	-	-	82.5	-	-	-	58.9**	-	-	-
EST-19	-	-	-	-	-	-	-	-	11.1	-	-	-	4.1	-	-	-
EST-18	-	-	-	-	-	-	-	-	7.9	-	-	-	-	-	-	-
EST-16	1.7	-	-	-	-	-	-	-	-	-	-	-	9.5	-	-	-
EST-14	33.3	48.0	46.4	31.2	40.0	36.0	32.1	28.1	36.5	59.7	35.9	35.9	61.6**	76.1*	46.3	26.8
EST-13	41.7	36.0	7.1	6.2	43.3	48.0	14.3	3.1	39.7	37.3	35.9	38.5	34.2	26.9	43.9	34.1
EST-12	20.0	8.0	-	-	18.3	18.0	-	-	9.5	10.4	12.8	17.9	13.7	6.0	14.6	17.1
EST-8	20.0	-	-	-	8.3	-	-	-	7.9	-	-	-	-	-	-	-
EST-7	55.0	50.0	-	-	55.0	44.0	-	-	22.2	53.7	-	53.8	30.1	55.2	-	53.6
EST-6	55.0	18.0	7.1	-	56.7	16.0	3.6	-	50.8	22.4	-	2.6	49.3	19.4	-	2.4
EST-5	31.7	-	-	-	20.0	-	-	-	15.9	7.5	-	-	20.5	3.0	-	-
EST-4	73.3	20.0	-	-	71.7	20.0	-	-	87.3	3.0	10.3	-	65.7**	3.0	4.9	-
EST-3	30.0	-	-	-	38.3	2.0	-	-	41.3	1.5	-	-	28.8	3.0	4.9	9.8
EST-2	38.3	-	-	-	21.7*	-	-	-	46.0	-	-	-	23.3**	-	-	-
EST-1	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
EST-A	-	-	-	-	1.7	2.0	-	-	-	4.5	-	-	-	-	-	-
EST-B	10.0	-	-	-	11.7	-	-	-	20.6	3.0	-	-	17.8	3.0	-	-
EST-C	13.3	-	-	-	10.0	-	-	-	17.5	1.5	-	-	8.2	4.5	-	-
EST-D	10.0	2.0	-	-	10.0	4.0	-	-	6.3	6.0	-	-	1.4	3.0	-	-

L4 = fourth instar larvae; P = pupae; F = adult females; M = adult males; N = number of mosquitoes analyzed; - = absence of the bands. Asteriscs indicate significant differences between percentages in C and T, in both discontinuous and continuous treatments (* = p ≤ 0.05; ** = p ≤ 0.01).

concerned because resistance affects or may even hamper control programs. In recent years, the barbiturate phenobarbital (PB), which promotes increase in the insecticide tolerance, has been considered an important tool in the study of

Table 5 - Effect of PB-treatment on esterase band activity. Mean values and standard deviation in pixels obtained by Image Analysis of the esterase bands EST-1, EST-13 and EST-14, in gels prepared with control (C) and PB-pretreated (T) mosquitoes from São José do Rio Preto.

Bands	Test	N	Pixel numbers $\overline{X} \pm S_{\overline{X}}$
EST-1	T	67	76.7 ± 3.9*
	C	67	56.2 ± 4.3
EST-13	T	18	174.2 ± 9.7*
	C	26	194.1 ± 4.2
EST-14	T	51	159.1 ± 5.8
	C	40	165.3 ± 6.0

N = number of mosquitoes whose bands were measured.
Significant differences in the comparison of pretreated and control band values (* = p ≤ 0.05).

resistance. The work described in this paper was carried out to provide information on the insecticide resistance mechanisms of *A. aegypti*, PB being used in tests with two Brazilian strains. One of them considered organophosphate-susceptible was collected from São José do Rio Preto (SJ) and the other, considered organophosphate-resistant was collected from Goiânia (GO) (Macoris *et al.*, 1995; 1999).

Data on mortality obtained in the comparisons of PB-pretreated and non-pretreated larvae exposed to the insecticide TE (the organophosphate normally used by Brazilian sanitary authorities in larval control) showed that PB also causes insecticide tolerance in *A. aegypti*. In the light of the numbers, mortality of PB-treated larvae decreased in every comparison (Table 1). Considering only the significant data, this tolerance was more efficient at 0.01 mg/L of TE, in which PB treatments differed from control in every test, in both populations. At this TE concentration, both PB-treatments (discontinuous, for 30 h or continuous from egg to larval stage (about one week) were efficient in providing protection against the insecticide. The PB-discontinuous treatment was also significantly efficient for SJ larvae at 0.005 mg/L TE while the continuous treatment at
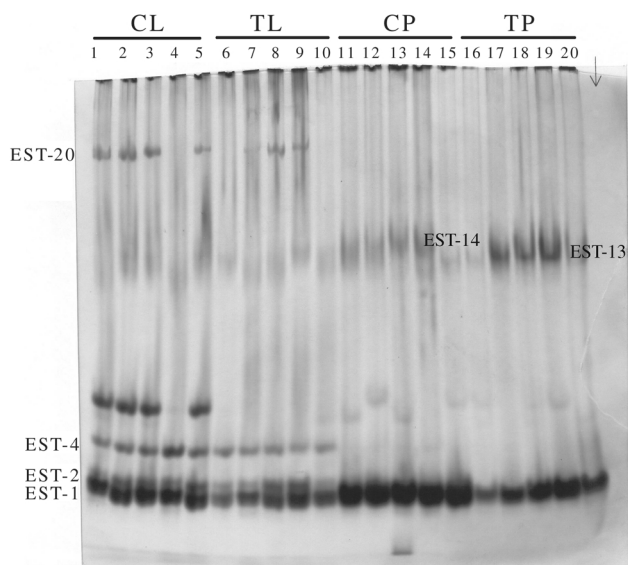
Figure 1 - A polyacrylamide gel showing esterase bands that presented changes in expression, in *Aedes aegypti* larvae and pupae submitted to continuous phenobarbital (PB) treatment and control. The results of five individuals per experiment are shown. CL = control larvae; TL = treated larvae; CP = control pupae; TP = treated pupae.

0.02 mg/L TE was efficient for both SJ and GO larvae. This data indicates that the duration of the PB treatment and the population that is submitted to the treatment, with its genetic features and previous environmental experience, are also important in the results.

Comparisons between SJ and GO PB-pretreated mosquitoes regarding sensitivity to TE reinforced previous observations made on *Drosophila melanogaster* and *Musca domestica* (Yu and Terriere, 1973; Hällström *et al.*, 1984; Lee and Scott, 1989; Scott and Lee, 1993; Fuchs *et al.*, 1994) in which PB induced a higher level of tolerance in insecticide-susceptible strains than in insecticide-resistant ones. Without PB pretreatment, the OP-susceptible SJ larvae were more sensitive to the insecticide than the OP-resistant GO larvae. After discontinuous or continuous PB-pretreatment, larvae from both populations showed a significant decrease in mortality, but those from the SJ population responded with greater intensity to PB and was the only population that showed significantly decreased mortality at 0.005 mg/L TE (Tables 1 and 2). Fuchs *et al.* (1994) explained the lower effect of PB on resistant *D. melanogaster* strains than on susceptible strains by suggesting that PB fails to induce the cytochrome isozymes (which are considered responsible for providing insecticide detoxification in this species) because the resistant strains have a higher basal level of such enzymes which enhances their capacity of degradation of PB.

Tolerance to PB has been mainly attributed to the induction of cytochrome P-450 and esterase isozymes (Carino *et al.*, 1994; Fuchs *et al.*, 1994; Kaliste-Korhonen *et al.*, 1998). In order to look for mechanisms involved in

the PB tolerance response of *A. aegypti* we tested these two possibilities by studying the effect of piperonyl butoxide (PBO) and the esterase patterns of PB-pretreated mosquitoes.

Treatment with PBO is a common procedure to detect cytochrome P-450 involvement in insecticide resistance because PBO inhibits mixed function oxidases and reduces the number of molecules available to interact with the insecticide and consequently reduces resistance. This effect was shown, for example, in *Drosophila* (Brun *et al.*, 1996; Dombrowski *et al.*, 1998) and *Musca domestica* (Liu and Scott, 1997).

In our study, when PB-non-pretreated larvae from São José do Rio Preto were pre-exposed to PBO and then to TE, percentage mortality was higher in PBO-treated larvae than in control, although the difference was not significant (Table 3). If we assume that there is some PBO activity, oxidases might partially explain the insecticide tolerance observed in PB-treated *A. aegypti*. In order to test this hypothesis, PB-pretreated SJ larvae were exposed to PBO, before or at the same time as TE. However, in both cases, significantly lower mortality rates (or higher levels of resistance) occurred, suggesting that oxidases are not involved at all. The reduction was considerably greater when PBO and TE were used simultaneously. One explanation might be that PB and PBO act in addition or synergistically, that is, PBO reinforces the effect of PB. Thus, presumably, oxidases are not responsible for PB-induced tolerance in the strains studied. Support for this finding comes from data on organophosphate resistance studies using PBO in another mosquito, *Culex pipiens*, showing that oxidases are not involved (Wirth, 1998). Two highly active esterases or one insensitive acetylcholinesterase were the mechanisms considered responsible for tolerance in the two *C. pipiens* strains studied. It seems that the absence of involvement of oxidases in PB tolerance might be predominant in mosquitoes.

Relation between esterase activity and resistance to OP is well documented for a large number of insects. Resistant mosquitoes from many places in the world have been characterized over the last few years, revealing amplification of genes responsible for several closely related esterases. In *Culex* mosquitoes insecticide resistance is considered to be due to the amplification of detoxifying genes, resulting in the overproduction of carboxylesterases which detoxify the insecticides by sequestration, that is, by rapid binding and slow turnover (Fournier *et al.*, 1987; Cuany *et al.*, 1993; Jayawardena *et al.*, 1994; Callaghan *et al.*, 1998). In *Culex pipiens*, the increase of OP resistance was ascribed to a series of genetic events occurring over time: the overproduction of the esterase A1, which appeared in or about 1972, the selection of a mutated gene for insecticide-insensitive acetylcholinesterase around 1978, and the overproduction of esterases A4-B4 and A2-B2, detected around 1986 (Pasteur and Richmond, 1996).
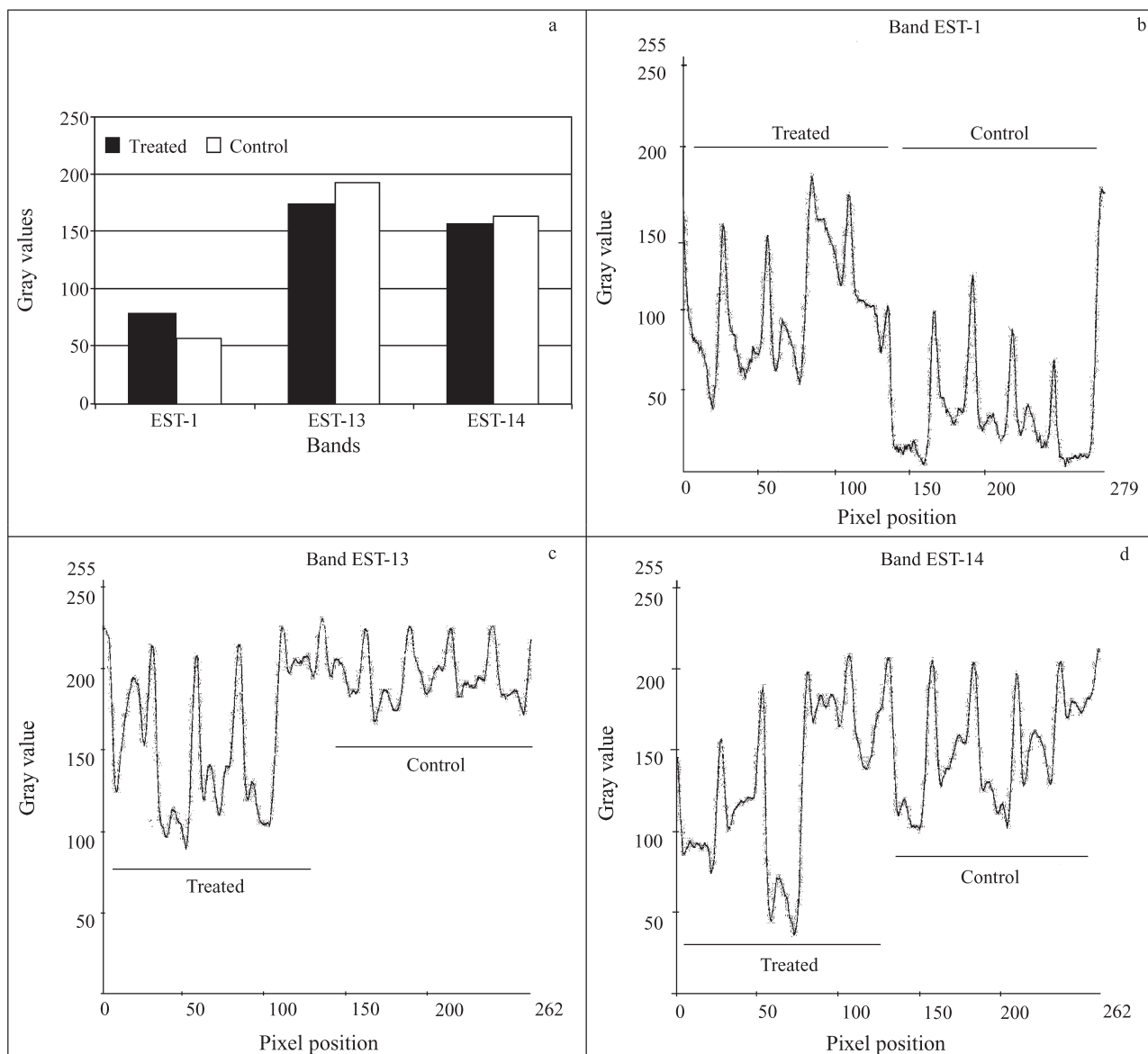
Figure 2(a-d) - (a) Distribution graph for staining degree mean values (in pixels) of esterase bands EST-1, EST-13 and EST-14, in continuous phenobarbital (PB) pretreated and control pupae from São José do Rio Preto. (b-d) Profile graphs of the same bands, obtained using the Global Lab Image Program.

Aedes aegypti mosquitoes from some regions also showed resistance to insecticides related with elevated esterase activity (Mourya *et al.*, 1993; Mazzarri and Georghiou, 1995; Vaughan *et al.*, 1998). In mosquitoes from São José do Rio Preto, which in the last 20 years have been submitted to control programs using different pyrethroid and organophosphorous insecticides, a carboxylesterase (EST-2) and a cholinesterase (EST-12) were considered as possibly involved in decrease of susceptibility (Lima-Catelani *et al.*, 2004).

In our study, a significantly increased expression of esterase bands EST-13 and EST-14 was observed in continuously PB-treated larvae and pupae (Tables 4 and 5; Figures 1 and 2). The frequency of PB-treated mosquitoes expressing the EST-14 band increased and also the number of those showing higher levels of activity was greater for esterase bands EST-13 and EST-14, being the difference in comparison with the control significant for EST-13.

The present results support the idea that PB-induced tolerance to TE in *A. aegypti* involves primarily the bands EST-13 and EST-14. Lima-Catelani *et al.*, 2004 included these enzymes in the group of cholinesterases which have been considered to be involved in resistance to organophosphorous insecticides in some insects (Smissaert, 1964; Devonshire, 1975; Fournier and Mutero, 1994; Baxter and Barker, 1998; Zhu and Gao, 1999). Besides, the esterase

band EST-12, which in the study of Lima-Catelani *et al.* (in press) showed changes of expression in mosquitoes from São José do Rio Preto when compared to mosquitoes from another population never exposed to insecticides, is also a cholinesterase that the same authors described as probably produced by an allele from the same locus which includes EST-13 and perhaps EST-14.

While the esterase bands EST-13 and EST-14 showed increased expression in PB-treated mosquitoes, bands EST-1, EST-2 and EST-20, which Lima-Catelani *et al.*, 2004 classified as carboxylesterases, showed the opposite effect suggesting that, in this species, esterase gene inactivation is also part of the response to PB.

Thus, resistance as well as tolerance-induction seem to be complex responses by the organisms involving the detoxifying genes, acting through mechanisms such as the selection of mutated genes, gene amplification and regulatory processes such as differential transcription activation or transcription enhancement of the detoxifying genes during development. Predominance of one or other of these processes is dependent on the inducing agent, the organism and its life history, as well as the environment. Knowledge of these mechanisms is important since they may eventually provide tools for interfering in the resistance process.

Previous studies on insecticide resistance of Brazilian *A. aegypti* populations carried out in our laboratory have indicated that mechanisms changing some esterase synthesis are involved in the process. In relation to the present study of PB-induced tolerance to TE in *A. aegypti,* esterase enzymes due to genes included in the same loci of the esterases found in those studies seem to be involved, supporting the idea of a common mechanism for insecticide resistance and induced tolerance by phenobarbital.

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