VARIATIONS IN THE FREQUENCY OF ESTERASE ISOENZYMES IN TWO STRAINS OF *Muscina stabulans* FALLEN (DIPTERA: MUSCIDAE) TREATED WITH TWO TYPES OF INSECTICIDES

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ABSTRACT**:** The effects of two types of insecticides, a pyrethroid and a carbamate, were analyzed in two *Muscina stabulans* strains after 10 selection cycles. Successive increases in the number of imagoes produced during the selection cycles were observed for the Sarapuí strain but not for the Monte Mor strain. The genetic effects of the insecticides were investigated after the selection cycles by analysis of the esterase isoenzyme compared to control populations not submitted to the selection tests. Twenty esterase isoenzyme patterns, divided into seven groups named A to G, were identified in the present populations. The populations treated with deltamethrine showed an increase in the frequency of group F isoenzymes and a reduction in group B enzymes. A reduction in isoenzyme frequency was observed in the populations treated with propoxur, except for isoenzyme 19 (group G), whose frequency significantly increased in the treated population. An increase in the number of gene amplifications for group E and D isoenzymes occurred in two strains.

Key words: Amplifications, deltamethrine, isoenzymes, propoxur, resistance**.**

VARIAÇÃO NA FREQUÊNCIA DE ISOENZIMAS ESTERÁSICAS EM DUAS LINHAGENS DE *Muscina stabulans* FALLEN (DIPTERA: MUSCIDAE) TRATADOS COM DOIS TIPOS DE INSETICIDAS

RESUMO: Os efeitos de dois tipos de inseticidas, um piretróide e um carbamato, foram analisados em duas linhagens de *Muscina stabulans* após 10 ciclos de seleção. Aumentos sucessivos nos números de imagos produzidos durante os ciclos de seleção foram observados para a linhagem de Sarapuí, mas não para a linhagem de Monte Mor. Os efeitos genéticos dos inseticidas foram investigados após os ciclos de seleção pela análise das isoenzimas esterásicas comparadas com as populações controle, não submetidas aos testes de seleção. O padrão das isoenzimas esterásicas identificadas nas populacões mostrou a existência de 20 tipos, as quais foram divididas em sete grupos denominados de A até G. As populacões tratadas com deltametrina mostraram um aumento na frequência de isoenzimas do grupo F e redução de proteínas do grupo B. Nas populacões tratadas com propoxur foi observada uma redução da freqüência de isoenzimas, exceto para isoenzima 19 (grupo G), cuja freqüência aumentou nas populações tratadas. Foi observado nas duas linhagens um aumento no número de amplificações gênicas para as isoenzimas dos grupos E e D.
Palavras-chave: Amplificações, deltrametrina, isoenzimas, propoxur, resistência.

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INTRODUCTION

Esterases represent a heterogenous group of enzymes that are widely distributed among organisms. The main function of these enzymes is the hydrolysis of carboxylic acids, but they may also act on other substrates such as peptides and amines (Walker & Macness, 1983). In animals, esterases are related to reproductive behavior (Johnson et al., 1968; Kambysellis et al., 1968; Mane et al., 1983; Karotam & Oakeshott, 1993), regulation of juvenile hormone levels (Jones et al., 1986, 1994), degradation of insecticides (Zerba et al., 1984; Kao et al., 1985), and digestive processes (Jones & Bancroft, 1986; Timmermans et al., 1994; Angentine & Jones, 1995).

The importance of esterases for the acquisition of resistance to many types of insecticides has been reported by several investigators (Devonshire & Moores, 1982; Bonning et al., 1991; Ketterman et al., 1992; Mutero et al., 1994; Badii & Almanza, 2007). In the aphid *Myzus percicae*, this fact is related to the presence of an esterase responsible for the hydrolysis of insecticide esters (Devonshire & Sawick, 1979). This mechanism is the result of the occurrence of gene amplifications originating from successive duplications of the structural gene of this esterase (Field et al., 1988). The same mechanism has been reported by Mouchés et al. (1986), Karunaratne et al. (1995) and Hemingway et al. (2000) for *Culex quinquefasciatus* and by Raymond et al. (2001) for *C. pipiens*. In the gels, gene amplifications manifest as an excessive enlargement of the normal original band. Organisms in which these bands are detected have shown a large number of descendants, surviving to insecticide applications and presenting an excessive increase in enzyme production.

The objectives of the present study were to investigate variations in the number of imagos produced by populations of two geographical strains of *Muscina stabulans* treated with two types of insecticides, a pyrethroid (deltamethrine) and a carbamate insecticide (propoxur), after 10 selection cycles, and then to analyze the molecular variations in esterase isoenzymes compared to control populations of the two strains not submitted to the selection tests.

MATERIALS AND METHODS

Specimens of the two strains were captured on hen laying houses in Monte Mor and Sarapuí, State of São Paulo. Each strain, named control strain, was initiated with 120 females and was housed separated in the chamber in special boxes for the maintenance of flies, with access to milk powder, sugar (1:1) and water.

The diet used for larval growth and egg collection had the following composition (Alves et al., 2004): 31.4 g wheat bran, 5.6 g yeast, 5.0 g powdered milk, 60 ml distilled water, 2.0 g yeast extract, 0.0050 g of each of the following five salts [Fe_2SO_4, $K_2HPO_2.3H_2O$, $(NH_4)SO_4$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$], 0.0050 g of a mixture of vitamins B_1, B_6 and B_{12}, and 0.1 μl of vitamin E.

Transparent plastic containers with a volume of 500 ml were used in the tests for mounting the experimental groups. An opening (3 x 4 cm) was made in the lid and covered with organza tissue to prevent imagos and larvae from escaping and to facilitate aeration. All experimental assays were performed in a chamber at a temperature of 25 ± 2ºC and relative humidity of 65 to 75% under a 12-h photoperiod.

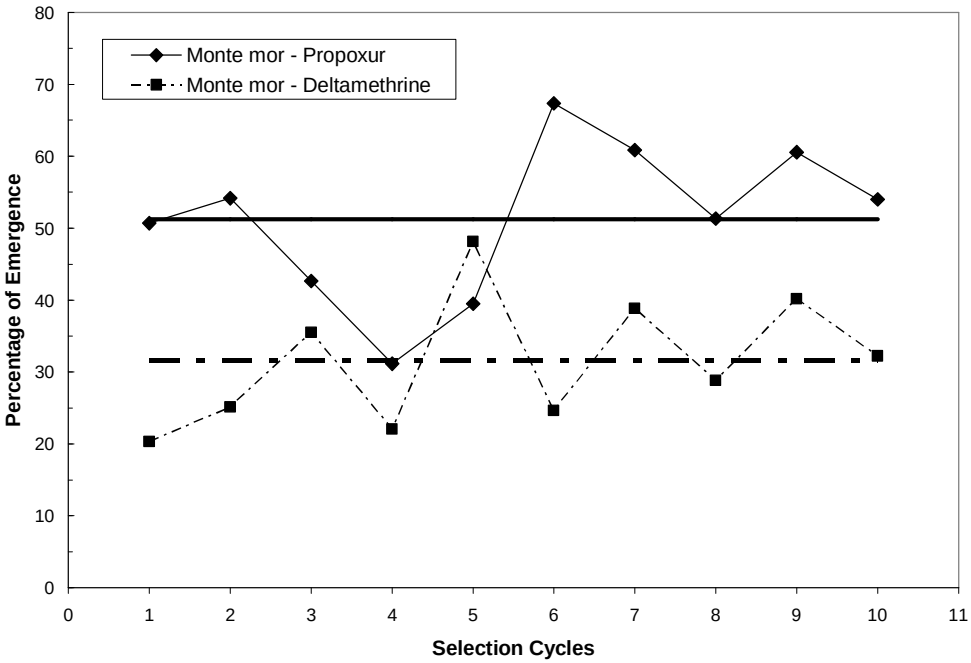
The genetic effects of the insecticides on the experimental populations were analyzed after 10 selection cycles of the two strains receiving a diet containing one of the two insecticides described below. The selection tests were carried out according to the following scheme: the eggs were obtained from the maintenance boxes by placing a Petri dish containing the diet inside the box. Next the dishes were collected and kept in the chamber for 3 days. Each experimental group was initiated with 200 larvae (3 days old) transferred to the transparent plastic container with the diet (150 ml) and the specific insecticide. The insecticide dose used for each group was based on the LD_{50} obtained for six other geographic strains of *M. stabulans*. Thus, 0.00023 g propoxur and 0.00540 g deltamethrine were independently applied to each plastic container. After emergence in the experimental groups, the flies were counted and transferred to a new box for maintenance of the population. This procedure was repeated ten times and, at the end, the flies were submitted to electrophoresis for the analysis of esterase isoenzymes.

Thus, four types of populations were formed: Monte Mor strain grown on a diet containing propoxur (Monte Mor-propoxur) and deltamethrine (Monte Mor-deltamethrine), and Sarapuí strain grown on either insecticide (Sarapuí-propoxur and Sarapuí-deltamethrine). Three replicates were organized for each population, for a total of 12 populations that passed through the selection cycles and six populations of the two strains that were not submitted to the selection cycles (controls).

The four populations submitted to the selection cycles and the control populations were submitted to polyacrylamide gel electrophoresis in a discontinuous system for the analysis of esterases. The solutions and buffers used were described by Ceron (1988), adapted from Davis (1964), and Laemmli (1970). A total of 120 flies (60 males and 60 females) were analyzed for each population. A negatoscope was used for interpretation of the gels.

RESULTS AND DISCUSSION

Polynomial regression applied to the analysis of percent emergence as a function of selection cycles (Figure 1) showed a progressive increase in the number of imago emergences with increasing selection cycles in the Sarapuí-deltamethrine ($F=10.37$; $P<0.05$) and Sarapuí-propoxur ($F=4.37$; $P<0.05$) populations, with this increase following a linear regression model. No difference in the number of emerged imagos as a function of selection cycles was observed for the Monte Mor-deltamethrine ($F=2.,20$; $P>0.05$) or Monte Mor-propoxur ($F=2.67$; $P>0.05$) populations.



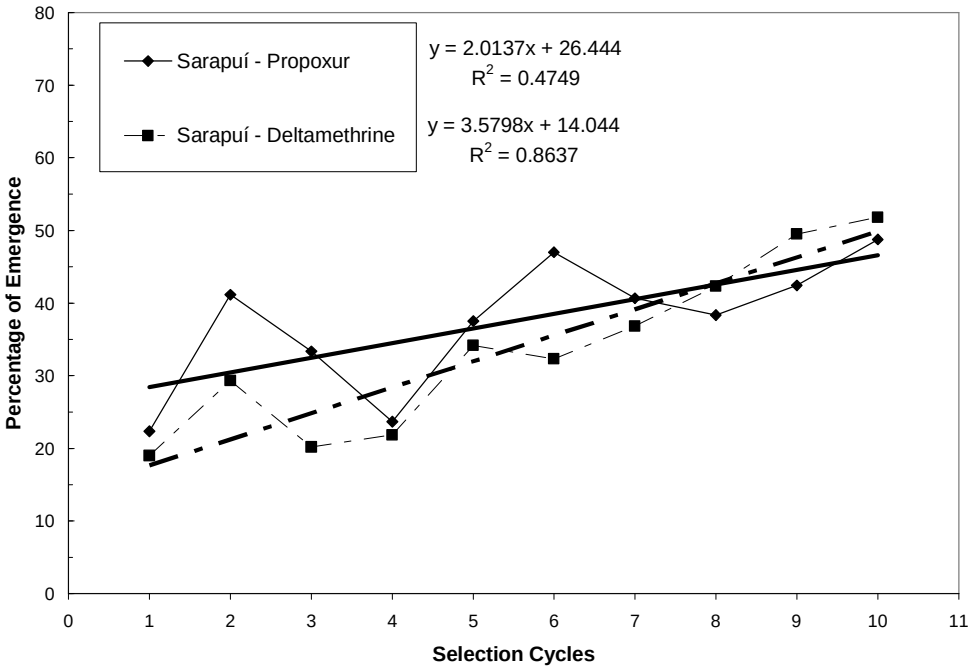


Figure 1. Mean percentage of emerged flies of the two *Muscina stabulans* strains during selection on the insecticides tested and the corresponding regression lines.

It is possible that the increase in the emergence of Sarapuí flies was associated with the evolution to insecticide resistance. According to Lomônaco & Prado (1994), under natural conditions Monte Mor flies are always subjected to the effects of insecticides, thus explaining the lack of an increase in the emergence of individuals during the selection cycles.

On the basis of band staining, thickness and migration, the esterase isoenzyme pattern of the *M. stabulans* strains was characterized by the presence of 20 types of bands (Figure 2), which were divided into seven groups, named A to G. Group F comprised the largest number of bands (13, 14, 15, 16 and 17). Band 1 migrated farthest into the gel and band 20 migrated

least. No differences in the esterase pattern were observed between males and females. The bands of group E (11 and 12) and group G (18, 19 and 20) presented staining specificity for ß-naphthyl acetate (red) and the other bands presented staining specificity for α-naphthyl acetate or both (dark).

The most common bands detected in the individuals of the populations were band 9 (D), band 2 (A), band 19 (G), band 1 (A), and band 12 (E). In group G, only band 19 was common, whereas the other bands were rare. Thus, the most common enzymes were generally found in groups A, D and E. The frequency of group B bands (3, 4 and 5) decreased in the two populations of the Monte Mor strain compared to the control population (Table 1). The same trend was observed for the Sarapuí populations, with a decrease in the frequency of group C bands (6 and 7) in the Sarapuí-propoxur population,

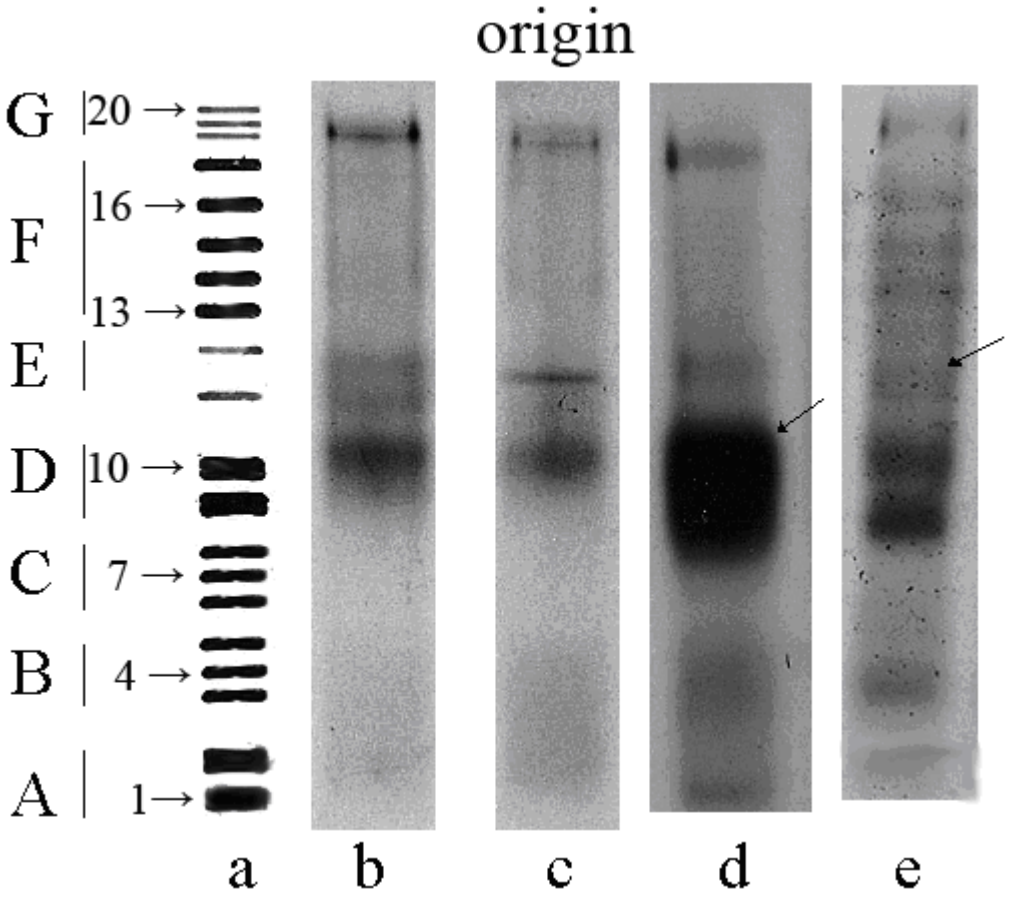


Figure 2- The column a presents a hypothetical representation of the *M. stabulans* zymogram, with all 20 types of esterase bands found in the populations analyzed, the band numbers and the group which the bands belong (A to G). The bands 11 and 12 of the group E are showed by the flies b and c. The fly d presents an amplification in the group D, and the fly e shows some bands of the group F, with an amplification in group E of isoenzymes.

Table 1- Number of *Muscina stabulans* flies presenting the different esterase bands found in the three types of populations of the two strains.

Groups	Band	Monte Mor populations			Sarapuí populations		
		Control	Propoxur	Deltamethrine	Control	Propoxur	Deltamethrine
A	1	50 b[1]	31 c	120 a	60 ab	73 a	52 b
	2	105 b	119 a	88 c	106 a	109 a	114 a
B	3	43 a	8 b	3 b	20 a	29 a	28 a
	4	36 a	14 b	10 b	47 a	34 ab	29 b
	5	50 a	23 b	26 b	48 a	27 b	28 b
C	6	34 b	32 b	80 a	25 a	5 b	18 ab
	7	44 a	55 a	21 b	53 b	12 c	75 a
	8	15 b	45 a	1 c	19 c	29 b	58 a
D	9	116 b	87 c	120 a	119 a	99 b	113 c
	10	16 c	75 a	46 b	32 c	50 b	90 a
E	11	52 a	0 b	0 b	79 b	71 b	103 a
	12	80 b	40 c	96 a	17 c	52 b	93 a
F	13	25 a	8 b	15 ab	16 c	32 b	67 a
	14	28 b	38 b	67 a	36 b	23 b	82 a
	15	52 ab	39 b	67 a	60 b	23 c	82 a
	16	76 a	40 b	71 a	46 b	23 c	81 a
	17	62 a	8 c	31 b	57 a	8 b	6 b
G	18	5 b	8 b	41 a	2 b	33 a	26 a
	19	87 b	88 b	120 a	108 b	113 a	82 c
	20	0 a	0 a	0 a	0 b	0 b	4 a

[1] Within the same strain, numbers in the same line followed by the same lower case letter did not differ from one another (chi-square test).

whereas in the Sarapuí-deltamethrine population the frequency of group C bands (7 and 8) seemed to have increased compared to the control population.

The frequency of group D bands (9 and 10) presented a significant increase in the Monte Mor-deltamethrine population. No pattern was observed for these bands in the Monte Mor-propoxur population. A similar result was obtained for group D bands in the Sarapuí strain, although these bands showed a high frequency in the two strains submitted to the selection cycles.

The selection tests showed a significant increase in the frequency of group F bands, except for band 17, in the populations treated with deltamethrine when compared to the control populations. In the propoxur populations, the frequency of these isoenzymes tended to decrease compared to the control and deltamethrine populations. Thus, the selection tests demonstrated that some isoenzymes increased in frequency whereas others decreased in the populations due to the selective pressure of the insecticides. Isoenzymes of group F and group D (9 and 10) played an important role in the deltamethrine populations. In the propoxur populations, the role of isoenzymes of group D plus bands 2 and 19, which were common in both strains, can be emphasized.

Pairwise comparison of the number of significantly more frequent bands between populations of the same strain showed the presence of seven bands in the control Sarapuí population that were significantly more frequent than in the propoxur population. The latter presented only six bands that were more frequent than in the control population. The deltamethrine population presented 11 bands whose frequency was higher than that of the propoxur population, and this population presented two bands whose frequency was higher compared to the deltamethrine population. The latter population presented a larger number of bands with significant frequencies (11) than the control population (5, for reverse comparison). Similar results were obtained for the Monte Mor strain (Table 1), except for the deltamethrine and control populations which presented the same number of significantly more frequent bands (8) that differed from one another. These results indicate a reduction in the diversity of esterases in the propoxur populations, whereas diversity seems to have increased in the deltamethrine populations over the selection cycles.

The Monte Mor strain presented a total of 264 amplifications (Table 2). A larger number of amplifications of group C isoenzymes were observed for the Monte Mor-deltamethrine population compared to the control and Monte Mor-propoxur populations. The Monte Mor-propoxur population presented 50 and 36 amplifications for groups D and E, respectively, whereas the Monte Mor-deltamethrine population presented 6 and 96 amplifications for the same groups. These results show that, despite a lack of progressive growth in the number of flies over the selection cycles, the Monte Mor strain responded positively to the effects of the insecticides by increasing the number of amplifications as compared to the control Monte Mor population.

The Sarapuí strain presented 24 amplifications more than the Monte Mor strain. An elevated number of amplifications for isoenzymes of groups D and E was observed in the Sarapuí-propoxur and Sarapuí-deltamethrine populations, with this number being higher in the Sarapuí-deltamethrine population compared to the other two populations of the same strain.

Table 2 shows the number of flies presenting amplifications in isoenzymes beyond one group, douple amplifications (duplications). In the Monte Mor-propoxur population, two flies presented amplifications of group C and E isoenzymes. This number was 30 in the Monte Mor populations submitted to the selection cycles. In the two control populations, 9 (Monte Mor) and 2 (Sarapuí) amplifications were observed.

. **Table 2. Number of amplifications found in the esterase groups of the three types of populations of the two *Muscina stabulans* strains**

Groups	Monte Mor populations			Sarapuí populations		
	Control	Propoxur	Deltamethrine	Control	Propoxur	Deltamethrine
A				2 a	0 a	0 a
C	2 b[1]	8 b	24 a	0 b	9 a	6 a
D	32 b	50 a	6 c	9 c	35 b	64 a
E	10 c	36 b	96 a	0 c	61 b	104 a
Total	44	94	126	11	103	174
Duplication	9 DE[2]	2 CE; 5 DE	20 CE; 3 DE	2 AD	4 CE; 15 DE	4 CE; 60 DE

[1] Within the same strain, numbers in the same line followed by the same lower case letter did not differ from one another (chi-square test).

[2] Individuals with double amplifications in two groups of isoenzymes.

All duplications found in the populations of the two strains, except for the two duplications observed in the control Sarapuí population, occurred together with group E isoenzymes, a fact indicating the importance of these amplifications in the acquisition of insecticide resistance. According to Mouchés et al. (1986), it seems likely that gene amplification is a common mechanism for the acquisition of insecticide resistance in insects.

The total number of duplications in the Sarapuí strain was 85, which was larger than that obtained for the Monte Mor populations. In spite of the reduction in the frequency of isoenzymes in the Sarapuí populations it was compensated by an increase in gene amplifications. This finding might be associated with an increased specific activity of esterases, a fact also observed by Takada & Murakami (1988) and Abdel-Aal et al. (1992).

An increase in isoenzyme diversity was observed in the Monte Mor strain, especially for group F isoenzymes, in addition to a significant number of gene amplifications in group D and E isoenzymes. This finding seems to indicate the use of more than one resistance mechanism (amplifications and an increased frequency of isoenzymes) as a protection against the toxic effects of deltamethrine.

CONCLUSIONS

The tests showed that Sarapuí-deltamethrine and Sarapuí-propoxur populations presented a progressive increase in number of imagoes with the tests of selecton, showing an answer to insecticides, not presented by Monte-Mor populations, that by the other hand, presented higher number of band duplications than Monte-Mor.

The isoenzymes of the group D and E showed the highest number of amplifications, indicating that were the most importance against the insecticides, likewise, to the Monte-Mor strains group F of isoenzymes were important by the flies to get resistence.

ACKNOWLEDGMENTS

We are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for granting a fellowship to the first author.

REFERENCES

ABDEL-AAL, Y.A.I.; LAMPERT E.P.; ROE, R.M.; SEMTNER P.J. Diagnostic esterases and insecticide resistance in the tobacco aphid *Mysus nicotiane* Blackman (Homoptera: Aphididae). **Pesticide Biochemistry and Physiology**, v.43, 1992, p.123-133.

ALVES, S.M.; BÉLO, M.; AL GAZI, A.D.F. Seleção de uma dieta para o desenvolvimento de larvas de *Muscina stabulans* FÁLLEN (Diptera: Muscidae) e testes com inseticidas. **Biodiversidade**, v.1, 2004, p.49-61.

ARGENTINE, J.A.; JAMES A.A. Characterization of a salivary gland-specific esterase in the vector mosquito *Aedes aegypti*. **Insect Biochemistry and Molecular Biology**, v.25, 1995, p.621-630.

BADII, M.H.; ALMANZA, V.G. Resistencia in insectos, plantas y microorganismos. **Cultura, Ciencia y Tecnogía**, v.4, 2007, p.9-25.

BONNINGET B.C.; HEMINGWAY, J.; ROMI, R.; MAJORI G. Interaction of insecticide resistance genes in field populations of *Culex pipiens* (Diptera: Culicidae) from Italy in response to changing insecticide selection pressure. **Bulletin of the Entomological Research**, v.81, 1991, p.5-10.

CERON, C.R. Padrão de esterases no desenvolvimento de *Drosophila mulleri*, *D. arizonesis* e seus híbridos (PhD Thesis) Instituto de Biociências, Universidade de São Paulo, 1988, 142 p.

DAVIS, B.J. Disc electrophoresis II. Methods and application to human serum proteins. **Annual New York Academy of Science**, v.121, 1964, p. 404-427.

DEVONSHIRE, A.L.; MOORES, G.D. (1982) A carboxylesterase with broad substrate specificity causes organophosphate, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). **Pesticide Biochemistry and Physiology**, v.1218, 1982, p.235-246.

DEVONSHIRE, A.L.; SAWICKI, R.M. Insecticide-resistant *Myzus persicae* as an example of evolution by gene duplication. **Nature**, v.280; 1979, p.140-141.

FIELD, L.M.; DEVONSHIRE, A.L.; FORD, B.G. Molecular evidence that insecticide resistance in peach-potato aphids (*Mysus persicae* Sulz.) results from amplification of an esterase gene. **Biochemical Journal**, v.251, 1988, p.309-312.

HEMINGWAY J.; COLEMAN, M.; PATON, M.; MCCARROL, L.; VAUGHAN, A.; DE SILVA, D. Aldehyde oxidase is coamplified with the world's most common *Culex* mosquito resistance associated esterases. **Institute of Molecular Biology**, v.9, 2000, p.93-99.

JOHNSON, F.M.; RICHARDSON, R.H.; KAMBYSELLI, M.P. Isozyme variability in species of the genus *Drosophila*. III. Quantitative comparison of the esterases from *D. aldrichi* and *D. mulleri*. **Biochemical Genetics**, v.1, 1968, p.239-247.

JONES, B.R.; BRANCOFT, H.R. Distribution and probable physiological role of esterases in reproductive, digestive and fat-body tissue of adult cotton boll weevil *Anthonomus grandis* Boh. **Biochemical Genetics**, v. 24, 1986, p.499-508.

JONES, B.R.; JONES, G.; RUDINICKA, M.; CLICK, A.J.; SPEEKRISHANA, S.C. (1986) High resolution isoelectric focusing of juvenile hormone esterase activity from haemolymph of *Tricoplusia ni* (Hubner). **Experientia,** v.42, 1986, p.45-47.

JONES. G.; VENKATARAMAN, B.R.; RIDLEY, B.; OMANOY, T.; TURNER, H. Structure, expression and gene sequence of a juvenile hormone esterase-related protein from metamorphosing larvae of *Ticlopusia ni*. **Biochemical Journal**, V.302, 1994, p.827-835.

KAMBYSELLIS, M.P.; JOHNSON, F.M.; RICHARDSON, R.H. Isoenzyme variability in species of the genus *Drosophila*. IV. Distribution of the esterases in body tissues of *D. aldrich* and *D. mulleri* adults**. Biochemical Genetics**, v.1, 1968, p. 249-265.

KAO, L.R.; NOTOYAMA, N.; DAUTERMAN, W.C. The purification and characterization of esterases from insecticide-resistant and susceptible house flies. **Pestcide Biochemistry and Physiology**, v.23, 1985. p. 228-239.

KAROTAM, J.; OAKESHOTT, J.G. (1985) Regulatory aspects of esterases 6 activity variation in sibling *Drosophila* species. **Heredity**, v. 71; p. 41-50.

KARUNARATNE, S.H.P.P.; HEMINGWAY, J.; DASSANAYAKA, G.I.J.V.; VAUGHAN, A. Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible *Culex quinquefaciatus* mosquitoes. **Journal of Biological Chemistry**, v.270, 1995, p.31124-31128.

KETTERMAN, A.J.; JAYAWARDENA, K.G.I.; HEMINGWAY, J. Purification and characterization of a carboxylesterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*. **Biochemical Journal**, v.287, 1992, p.355-360.

LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. **Nature**, v.22, 1970, p.680-685.

LOMÔNACO, C.; PRADO, A.P.Estrutura comunitária e dinâmica populacional da fauna de dípteros e seus inimigos naturais em granjas avícolas. **Sociedade Entomológica do Brasil**, v.23, 1994, p.71-80.

MANE, S.D.; TOMPKINS, L.; RICHMOND, R.C. Male esterase 6 catalyses the synthesis of a sex pheromone in *Drosophila melanogaster* females. **Science**, v.222, 1983, p. 419-421.

MOUCHÉS C.; PASTEUR, N.; BERGE, J.B.; OLIVER, H. Amplification of an esterase gene is responsible for insecticide resistance in California *Culex* mosquito. **Science**, v.233, 1986, p.778-780.

MUTERO, A.; PRALAVORIO, M.; BRIDE, J.M.; FOURNIER, N. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. **Proceeding of the National Academic Science**, v.91, 1994, p.5922-5926.

TIMMERMANS, M.Y.J.; TEUCHY, H.; KUPPERS, L.P.M. The cDNA sequence encoding bovine pregastric esterase. **Gene,** v.147, 1994, p. 259-262.

WALKER, C.H.; MACKNESS, M.I. Esterases problems of identification and classification. **Biochemical Pharmacology**, v.32, 1983, p. 3265-3269.

ZERBA, E.N.; WOOD, E.J.; MALKENSON, N.C. Isolation and characterization of an esterase of *Triatoma infestans* with a critical role in the degradation of organophosphorus esters. **Insect Biochemistry**, v.14, 1968, p. 481-486.