ONTOGENETIC PATTERNS AND GENETIC VARIATION IN *Anopheles* (*Anopheles*) *intermedius* CHAGAS, 1908 AND *Anopheles (Anopheles) mattogrossensis* LUTZ & NEIVA, 1911 (DIPTERA: CULICIDAE) IN THE BRAZILIAN AMAZON

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ABSTRACT

Changes in the expression of genes were observed during development in populations of *Anopheles (Anopheles) intermedius* and *Anopheles (Anopheles) mattogrossensis*. Esterase showed seven zones of activity: EST1 was present in all developmental stages of both species; EST2 was observed only in larvae of *A. intermedius* and larvae and pupae of *A. mattogrossensis*, with greater activity in pupae; EST3 and EST5 were present in all developmental stages, with greater intensity in larvae; EST4 and EST6 showed weak activity in larvae of *A. mattogrossensis* and was not found in *A. intermedius*. Leucine aminopeptidase showed four zones of activity, of which LAP1 and LAP2 were found in all stages of *A. intermedius*, with highest activity in larvae, and in larvae only of *A. mattogrossensis*. LAP3 was detected in all stages of *A. mattogrossensis* and in larvae only of *A. intermedius*. LAP4 was detected only in larvae and pupae of *A. mattogrossensis*, with greater intensity in pupae. α–Glycerophosphate dehydrogenase showed a single zone of activity, detected in older fourth-instar larvae and becoming more intense from the pupal stage onwards.

Key words: *Anopheles intermedius*, *Anopheles mattogrossensis*, ontogeny, isozymes, malaria.

RESUMO

Padrões ontogenéticos e variação genética em *Anopheles (Anopheles) intermedius* Chagas, 1908 e *Anopheles (Anopheles) mattogrossensis* Lutz & Neiva, 1911 (Diptera: Culicidae) da Amazônia brasileira

Foram observadas modificações na expressão gênica durante o desenvolvimento de *Anopheles intermedius* e *Anopheles mattogrossensis* do subgênero *Anopheles*. A esterase revelou sete regiões de atividade, sendo a EST1 presente durante todo o desenvolvimento das duas espécies; a EST2 observou-se apenas em larvas de *A. intermedius* e em larvas e pupas de *A. mattogrossensis*, com maior atividade em pupas; as EST3 e EST5 revelaram-se em todos os estágios, mostrando maior intensidade em larvas; as EST4 e EST6 foram detectadas com fraca atividade em *A. intermedius* durante todos os estágios, porém em *A. mattogrossensis* apresentaram forte atividade em pupas e fraca em larvas; a EST7 revelou-se apenas em pupas de *A. mattogrossensis*, não sendo observada em *A. intermedius*. A leucina aminopeptidase revelou quatro regiões de atividade, das quais, LAP1 e LAP2 foram observadas durante todo o desenvolvimento de *A. intermedius*, com maior atividade em larvas, e em *A. mattogrossensis* reveladas apenas em larvas; a LAP3 detectou-se em todos os estágios de *A. mattogrossensis* e somente em larvas de *A. intermedius*; a LAP4 foi somente detectada em larvas e pupas de *A. mattogrossensis*, com maior intensidade em pupas. A α–GPDH apresentou uma única região de atividade, revelada em larvas de 4º estágio velhas e a atividade é intensificada a partir do estágio de pupa, em ambas espécies.

Palavras-chave: *Anopheles intermedius*, *Anopheles mattogrossensis*, ontogenia, isoenzimas, malária.

INTRODUCTION

Studies of the electrophoretic patterns of proteins and enzymes during ontogeny of different organisms provide data about differential gene action, enabling researchers to determine the exact moment when a specific gene becomes active in the synthesis of the corresponding enzyme (Wagner & Selander, 1974).

Several studies have pointed out that some enzymes are modified during the development either in terms of band intensity or in terms of the appearence of new forms and the desappearence of others (Wright & Shaw, 1969; O'Brien & MacIntire, 1972; Vedbrat & Whitt, 1975; Santos, 1979; Santos *et al.*, 1985; Santos *et al.*, 1996a,b; Scarpassa, 1988; Scarpassa *et al.*, 1992; Maia & Santos, 1999). These studies, when taken as whole, enable a deeper understanding of how the genes in mosquitoes act and are expressed. The ontogeny changes provide a special insight that can be useful if accompained with the study of the larvicidal effects in nature. Besides, conventional morphology seldom predicts discriminating characters in the first development stages, thus the enzymatic variants are very useful.

According to Vedbrat & Whitt (1975) the appearence and disappearence of a specific enzyme or isozyme may be due to: 1) the substitution of one type of cellular pathway for another; 2) the triggering or stopping of the activity of already existing enzymes; 3) or suppression or expression of a specific gene locus.

In this study we present the electrophoretic patterns of gene manifestation of esterase, leucine aminopeptidase and α-glycerophosphate dehydrogenase during the development of *Anopheles intermedius* and *Anopheles mattogrossensis* of the *Anopheles* subgenus

MATERIAL AND METHODS

The mosquitoes used in this study were obtained from natural populations of Janauarilândia in the Amazon State, and in Macapá in the State of Amapá. Individuals were studied by electrophoresis in three different stages of development, i. e., 4 th-instar larvae (young: 12 hours; after young: 24 hours and old: after 30 hours), pupae and adults. Larvae were reared by the method of Santos *et al.* (1981), slightly modified. For isozymes analysis, the material was collected into cuvettes and stored frozen at –70ºC. Samples were homogenized with the aid of a glass stick in 15 ul of 0.5% 2-beta-mercaptoetanol (v/v) for larvae and 20 ul for pupae and adults, on concave porcelain plates kept on ice. After the homogenization, thin paper pieces measuring 2 x 2 cm were placed on the plates containing the samples, which were soaked up with Whatman n. 3 filter paper. These papers pieces were inserted vertically into the gel.

Three enzimatic systems were studied: esterases, leucine aminopeptidase and α-glycerophosphate dehydrogenase, at 12.5% starch gel. Buffer solutions and reaction mixtures were prepared by the methods of Santos *et al.* (1985) and Santos *et al.* (1996 a).

RESULTS

Esterases

Analysis of esterases during the ontogenetic development showed the occurrence of seven activity bands changing in staining intensity according to each stage (Figs. 1 and 2).

Esterase 1 was detected in all development stages in both species. EST2 was only detected in larvae and EST4 and EST6 were detected in all phases of development, in *A. intermedius* EST2, EST4 and EST6 were detected in larvae and pupae with higher activity in pupae in *A. mattogrossensis*. EST3 and EST5 showed a similar profile in both species during all phases of development with high activity in larvae and low activity in pupae and adults. A more cathodical band in *A. mattogrossensis* population denominated EST7 only detected in pupae, was also observed.

Most regions observed during development presented variations in the two species even though EST2 was only monomorphic for *A. mattogrossensis* and EST3 for *A. intermedius*.

EST1 showed two codominant alleles: *EST1*A* and *EST1*B*, which permitted the presence of three distinct phenotypes: EST1 A, EST1 AB and EST1 B. The latter one was not detected in the population of *A. intermedius*. The heterozygote profile showed two well defined bands, suggesting it to be a monomeric enzyme.

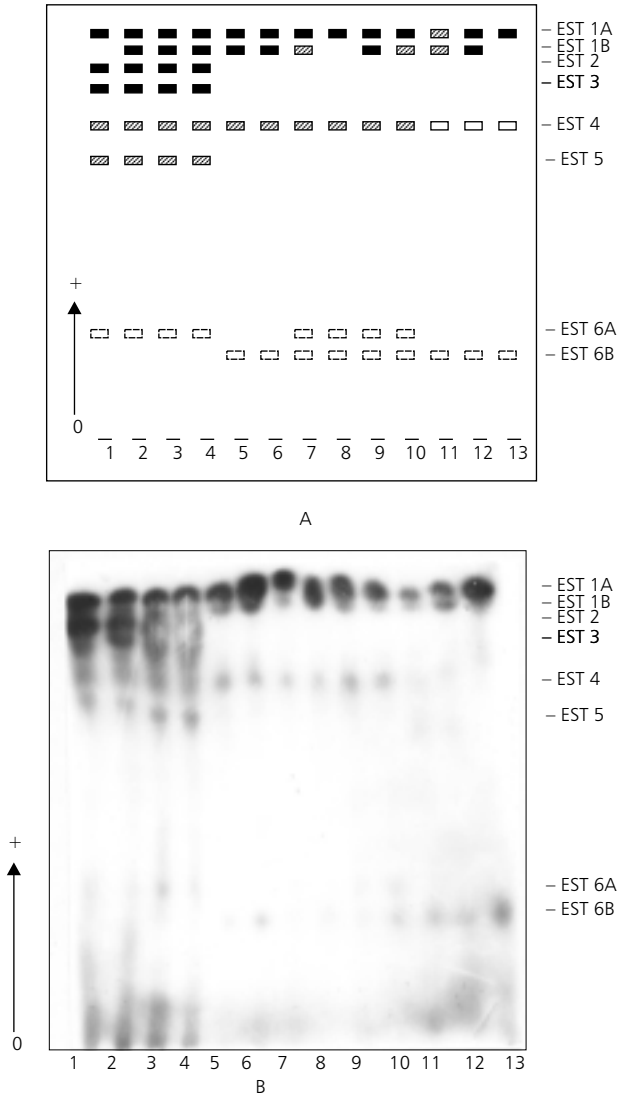

Fig. 1 — Electrophoretic profiles of esterases during the ontogenetic development of *Anopheles intermedius*. Tris-citrate-borate buffer system, pH 8.0. Starch gel electrophoresis. (A) Scheme. (B) Photography. Samples 1 to 4 = 4 th-instar larvae; 5 to 8 = pupae; 9 to 13 = adults.

EST2 presented variation of more than two alleles in *A. intermedius*. EST3 showed two codominant alleles – *EST3*A* and *EST3*B* for the *A. mattogrossensis* population, yet it was monomorphic for *EST3*A* allele in *A. intermedius*. The heterozygote profile presented two intense bands suggesting a monomeric structure for this protein. For EST4, EST5, EST6 and EST7 variation was observed in two alleles, whose heterozygotes showed two bands with the same staining intensity.

Leucine aminopeptidase

The electrophoretic patterns of leucine aminopeptidase isozymes during the ontogeny of *A. intermedius* and *A. mattogrossensis* showed four activity bands (Figs. 3 and 4). LAP1 and LAP2 were observed in all development stages in *A. intermedius*, with greater activity in larvae. In *A. mattogrossensis* it was present only in larvae. LAP3 was observed only in *A. intermedius* larvae, though it was present in all development phases of *A. mattogrossensis*.

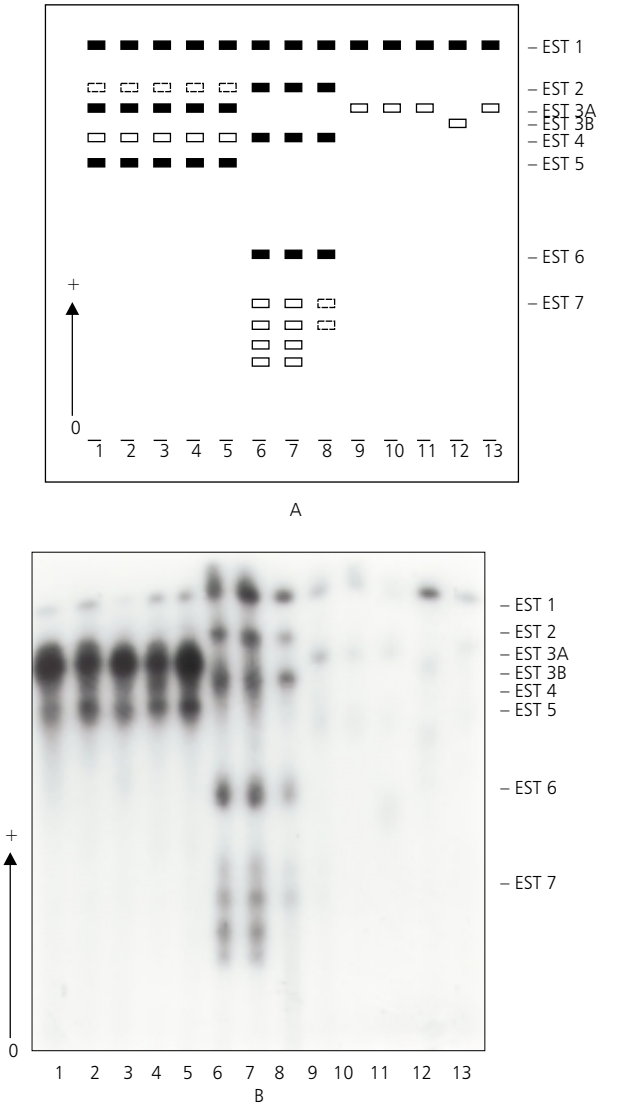

Fig. 2 — Electrophoretic profiles of esterases during the ontogenetic development of *Anopheles mattogrossensis*. Starch gel electrophoresis. Tris-citrate-borate buffer system, pH 8.0. (A) Scheme. (B) Photography. Samples 1 to 5 = 4 th-instar larvae; 6 to 8 = pupae; 9 to 13 = adults.

LAP4 it was detected in larvae and pupae of *A. mattogrossensis* with larger intensity in the latter. This band was not detected in *A. intermedius*, possibly due to the small number of analyzed individuals. A more cathodic band, very close to the origin, with moderate to weak intensity, was observed in both species.

LAP1, which was more anodic, presented greater variation and intensity and stained with three distinct phenotypes – LAP1 A, LAP1 AB and LAP1 B, in the population of *A. intermedius*, and only LAP1 A and LAP1 AB in *A. mattto-grossensis*.

These phenotypes result from the combination of two codominant alleles – *LAP1*A* and *LAP1*B*, the first one being the most frequent. LAP2, which also varied in both species, showed two codominant alleles – *LAP2*A* and *LAP2*B*. Heterozygotes profiles show two bands suggesting the enzyme having a monomeric structure.

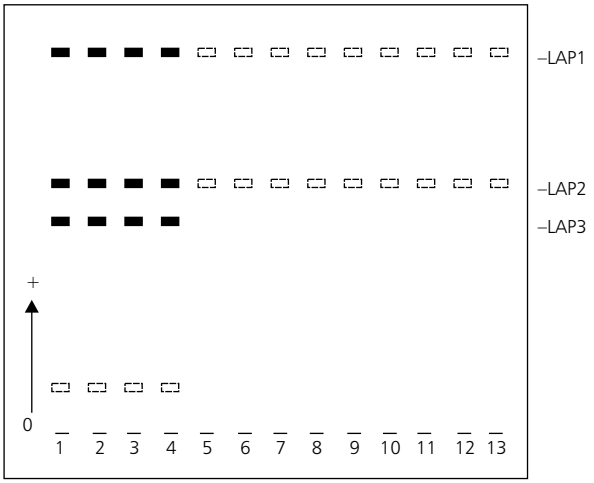

Fig. 3 — Electrophoretic profiles scheme of leucine aminopeptidase during the ontogenetic development of *Anopheles intermedius*. Starch gel electrophoresis. Tris-citrate-borate buffer system, pH 8.0. Samples 1 to 4 = 4 th-instar larvae; 5 to 8 = pupae; 9 to 13 = adults.

LAP3 also showed polymorphism, with two alleles – *LAP3*A* and *LAP3*B*. In the population of *A. intermedius* the phenotypes LAP3 A, LAP3 AB and LAP3 B were observed, whereas only LAP3 A and LAP3 AB were detected in *A. mattogrossensis*. The isozyme profile of the heterozygote, two equally stained bands, suggest that protein has a monomeric structure.

α-*Glycerophosphate dehydrogenase*

The electrophoretic patterns of α–glycerophosphate dehydrogenase during ontogenetic development, start to be detected at weak intensity in strongly pigmented 4th-instar. Staining intensity increased in the pupal phase and continued during the adult stage. The same pattern was observed in both species (Fig. 5).

The α–GPDH profile consisted of a single electronegative activity band close to the origin. These data suggest that the genetic control of this enzyme occurs through one monomorphic locus α–GPDH.

Table I shows comparative study of activity of enzyme analised during ontogenesis in mosquito species – Esterases, LAP and α–GPDH.

DISCUSSION

Changes in gene expression during ontogenetic development of *A intermedius* and *A. mattogrossensis* were observed in the three enzymatic systems. The profile of EST3 and EST5 with the higher larvae activity appear to correspond with a decrease in the metabolic activity rate, metabolism being intensive in larvae, and decreasing for the following stages. These results are in agreement with Scarpassa (1988), who described seven activity bands for *Anopheles nuneztovari* of Tucurui, noting that esterases of this pattern are related to metabolic activity during development.

EST2, present in the larvae of *A. intermedius*, appears to be associated with the larval digestive system and metabolism, differing from that of adult. Larvae feeding on micro-organisms, algae, protozoa and similar foods may require these esterases, while adults feeding on sugary substances do not (VedBrat & Whitt, 1975).

EST4 and EST6 of *A. intermedius* present during all the development stages, could be related to more generalized functions than those recorded in a specific stage of development. It is thought that these esterases are related to the break down of lipids, used as an energy source. Freyvogel *et al.* (1968), showed that lipids probably play an important role in blood digestion for *Aedes aegipty* and *Anopheles stephensi*. The fact that esterases are non-specific in their activity implies that they might function in the regulation of lipids, as suggested by the high levels of esterase found in the stomach epithelium of these mosquitoes.

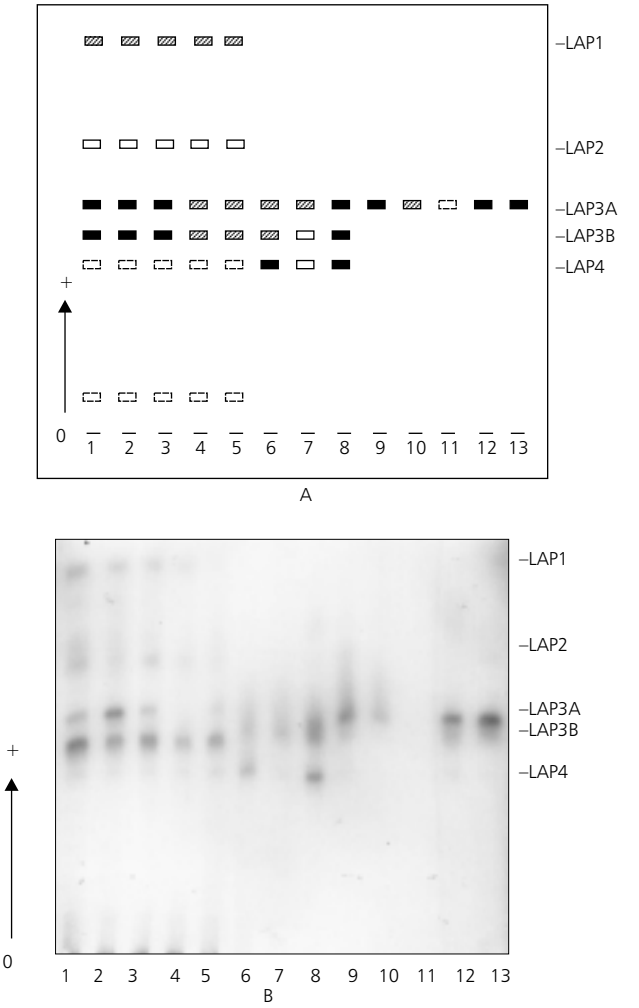

Fig. 4 — Electrophoretic profiles of leucine aminopeptidase during the ontogenetic development of *Anopheles mattogrossensis*. Starch gel electrophoresis. Tris-citrate-borate buffer system, pH 8.0. (A) Scheme. (B) Photography. Samples 1 to 5 = 4 th-instar larvae; 6 to 9 = pupae; 10 to 13 = adults.

The appearence of EST2, EST4 and EST6 with a higher activity in *A. mattogrossensis* pupae suggest they may serve a regulatory function on ecdysone levels, postulating that this isozyme form in the pupal stage participates in the metabolism of this hormone. It was suggested by Whitmore *et al*. (1972) that carboxilesterase may play an important role in regulating the levels of juvenile hormone.

This hormone functions by favoring the expression of larval characteristics and is normally present in the pupae. According to these authors, the induction of the enzymes is capable of degrading the hormone, a mechanism whereby insects can assure normal metamorphosis. Study of these enzymes may have important implications for those interested in the use of juvenile hormone and its analogs as insect-controlling agents, and suggests that these organisms have a "biochemical pool" that permit the degradation of foreign molecules (such as DDT) and the regulation of endogenous hormones when necessary.

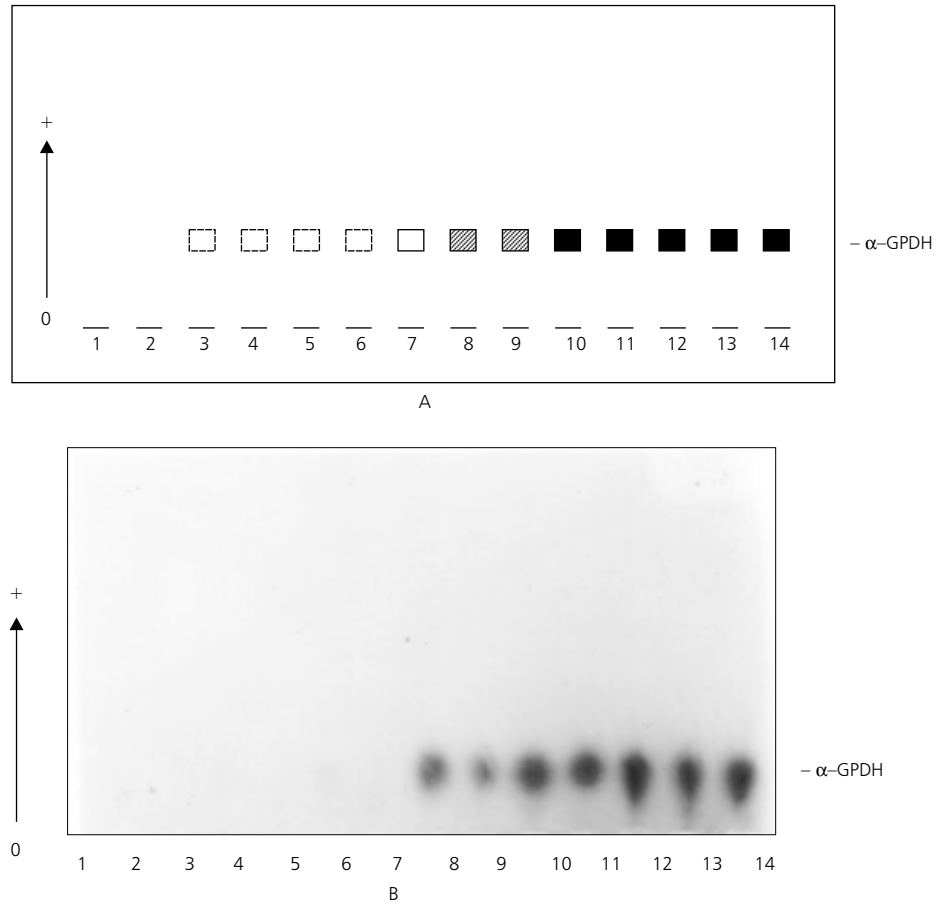

Fig. 5 — Electrophoretic profiles of a-glycerophosphate dehydrogenase during the ontogenetic development of *Anopheles mattogrossensis*. Starch gel electrophoresis. Tris-phosphate buffer system, pH 7.4. (A) Scheme. (B) Photography. Samples 1 to 5 = 4 th-instar larvae; 6 to 9 = pupae; 10 to 14 = adults.

Maia (1997) and Maia & Santos (1999) detected four activity regions for esterases during ontogeny of *Anopheles albitarsis*; EST1 was seen in the 4th-instar old larvae and pupae, EST2 and EST4 during all developmental stages, and EST3 was only detected in 4th-instar larvae. Similar results were obtained by Santos (1992) and Santos *et al.* (1996a,b) who detected five activity regions during ontogeny in *Anopheles darlingi*: EST1 and EST2 showed the highest staining intensity in the larval stages, whereas EST3 and EST4 were most deeply stained in pupae and adults; EST5 was detected during all developmental stages.

Several authors reported multiple bands of esterase activity in mosquitoes and they concluded that these bands are the result of genetic control of more than one locus. Among others we can mention Freyvogel *et al.* (1968) for *Anopheles freeborni, A. stephensi, Ae. aegypti* and *Culex tarsalis;* Vedbrat & Whitt (1975) for *Anopheles albimanus* and Narang *et al.* (1979) for *Anopheles aquasalis*.

The results concerning ontogeny of leucine aminopeptidase in *A. intermedius* and *A. mattogrossensis* are similar to those obtained by Maia & Santos (1999) in *A. albitarsis*. This author detected four activity regions: LAP1 and LAP2 was observed in the 4th-instar larvae; LAP3, was found only in pupae and adults; LAP4 was detected throughout development. However, six activity regions for this enzyme were observed in *A. nuneztovari* and *A. darlingi.* For the former species, Scarpassa (1988) and Scarpassa *et al*. (1992) reported for the population of Tucuruí (Pará) that LAP1 and LAP5 were detected at all developmental stages, LAP2 and LAP4 showed highest activity in larvae and reduced activity in pupae and adults, while LAP3 and LAP6 were characteristic of the last two stages. For *A. darlingi* Santos (1992) and Santos *et al.* (1996b) observed more activity of LAP1, LAP2 and LAP5 in larvae, while that for LAP3 was detected in pupae and adults, and LAP4 was restricted to pupae. The LAP4 of *A. mattogrossensis* detected in this study, may correspond with the LAP4 of *A. darlingi* revealed in the pupae stage, suggesting it may play a function in larval tissue histolysis at the time when the differentiation of adult tissues occurs.This hypothesis for the greater activity in pupae agrees with Sakai *et al.* (1969) and Pataryas *et al.* (1971) who reported the participation of the exopeptidases controlled by the *LAP-D* locus in the histolysis of larval tissues in the pupal stages of *Drosophila melanogaster.*

The electrophoretic patterns of α–glycerophosphate dehydrogenase isozymes during the ontogeny of *A. intermedius* and *A. mattogrossensis* – showing weak staining intensity in 4th-instar larvae with increasing activity in pupae and adults – are similar to those obtained by Narang *et al.* (1979), Santos *et al.* (1996b) and Maia & Santos (1999). These authors detected only one region, for this enzyme, whose activity increased until the adult stage of *A. aquasalis*, *A. darlingi* and *A. albitarsis*. However Scarpassa (1988) reported two forms of the enzyme in *A. nuneztovari*: α–GPDH1 appear in old pupae and adults with more staining intensity in the latter, and is controlled by two codominant alleles whose heterozygotes suggest that the enzyme has a dimeric structure; α–GPDH2 was observed only in larvae, without allelic variation. Similar results were found by Mukiama (1980) who detected two loci of α–GPDH during ontogenesis of *Ae. aegypti*: α–GPDH1 in larvae and pupae, and α–GPDH2 only in the adult stage.

Tadano (1984) reported polymorphism in *Aedes albopictus* with one locus that showed activity only in adult phase. Another band less thick than usual was occasionally detected in larvae and pupae. Palabost-Charles (1980) also observed one polymorphic locus with two alleles in *D. melanogaster*. In this same specie, Wright & Shaw (1969) described three loci in the adult phase and verified that α–GPDH1 was concentrated in the thorax, while α–GPDH3 was found in the head and abdomen. According to these authors, the occurrence of α–GPDH1 in the adult's thorax, where the flight muscles are located, suggests that the enzyme carries out two functions: 1) regeneration of NAD for the continuous glycolisis, and 2) production of energy needed for flight. The latter hypothesis is supported strongly by the fact that mutants defficient in the activity of α–GPDH cannot begin or maintain the flight (O'Brien & MacIntire, 1972). It is possible that the α–GPDH locus of the species treated in this paper corresponds to the α–GPDH1 locus of *D. melanogaster*, because the greatest activity of the enzyme was observed in the adult stage. On the other hand, this enzyme was polymorphic in some organisms, suggesting that α–GPDH it is not involved in the production of energy. Likewise, Zera (1981) reported high levels of polymorphism of this enzyme in aquatic hemipterous, noting that this may be the result of reductive selection pressure about this locus.

Data shown in Table 1 allows a general view of the three enzymes in the mosquito species studied to the present. Each species a characteristic ontogenetic profile within the range of the subgenus to which it belongs. The activity pattern of each enzyme is result of the role that it has according to its developmental stage.

TABLE 1

Comparative study of activity of Esterases, LAP and α–GPDH during ontogenesis in mosquito species.

Enzym	Isozyme	Enzyme activity in ontogenesis			Species	References
		L	P	A		
Esterase	1	+	+	+	*A. nuneztovari*, *A. intermedius* and *A. mattogrossensis*	Scarpassa (1988) and Diaz Rodriguez (1998).
		+	+	–	*A. albimanus*, *A. darlingi* and *A. albitarsis*	Vedbrat & Whitt (1975), Santos *et al.* (1996a, b) and Maia & Santos (1999).
	2	+	–	–	*A. intermedius*	Diaz Rodriguez (1998)
		+	+	–	*A. nuneztovari*, *A. darlingi*, and *A. mattogrossensis*	Scarpassa (1988), Santos *et al.* (1996a, b) and Diaz Rodriguez (1998).
		+	+	+	*A. albimanus* and *A. albitarsis*	Vedbrat & Whitt (1975) and Maia Santos (1999).
	3	+	–	–	*A. nuneztovari*	Scarpassa (1988).
		+	+	–	*A. albitarsis*	Maia & Santos (1999).
		+	+	+	*A. intermedius* and *A. mattogrossensis*	Diaz Rodriguez (1998)
		–	+	–	*A. albimanus*	Vedbrat & Whitt (1975).
		–	+	+	*A. darlingi*	Santos *et al.* (1996a, b).
	4	+	+	–	*A. nuneztovari* and *A. mattogrossensis*	Scarpassa (1988) and Diaz Rodriguez (1998).
		+	+	+	*A. albimanus*, *A. intermedius* and *A. albitarsis*	Vedbrat & Whitt (1975), Diaz Rodriguez (1998) and Maia Santos (1999).
		–	+	+	*A. darlingi*	Santos *et al.* (1996b)
	5	+	–	–	*A. albimanus*	Vedbrat & Whitt (1975).
		+	+	+	*A. nuneztovari*, *A. darlingi*, *A. intermedius* and *A. mattogrossensis*	Scarpassa (1988), Santos *et al.* (1996a, b) and Diaz Rodriguez (1998).
	6	+	+	–	*A. nuneztovari* and *A. mattogrossensis*	Scarpassa (1988) and Diaz Rodriguez (1998).
		+	+	+	*A. albimanus* and *A. intermedius*	Vedbrat & Whitt (1975) and Diaz Rodriguez (1998).
	7	+	–	–	*A. albimanus*	Vedbrat & Whitt (1975)
		+	+	+	*A. nuneztovari*	Scarpassa (1988)
		–	+	–	*A. mattogrossensis*	Diaz Rodriguez (1998)
	8	+	+	+	*A. albimanus*	Vedbrat & Whitt (1975).
	9	–	+	–	*A. albimanus*	Vedbrat & Whitt (1975).
LAP	1	+	–	–	*A. mattogrossensis* and *A. albitarsis*	Diaz Rodriguez (1998) and Maia & Santos (1999).
		+	+	+	*A. nuneztovari*, *A. darlingi*, and *A. intermedius*	Scarpassa *et al.* (1992), Santos *et al.* (1996b) and Diaz Rodriguez (1998).

TABLE 1 (*continued*)

Enzym	Isozyme	Enzyme activity in ontogenesis			Species	References
		L	P	A		
LAP	2	+	–	–	*A. darlingi*, *A. mattogrossensis* and *A. albitarsis*	Santos *et al.* (1996b), Diaz Rodriguez (1998) and Maia Santos (1999).
		+	+	+	*A. nuneztovari* and *A. intermedius*	Scarpassa *et al.* (1992) and Diaz Rodriguez (1998).
	3	+	–	–	*A. intermedius*	Diaz Rodriguez (1998).
		+	+	+	*A. mattogrossensis*	Diaz Rodriguez (1998).
		–	+	+	*A. nuneztovari*, *A. darlingi*, and *A. albitarsis*	Scarpassa *et al.* (1992), Santos *et al.* (1996b) and Maia & Santos (1999).
	4	+	+	–	*A. mattogrossensis*	Diaz Rodriguez (1998).
		+	+	+	*A. nuneztovari*, *A. intermedius* and *A. albitarsis*	Scarpassa *et al.* (1992), Diaz Rodriguez (1998) and Maia Santos (1999).
		–	+	–	*A. darlingi*	Santos *et al.* (1996b).
	5	+	+	+	*A. nuneztovari*	Scarpassa *et al.* (1992).
		+	–	–	*A. darlingi*	Santos *et al.* (1996b).
	6	+	+	+	*A. darlingi*	Santos *et al.* (1996b).
		–	+	+	*A. nuneztovari*	Scarpassa *et al.* (1992).
α GPDH	1	+	+	+	*A. darlingi*, *A. intermedius*, *A. mattogrossensis* and *A. albitarsis*	Santos *et al.* (1996b), Diaz Rodriguez (1998) and Maia Santos (1999).
		+	+	–	*Ae. aegypti*	Mukiama (1980)
		–	+	+	*A.aquasalis* and *A. nuneztovari*	Narang *et al.* (1979) and Scarpassa (1988).
		–	–	+	*Ae. albopictus*	Tadano (1984).
	2	+	–	–	*A. nuneztovari*	Scarpassa (1988)
		–	–	+	*Ae. aegypti*	Mukiama (1980)

L = larvae; P = pupae; A = adult
+ = activity; – = no activit

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