ELECTROPHORETIC ANALYSIS OF 11 ENZYMES IN NATURAL POPULATIONS OF *Anopheles (N.) darlingi* ROOT, 1926 (DIPTERA: CULICIDAE) IN THE AMAZON REGION.

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ABSTRACT — Of eleven proteins analyzed in four Amazonian populations, the esterases showed the greatest variation, with five activity zones. EST1, EST2 and EST5 showed variation in each of the populations studied. EST1 and EST2 are each controlled by two, and EST5 by four, codominant alleles. LAP presented six activity zones, with codominant variation in **LAP5** and **LAP6**. ∝—GPDH was monomorphic with one activity band on starch gel and two on polyacrylamide gel. IDH presented two activity zones, with variation in the IDH1 region. PGM had a single activity zone, with variation in all populations. The Ariquemes populations showed five alleles and the other populations three, all of then codominant. Three activity zones with two codominant alleles were observed for ODH. Aldehyde Oxidase showed two activity zones, with variation in AO1 only in the Ariquemes and Porto Velho/Samuel populations. 6-PGDH showed only one activity zone and variation only in the Ariquemes population. The remaing systems - XDH, G-6-PDH and GDH, was monomorphic.

Key words: *Anopheles darlingi*, isozymes, electrophoresis

Análise Eletroforética de 11 Enzimas em Populações Naturais de *Anopheles* (*N.*) *darlingi* Root, 1926 (Diptera: Culicidae) na Região Amazônica.

RESUMO — Dos 11 sistemas protéicos analisados, em quatro populações da Amazônia, as esterases foram as que apresentaram maior variação, com cinco zonas de atividade. EST1, EST2, e EST5 mostraram variação genética determinada em todas populações estudadas, sendo que as duas primeiras são resultantes do controle de dois alelos e a EST5 de quatro, todos codominantes. A LAP consiste de seis zonas de atividade e com variação apenas nos loci **LAP5** e **LAP6**, ambos codominantes. A ∝—GPDH mostrou-se monomórfica com uma zona de atividade em gel de amido e duas em poliacrilamida. A IDH apresentou duas zonas de atividade, com variação na região da IDH1. A PGM consiste de uma única zona de atividade e com variação em todas as populações estudadas. A população de Ariquemes revelou cinco alelos e as demais três, todos codominantes. Para a ODH, foram observadas três zonas de atividade com dois alelos codominantes. A enzima AO apresentou duas zonas de atividade, sendo que a AO1 mostrou-se variável apenas em Ariquemes e Porto Velho/Samuel. 6-PGDH possui apenas uma zona de atividade e variação apenas em Ariquemes. Os demais sistemas - XDH, G-6-PDH e GDH - mostraram-se monomórficos.

Palavras-chave: *Anopheles darlingi*, isoenzimas, eletroforese

INTRODUCTION

Studies on the protein variability of *Anopheles* have shown that in this group of mosquitoes, most isoenzymatic patterns are of autosomal codominant inheritance (BIANCHI & RINALDI, 1970; BULLINI *et al.*, 1971a; NARANG & KITZMILLER, 1971a, b; 1973a, b; NARANG & SEAWRIGHT, 1988; KAISER *et al.*, 1988; NARANG *et al.*, 1979a, b; 1989a, b; 1990; SANTOS *et al.*, 1992).

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Studies on the enzymatic polymorphism of *Anopheles* have been performed by (SANTOS, 1979; NARANG *et al.*, 1979a, b; SANTOS *et al.*, 1985) in populations of BR-174 (Manaus/Boa Vista). These authors found that, of the systems studied, the esterases were the most variable, with complex electrophoretic profiles involving six molecular forms. It was possible to estimate the number of males that had mated with a single female by analyzing the frequency of two alleles among the progeny of a single egg batch (SANTOS *et al.*, 1981a).

In this paper we report the electrophoretic patterns of eleven proteins analyzed in 4th-instar larvae and adults of four natural populations of *A. darlingi*. The number of activity zones and of loci involved were determined and possible allelic variants were identified.

MATERIAL AND METHODS

Samples on *A. darlingi* were obtained from natural populations of different Amazonian localities, i.e., Ariquemes, Porto Velho/ Samuel (RO), Manaus(AM), and Cachoeira Porteira(PA). Samples were collected in the adult stages. After capture, the females were fed chicken blood (*Gallus gallus*) and then placed in individuall cups for oviposition. After hatching, the larvae of each clutch were maintained in a sectarium at 26° ± 1°C in separate trays until adult emergence according to the method of SANTOS (1979) and SANTOS *et al.* (1981b). Electrophoretic analyses were carried out on fourth-instar larvae, pupae and adults. Individual samples were homogenized in 0,5% ß-mercaptoethanol and Whatman n°3 filter paper was used to absorb the supernatant for horizontal gel electrophoresis. Three types of gel were used, i.e., 12% starch (SMITHIES, 1955), starch-agarose at respective concentrations of 2% and 1%, and 7% polyacrylamide gel. Buffer solutions and reaction mixtures were prepared by the methods of HARRIS & HOPKINSON (1976) LIMA & CONTEL (1990) and SANTOS (1992).

Table 1 summarizes the electrophoretic conditions used for the study of eleven protein systems.

RESULTS AND DISCUSSION

Esterases

The electrophoretic pattern of *A. darlingi* esterases obtained during development using fluorogenic substrates (4-methylumbelliferyl acetate and propionate) and naphthol ester (∝–naphthyl acetate and propionate) revealed five electrophoretic activity zones being the esterases 1, 2 and 5 more deeply stained during the larval stages. Esterase 3 and 4 were characteristic of pupae and adult. The isozyme profiles obtained with the four substrates were quite similar, ∝–naphthyl propionate and 4-methylumbelliferyl propionate being the most efficient (Fig. 1). SANTOS (1979) tested seven naphthol esters in this species by polyacrylamide gel electrophoresis and detected a very similar electrophoretic profile with six activity zones. Even on these substrates, specificity was not observed. Nevertheless, a difference in esterases isozymes specificity for substrates was observed by other authors in

Tabela 1. Electrophoretic conditions utilized for the study of different proteins in *Anopheles darlingi*.

Enzyme	Buffers		Gel	Hours	V/cm
	Bridge	Gel			
	Borate	Tris-citrate	Starch	6; 16	8; 1.5
EST, LAP	pH 8.0	pH 8.0			
	0.3 M	0.193 M			
	Tris-phosphate	Tris-phosphate	Starch	18	4.5
∝–GPDH	pH 7.4	pH 7.4	Polycrylamide	4	4.5
	0.1 M	0.05 M			
	Phosphate-citrate	1:40 dilution	Starch	17	3
IDH	pH 5.9	0f bridge			
	0.395 M	buffer			
	TEMM	1: 15 dilution	Starch-agarose	5	6
PGM	pH 7.4	of bridge			
	0.22 M	buffer			
	Tris-HCL	Tris-HCL	Starch	16	2.5
GDH	pH 8.6	pH 8.6			
	0.3M	0.002M			
G-6-PDH,	Tris-citrate	Tris-citrate	Starch	4	4.5
6-GPDH	pH 6.9	pH 7.1			
	0.175M	0.012M			
ODH, AO,	Lithium-borate	Tris-citrate	Polyacrylamide	6	5.5
XDH	pH 8.2	pH 8.45			
	0.23M	0.083M			

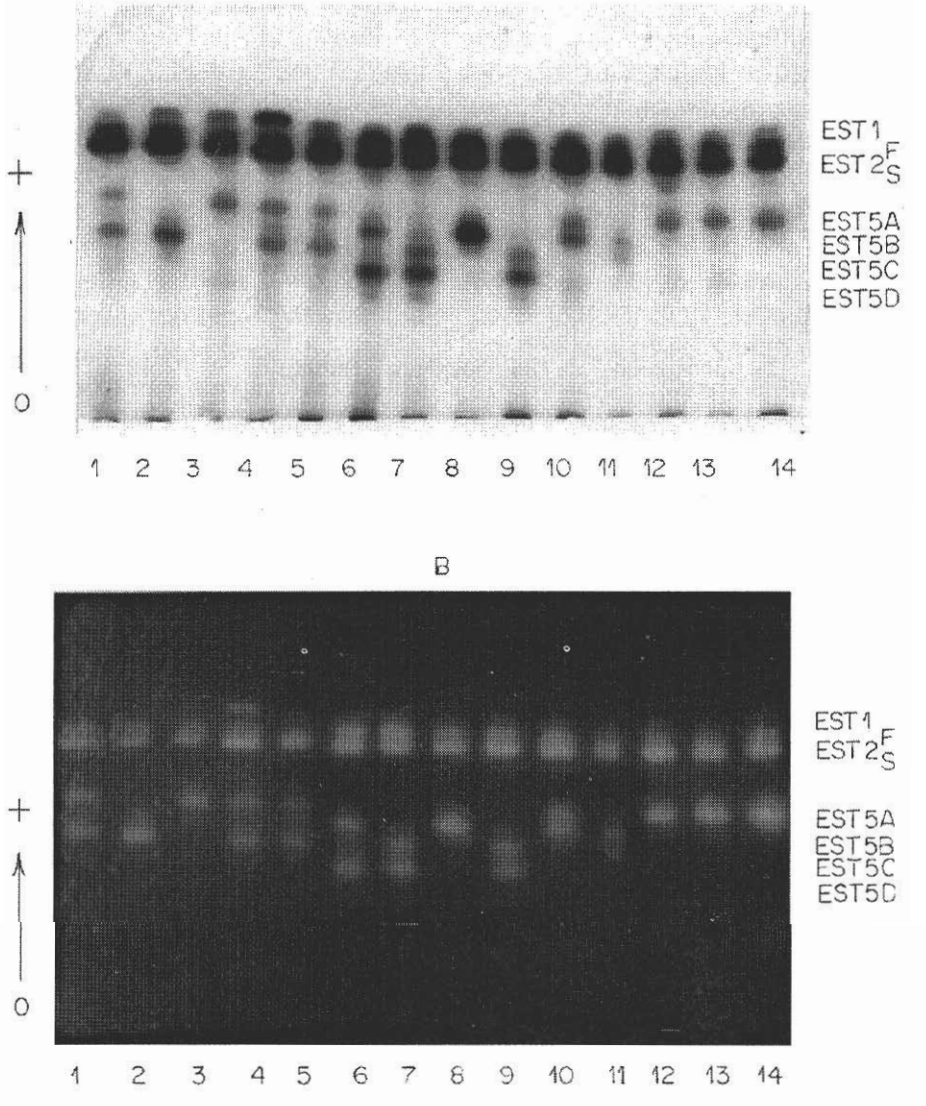

Figure 1. Electrophoretic profiles of esterases in 4th-instar larvae of *Anopheles darlingi*. Tris-citrate-borate buffer system, pH 8.0 . (A) ∝–naphthyl propionate and (B) 4-methylumbelliferyl propionate, showing the phenotypes of the **EST5** locus. Samples 3, 12, 13 and 14, EST5 A; sample 10, EST5 AB; samples 1, 4 and 5, EST5 BC; sample 6, EST5 BD; sample 2, EST5 C; samples 7 and 9, EST5 CD.

insects and other organisms, TREBATOSKI & HAYNES (1969) observed this difference in twelve species of mosquitoes, HOPKINSON *et al.* (1973) and COATES *et al.* (1975) in human erythrocytes, FALCÃO & CONTEL (1990) in species of stingless bees, and LIMA & CONTEL (1990) in *Spodoptera frugiperda.*

Of the five isozymes detected, EST1, EST2 and EST5 showed variation in four populations analyzed. The electrophoretic phenotypes observed for EST1 and EST2 suggest that each is genetically controlled by a locus with two codominant alleles (**EST1*F** and **EST1*S**, **EST2*F** and **EST2*S**). The heterozygous individuals presented two bands for the two loci, suggesting that these enzymes have a monomeric structure.

Similar results were observed for the **EST1**, **EST2** and **EST4** loci of *Anopheles triannulatus* (SANTOS *et al.*, 1992), **EST1** and **EST2** of *Anopheles nuneztovari* (SCARPASSA, 1988) and **EST2** of the *A. darlingi* population along the Manaus - Boa Vista Highway/BR-174 (SANTOS *et al.*, 1985) when the authors reported a genetic mechanism for each locus constituted by two codominant alleles.

The electrophoretic profiles for EST5 showed variation in the population studies with ten phenotypes. Of these possible phenotypes, EST5 C, EST5 CD and EST5 D were not detected in the Manaus population. Analysis of the data suggests that locus **EST5** has four alleles - **EST5*A**, **EST5*B**, **EST5*C** and **EST5*D** - of codominant action. The pattern of two bands observed in the heterozygotes suggests that EST5 presents a monomeric structure. A similar mechanism was found in the **EST3** and **EST5** loci of *A. triannulatus* (SANTOS *et al.*, 1992) and in the **EST6** locus of *A. nuneztovari* (SCARPASSA, 1988). The authors detected six phenotypes for these loci resulting from the action of three codominant alleles.

Leucine aminopeptidase

Leucine aminopeptidase consisted of six activity zones present during the life cycle of *A. darlingi.* The figure 2 shows LAP1, LAP2, LAP5 and LAP6, detected in 4th-instar larvae. LAP3 was especific for pupae and adults, LAP4 was detected only in pupae. Of the six bands detected, only LAP5 and LAP6 presented variations. In spite of difficulties in the interpretation of their phenotypes, the data suggest that loci **LAP5** and **LAP6** possess two codominant alleles each, denoted LAP5*F, LAP5*S, LAP6*F and LAP6*S, respectively. The two bands in the LAP5 and LAP6 forms may be interpreted as indicating heterozygous phenotypes for monomeric proteins.

Of the four populations in which the LAP system was studied, the Manaus population showed apparent monomorphism for the six loci analyzed. However, the data obtained were not sufficiently conclusive because of the small number of samples for this population.

Low variation was found in *A. darlingi* for this enzyme, in agreement with data in the literature for the majority of *Anopheles* species of the subgenus *Nyssorhynchus* (NARANG *et al.*, 1979a, 1979b; STEINER *et al.*, 1980; CONTEL, *et al.*, 1984;

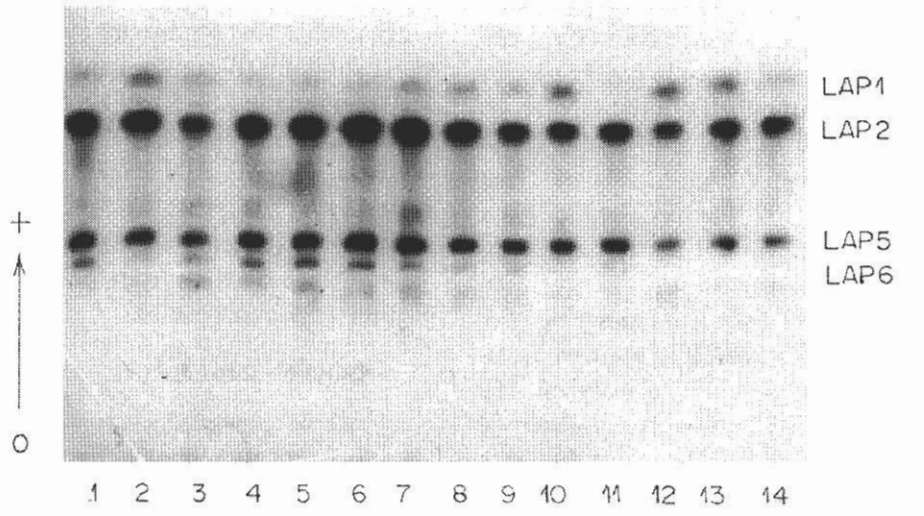

Figure 2. Electrophoretic profiles of leucine-aminopeptidase in 4th-instar larvae of *Anopheles darlingi* showing low variation. Tris-citrate-borate buffer system, pH 8.0 .

SCARPASSA *et al.*, 1992).

For Ariquemes (RO) and Highway PA-422 (PA) populations, CONTEL *et al.* (1984) reported that the three loci detected were monomorphic in two populations. In a population of *A. nuneztovari* from Tucuruí (PA), SCARPASSA *et al.* (1992) detected variability only for LAP5. STEINER *et al.* (1980) reported only the **LAP-F** locus in adult stage of this species in populations from Surinam and Venezuela with variations in both populations wich correspond the LAP3 detected by SCARPASSA (1992). In *Anopheles aquasalis*, four loci were described, with variation one for them (NARANG *et al.*, 1979b).

∝-Glycerophosphate dehydrogenase

The electrophoretic ∝-GPDH patterns of *A. darlingi* showed differences in adults according to the type of support utilized. On starch gel, the profile consisted of a single activity region (Fig. 3a). On polyacrylamide gel, the enzymes showed two activity zones very close to the origin, denominated ∝-GPDH1 and ∝-GPDH2 according to electrophoretic mobility (Fig. 3b). Both zones consist of only one activity band, but sometimes another band of small intensity in the more anodal zone denominated ∝-GPDH1 was revealed, possibly a secondary isozyme. The analysis of adult extracts revealed that this enzyme is monomorphic in the four populations analyzed. These results are partly similar to those observed by SCARPASSA & TADEI (1993) in the population of *A. nuneztovari* from Tucuruí (PA). These authors also described two activity zones for ∝-GPDH on starch gel, the most anodal denominated ∝-GPDH1 being specific to pupae and

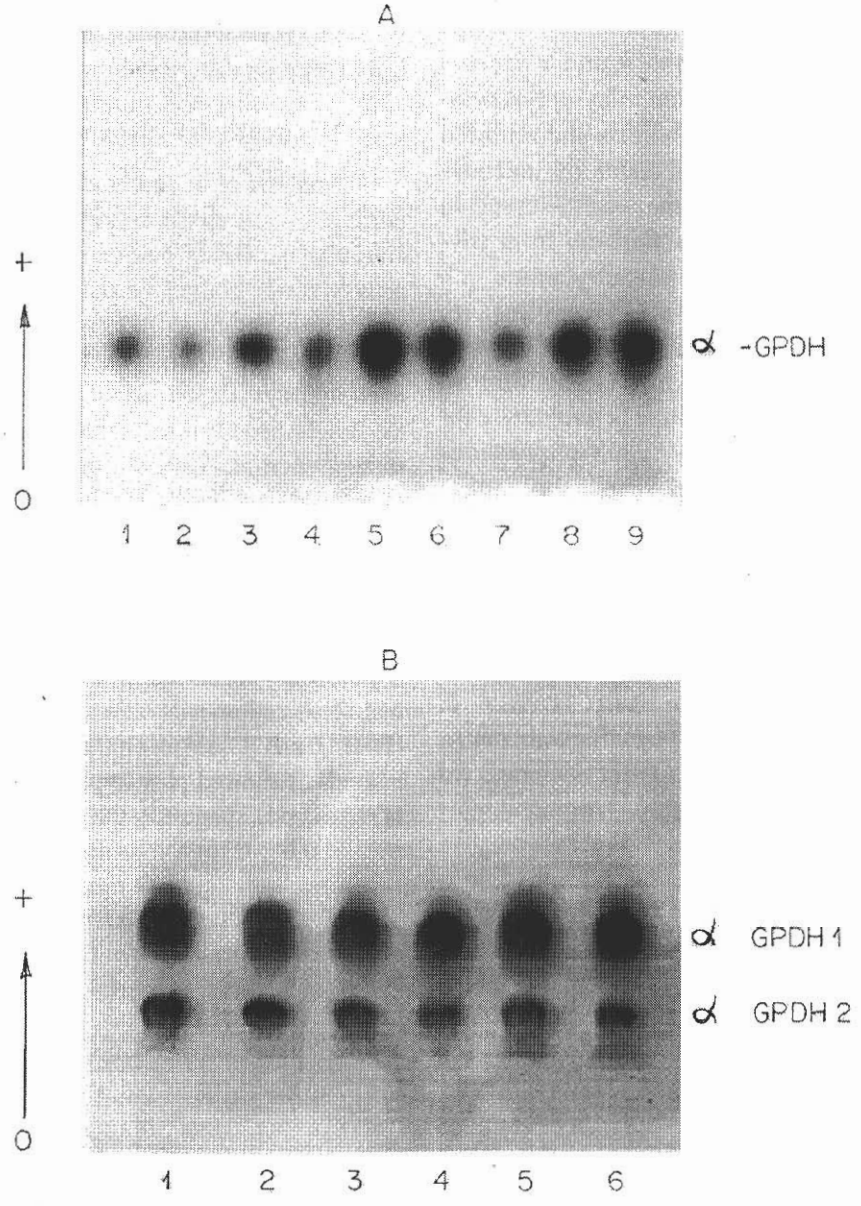

Figure 3. Electrophoretic profiles of ∝–glycerophosphate dehydrogenase in adults of *Anopheles darlingi*. Tris-phosphate buffer system, pH 7.4 . (A) Starch gel electrophoresis, (B) Polyacrylamide gel electrophoresis.

adults. NARANG *et al.* (1979a) studying this same species in populations from Manaus, also found two ∝-GPDH zones in adults by polyacrylamide gel electrophoresis and reported that each one was coded by independent loci. The same was observed in a population of *A. darlingi* from BR-174 analysed on polyacrylamide gel. In this population, NARANG *et al.* (1979a) detected only one activity zone in adult stage. STEINER *et al.* (1980) also found one activity zone for the *A. nuneztovari* adult populations from Surinam and Venezuela.

The ∝-GPDH enzyme of *A. darlingi* was monomorphic in the four populations studied. Considering the data in the literature, this enzyme varies little (JOHNSON, 1974; POWELL, 1975; LIMA & CONTEL, 1990; MACHADO & CONTEL, 1991). In *A. nuneztovari*, SCARPASSA & TADEI (1993) detected only three individuals with the ∝-GPDH S/ ∝-GPDH F phenotype out of 50 analyzed, and none showed the ∝-GPDH F/ ∝-GPDH F phenotype.

Isocitrate dehydrogenase

Analysis of 4th-instar larvae of *A. darlingi* revealed an electrophoretic pattern consisting of two isocitrate dehydrogenase activity zones, both of anodal migration, which were designated IDH1 and IDH2. The last one is monomorphic with a weak intensity band. The migration is closed to the slowest band in IDH1, with occasional superpositions, and suggest the existence of null alleles (Fig. 4). The IDH1 region presented variation in the four populations analyzed, with six phenotypes. Some of these phenotypes are ilustrated in Figure 4. The variations observed suggest that IDH1 is genetically controlled by a single locus with three codominant alleles: **IDH1*A**, **IDH1*B** and **IDH1*C**. The heterozygous individuals showed phenotypes consisted of three bands, suggesting that the enzyme

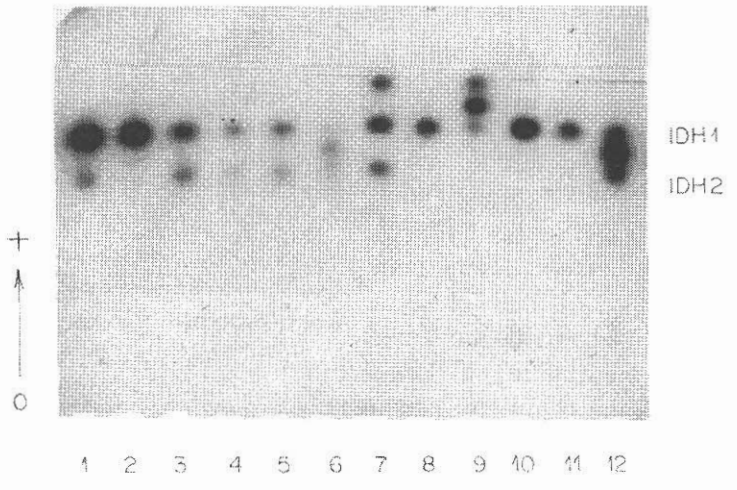

Figure 4. Electrophoretic profiles of isocitrate dehydrogenase in 4th-instar larvae of *Anopheles darlingi*. Phosphate-citrate buffer system, pH 5.9 . Phenotypes of the **IDH1** locus. Samples 1, 2, 3, 4, 5, 8, 10 and 11, IDH1 B; samples 6 and 12, IDH1 BC; sample 7, IDH1 AC; sample 9, IDH1 AB.

may be dimeric.

In the analysis of the Porto Velho/ Samuel population, the presence of allele **IDH1*A** was not detected. In the Cachoeira Porteira and Manaus populations the IDH1 A and IDH1 C phenotypes were not detected, whereas in Ariquemes only phenotype IDH1 A was not observed. Similar results were found in populations of *Anopheles stephensi* from Pakistan by VAN DRIEL *et al.* (1987) when 11 progenies were analyzed and the allele **IDH1*A** was not detected in four of them. The IDH2 region was monomorphic in all populations studied and consisted of only one weakly staining band located very close to the slowest IDH1 band. Analysis of these two regions was also hampered by occasional overlapping.

These results disagree, in part, with those obtained by ROSA-FREITAS *et al.* (1992) for three *A. darlingi* populations, the authors reported three alleles for the **IDH2** locus, two of them having low frequencies (0.0455 and 0.1591).

Phenotypic variants of IDH1 and IDH2 have been observed in the populations of *A. nuneztovari* from BR-174 (SCARPASSA, personal communication) and from Manaus for the **IDH2** locus (NARANG *et al.* 1979a).

Phosphoglucomutase

The electrophoretic pattern detected for phosphoglucomutase in 4th-instar larvae of *A. darlingi* consisted of a single activity zone of anodal migration. In the population analysis, PGM revealed phenotypic variants differing among the population studied. The data suggest that these phenotypes are genetically controlled by one locus with five codominant alleles: **PGM*A, PGM*B, PGM*C, PGM*A1** and **PGM*B1**. Of the possible phenotypes resulting from the combination of the alleles, we did not detect PGM A1, PGM A1B, PGM A1C or PGM B1C. Heterozygous individuals presented two bands of equal staining intensity. Only the **PGM*A** and **PGM*B** alleles were detected in Ariquemes. The other populations showed similar electrophoretic patterns with six phenotypes resulting from the control of three alleles, **PGM*A, PGM*B** and **PGM*C** (Fig. 5). These data are in accordance with those obtained BULLINI *et al.* (1971b, 1971c) in *A. stephensi* and CHARLWOOD *et al.* (1985) in *Anopheles punctulatus* Donitz, who also detected one activity band with polymorphism consisting of three alleles for the first species and four alleles for the second. ROSA-FREITAS *et al.* (1992) studied PGM in one population of *A. darlingi* from the Amazon region and two from the South of Brazil and detected four alleles, three of them segregating in each population.

Octanol dehydrogenase

Octanol dehydrogenase presented three activity zones denominated ODH1, ODH2 and ODH3. ODH1 showed variations in the populations from Ariquemes, Porto Velho/Samuel and Manaus, with three phenotypes in which heterozygous individuals consisted of three activity bands, suggesting that the protein could be dimeric (Fig. 6a). The

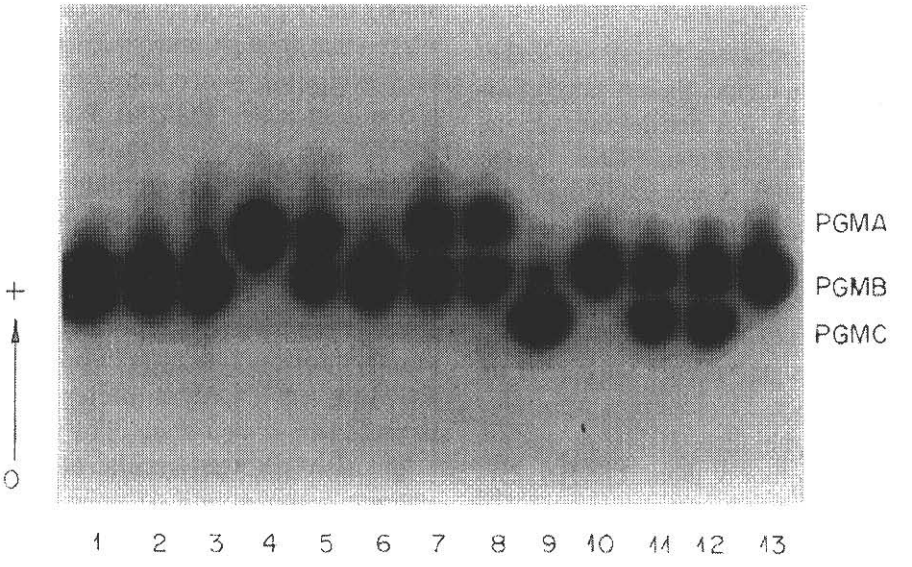

Figure 5. Electrophoretic profiles of phosphoglucomutase in 4th-instar larvae of *Anopheles darlingi*. TEMM buffer system, pH 7.4 . Electrophoretic phenotypes of PGM in the Manaus population. Sample 4, PGM A; samples 5, 7 and 8, PGM AB; samples 1, 2, 3, 6, 10 and 13, PGM B; samples 11 and 12, PGM BC; sample 9, PGM C.

phenotypic variants detected for ODH1 may be determined by two codominant alleles at the **ODH1** locus, **ODH1*F** and **ODH1*S**. In the population from Cachoeira Porteira, only individuals showing the ODH1 S phenotype were detected (Fig. 6b). The observation of the phenotypes suggest the existence of a null allele - **ODH1*O**. The null allele was not detected in the population from Manaus.

The **ODH2** locus showed variability in all populations and notwithstanding difficulties in the interpretation of their phenotypes, the data suggest that there exist three alleles - **ODH2*F**, **ODH2*S** and **ODH2*O**.

Locus **ODH3** showed variability in all populations except that from Manaus. The phenotypic variation detected suggests that this locus has three alleles: **ODH3*F**, **ODH3*S** and **ODH3*O**. Similar data were observed by NARANG *et al.* (1979a) in a population of *A. darlingi* from BR-174 (Manaus/Boa Vista) and in a population of *A. nuneztovari* from Manaus. In both species, the authors reported three actvity zones. In the first population, polymorphism was only detected at the **ODH2** locus, whereas in the second, polymorphism was detected only at the **ODH1** locus. At both loci, these authors detected three codominant alleles. In *A. aquasalis*, NARANG *et al.* (1979b) also reported three **ODH** loci, but all were monomorphic. In the *A. darlingi* populations studied here, the **ODH1** locus from the Cachoeira Porteira population and the **ODH3** locus from Manaus were monomorphic. In the genus *Anopheles*, NARANG and KITZMILLER (1971b) reported that for *Anopheles punctipennis*

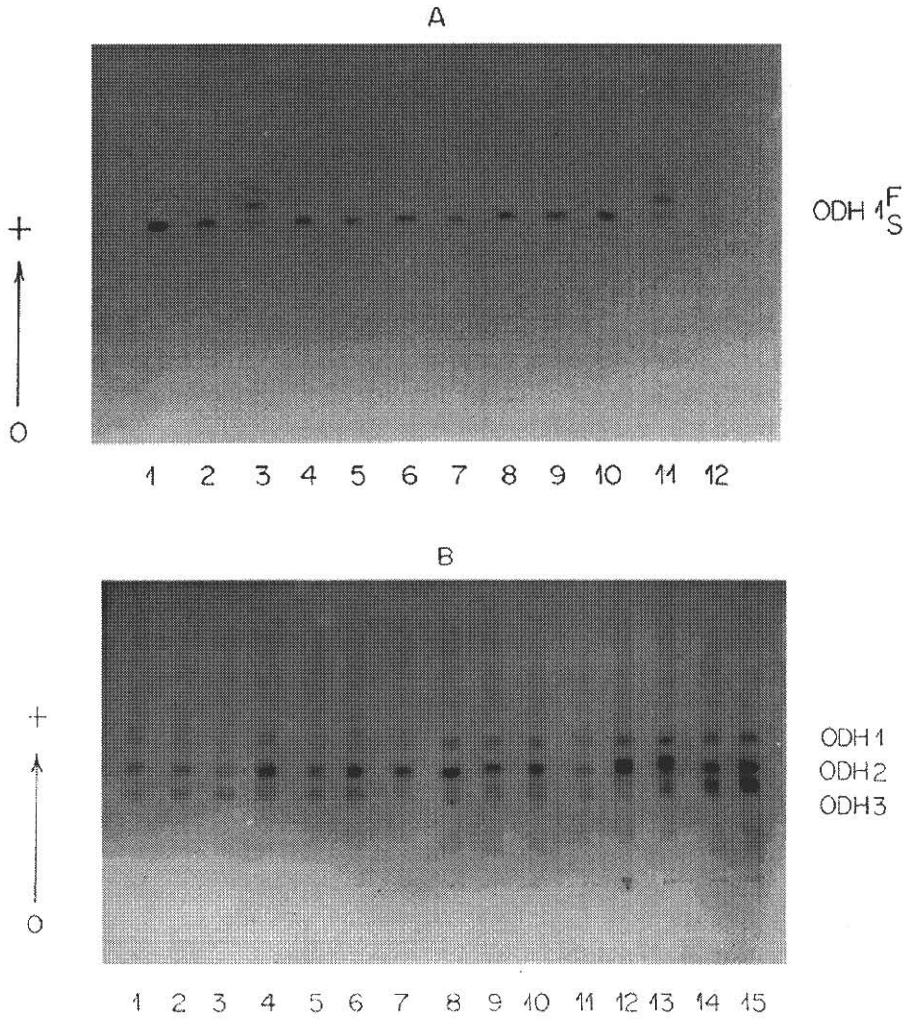

Figure 6. Electrophoretic profiles of octanol dehydrogenase in 4th-instar larvae of *Anopheles darlingi*. Polyacrylamide gel electrophoresis. Tris-citrate, pH 8.4 and lithium-borate, pH 8.2, buffer system. (A) Heterozygous phenotype (ODH1 FS) for the **ODH1** locus in samples 3, 11 and 12. (B) **ODH1**, **ODH2** and **ODH3** loci, with variation at the **ODH1** locus in the Ariquemes population (1 to 7 - ODH1 FS phenotype) and only the ODH1 S phenotype in the Cachoeira Porteira population (8 to 15).

the electrophoretic profile of ODH consisted of only one activity band with three phenotypes controlled by two codominant alleles. Similar results were found in *Anopheles culicifacies* by AHMAD *et al.* (1978).

Aldehyde oxidase

Aldehyde oxidase consisted of two main activity zones: AO1 and AO2. A third zone was detected in the Ariquemes and Cachoeira Porteira populations, but it was not considered in the populations studied.

AO1 showed variability only in Ariquemes and Porto Velho/Samuel, and consisted of three phenotypes. The phenotypic variants detected suggest that this locus may have two codominant alleles, **AO1*F** and **AO1*S**, with heterozygous individuals showing three bands suggesting a dimeric structure (Fig. 7a). Null alleles were also detected at this locus. Analysis of the Cachoeira Porteira and Manaus populations did not show variations for the AO1 locus, but only the **AO1*F** allele was observed (Fig. 7b).

AO2 showed variations in all populations analyzed, with a pattern consisting of two or three bands in the heterozygotes, which may be interpreted as secondary changes, i.e., post-translational changes. Analysis of these phenotypes suggests that the genetic control of this enzyme occurs through two codominant alleles - **AO2*F** and **AO2*S**. A similar pattern was observed in a population of *A. darlingi* from BR-174 (Manaus/Boa Vista) and a population of *A. nuneztovari* from Manaus (NARANG *et al.*, 1979a). In both species, the authors reported three activity zones, with variations, in which the most anodic (AO1) had low activity, which was visualized only after a prolonged time. In *A. darlingi* studied here, it was the AO3 zone that presented low activity and that was not visualized in most analyses.

6-Phosphogluconate dehydrogenase

A single activity zone of phosphogluconate dehydrogenase was detected (Fig. 8). Variation for this enzyme was found only in the Ariquemes population. The phenotypes observed suggest the existence of two alleles: **6-PGDH*F** and **6-PGDH*S**. The populations from Porto Velho/Samuel, Cachoeira Porteira and Manaus were monomorphic.

The data of variation in heterozygotes showed three bands, indicating that this protein has a dimeric structure, the same as that reported by CHENG & HACKER (1976) in *Culex p. quinquefasciatus*. SCARPASSA (personal communication) detected three alleles for the **6-PGDH** locus with segregation for only two in each population of *A. nuneztovari* from amazon region.

Xanthine dehydrogenase

Under the electrophoretic conditions used, two activity zones of xanthine dehydrogenase were observed: XDH1 and XDH2. These two regions were monomorphic in all populations analyzed and were represented by only one band (Fig. 9). XDH1 presented low activity and was detected after a prolongated period. XDH2 is the most prominent and presents a band of more intense activity. These results are in agreement with those reported by NARANG *et al.* (1979a)

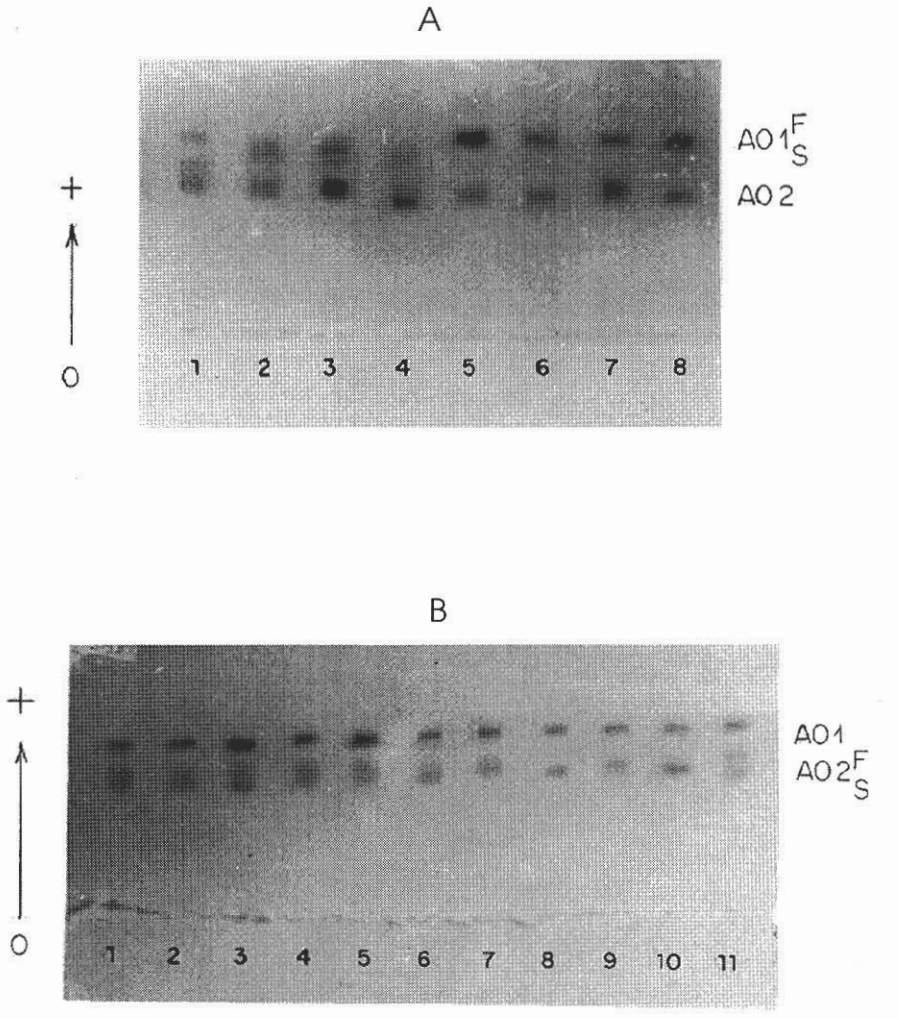

Figure 7. Electrophoretic profiles of aldehyde oxidase in 4th-instar larvae of *Anopheles darlingi*. Polyacrylamide gel electrophoresis. Tris-citrate, pH 8.4 and lithium-borate, pH 8.2, buffer system. (A) Variation for AO in the Porto Velho/Samuel population. Phenotypes for the **AO1** locus. Samples 1, 5, 6, 7 and 8, AO1 F; samples 2 and 3, AO1 FS; sample 4, AO1 S. (B) **AO1** locus showing monomorphism in the Cachoeira Porteira population. **AO2** locus showing variation: samples 1, 2, 3, 4, 5 and 11 - AO2 FS phenotype.

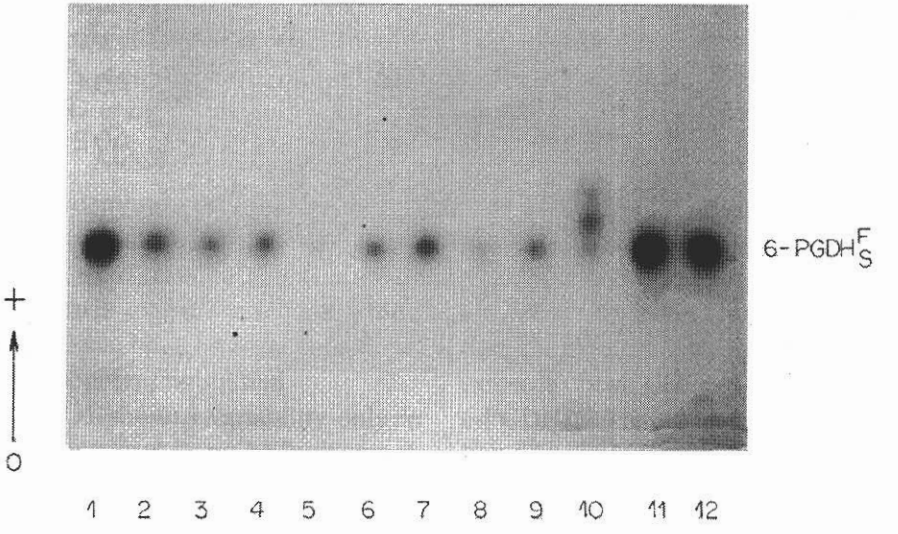

Figure 8. Electrophoretic profile of phosphogluconate dehydrogenase in 4th-instar larvae of *Anopheles darlingi*. Tris-citrate, pH 7.1 and Tris-citrate, pH 6.9, buffer system. Variation for 6-PGDH in the Ariquemes population, sample 10, 6-PGDH FS.

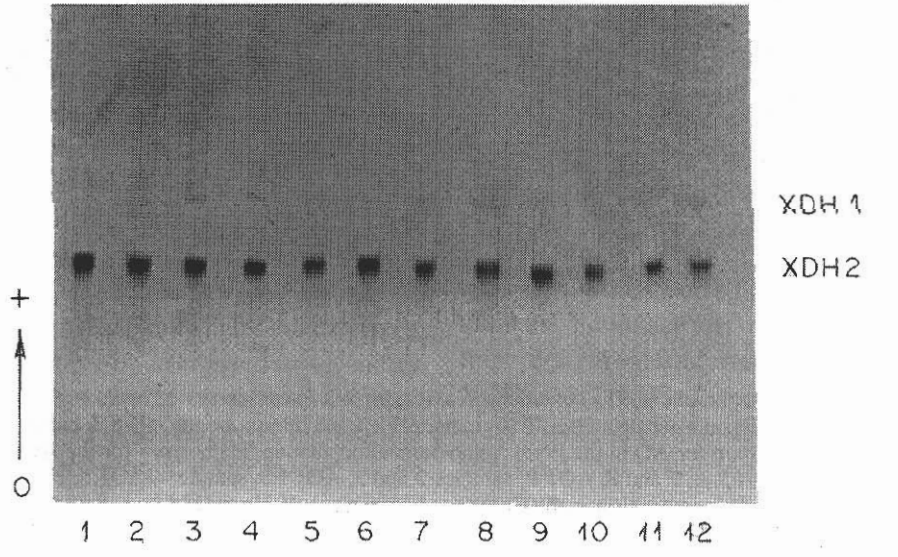

Figure 9. Electrophoretic profile of xanthine dehydrogenase in 4th-instar larvae of *Anopheles darlingi*. Polyacrylamide gel electrophoresis. Tris-citrate, pH 8.4 and lithium-borate, pH 8.2, buffer system.

for the *A. darlingi* population from BR-174. These authors detected three activity zones without variation, in which only the XDH2 zone was considered, since XDH1 and XDH3 showed low activity.

The other systems analyzed in the present study - glucose dehydrogenase and glucose-6-phosphate dehydrogenase - revealed only one activity band, without variations in the populations.

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