BIOLOGY OF AMAZONIAN MOSQUITOES. III. ESTERASE ISOZYMES IN ANOPHELES DARLINGI.

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SUMMARY

Six esterase isozymes were studied during the development of ***Anopheles darlingi*** *by using polyacrylamide gel electrophoresis and two different substrates, α-naphthyl-acetate and α-naphthylpropionate. Esterases 5 and 6 were detected in all developmental stages; esterases 1 and 2 were more intensively stained in larvae, while esterases 3 and 4 were better visualized in pupae and adults. Strong differences in intensity of some of the isozymes were observed during the pupal stage.*

*Four out of the six isozymes showed variation in the electrophoretic mobility. Esterase-2 was choosed for genetic studies, because was the best stained isozyme in the gels. Two codominant alleles (**Est2*S** and **Est2*F**) code for this polymorphic system, with the **Est*S** frequency equal to 0.521. Phenotypic distribution is in agreement with Hardy-Weinberg expectations.*

INTRODUCTION

Anopheles darlingi is a mosquito species closely associated with man in the Amazon region. Thus it is important to obtain data on its biology, ecology, ethology and genetics. In previous papers we have reported data on the biology and ethology of this mosquito (Santos **et al.**, 1981a; 1981b). In the present paper we study six esterase isozymes, particularly the genetic aspects of Esterase 2.

Thus far, only one description of **A. darlingi** isozymes has been published (Narang **et al.**, 1979b), whereas an increasing number of polymorphic isozymic systems has been described in other species such as **stephensi** (Bianchi, 1969; Bullini et al., 1971 ; Iqbal **et al.**, 1973), **punctipennis** (Narang & Kitzmiller, 1971a; 1971b), **aquasalis** (Narang **et al.**, 1979a), **nuneztovari** (Narangi **et al.**, 1979b), **albimanus** (VedBrat &

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Whitt, 1976) and **culicifacies** (Dubash **et al.**, 1982), among others.

The objective of the present study was to analyse esterase isozymes in **A. darlingi** during different developmental stages, and to estimate the gene frequencies in a sample collected in the Amazon region. An interesting result that was already published is the estimate of the number of males that mate with a determined female by means of the allele frequencies.

MATERIAL AND METHODS

The especies used in this study, **Anopheles darlingi**, was collected at two different localities (Km 137 and Km 182) along the Manaus-Boa Vista Highway (BR, 174). All developmental stages were used for the ontogenetic study. Larvae were reared by the methods of Freire & Faria (1947) and Rabbani **et al.** (1976), slightly modified.

Individuals were studied by electrophoresis at all developmental stages. 1st, 2nd and 3rd-instar larvae were respectively, pooled together, whereas 4th-instar larvae, pupae and adults were homogenized individually. Larvae and pupae were homogenized in 15 ul distilled water, and adults in 20 ul distilled water. The homogenate was centrifuged at 3,500 rpm for 5 minutes at room temperature. Several buffer systems were tested and Tris-HCl, pH 8.6 was selected (Smith **et al.**, 1971, slightly modified). Electrophoresis was carried out by the technique of Stainer & Johnson (1973), with some modifications, on polyacrylamide gel. 7 g Cyanogum-41 were dissolved in 100 ml 0.02 M Tris-HCl buffer, pH 8.6, and the reaction was catalyzed by the stepwise addition of 0.1 ml TMED and 0.43 ml 10% ammonium persulfate. Electrophoresis was carried out at 4º for a period of approximately 3 hours with a difference of 170 V between the ends of the gel, until the dye used as a migration control (Bromophenol Blue) had migrated approximately 10 cm from the origin.

Esterase activity was characterized using naphthol esters by the methods of Stainer & Johnson (1973): 20 mg Fast Blue RR and 2 ml of substrate solution (1 g ester dissolved in 100 ml 1:1 water: acetone, v/v). The following substrates were tested : α-naphthyl acetate, propionate, stearate and laurate; β-naphthyl acetate, propionate and stearate.

Only 4th-instar larvae were used to determine possible genetic variability and, consequently, gene frequency, since their isozymes showed the best resolution. We analysed 143 larvae from 15 ovipositions selected at random.

RESULT

Six regions of esterase activity, all of them electronegative, were detected using different naphthol ester substrates. They were denominated esterase-1 through 6 according to electrophoretic mobility, the lower numbers being given to the esterases of higher mobility (Figure 1).

ESTERASE ISOZYMES OF **ANOPHELES DARLINGI** DURING ONTOGENETIC DEVELOPMENT

The results of the electrophoretic analysis of enzymes having esterase activity in **Anopheles darlingi** during the different developmental phases, detected with α-andβ-naphtyl acetate and α- and β- naphtyl propionate, are described bellow. The isozyme profiles obtained with the four substrates were quite similar, and similarity was higher when α-naphthyl acetate and proprionate were used.

Esterases 1 and 2 showed the highest staining intensity in the larval stages,but reduced staining in pupae and adults with all four substrates. No esterase-2 activity was detected in pupae when β-naphthyl propionate was used. The opposite occurred with esterases 3 and 4, which were most deeply stained in pupae and adults. No esterase -3 or esterase-4 activity was detected in 1st instar larvae when α-naphthyl acetate was used, or in any larval stage with β-naphthyl acetate and propionate.

Esterases 5 and 6 were detected during all developmental stages when α-naphthyl acetate and propionate were used, but only in some stages with the other two substra - tes.

Esterase -1 showed growing staining intensity in the larval stages, with high intensity in pupae and reduced (many times absent) intensity in adults. Esterase - 2 had excelent staining intensity in the larval stages, and esterases 3 and 4 in general had growing staining intensity from the larval stages to the adult phase regardless of the substrate used. Esterase 5 and 6 were the weakly stained with any of the substra - tes, and in many analyses were not visualized at all in the gel when β-naphthyl aceta te and propionate were used.

Significant changes in staining intensity occurred during the pupal stage for so me of the isozymes. Esterase 3 and 4 showed increased staining intensity at pupation and remained well visible throughout the pupal stage (Figure 2).

Esterases 1 and 2 (the most intensely stained isozymes in the larval analyses) continued to show considerable staining during pupation. However, the intensity decre ased after about 10 hours, and neither esterase was detected approximately 20 hours after pupation. On the other hand, a more detailed analysis of these data seems to show that esterase-1 and esterase-2 appear with higher intensity during the 4th larval stage, disappear in older pupae (dark pupae) and reappear with low intensity in adults. This phenomenon needs further detailed investigation.

Pupal analysis showed that, as pupae grow older, the electrophoretic profile of esterase isozymes is very similar to that detected in adults. No differences were detected between adult males and females.

ELECTROPHORETIC VARIANTS OF **ANOPHELES DARLINGI**

The isoesterase profile of **Anopheles darlingi** is complex, with six molecular forms whose staining intensity vary according to development. Variation in electrophoretic mobility was observed in four of these six esterases.

Esterase-1: most larval and pupal analyses showed one or two bands for this enzyme. In some individuals, the electrophoretic profiles were characterized by the appearance of three bands. This is typical of heterozygous for a dimer enzyme, whose synthesis depends on two codominat alleles. However, our observations with respect to this variability cannot be considered definitive, both because of the small number of analyses and because of the existance of an overlap between the showest band of this typical heterozygote profile and the fastest band of the form denominated esterase-2.

Esterase-2: of all isozymes detected at the different developmental stages, esterase-2 showed the sharpest variation, with three different phenotypes perfectly distinguishable on the gel. The first, represented by a single band of fast mobility, was designated esterase-2F; the second, also represented by a single band, but of slow mobility, was denominated esterase-2S, and the third, with two bands running simultaneously and showing the same mobility as the bands that occurred individually in the phenotypes described earlier was denominated esterase-2FS(Figure3). This isozyme form was also studied in some of its genetic aspects. Preliminary analyses were also done using a fluorogenic substrate (4-methylumbelliferyl propionate), which also reveals polymorphic variation in esterase-2. This type of analysis with a fluorogenic substrate was done more in depth in the study of an **A. darlingi** sample collected from other sites in the Amazon region (Contel **et al.**, 1984).

Esterases 3 and 4: staining intensity was very low in larvae, and this prevented a more precise analysis of the possible variants of these two isozymes. In pupae and adults, esterase-3 shows one to two bands with different electrophoretic mobility (slow and fast) which are located very close to the esterase-4 region. This (esterase-4) is frequently represented by a single band with variations in electrophoretic mobility. Another band less thick than the common one was occasionally detected in the esterase-4 region, suggesting that it is probably a secondary isozyme. Analysis of these two regions was also hampered by occasional overlapping.

Esterases 5 and 6: although there was evidence of electrophoretic variants of these two enzymes in 4th-instar larvae it was not possible to establish whether the type of control had genetic basis because the two isozymes were weakly stained on the gels.

GENETIC DATA AND ESTIMATES OF THE GENE FREQUENCIES OF THE POLYMORPHIC ESTERASE-2 SYSTEM

The results obtained (three distinct electrophoretic phenotypes) suggest that esterase-2 is controlled by two alleles at the same locus, **Est2*S and Est2*F**. The **Est2*F** allele determines the esterase with fast mobility and **Est2*S** the esterase with slow mobility. In heterozygotes, these alleles are expressed in a codominant manner thus producing esterase-2FS, characterized by the presence of two bands, a slow one and a fast one (Figura 3). The genotypes corresponding to the three phenotypes observed were denoted **Est*2S/Est*2S, Est2*F/Est2*S and Est2*F/Est*2F.**

Our results indicate that the frequency of the **Est2*S** allele is 0.521. We also observed that phenotype distribution agrees with the distribution predicted by random mating, as shown in Table 1.

DISCUSSION AND CONCLUSIONS

The analysis of several isozyme systems in **Anopheles** has indicated that these *mosquitoes are very useful for the study of biochemical genetics and development* (Ved-Brat & Whitt, 1976). A relatively large number of esterases has been visualized in mosquitoes by gel electrophoresis. Freyvogel **et al.** (1968) have shown 19 esterase bands in **Anopheles stephensi** and 24 in **Culex tarsalis**. Nine esterase forms have been recognized in **Anopheles albimanus** (VedBrat & Whitt, 1975). Briegel & Freyvogel (1971) have detected 12 esterase forms in Culex **pipiens fatigans,** and Narang **et al.** (1979b) detected 7 esterase forms in **A. nuneztovari.**

In the present study, 6 esterase forms were visualized during the development of A. **darlingi**, some of which indicating allelic variation and changes in gene expression. Esterases 1 and 2, that stained more deeply in larvae, revealed cyclic expression; esterases 3 and 4, that stained more deeply in pupae and adults, were rarely present in larvae, and esterases 5 and 6 present at all developmental stages, weakly stained in pupae and adults.

Similar results have been reported for enzymes of **Drosophila** and other insects, such as isocitrate dehydrogenase, hexokinase, desoxyribonuclease, alkaline phosphatase, *esterase, amylase, glucose-6-phosphate dehydrogenase, and aconitase* (Wagner & Selander, 1974).

As far as the cyclic expression of the gene controlling esterase-1 synthesis, similar results were obtained by VedBrat & Whitt (1975) in **Anopheles albimanus**: the enzymes showed a very weak band before pupation, was not detected on the gel during the first 2 to 3 hours of the pupal stage and was visualized up to 18 hours after pupation. The authors explained this fact in terms of the regulation of ecdysone concentration, postulating that this isozyme form participates in ecdysome metabolism. It is possible that esterases 1 and 2 in the pupal stage of A. **darlingi** have some regulatory function on ecdysone levels. The appearance of this isozyme during the adult phase may be interpreted to be due to its participation in lipid degradation. In contrast to the behavior of esterases 1 and 2, esterases 3 and 4 are more deeply stained in pupae and adult, thus suggesting they may play a role in the maintenance of low levels of juvenile hormone. This hormone functions by favoring the expression of larval characteristics and normally is not present in pupae or adults, or is present at very low concentrations (*Williams, 1961, quoted by Downer, 1975). Similar results were obtained in* **Hyalophora gloveri** by Whitmore **et al.** (1972), who were led to propose that these esterases are important in the maintenance of low levels of juvenile hormones, a mechanism whereby insects can assure normal metamorphosis. Pantelouris and Downer (1969) also detected carboxyl esterase forms in **Drosophila immigrants** that were more deeply stained in pupae and adults. These data may have implications for those interested in the use of juvenile hormone and its analogs as insect-controlling agents. Whitmore **et al.** (1972) suggest that insects have a biochemical repertory that not only permits the degradation of foreign molecules (such as DDT, for example) but turns them resistant by means of their own hormones when necessary. Gilmour (1965, quoted by chambers, 1973) advanced the hypothesis that some insect carboxylesterases represent an important defense against organophosphates and possible other insecticides. Pasteur & Sinegre (1975) showed an increase in the frequency of the Est-$2^{0,64}$ allele in populations of **Culex pipiens** that were less sensitive to an organaphosphate insecticede.

Esterases 5 and 6, which are present in all developmental stages, may have a more generalized function than the esterases that are characteristics of a particular developmental stage. Esterases that are present throughout development have reported in **Anopheles albimanus** (VedBrat & Whitt, 1975), **Drosophila** (Pantelouris **et al.**, 1969) Berger & Canter, 1973), and **Plodia interpunctella** (Pataryas **et al.**, 1970). Our data did not demonstrate the existence of isozyme forms that appear in a single phase of development only, in contrast to the data reported by VedBrat & Whitt (1976) in **Anopheles albimanus**, where some isozymes appear only in larvae and others in pupae. Qualitative differences in the electrophoretic profile of esterases during the various developmental phases have been also observed in other insects, such as bees, for example (Contel & Mestriner, 1975). In the present study, no qualitative differences were detected in the isozyme forms of males and females, in agreement with observations by Briegel & Freyvogel (1971) in **Aedes aegypti**.

Most of the six esterase isozymes of **A. darlingi** showed electrophoretic variants. The variation observed for this enzyme group is consistent in a wide variety of organisms: **Apis mellifera** (Mestriner & Contel, 1972), **Fundulus heteroclitus** (Holmes & Whitt, 1970), **Drosophila willistoni** (Ayala **et al** ., 1972) **Peromyscus leucopus** (Wilmot & Underhill, 1973), **Drosophila pseudoobscura** (Lewontin & Huddy, 1966), **Colias eurytheme** (Burns & Johnson, 1967), **Anopheles albimanus** (VedBrat & Whitt, 1975), **Aedes aegypti** (Saul **et al.**, 1976), and **Anopheles punctipennis** (Narang & Kitzmiller, 1971a and 1971b).

Esterase-1 showed electrophoretic variants in most of the larvae studied. One or two regions, and occasionally three, indicating activities, were frequently visualized. The appearance of three bands in the esterase-1 form may be interpreted to be the heterozygous for phenotype a protein with dimeric structure.

Esterase-2 variants consisting of three distinct phenotypes were also observed at different developmental stages: a phenotype with a single, fast band; a second one with a single, slow band, and a third one with two simultaneous bands, one of which was fast, and the other slow. These data suggest that the genetic control of these enzyme occurs through two codominant alleles and that the enzyme has a monomeric structure.

In most of the pupal and adult analyses, esterase-3 showed two variants, a fast one and a slow one. However, as previously commented, the interpretation of this variability is difficult, mainly because of the overlapping of esterase-3 and esterase-4 bands, a phenomenon already observed in **A.nuneztovari** by Narang **et al.** (1979a). More conclusive data may be obtained by using different types of substrates and inhibitors. The use of these substances and of other kinds of buffers may provide data for better caracterization of the different isozymes. As already indicated, we are now using a larger number of substrates.

The variation observed with respect to esterases 5 and 6 suggests genetic control rather than secondary changes, i.e., post-translational changes. However, the analysis of this variation was difficult because these isozymes stained very weekly in the gel. This is a question we intend to approach in later studies.

The analyses of 4th-instar larvae with respect to the **Est2** locus suggest that the three electrophoretic variants are phenotypic expressions of the activities of two alleles, **Est2*S and Est2*F** that are located on an autosome and are expressed codominantly in heterozygotes. The frequencies of these alleles were practically identical (**Est2*S** = 0.521) and the observed phenotype distribution in the population agreed with the expected theoretical distribution. The isozyme profile of the heterozygote, two equally stained bands, suggest that esterase-2 has a monomeric structure.

The results reported in the present paper on the basis of the gene frequencies observed for the **Est2*F** and **Est2*S** alleles and of the possible types of mating also demonstrated that **Anopheles darlingi** females are monogamous, or, even if a female mates with two males she will be fecundated by the spermatozoa of only one. (Santos **et al.**, 1981a). In that occasion, the χ^2 value obtained by the authors, when the hypothesis of a female being fertilized by more than one male was considered, was highly significant ($P < 0.001$). When the hypothesis of a single male fertilizing a female was considered, the χ^2 values showed that the differences obtained were not statistically different ($0.30 < P < 0.50$) form those expected. Therefore, they suggested that each female is fertilized by one male only.

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RESUMO

Seis isoenzimas de esterase foram estudadas durante o desenvolvimento de ***Anopheles darlingi*** *usando eletroforese em gel de poliacrilamida e dois substratos diferentes, α-naftilacetato e α-naftilproprionato. Esterases 5 e 6 foram detectados em todos os estágios do desenvolvimento, esterases 1 e 2 foram coradas com maior intensidade em larva, enquanto esterases 3 e 4 foram melhor visualizadas em pupa e adulto. Foram observadas grandes diferenças na intensidade de algumas isoenzimas durante o estágio de pupa.*

*Quatro das seis isoenzimas revelaram variações na mobilidade eletroforética. A esterase 2 foi a escolhida para estudos genéticos, em virtude de ter sido a isoenzima que apresentou melhor coloração no gel. Dois alelos codominantes (**Est 2*S** e **Est 2*F**) codificam para este sistema polimórfico, com a frequência de **Est*S** igual a 0,521. A distribuição fenotípica está de acordo com o esperado em Hardy - Weinberg.*

Table 1 - Observed and expected phenopypic frequencies in **Anopheles darlingi**. Esterase-2 system.

Phenotypical Classes	observed	Expected	χ^2
Est2 S	41	38.82	0.147
Est2 F	67	71.37	0.267
Est2 S-F	35	32.81	0.122
	143	143.00	0.536

Est2*S = 0.521
Est2*F = 0.479

P = 0.444

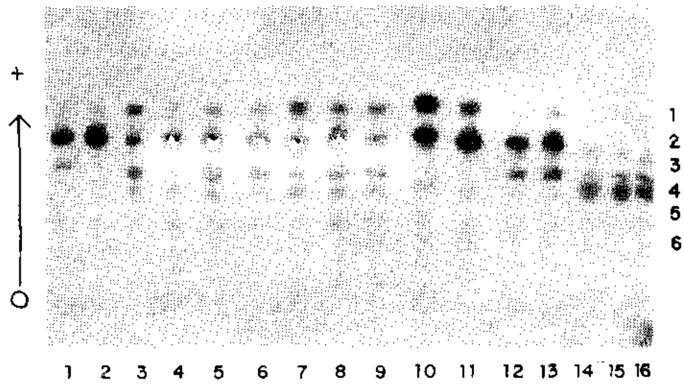

FIG. 1 - Anopheles darlingi esterase isozymes visualized on gel stained at pH 8.6 using α-naphthyl acetate as substrate. Slots 1 to 13 were filled with 4th instar larvae extracts, and the others were filled with pupae extracts.

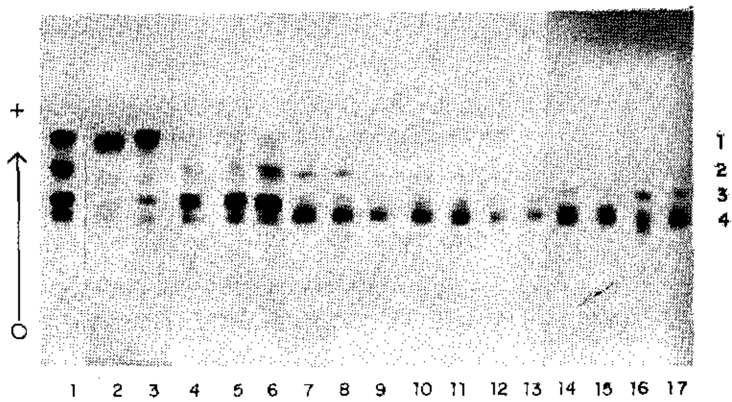

FIG. 2 - Esterase isozymes from pupae with different ages: 3 hrs (sample 1), 5 hrs (samples 2 and 3), 8 hrs (4-6), 12 hrs (7-9), 15 hrs (10-13), 20 hrs (14 and 15), and 24 hours (sample 16 and 17).

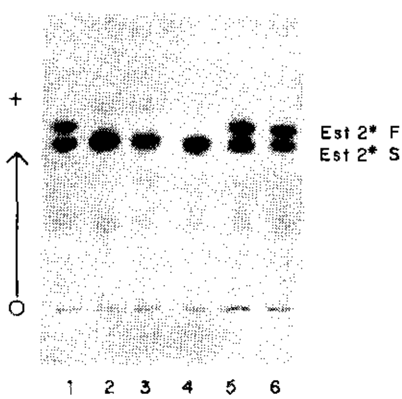

FIG. 3 - Genetic variation observed on esterase-2 isozymes from 4th-instar larvae extracts. Two phenotypes are show that correspond to genotypes Est2*S/Est2*S (samples 2, 3 and 4) and Est2*S/Est2*F.

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