CUTICULAR HYDROCARBONS, ISOENZYMES AND BEHAVIOR OF THREE POPULATIONS OF *ANOPHELES DARLINGI* FROM BRAZIL

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ABSTRACT. Three populations of *Anopheles darlingi* were studied for cuticular hydrocarbons, isoenzymes and patterns of peak biting activity. Differences were found in specimens from Costa Marques, a malaria endemic area; Dourado, a site with a very exophilic population and Juturnaíba, located near the type locality. Twelve hour collections from sunset to sunrise showed that *An. darlingi* from Costa Marques had a bimodal biting activity profile with a major peak at sunset and a minor peak at sunrise. At Dourado, the pattern was trimodal, with peaks at both morning and evening periods of twilight and near midnight. The Juturnaíba population showed a slight increase in activity near 2000 and 0100 h. Nei's genetic distances, determined by isoenzyme electrophoresis between pairs of populations, were low ($D \leq 0.049$). Using discriminant analysis for the cuticular hydrocarbons, 92.4% of the specimens from Costa Marques, 91.2% of the specimens from Dourado and 61.3% from Juturnaíba were correctly identified. Cuticular hydrocarbon and isoenzyme results matched very well: the smaller the Nei's distance, the more misidentifications occurred in the jackknife estimator used in the cuticular hydrocarbon analysis. This is the first report of cuticular hydrocarbon analysis in combination with isoenzymes to investigate neotropical anopheline species.

INTRODUCTION

Anopheles darlingi Root has long been considered the primary malaria vector for most of Brazil (Davis 1931, Davis and Kumm 1932, Shannon 1933, Deane et al. 1948, Rachou 1958) as well as contributing to malaria endemicity in parts of Mexico, Central America, Venezuela, Ecuador, Peru, Bolivia and the Guianas. *Anopheles darlingi* is widely distributed from southern Mexico to northern Argentina and from the western side of the Andes to the Atlantic coast.

Based on differences in behavior, isoenzymes and chromosomal patterns, *An. darlingi* is believed to be a complex of cryptic species (World Health Organization 1984, 1988). However, no differential morphological characters have been found in any of the life stages that provide a basis for a subspecific classification (Lane 1953). Misidentification of *An. darlingi* eggs by Root (1926) (eggs pictured were *An. albitarsis* Lynch-Arribálzaga) led to the description of *An. darlingi* var. *paulistensis* (Galvão et al. 1937) as well as *An. albitarsis* var. *limai* (Galvão and Lane 1937). Both varieties were subsequently synonymized with *An. albitarsis* by Causey et al. (1942).

Larval polytene chromosomal studies revealed many polymorphisms in *An. darlingi*. Kreutzer et al. (1972) described 9 inversion polymorphisms in 2 populations. Populations from northern Brazil (Manaus, Amazonas State) exhibited a greater degree of polymorphism as compared with those from southern Brazil (Dourado, referred in this paper as Araraquara, São Paulo State). A pattern in autosome 2, exclusive to the Dourado population, could be used as a differentiating character (Kreutzer et al. 1972).

With the aim of studying the existence of cryptic species in *An. darlingi*, three populations displaying distinct behavior and/or chromosomal patterns were studied by peak biting activity, isoenzyme electrophoresis and cuticular hydrocarbons.

MATERIALS AND METHODS

Twelve-hour human bait collections were carried out in 3 localities in Brazil: Costa Marques (12°26′S, 64°13′W, June 1989), Rondônia State; Dourado (22°06′S, 48°19′W, April 1989), São Paulo State and Juturnaíba (22°38′S, 42°18′W, February–March 1989), Rio de Janeiro State. In Costa Marques, where malaria is endemic, only outdoor collections were made since unsprayed or unscreened houses were not available. Similarly, due to the highly exophilic behavior of the Dourado population and the lack of houses at this site, collections also were conducted only outdoors. The populations of Costa Marques and Juturnaíba were related to malaria transmission. In Juturnaíba, 60 km from the type locality, mosquitoes were collected indoors resting on the walls. The population found in Juturnaíba occurs near a Biological Reserve (Poço das Antas, Silva Jardim).

Wild-caught females were placed in paper cartons and transferred to liquid nitrogen for isoenzyme studies. A portion of the females was main-

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tained in 100% isopropanol for DNA analyses. For cuticular hydrocarbons, females were killed by freezing and transferred to glass vials containing silica gel. For morphological studies, some females were transferred to individual oviposition tubes to lay eggs for progeny rearing and subsequent morphological examination.

The procedure for cuticular hydrocarbon analysis was that of Phillips et al. (1988) except individually dried females were immersed in hexane for 1 min, after which the extract was allowed to evaporate and resuspended with 2 μl of hexane containing 10 ppm pentadecane. The statistical analyses were detailed in Kamhawi et al. (1987). Voucher specimens were deposited in the Costa Lima Collection, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

Isoenzymatic analysis were carried out as described in Rosa-Freitas et al. (1990) for 10 *loci*, i.e., ME (E.C. 1.1.1.37.), IDH (E.C. 1.1.1.42.), HK (E.C. 2.7.1.1.), PGM (E.C. 2.7.5.1.), PEPD (E.C. 3.4.13.9.), FUM (E.C. 4.2.1.2.), MPI (E.C. 5.3.1.8.), GPI (E.C. 5.3.1.9.), MDH-1 and MDH-2 (E.C. 1.1.1.37.).

RESULTS

Costa Marques is located in a malaria endemic area and the vector is related to local malaria transmission (Klein and Lima 1990). The Dourado adult population is exophilic. Principal larval habitats are pools along the Jacaré-Pepira River as described in detail by Forattini (1987). The population was considered as having very polymorphic larval chromosomal banding patterns (Kreutzer et al. 1972). Juturnaíba is located on the edge of the Juturnaíba Lake formed by a dam on the São João River. This population was probably involved in malaria transmission up to the 1950s when malaria was widespread in the Rio de Janeiro lowlands and even in the 1970s when dozens of cases were reported locally.

The 12-h captures showed distinct patterns in peak biting activity for the 3 localities (Fig. 1). The Costa Marques population displayed a bimodal cycle with a major peak at sunset and a minor one at sunrise. Dourado had a trimodal cycle, with the main peaks at both morning and evening twilight periods and a minor peak at 2300 h. Juturnaíba showed no crepuscular peaks but there was a slight increase in activity at 2000 and 0100 h.

For the hydrocarbon analysis 196 females were processed; 66 females for Costa Marques, 68 for Dourado and 62 for Juturnaíba. Stepwise discriminant analysis (Dixon 1988) was used to analyze the 37 peaks (Fig. 2, Table 1) of the hydrocarbon profiles. The F-ratio criterion which controls the inclusion of variables into the discriminant function was set to 10, a conservative value, and the percentage of correct identifications gauged using a jackknife estimator (Table 2, Fig. 3).

Nei's genetic distances (D) (Nei 1972) between populations were calculated using results found for 10 isoenzymic *loci* (Table 3). The enzymes MDH, HK, GPI and IDH displayed 2 *loci*. For HK, GPI and IDH only the second *loci* were scored in the analysis. Three enzymes, MDH-2, FUM and GPI-2, were monomorphic. The highest D was 0.049, between the Costa Marques and Dourado populations. A distance of 0.024 separated Dourado from Juturnaíba while Juturnaíba and Costa Marques were separated by 0.018 (Table 4). These values fall within the range of intrapopulational variation (Avise and Smith 1974, Steiner et al. 1982, Bullini 1982).

DISCUSSION

Repeated attempts to collect *An. darlingi* at the type locality, Porto das Caixas (22°42'S, 42°53'W), were unsuccessful. Juturnaíba, 60 km from the type locality, was the closest site where *An. darlingi* could be found. The population in the type locality is believed to be extinct due to widespread insecticide spraying campaigns carried out in the 1950s in the Rio de Janeiro lowlands.

Different patterns of biting activity and host preference indicate that *An. darlingi* populations are not homogeneous throughout their wide distribution. Chromosomal and isoenzymatic studies also indicate a high degree of heterogeneity. No morphological characteristics for separation have been found so far.

Generally, haematophagic behavior is the most variable trait of the species. It can be variable in terms of endophilic and exophilic behavior, host preference and peak biting activity. Most populations of *An. darlingi* are highly anthropophilic and endophilic (Deane et al. 1948, Rozendaal 1989). Anthropophilic populations biting outdoors have also been observed in the Amazon region, mainly in the "garimpo" mines as well as agricultural and Amerindian settlements (Lourenço-de-Oliveira 1989; Lourenço-de-Oliveira et al. 1988,[2] 1989). In Suriname, Hudson (1984) observed specimens biting

[2] Lourenço-de-Oliveira, R., T. F. Silva, M. Arle and M. G. Castro. 1988. Circadian haematophagic activity of *Anopheles darlingi* in Rondonia State, Brazil. II Simposio sobre Malaria. II Reunião Nacional de Pesquisadores em Malaria, São Paulo (Unpublished abstract, no. 15, 1 p.).

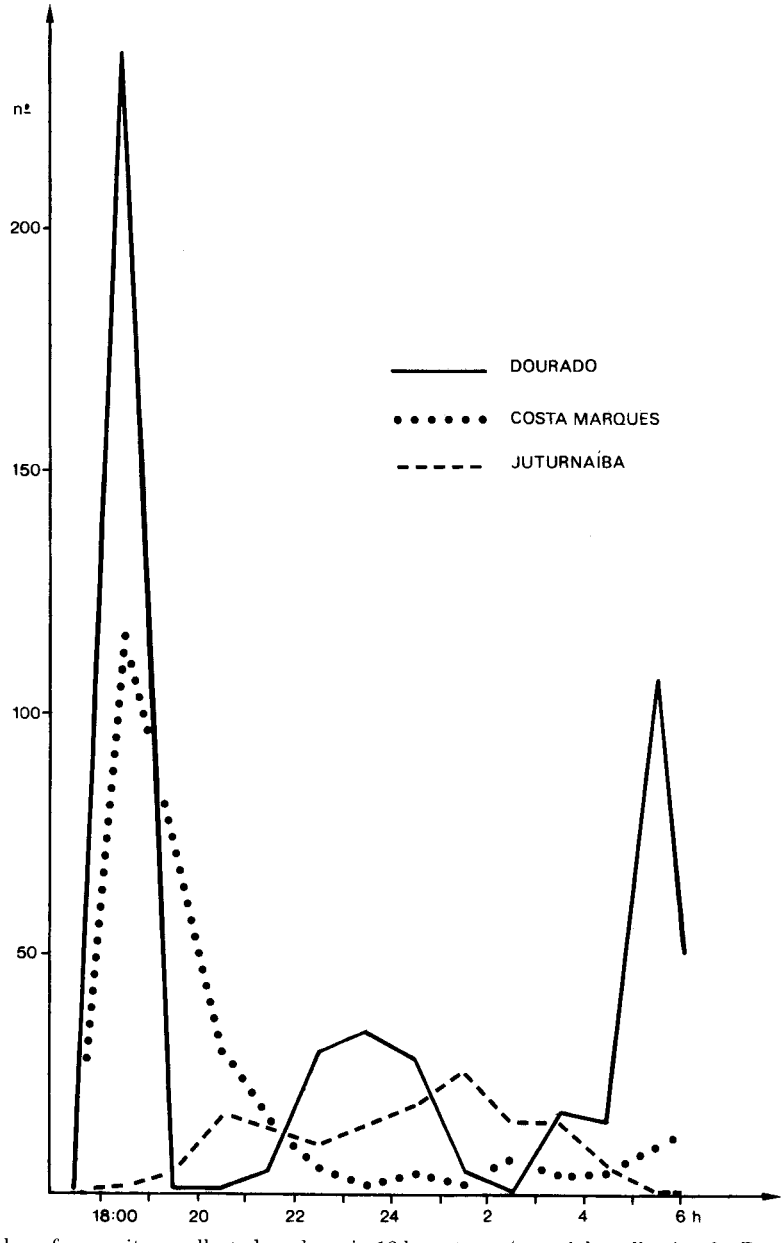

Fig. 1. Number of mosquitoes collected per hour in 12 h captures (one night collection for Dourado and Costa Marques; three nights average for Juturnaíba) for determining peaks of haematophagic activity of 3 populations of *Anopheles darlingi*.

both outdoors on animals and indoors. Forattini (1987) studied the behavior of the chromosomally distinct population described by Kreutzer et al. (1972) as being from Araraquara. The population was actually collected by Kreutzer et al. (1972) in Dourado. Forattini collected in Dourado county, where Kreutzer's population was actually sampled. Forattini (1987) concluded that the Dourado population was exophilic with bimodal activity peaks at sunset and sunrise. Exophilic populations with zoophilic behavior have also been observed in French Guiana (Pajot et al. 1977) and in the region of the São Francisco River in Brazil (Guedes et al. 1953). Peaks of feeding activity can vary greatly and unimodal, bimodal and trimodal cycles have been observed for *An. darlingi* from different localities (Fig. 4, Table 5).

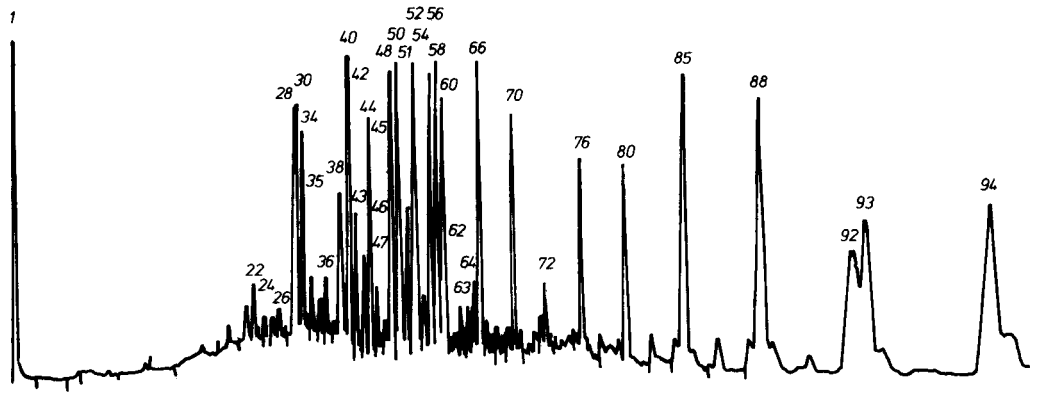

Fig. 2. Peak numbers used in the hydrocarbon profiles obtained by gas chromatography of *Anopheles darlingi* specimens. Peak number 1 is the internal standard, pentadecane.

Table 1. Cuticular hydrocarbon peaks found in 3 populations of *Anopheles darlingi*.

Peak number*	Retention time	Costa Marques	Dourado	Juturnaíba
1	3.01 (0.05)			
22	7.84 (0.07)	2.4 (4.3)	2.9 (4.4)	1.9 (3.8)
24	8.00 (0.05)	5.3 (4.5)	5.2 (4.5)	6.3 (4.1)
26	8.61 (0.05)	2.0 (3.9)	1.5 (3.4)	2.4 (4.1)
28	8.83 (0.05)	6.9 (4.5)	6.6 (4.9)	7.8 (4.1)
30	8.86 (0.05)	2.5 (4.2)	9.1 (2.7)	2.9 (4.5)
34	9.00 (0.05)	8.6 (3.2)	9.8 (0.5)	9.3 (2.2)
35	9.15 (0.01)	0	0	2.2 (4.0)
36	9.47 (0.06)	1.9 (3.7)	2.1 (3.8)	2.5 (4.0)
38	9.78 (0.05)	7.7 (3.9)	7.3 (4.3)	7.6 (4.0)
40	9.93 (0.05)	10.2 (0.5)	10.3 (0.5)	10.2 (0.5)
42	10.08 (0.05)	1.6 (3.4)	6.6 (4.2)	3.9 (4.5)
43	10.25 (0.05)	0.8 (2.5)	4.2 (4.5)	2.0 (3.8)
44	10.37 (0.07)	8.3 (3.1)	9.3 (1.2)	9.0 (2.1)
45	10.51 (0.03)	1.6 (9.5)	2.1 (3.8)	1.7 (3.5)
46	10.64 (0.03)	5.3 (4.8)	0.3 (1.5)	2.0 (3.8)
48	10.80 (0.05)	10.0 (0.5)	10.1 (0.5)	10.0 (0.6)
50	10.94 (0.05)	10.0 (1.4)	10.4 (0.6)	10.4 (0.5)
51	11.10 (0.05)	2.7 (4.5)	4.5 (4.7)	2.1 (3.9)
52	11.28 (0.05)	11.3 (0.7)	11.6 (0.6)	11.3 (0.6)
54	11.48 (0.06)	4.1 (4.7)	4.2 (4.7)	3.1 (4.4)
56	11.61 (0.05)	7.3 (3.7)	7.9 (3.3)	8.1 (3.2)
58	11.74 (0.05)	10.0 (1.4)	9.6 (2.1)	10.0 (1.4)
60	11.86 (0.05)	8.3 (3.6)	9.0 (2.9)	9.0 (2.4)
62	12.03 (0.04)	1.8 (3.6)	0	0.3 (1.6)
63	12.19 (0.11)	1.5 (3.5)	0.8 (2.6)	0.9 (3.0)
64	12.49 (0.08)	4.1 (4.6)	3.0 (4.3)	3.8 (4.5)
66	12.61 (0.05)	7.4 (4.0)	8.7 (3.2)	7.5 (4.0)
68	12.90 (0.21)	0.1 (1.1)	0	0.1 (1.2)
70	13.32 (0.05)	4.9 (4.8)	7.0 (4.5)	5.2 (4.6)
72	13.92 (0.06)	1.8 (3.8)	0.7 (2.5)	0.6 (2.4)
76	14.72 (0.06)	10.0 (1.4)	8.1 (4.4)	9.6 (2.2)
80	15.66 (0.01)	9.8 (1.8)	8.4 (4.0)	10.1 (1.4)
85	16.87 (0.12)	10.0 (1.9)	9.2 (3.4)	10.3 (1.4)
88	18.51 (0.14)	9.5 (3.6)	9.1 (4.1)	10.3 (2.8)
92	20.37 (0.41)	6.8 (5.3)	5.4 (5.6)	6.6 (5.6)
93	20.83 (0.55)	3.4 (5.1)	3.1 (5.0)	8.2 (4.9)
94	23.36 (0.24)	6.5 (5.5)	4.3 (5.4)	7.9 (5.1)

* Mean relative area of 37 peaks; retention time in minutes, standard deviation in parentheses. Peak number 1 is the internal standard, pentadecane.

Table 2. Number of specimens correctly classified (expressed as percentage) using cuticular hydrocarbon analysis for 37 peaks and jackknife estimator for 3 populations of *Anopheles darlingi*.

Populations	Total specimens	Costa Marques	Dourado	Juturnaíba	Percent correct
Costa Marques	66	61	3	2	92.4
Dourado	68	3	62	3	91.2
Juturnaíba	62	19	5	38	61.3
Total	196	83	70	43	82.1

Table 3. Gene frequencies at 7 enzyme *loci* in 3 populations of *Anopheles darlingi*.

Enzyme	Alleles	Collection site		
		Costa Marques	Dourado	Juturnaíba
PGM	76	0.2576	0.0417	0.0278
	98	0.1667	0.0000	0.0000
	100	0.5758	0.8333	0.7777
	121	0.0000	0.1250	0.1944
	n	66	96	72
PEP D	87	0.0278	0.1774	0.0000
	96	0.0833	0.4032	0.1875
	100	0.8889	0.4194	0.8125
	n	36	62	32
MPI	88	0.0714	0.0513	0.0500
	100	0.3392	0.4744	0.6000
	119	0.5000	0.4487	0.3333
	125	0.0714	0.0256	0.0167
	144	0.0178	0.0000	0.0000
	n	56	78	60
ME	93	0.0056	0.1143	0.0625
	100	0.6852	0.4571	0.7083
	104	0.2222	0.4286	0.2292
	120	0.0370	0.0000	0.0000
	n	54	70	48
IDH-2	93	0.0455	0.0536	0.0000
	100	0.7955	0.9464	0.9706
	108	0.1591	0.0000	0.0294
	n	44	56	34
HK-2	86	0.0250	0.1333	0.0000
	100	0.9500	0.8500	1.0000
	111	0.0250	0.0167	0.0000
	n	40	60	36
MDH-1	50	0.0128	0.0612	0.0000
	100	0.9872	0.9388	1.0000
	n	78	98	68

* *MDH-2*, *FUM* and *GPI-2* were monomorphic in all samples.
** *n* = number of alleles sampled.

Table 4. Comparison between isoenzymatic and cuticular hydrocarbon analyses of *Anopheles darlingi* specimens from 3 populations.

Populations	Nei's genetic distance	% specimens incorrectly identified by cuticular analysis
Costa Marques—Dourado	0.049	4.7
Dourado—Juturnaíba	0.024	7.4
Juturnaíba—Costa Marques	0.018	17.5

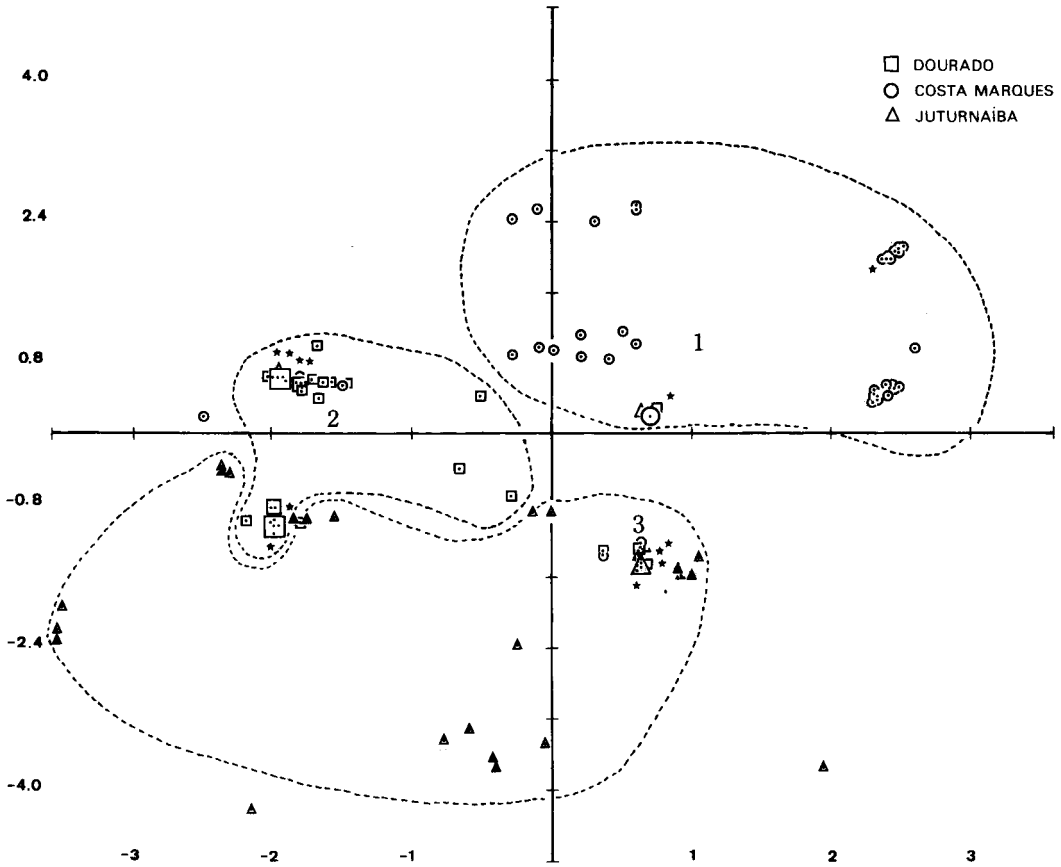

Fig. 3. Discriminant function plot of the canonical variables obtained for cuticular hydrocarbon profiles of 3 *Anopheles darlingi* populations. Numbers 1, 2 and 3 give the centroids for Costa Marques-CMQ (66 specimens), Dourado-DOU (68) and Juturnaíba-JUT (62), respectively. * Overlapping points: X = 2.4 (±0.2), Y = 2.0 (± 0.2) for 6 specimens-CMQ, 0.7 and 0.2 (24 specimens-CMQ and 2 specimens-DOU), 0.6 and −1.2 (2-JUT), 0.6 and −1.3 (5-JUT), 0.6 and −1.4 (11-JUT), 0.6 and −1.5 (3-JUT and 1-DOU), −2.0 and 0.6 (22-DOU + 1-JUT), −1.9 and 0.6 (11-DOU), −1.6 and 0.6 (4-DOU), −1.8 and 0.6 (4-DOU), −1.9 and −1.0 (4-DOU), −2.0 and −1.1 (2-DOU).

Three different peak biting activity patterns were observed for the 3 populations of *An. darlingi* studied. Clear peaks were not seen in the indoor collections at Juturnaíba although there was an increase in activity during the night and the first hours of the morning. This might have been due to low population density at the time of the year collections were made, February and March, when temperatures were low (24°C average) and rain was common. Other reports on peak activity agree with the observations made from outdoors collections at Dourado (trimodal) and Costa Marques (bimodal crepuscular cycle) (Forattini 1987, Klein and Lima 1990). Elliott (1972) and Klein and Lima (1990) demonstrated that peak biting patterns can differ seasonally. They also vary according to the type of (indoor/outdoor) collection. At El Pescado, Colombia, Elliott (1972) found a unimodal cycle from 2200 to 0100 h in June–August 1965, both indoors and outdoors. During the moderate density period, January, March–May and September, the outdoor peak was between 2000–2100 h and an indoor peak 1 h later. In El Pescado there also was a minor peak at 0200–0300 h. In the low density period, February and October–December, outdoor peaks were at 1900–2000 h and at sunrise. Indoor peaks of activity were at sundown and at 2200 and 0200 h. On all occasions outdoor biting patterns were either greater in number or the same as indoors. Klein and Lima (1990) studied the Costa Marques population during July 1986 to December 1987. In the period July–September 1986, they found a trimodal cycle with peaks at both morning and evening periods of twilight and a minor one

Table 5. Peak biting patterns of *Anopheles darlingi* populations.

Locality	Pattern	Peaks (h)	Reference	Type of collection	Figure 4
Columbia	Unimodal*	22:00-24:00	Elliott (1972)	Outdoors and indoors	1
Peru	Trimodal	Sunset, sunrise + 02:00	Elliott (1972)	Outdoors and indoors	2
French Guiana	Trimodal	Sunset, sunrise + 01:00-02:00	Pajot et al. (1977)	Outdoors	3
Suriname	Unimodal	22:30-23:30	Hudson (1984), Rozendaal (1989)	Outdoors and indoors	4
Brazil					
Belém and Aurá Pará State	Trimodal	Sunset, sunrise + 24:00	Deane et al. (1948)	Outdoors and indoors	5
BR 174 Manaus-Boa Vista Highway, km 137, Pará State	Unimodal	20:00-24:00 and 24:00-03:00	Hayes and Charlwood (1979)	Indoors and outdoors	6
	Unimodal	20:00-21:00	Charlwood and Hayes (1978)	Outdoors	
Uauaris, Roraima State	Unimodal	24:00-02:00	Charlwood and Hayes (1978)	Outdoors	7
Floresta Ituxi River, Amazonas State	Bimodal	Sunset + sunrise	Roberts et al. (1987)	Outdoors and indoors	8
Porto Velho, Rondônia State	Trimodal	Sunset, sunrise + 24:00	Deane et al. (1948)	Outdoors and indoors	9
Jarú, Rondônia State	Trimodal	Sunset + sunrise + 20:00-21:00	Charlwood and Alecrim (1989)	Outdoors	10
Costa Marques, Rondônia State	Bimodal*	Sunset + sunrise	Klein and Lima (1990)	Outdoors and indoors	11
Aripuanã, Mato Grosso State	Bimodal	20:00 + sunrise	Charlwood and Hayes (1978)	Outdoors	12
	Bimodal	Sunset + sunrise	Charlwood and Wilkes (1979)	Outdoors	
Dourado, São Paulo State	Bimodal	Sunset + sunrise	Forattini (1987)	Outdoors	13
Juturnaíba, Rio de Janeiro State	Unimodal	20:00-01:00	Present study	Indoors	14

* Patterns vary according to season.

Fig. 4. Localities where collections of *Anopheles darlingi* were made in Brazil and peak biting patterns of several populations based on literature data. Numbers on the figure refer to localities given in Table 4.

around 2100 h, both outside and inside the houses. This may have been affected by rains occurring around 1900–2000 h. From October to December in the same year the pattern was reduced to a bimodal crepuscular cycle. This cycle was repeated from July to September of the following year, 1987. The minor night peak moved to around midnight in January–March, 1987. The patterns observed were almost the same indoors and outdoors with a preponderance of mosquitoes collected outdoors over indoor collections. The pattern observed by us corresponded to a bimodal cycle with a major peak at sundown and a minor peak around sunrise in June, 1989. Apparently for this locality the lower the density, the fewer the peaks that can be observed. In Costa Marques, the use of DDT house spraying and malathion fogging may have reduced *An. darlingi* density.

Charlwood and Alecrim (1989) marked mosquitoes biting at sundown in Jarú, Rondônia State, Brazil and recaptured them biting during sunrise. This observation showed that there was no subgrouping within the population by biting times.

Although only one night collection was made for Costa Marques and Dourado, previously, long term studies have been conducted with similar results (Forattini 1987, Klein and Lima 1990). Based on literature data (Fig. 4, Table 5), no clear delimitations can be made in the geographical distribution of the distinct peak biting activities reported for the different populations. Whether the difference in peak biting activity is a polymorphic characteristic of the species or if it is density dependent remains to be clarified.

Specimens from 5 Brazilian populations of *An. darlingi* were analyzed isoenzymatically by

Steiner et al. (1982). The genetic distance between the population from Salvador, Bahia and the other 4 localities was high enough ($D \geq 0.59$) to warrant specific status for this population. In contrast, the populations we examined were not significantly different. Nei's genetic distances were in the range of intrapopulational variation ($D \leq 0.049$) according to Avise and Smith (1974), Steiner et al. (1982) and Bullini (1982).

Cuticular hydrocarbon analysis has been used for studying species complexes of medically important Diptera such as anophelines (Carlson and Service, 1979, 1980; Milligan et al. 1986), sandflies (Kamhawi et al. 1987) and blackflies (Phillips et al. 1985). This technique has successfully identified sibling species as well as described the function of cuticular hydrocarbons in mate recognition (Phillips et al. 1988).

In our study using cuticular hydrocarbons data, peak 35 was only found in Juturnaíba specimens while peaks 62 and 68 were not present in Dourado specimens. Specimens from Dourado and Costa Marques could be correctly identified with >90% success, indicating a high degree of dissimilarity between their profiles. Juturnaíba showed poor separation, its profiles were misidentified 40% of the time (Table 2). This corresponds to the isoenzyme patterns. Juturnaíba gave the smallest genetic distance ($D = 0.018$ from Costa Marques and $D = 0.024$ from Dourado). Costa Marques and Dourado had the largest ($D = 0.049$) (Table 4).

Based on our results, none of the 3 populations can be considered as separate species. The peak biting activity appears to be a polymorphic character which can be influenced by extrinsic environmental factors and thus may not be a useful taxonomic character. However, the clear dissimilarity between the Costa Marques and Dourado populations, shown by their cuticular hydrocarbons, which was to some extent corroborated by their Nei's genetic distance, indicates a degree of intraspecific population structure that requires further investigation.

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REFERENCES CITED

Avise, J. C. and M. H. Smith. 1974. Biochemical genetics of sunfish. II—Genic similarity between hybridizing species. Am. Nat. 108:458–472.

Bullini, L. 1982. Enzyme variants in the identification of parasites and vectors: methodological aspects of electrophoretic approach, pp. 53–69. *In*: New approaches to the identification of parasites and their vectors. Tropical Diseases Series 5. World Health Organization, Geneva.

Carlson, D. A. and M. W. Service. 1979. Differentiation between species of the *Anopheles gambiae* Giles complex by analysis of cuticular hydrocarbons. Ann. Trop. Med. Parasitol. 73:589–592.

Carlson, D. A. and M. W. Service. 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. Science 207:1089–1091.

Causey, O. R., L. M. Deane and M. P. Deane. 1942. Note clarifying the status of *Anopheles albitarsis* and *Anopheles darlingi*. Proc. Entomol. Soc. Wash. 44:122–126.

Charlwood, J. D. and W. A. Alecrim. 1989. Capture-recapture studies with the South American malaria vector *Anopheles darlingi* Root. Ann. Trop. Med. Parasitol. 83:569–576.

Charlwood, J. D. and J. Hayes. 1978. Variações geográficas no ciclo de picadas do *Anopheles darlingi*, Root no Brasil. Acta Amaz. 8:601–603.

Charlwood, O. R. and T. J. Wilkes. 1979. Studies on the age composition of samples of *Anopheles darlingi*, Root in Brasil. Bull. Entomol. Res. 69:337–342.

Davis, N. C. 1931. A note on the malaria carrying anophelines in Belem, Para and in Natal, Rio Grande do Norte, Brazil. Rev. Malariol. 10:43–51.

Davis, N. C. and H. W. Kumm. 1932. Further incrimination of *Anopheles darlingi*, Root as a transmitter of malaria. Am. J. Trop. Med. 12:93–95.

Deane, L. M., O. R. Causey and M. P. Deane. 1948. Notas sobre a distribuição e a biologia dos anofelinos das Regiões Nordestina e Amazônica do Brasil. Rev. Serv. Esp. Saude Publica 1:827–965.

Dixon, W. J. 1988. BMDP Statistical Software Manual (1988 software release). Vol. 1. University of California Press, Berkeley.

Elliott, R. 1972. The influence of vector behaviour on malaria transmission. Am. J. Trop. Med. Hyg. 21:755–763.

Forattini, O. P. 1987. Comportamento exófilo de *Anopheles darlingi* Root em Região Meridional do Brasil. Rev. Saude Publica Sao Paulo 21:291–304.

Galvão, A. L. A. and J. Lane. 1937. Notas sobre os *Nyssorhynchus* de São Paulo. VII. Estudo sobre as variedades deste grupo com descrição de *Anopheles* (*Nyssorhynchus*) *albitarsis* var. *limai*. An. Fac. Med. Univ. Sao Paulo 13:211–238.

Galvão, A. L. A., J. Lane and R. R. Correa. 1937. Notas sobre os *Nyssorhynchus* de Sao Paulo. V. Nota sobre os *Nyssorhynchus* de Novo Oriente. Rev. Biol. Hig. 8:37–45.

Guedes, A. S., J. R. Freitas and S. B. Xavier. 1953. Contribuicao ao conhecimento da distribuicao geografica dos anofelinos e algumas observacoes sobre a biologia do *Anopheles darlingi*. Rev. Bras. Malar. Doencas Trop. 5:157–165.

Hayes, J. and J. D. Charlwood. 1979. Dinâmica estacional de uma população de *Anopheles darlingi*, numa área endêmica de malária no Amazonas. Acta Amaz. 9:79–86.

Hudson, J. E. 1984. *Anopheles darlingi*, Root in the Surinam rain forest. Bull. Entomol. Res. 74:129–142.

Kamhawi, S., D. H. Molyneux, R. Killick-Kendrick, P. J. M. Milligan, A. Phillips, T. J. Wilkes and M. Killick-Kendrick. 1987. Two populations of *Phlebotomus ariasi* in the Cevennes focus of leishmaniasis in the south of France revealed by analysis of cuticular hydrocarbons. Med. Vet Entomol. 1:97–102.

Klein, T. A. and J. B. P. Lima. 1990. Seasonal distribution and biting patterns of Anopheles mosquitoes in Costa Marques, Rondonia, Brazil. J. Am. Mosq. Control Assoc. 6:700–707.

Kreutzer, R. D., J. B. Kitzmiller and E. Ferreira. 1972. Inversion polymorphism in the salivary gland chromosomes of *Anopheles darlingi*. Mosq. News 32:555–556.

Lane, J. 1953. Neotropical Culicidae. Vol. I, Univ. São Paulo, São Paulo.

Lourenço-de-Oliveira, R. 1989. Some observations on the mosquitoes of indian settlements in Xingu National Park, Mato Grosso State, Brazil, with emphasis on malaria vectors. Rev. Bras. Biol. 49:393–397.

Lourenço-de-Oliveira, R., A. E. Guimarães, M. Arle, T. F. Silva, M. G. Castro, M. A. Motta and L. M. Deane. 1989. Anopheline species, some of their habits and relation to malaria in endemic areas of Rondônia State, Amazon Region of Brazil. Mem. Inst. Oswaldo Cruz Rio 84:501–514.

Milligan, P. J. M., A. Phillips, D. H. Molyneux, S. K. Subbarao and G. B. White. 1986. Differentiation of *Anopheles culicifacies* Giles sibling species by analysis of cuticular components. Bull. Entomol. Res. 76:529–537.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106:283–292.

Pajot, F. X., F. Le Pont, J. F. Molez and N. Degalier. 1977. Agressivite' d'*Anopheles darlingi* Root, 1926 en Guyane Française. Cah. O.R.S.T.O.M. Ser. Entomol. Med. Parasitol. 15:15–22.

Phillips, A., J. F. Walsh, R. Garms, D. H. Molyneux, P. J. M. Milligan and G. H. Ibrahim. 1985. Identification of adults of the *Simulium damnosum* complex using hydrocarbon analysis. Tropenmed. Parasitol. 36:97–101.

Phillips, A., P. J. M. Milligan, G. Broomfield and D. H. Molyneux. 1988. Identification of medically important Diptera by analysis of cuticular hydrocarbons, pp. 39–59, *In*: M. W. Service (ed). Biosystematics of Haematophagous Insects, Systematics Assoc. Special Volume 37, Clarendon Press, Oxford.

Rachou, R. G. 1958. Anofelinos do Brasil: comportamento das espécies vetoras de malária. Rev. Bras. Malariol. Doencas Trop. 10:145–181.

Root, F. M. 1926. Studies on Brazilian mosquitoes. I. The anophelines of the *Nyssorhynchus* group. Am. J. Hig. 6:684–717.

Rosa-Freitas, M. G., L. M. Deane and H. Momen. 1990. A morphological, isoenzymatic and behavioural study of ten populations of *Anopheles* (*Nyssorhynchus*) *albitarsis* Lynch-Arribalzaga, 1878 including from the type-locality, Baradero, Argentina. Mem. Inst. Oswaldo Cruz Rio 85:275–289.

Roberts, D. R., W. D. Alecrim, A. M. Tavares and M. G. Radke. 1987. The house-frequenting, host-seeking and resting behavior of *Anopheles darlingi* in Southeastern Amazonas, Brazil. J. Am. Mosq. Control Assoc. 3:433–441.

Rozendaal, J. A. 1989. Biting and resting behavior of *Anopheles darlingi* in the Suriname rainforest. J. Am. Mosq. Control Assoc. 5:351–358.

Shannon, R. C. 1933. Anophelines of the Amazon Valley. Proc. Entomol. Soc. Wash. 35:117–143.

Steiner, W. W. M., S. Narang, J. B. Kitzmiller and D. L. Swofford. 1982. Genetic divergence and evolution in neotropical Anopheles (subgenus *Nyssorhynchus*), pp. 523–551. *In*: Recent developments in the genetics of insect disease vectors/Stipes Publ./Urbana, IL.

World Health Organization. 1984. Malaria vector species complexes and intra-specific variations: relevance for malaria control and orientation for further research. Report of an informal consultation of the Scientific Working Group on Applied Field Research in Malaria. Bangkok. TDR/FIELDMAL-SWG 930/84.3.

World Health Organization. 1988. Report on a technical consultation on research in support of malaria control in the Amazon Basin. TDR/FIELDMAL/SC/AMAZ/88.3.