

ORIGINAL ARTICLE

***Anopheles* DIVERSITY AND MALARIA
INFECTION IN THE COCAIS ZONE, STATE OF
MARANHÃO, BRAZIL**

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ABSTRACT

A survey of the *Anopheles* species was conducted in the municipality of São Mateus, in the state of Maranhão. Species hematophagy habits, the influence of seasonality as well as the vector's infection by *Plasmodium* were studied. The female *Anopheles* mosquitoes were captured between February and December 2010 and in March 2012, from 18h00 to 21h00, in intra and peridomiciles. PCR was used for the infection studies. A total 615 specimens of *Anopheles* were captured, of which 223 (36.2%) in the locality of Piquizeiro and 392 (63.8%) on the Retiro Velho Farm belonging to the species *An. nuneztovari*, *An. darlingi*, *An. triannulatus*, *An. albitarsise*, and *An. oswaldoi*. In Piquizeiro most specimens were captured in the intradomiciliary area, while on the Retiro Velho Farm they were mostly found in the peridomiciliary area. The results show the influence of seasonality and time of capture in the hematophagy frequency of the captured *Anopheles*. The negative result for *Plasmodium* infection demonstrates a reduction in the number of malaria cases in the State during the study period.

KEY WORDS: Malaria; *Anopheles*; *Plasmodium*; infection; mosquitoes; control.

RESUMO

Diversidade de *Anopheles* e infecção por malária na Zona dos Cocais, Estado do Maranhão, Brasil

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Realizou-se um levantamento das espécies de *Anopheles* no município de São Mateus, estado do Maranhão. Foram estudados os hábitos da espécie, a hematofagia, a influência da sazonalidade, assim como a infecção do vetor por *Plasmodium*. Os mosquitos fêmeas de *Anopheles* foram capturados entre fevereiro e dezembro de 2010 e em março de 2012, das 18h às 21h, no intra e peridomicílio. A PCR foi utilizada para os estudos de infecção. Um total de 615 espécimes de *Anopheles* foi capturado, sendo 223 (36,2%) na localidade de Piquizeiro e 392 (63,8%) na Fazenda Retiro Velho, distribuídos entre as espécies *An. nuneztovari*, *An. darlingi*, *An. triannulatus*, *An. albitarsis* e *An. oswaldoi*. No Piquizeiro, a maioria foi capturada na área intradomiciliar e, na Fazenda Retiro Velho, no peridomicílio. Os resultados mostram a influência da sazonalidade, o tempo de captura e frequência hematofágica dos *Anopheles* capturados. A análise da infecção negativa para *Plasmodium* demonstra a redução dos casos de malária no estado no período de estudo.

DESCRITORES: Malária; *Anopheles*; *Plasmodium*; infecção; mosquitos; controle

INTRODUCTION

Malaria is caused by a protozoan from the genus *Plasmodium* transmitted through the bite of infected female *Anopheles* mosquitoes. About 3.3 billion people are at some risk of contracting the disease (27). Most deaths are caused by the species *Plasmodium falciparum* among young children and pregnant women (13).

In Brazil, more than 99% of all cases occur in the Amazon region, of which *An. darlingi* is the main vector (10, 27). In the state of Maranhão, despite the reduction in the number of cases during recent years, the disease continues to be a public health issue with great impact in the lower socioeconomic strata of the population (14).

There is a need for continued and reinforced malaria control programs, especially those regarding entomological surveillance as they provide information about parameters regarding humans/vector contact. This knowledge is important for establishing control strategies that are specific for each locality, since malaria is a focal disease (12, 26).

Malaria transmission involves an agent, a host, and the environment (18, 27). Its specificity is related to the presence of anophelines, sensitive or not to infection by plasmid-vectorial capacity (13, 14). Therefore, identifying the *Plasmodium* species present in the mosquito is essential for malaria control (12, 14, 26).

In Maranhão, medium and high risk areas are situated in the west and in the border zone with the State of Pará. However, after 2005, an increase in the number of cases in municipalities in the central and eastern regions of the state was noted, especially in São Mateus, classified as a malaria high risk area (7, 11, 15, 17, 18, 22). Therefore, in order to determine the areas with the highest risk of malaria transmission in the state, it is necessary to conduct broader studies on the entomology of malaria vectors, assessing diversity, abundance, seasonality, and disease transmission ability of the *Anopheles* species.

MATERIALS AND METHODS

Study area

The area chosen for the development of this study was the municipality of São Mateus, coordinates: 04°03'53.9"S/44° 47'59.E"W, in the central region of the state of Maranhão. It is mostly known as Zona dos Cocais (Palm grove zone), due to the abundance of babaçu palms (*Attalea speciosa* Mart. Ex. Spreng). The municipality is part of the *Médio Mearim* micro-region, and is characterized by a hot-humid tropical climate. The rainfall indices vary from 1,200 mm³ to 1,600 mm³ (5). The vegetation consists of evergreen open forest, in an area that, although it is not within the limits of the higher risk region, presents annual outbreaks of malaria, and where little is known about the anophelines (2). The study area is 783 km², and presents Cerrado and Amazonian biomes. There are 39,622 inhabitants, mostly in trade and agricultural activities (5).

Sampling was conducted in two localities situated in the countryside of São Mateus: locality of Piquizeiro and the Retiro Velho Farm. The houses in both sites are typically rural adobe buildings. The residents are engaged mainly in fishing, hunting, and subsistence agriculture.

Sampling methods and period

The female *Anopheles* mosquitoes were captured using the human-bait attraction method. This was conducted by collectors wearing long sleeved shirts, long pants, and long black socks, to avoid direct contact with the mosquitoes. Pants were rolled up to the knees, so the ankles would attract the female *Anopheles* mosquitoes. The mosquitoes were collected while landing for blood feeding, with the aid of Castro collectors and an oriented light source.

The collectors stayed in the intra and peridomiciliary environments from 18h00 to 21h00. There was a collector swap within the environments at every hour. The anophelines captured were placed in entomological cages separated according to time and environment in appropriate conditions to preserve the humidity and temperature needed to keep the mosquitoes alive. At the laboratory, the mosquitoes were sacrificed using ethyl acetate, and then sorted and identified using dichotomous keys (3, 9). Later, the species were sent to the Malaria and Dengue Vectors Laboratory (INPA-AM) for identification confirmation. After species confirmation, the samples were preserved in freezers, at -20°C.

In the locality of Piquizeiro, sampling was conducted from February to December 2010. During the drought period (July to December), sampling took place every two months due to the usual decrease in the number of specimens

collected throughout the period. The samples were used to build information on specie distribution, hematophagic frequency (time and environment of capture), and seasonality influence.

On the Retiro Velho Farm, sampling was conducted in March 2012, when there was a suspicion of malaria transmission. The specimens were taken to the Medical Entomology laboratory of the Oswaldo Cruz Foundation (FIOCRUZ), at the René Rachou Research Center, Belo Horizonte, MG for the molecular study and detection of infection by *Plasmodium* species.

Detection of infection in anopheline species using polymerase chain reaction - PCR

The samples collected were analyzed by the PCR method to detect the presence of *Plasmodium*, as previously described (1). *Anopheles* specimens were sorted by species, date, time, and environment collection (peri and intradomiciliary), and then put into 1.5 mL tubes, every tube containing up to five females. For DNA extraction, the mosquitoes were macerated and homogenized in a 200 µL DNAzol solution and then centrifuged at 12,000 rpm for 10 minutes, at 4°C. Subsequently, 150 µL of the supernatant of each sample was transferred to new tubes, to which 200 µL of cold absolute ethanol was added. The tubes were inverted for about 30 seconds and then put on ice for five minutes.

The samples were then centrifuged for two minutes at 12,000 rpm and at 4°C. The supernatant was then discarded by pouring it out of the tubes. For washing the DNA, 500 µL of cold ethanol at 70%, diluted in Diethyl pyrocarbonate 9 (DEPC) water, was added. The tubes were later homogenized and kept in the vertical position for one minute for DNA sedimentation. The samples were again centrifuged at 12,000 rpm for four minutes at 4°C, and the supernatant subsequently discarded. The tubes were left open to dry for about two hours. After drying, the DNA was eluted in 10 µL of EDTA buffer (ethylene-diamine-tetraacetic acid) for solubilization. The samples were then stored at -70 °C. The extraction of the DNA of *Plasmodium* sp. was conducted in accordance with the Kit DNAzol.

In order to detect the presence of *Plasmodium* sp. in the samples, PCR was conducted using the GoTaq (Promega) enzyme with the following primers: rPLU5: 5'CCTGTTGTTGCCTTAAACTTC3' and rPLU6 5'TTAAAATTGTTGCAGTTAAAACG3', to amplify the DNA fragment of 1200 pb according to Snounou et al. (1993) (24).

The results obtained by PCR were observed in 6% acrylamide gel. The gels were subjected to electrophoresis (110V) using TBE 1 X buffer (Tris-borate, boric acid and EDTA) for running. The gel was fixed for 10 minutes using fixing solution (2% acetic acid, 4% ethylic alcohol v/v). Next, it was colored with silver nitrate solution (1 g/500 mL of distilled water) for 10 minutes and washed twice for one minute with distilled water. The gels were

then incubated with a 3% NaOH solution (15 g/500 mL of distilled water) and 200 µL of formaldehyde until the bands were revealed. The reaction was interrupted by adding a fixing solution. The gels were visualized and photographed using a digital camera.

Statistical analysis

The statistical analysis was run using the software Epi-Info. The Chi-squared test was used to compare percentages for categorical variables. The significance level adopted was 5%.

Ethics

The present study was approved by the HU-UFMA Research Ethics Committee (CEP) under permit n°0004578/2010-10.

RESULTS

A total of 615 mosquitoes from the genus *Anopheles* were captured. An amount of 223 specimens were collected in the locality of Piquizeiro. These consisted of five species from the subgenus *Nyssorhynchus* (Blanchard, 1902): *An. albitarsis*, *An. darlingi*, *An. nuneztovari*, *An. oswaldoi* and *An. triannulatus*. The species *An. nuneztovari* was the most frequent in the study site, with 106 specimens (47.5%). Most species were captured in the intradomiciliary area: 139 (62.3%), except for *An. triannulatus*, that numbered 13 (59.1%) specimens captured in the peridomiciliary area. Yet, there was no statistically significant difference when relating the species with the capture environment (p=0.174) (Table 1).

Table 1. Frequency of female *Anopheles* mosquito captured in the peridomiciliary and intradomiciliary areas in Piquizeiro

Species of <i>Anopheles</i>	Sampling environment				Total		p value ¹
	Peri		Intra		n	%	
	n	%	n	%			
<i>An. albitarsis</i>	2	40.0	3	60.0	5	2.3	0.174
<i>An. darlingi</i>	29	33.0	59	67.0	88	39.5	
<i>An. nuneztovari</i>	40	37.7	66	62.3	106	47.5	
<i>An. oswaldoi</i>	0	0.0	2	100.0	2	0.9	
<i>An. triannulatus</i>	13	59.1	9	40.9	22	9.9	
Total	84	37.7	139	62.3	223	100.0	

¹Chi-squared test. Comparison between the variables 'species of *Anopheles*' and 'sampling environment'.

When comparing the two sampling environments, a statistical difference ($p=0.007$) in the hours with higher frequency of mosquito capture was observed. In the intradomiciliary area, there was a higher frequency in the first two hours (40.5% and 41.7%, respectively), while in the peridomiciliary area, almost 60% of the captures occurred in the first hour (Table 2).

Table 2. Frequency of female *Anopheles* mosquito captured in the different time intervals and sampling environments, in Piquizeiro

Variable	Sampling environment				Total		p value ¹
	Intra		Peri		n	%	
	n	%	n	%			
Time of capture							0.007**
18h00-19h00	34	40.5	80	57.6	114	51.1	
19h00-20h00	35	41.7	31	22.3	66	29.6	
20h00-21h00	15	17.9	28	20.1	43	19.3	
Total	84	37.6	139	62.4	223	100.0	

¹Chi-squared test. Comparison between the variables 'sampling environment' and 'time of capture'. ** $p \leq 0,01$.

On the Retiro Velho Farm, 392 specimens of female *Anopheles* mosquito were collected. These consisted of the same five species found in Piquizeiro. The species *An. albitarsis* was the most frequent in the study site, comprising 183 specimens (46.8%). Most species were captured in the peridomiciliary area: 246 (62.7%), except for *An. oswaldoi*, with 2 (66.7%) specimens captured in the intradomiciliary area. Yet a statistical difference was noted when relating the species with the capture environment ($p=0.0026$), where the peridomiciliary area proved more relevant (Table 3).

Table 3. Frequency of females *Anopheles* mosquito captured with human-bait attraction method in the peridomiciliary and intradomiciliary areas on the Retiro Velho Farm

Species of <i>Anopheles</i>	Sampling environment				Total		p value ¹
	Peri		Intra		n	%	
	n	%	n	%			
							0.0026**
<i>An. albitarsis</i>	108	59.1	75	40.9	183	46.8	
<i>An. darlingi</i>	20	57.1	15	42.9	35	8.9	
<i>An. nuneztovari</i>	80	62.0	49	38.0	129	32.9	
<i>An. oswaldoi</i>	1	33.3	2	66.7	3	0.7	
<i>An. triannulatus</i>	37	88.0	5	12.0	42	10.7	
Total	246	62.7	146	37.3	392	100.0	

¹Chi-squared test. Comparison between the variables 'species of *Anopheles*' and 'sampling environment'. ** $p \leq 0,01$.

When comparing the two sampling environments on the Retiro Velho Farm, a statistically significant difference was noted ($p=0.0305$) in the hours with higher frequency of mosquitoes captured. In the intradomiciliary area, there was a higher frequency in the third collection hour (42.9%), and in the peridomiciliary area, the same occurred with 57.1% of captures between 20h00 and 21h00 (Table 4).

Table 4. Distribution of the frequency of females *Anopheles* mosquito captured in the different time intervals and sampling environments, on the Retiro Velho Farm.

Variable	Sampling environment				Total		p value ¹
	Intra		Peri		n	%	
	n	%	n	%			
Time of capture							0.0305*
18h00-19h00	31	39.2	48	60.8	79	20.1	
19h00-20h00	39	28.6	97	71.4	136	34.6	
20h00-21h00	76	42.9	101	57.1	177	45.1	
Total	146	37.2	246	62.8	392	100.0	

¹ Chi-squared test. Comparison between the variables 'sampling environment' and 'time of capture'. * $p \leq 0,05$.

Infection by *Plasmodium* in any of the specimens collected was not detected (Figure 1).

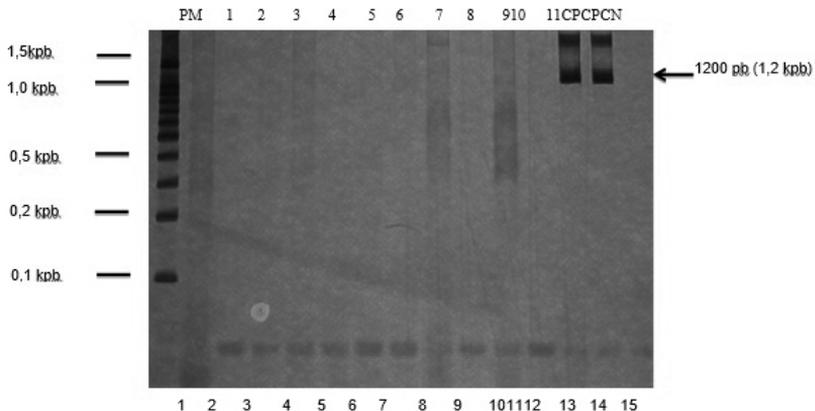


Figure 1. Determination of the *Plasmodium* sp infection, during the analysis of pools of *Anopheles*, through a PCR assay.

Subtitle: 1: MW (standard molecular weight 100pb); 2 to 12: samples of mosquitoes; 13 and 14: positive control (+), and 15: negative control (-). The arrow indicates the amplicon of 1,200pb relative to the positivity to *Plasmodium* sp detected only in the positive control.

DISCUSSION

Five species were captured in Piquizeiro and on the Retiro Velho Farm, which partly corroborates the results obtained by other authors (15) who studied the distribution of *Anopheles* associated to humans in Buriticupu, in Amazonian Maranhão. There, seven species of anophelines were found, four of which were also found in this study. The seven species were: *An. argyritasis*, *An. oswaldoi*, *An. nuneztovari*, *An. rangeli*, *An. triannulatus*, *An. darlingi*, and *An. evansae*. Nevertheless, one of the species in this study, *An. albitarsis*, was not captured then, being found in this study near humans thanks to environmental changes such as artificial irrigation (16). In this study there were few captures of the species *An. oswaldoi*. This is due to the different degrees of anthropophily found among anophelines, since not all species are capable of reaching human habitations, remaining in the wild, which is their usual habitat (11, 16, 17).

The fact that most anophelines were captured in the intradomiciliary area (62.3%) in the locality of Piquizeiro, and in the peridomiciliary area (62.7%) on the Retiro Velho Farm, with a higher occurrence of *An. nuneztovari* (106) and *An. darlingi* (88) in the first locality, and *An. albitarsis* (183) and *An. nuneztovari* (129) in the second environment, demonstrates endophilic behavior in these species. The species *An. nuneztovari*, although not considered a primary vector of malaria in Brazil, is frequently found to be infected by *Plasmodium* (16). There are studies in which such mosquitoes presented considerable anthropophily and endophilic behavior. This fact may be attributed to the people's habit of having pets such as dogs and chickens near or even inside their homes, leading the mosquitoes to acquire intradomiciliary habits (21).

An. darlingi, the second most frequent species in the locality of Piquizeiro, is considered frequently anthropophilic and endophilic in Brazil (4, 6, 8) and presents survivor rates higher than the other anophelines present in the Brazilian Amazon (3, 9, 17). This species is very susceptible to human plasmids and adapts very well to environmental changes. This fact favors an increase in population density and, consequently, a permanently high level of endemicity (16).

Regarding the frequency of species in relation to the time of capture, there was a statistically significant difference between the locality of Piquizeiro ($p=0.007$) and the Retiro Velho Farm ($p=0.0305$). Besides, there was a reduction in the frequency of all species at nightfall. A study in São Mateus detected anophelines with the same hematophagic behavior, and higher capture rates from 18h00 to 20h00. Other studies conducted in Maranhão (19, 28) verified that dusk is the crucial period for anopheline female blood feeding, which can also be extended into the first hours of the night. This is a critical fact regarding human exposure to the vectors, since most people in the rural area, settle down

at home with doors and windows open (7, 19).

In São Luís and in Pinheiro, municipalities in the Maranhão state, high densities of the species *An. Albitarsis* were found only in the first hour of sampling (14), testifying the results from Piquizeiro (São Mateus) where the five specimens of *An. albitarsis* were collected from 18h00 to 20h00. This species had already been found in several municipalities in Maranhão (19) using a number of breeding sites, which explains its wide distribution. Nevertheless, the species is rarely found to be infected. Conversely, the species *An. darlingi* was captured during all the sampling hours, presenting a decrease in density at dusk. This behavior has already been described in other studies (25, 28). The species *An. nuneztovari* presented the same behavior, differing from the findings in other studies in the Amazonian Maranhão, in which the species was present only during the first hours of the night. And although it is a secondary vector of malaria transmission, *An. nuneztovari* has been found infected by *P. malariae* in a study conducted in Anajás, PA (20).

Regarding the peridomiciles, the predominance of captures was in the first hour of sampling ($p=0.007$) in Piquizeiro and in the third hour of capture ($*p=0.0305$) on the Retiro Velho Farm, in consonance with other studies (19, 28) previously conducted. This is an important finding regarding control measures, because people in the localities studied tend to be around the houses at that critical time, exposing themselves to mosquito bites.

Of all *Anopheles* species collected in both study regions, not one was infected by *Plasmodium*. The negative results show that *Plasmodium* is not circulating in the sampling area, which can be related to the disease control measures in the state (10, 27). In 2009, 3,991 cases were reported in Maranhão. In 2010, there were 3,904 cases (0.72% of the total number of notifications in the Legal Amazon). However, according to SIVEP (23), the state is still vulnerable and susceptible due to the proximity to high transmission rate areas. In 2010, 40% of the cases came from those areas.

This research demonstrates that the study area has a relevant diversity of species of the genus *Anopheles*, including the main vector of malaria in Brazil, *An. darlingi*. Thus, important information regarding behavioral parameters of anophelines was obtained. With this information it is possible to apply control measures to avoid the proliferation of the mosquito populations. The data also shows that the malaria surveillance program must be continuous and that control actions should be supported by entomological results from areas with broader studies. This allows the understanding of adaptive aspects of malaria vectors and their dynamics occurrence in different ecosystems within the state of Maranhão, which should be used in the implementation of control measures for vectors in malaria risk areas.

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