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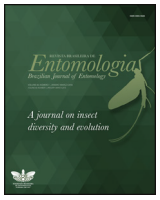

Blood-feeding behavior of *Anopheles* species (Diptera: Culicidae) in the district of Ilha de Santana, state of Amapá, eastern Brazilian Amazon

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ABSTRACT

The present study aimed identifying the behavioral patterns of *Anopheles* species as well as to estimate the parity rate and natural infection analysis for *Plasmodium* species in the district of Ilha de Santana, state of Amapá, Brazil. The samples were obtained in four and 12-hours collections. In the intradomiciliary and peridomiciliary conditions and also in environments with the presence of animals from January/2017 to December/2018. The entomological parameters evaluated were human biting rate (HBR); Indexes of Anthropophily (I_A) and Zoophily (I_Z); Parity Rate (PR); Natural Infection Rate (NIR); Monthly and annual entomological inoculation rate (EIR). A total of 1,330 *Anopheles* specimens were collected, distributed in nine species. All captured species showed preference biting in outdoor enviroment. *Anopheles darlingi* was the most frequent species collected in indoor environment and the most anthropophilic (I_A = 0.39) compared with the remaining species captured. It was also the unique species positive for *Plasmodium vivax*, had the highest anthropophily degree, highest biting activity and HBR in the first hours with a hight rate of parous females. *Anopheles nuneztovari* s.l. was the most zoophilic species (I_Z = 0.65). These findings suggest that *A. darlingi* is the main malaria vector in the studied area. *Anopheles albitarsis* s.l. was the second species more anthropophilic (I_A = 0.31) and revealed a stable pattern with a biting activity peak after sunset, consequently this species may contribute with malaria transmission in area.

Introduction

Malaria transmission in South America occurs in all Amazonian countries (Brazil, Bolivia, Peru, Ecuador, Colombia, Venezuela, Guyana, Suriname and French Guiana) (MS, 2020a). In Brazil, approximately 68 species of Anopheles Meigen, 1818 have already been reported, of which approximately 33 occur in the Brazilian Amazon, and 23 species have already been reported in the state of Amapá (Deane et al., 1948; Deane et al., 1971; Bergo et al., 2007; Galardo et al., 2015; Barbosa et al., 2016; WRBU, 2020). These species are distributed in five subgenera, with the ones with the greatest epidemiological importance in the subgenera *Kerteszia* Theobald, 1905 and *Nyssorhynchus* Blanchard, 1902 (Hiwat and Bretas, 2011). The latter includes the main species involved in the transmission of human malaria in the Brazilian Amazon region, including *Anopheles darlingi* Root, 1926 and some members of the Albitarsis and Nuneztovari complexes (Hiwat and Bretas, 2011).

Anopheles darlingi is the main vector of *Plasmodium* species that cause the human malaria in the Brazilian Amazon, with broad behavioral plasticity (ranged from endophilic to exophilic and ranged from anthropophilic to zoophilic), increasing the complexity of the transmission dynamics of this disease (Santos et al., 2009). The degree of anthropophily is an essential condition for anopheline species to be considered an important vector of human malaria (Gouveia de Almeida, 2011) and, within the same taxon, the degree of anthropophyly may vary according to the region (Forattini, 2002).

Variations in the behavioral patterns of anopheline species are influenced by external factors, such as ecological, environmental, and demographic. Human actions can also exert selective pressure on vector populations, benefiting them under new conditions (Kuwabara, 2008). For example, the deforestation and anthropization influence the local density of *A. darlingi*, which show high adaptive plasticity and degree of synanthropism (Vittor et al., 2006; Gomes et al., 2008). In addition, behavioral changes from endophily to exophily, resulted from the use of indoor residual spraying (IRS), are the selection of vector behavior and/or physiological resistance to insecticides (Tadei, 1987; Glunt et al., 2015; Ranson and Lissenden, 2016; Prussing et al., 2018).

Behavioral and ecological patterns of anopheline vectors can vary in space and time, including seasonal changes. Thus, local control strategies must be adequately planned according to the characteristics of the vectors (MS, 2019a). Periodic studies evaluating behavioral

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patterns determined by the species composition of each area, density, interspecific interactions, biting behavior, intensity of human contact and female longevity are essential to monitor changes that may be associated with climate and environmental determinants (Barbosa et al., 2016; MS, 2019a).

The behavior of the species involved in malaria transmission influences the local epidemiological pattern (Barbosa et al., 2016). The same population may show behavioral variations due to external changes, increasing the complexity of the disease transmission dynamics, as have been observed in *A. darlingi* populations (Voorham, 2002; Santos et al., 2009). Economic development, exploitation of forest products and migratory flow have driven the decrease in forest cover in the Brazilian Amazon. Deforestation is an important risk factor for the emergence of malaria transmission (Tadei et al., 1998; Chaves et al., 2018). These changes affect the development and proliferation of mosquitoes, in addition to socioeconomic factors associated with low Human Development Index (HDI) (migration, housing, population density, and income), as well as environmental (hydrology, climate, topography, and vegetation), biological (life cycle of vectors and pathogens and population immunity) and medical-sanitary components (health system effectiveness) (PAHO, 2009; Tadei et al., 2017; Silva et al., 2010). Despite advances in treatments with new drugs and attempts to develop vaccines, strategies to combat vectors, as well as land use and development planning are still lacking (Li et al., 2016; WHO, 2017; Chaves et al., 2018).

In 2019, an estimated 229 million malaria cases occurred worldwide and approximately 139 million people are at risk of contracting the disease in the Americas (WHO, 2020). In the Americas, Brazil, Colombia, and Venezuela combined account for 86% of the estimated cases of malaria. In Brazil, approximately 99.8% of them occur in the Amazonian region and the states with most malaria cases are: Acre, Amazonas, Amapá, Pará, Rondônia, and Roraima (WHO, 2020; MS, 2020b). In 2018 alone, ~194,512 malaria cases were reported in Brazil, of which 10,008 cases occurred in Amapá (MS, 2019b).

In the municipality of Santana, state of Amapá, 2,811 cases of malaria were reported in 2018, with an Annual Parasite Index (API) of 23.5. In the same year, 570 cases were reported in the district of Ilha de Santana, with an API of 241.3; thus, considered a high-risk area of malaria transmission (API ≥ 50) (MS, 2019b).

The present study aimed identifying the behavioral patterns of *Anopheles* species as well as to estimate the parity rate and natural infection rate for *Plasmodium* species in the district of Ilha de Santana, state of Amapá, an area where occur malaria transmission.

Materials and methods

Specimens of *Anopheles* species were collected in the district of Ilha de Santana, municipality of Santana, state of Amapá, Brazil. This district is located on the banks of the Amazon River, between the geographical coordinates of 00º04'00"and 00º06'00"S and 51º08'00" and 51º12'30"W, and comprises an area of 20.06 km^2 with an estimated population of 3,226 inhabitants (Madeira and Simões, 1972; Valente et al., 1998; IBGE, 2020). The municipality of Santana has an area of 1,541,224 km^2 and is located in the southern region of the state of Amapá, 25 km from the capital Macapá, bordering the municipalities of Macapá, Mazagão and Porto Grande (IBGE, 2013). The main economic activity of the district of Ilha de Santana is based on the primary sector, with most residents working in agricultural activities (fruits and vegetables) and in the production of fruit pulps, especially of two important products of the açaí palm (*Euterpe oleracea* Mart): fruits and palm hearts (Valente et al., 1998).

Adult anophelines were collected from January 2017 to December 2018, covering the rainy and dry seasons. Active collections were carried out using the Protected Human Attraction Technique (PHAT), with a team formed by six experienced and properly trained collectors for this purpose. All team members used Personal Protective Equipment - PPE's, in comply with safety guidelines on the basic material required for sampling activities of the "Guide for Planning Anopheline Sampling by the Protected Human as Attraction Technique (PHAT) and Monitoring Health Risks of the Professional Collector" (MS, 2019a). Anopheline specimens (females only) were collected with the aid of a flashlight, small insect collecting net, and a mouth aspirator, when trying to land on collectors, following Forattini et al. (1999).

The selection of sampling sites followed the criteria: (I) locations with the highest number of reports of malaria cases and (II) locations with the higher demographic density (IBGE, 2017). Sampling days were determined according to the lunar phases (Horsfall, 1943). Collections were carried out for four and 12 continuous hours and in habitats with the presence of animals (pigs, chickens, and/or cattle).

The collections were divided into three categories: the first one evaluated the levels of endophily and exophily. Collections were carried out concomitantly with one collector indoors (inside the dwelling - intradomiciliary) and another outdoors (within a radius of up to ~30m away from the dwelling - peridomiciliary) from 18:00 to 22:00 hours in three different dwellings on the same day. The second category had the main goal of evaluating human biting rate and hourly biting activity in the peridomiciliary area from 18:00 to 6:00 hours. Three collectors participated in these collections, taking turns every two hours. Finally, the third category assessed parameters of anthropophily and zoophily in environments with pigs, chickens and/or cattle (extradomiciliary area) and in the peridomiciliary area, with two collectors, one collector in each area simultaneously from 18:00 to 22:00 hours. The three categories totaled 52 collection sites (Fig. 1) (Table 1S, supplementary file).

All mosquitoes captured were placed in plastic cups and properly labelled with date, hour, site, and sampling method. Following Forattini (1962), mosquitoes were then transported in tightly closed isothermal boxes to the Laboratory of Arthropods of the Federal University of Amapá, where the morphological identification and dissection of ovaries were carried out to estimate parity rate. Anophelines were killed in a freezer at -20ºC and then identified under a stereo microscope Zeiss Stemi DV4, with the aid of dichotomous keys of Faran and Linthicum (1981), Consoli and Lourenço-de-Oliveira (1994), and Forattini (2002). The nomenclature used was proposed by Guimarães (1997). Damaged specimens that could not be identified were grouped as *Anopheles* species.

The collected individuals belonging to species complexes were subjected to molecular analysis to identify their members, using the DNA barcode region (Folmer region) of the *COI* gene of mitochondrial DNA (data no shown). From each species recognized as complex, a sample of 3% of the total captured was taken for analyzes. Other species were also sequenced to confirm their occurrence in the study area.

Endophily and Exophily

The four-hour collections carried out inside (indoors) the dwelling (intradomiciliary) and outdoors (peridomiciliary) were used to estimate the levels of endophily and exophily of the species. The variable level considered the number of mosquitoes (abundance) collected in each environment, analyzing the absolute and relative frequency.

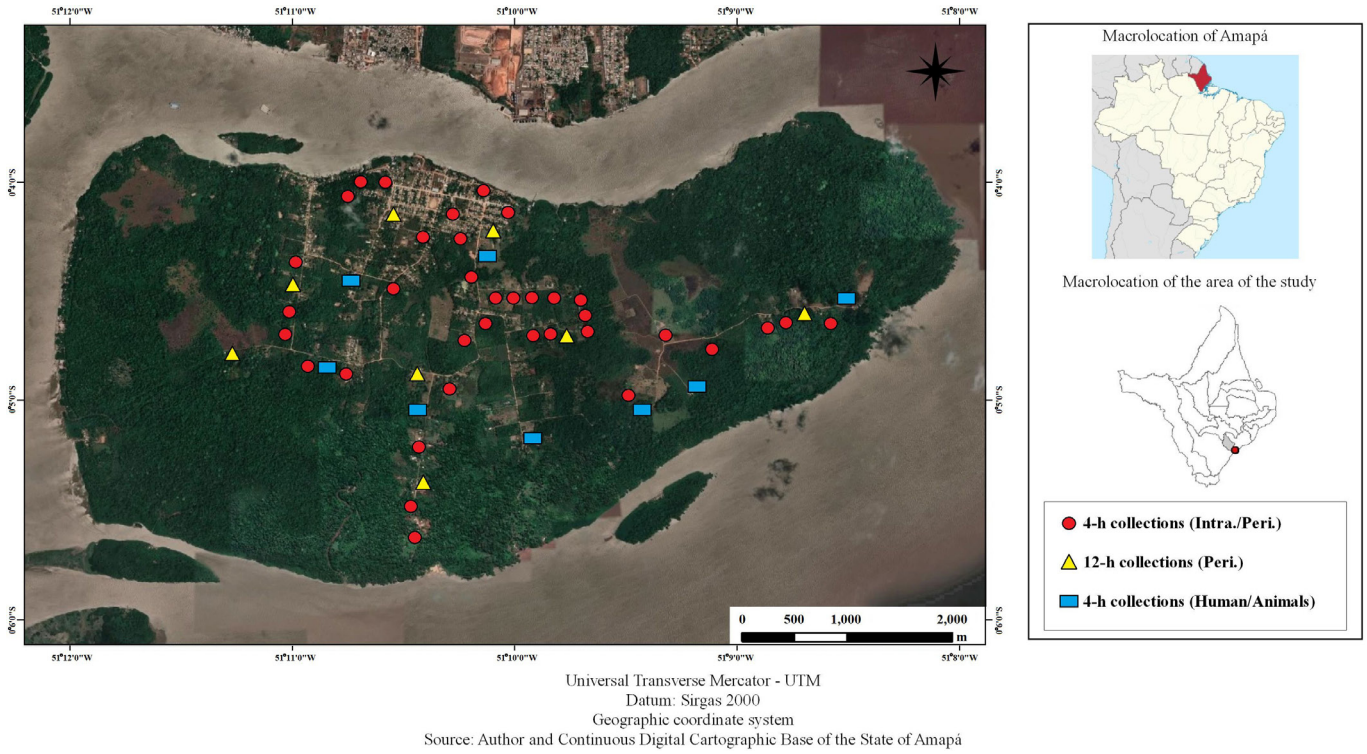

Figure 1 Map indicating the location of the district of Ilha de Santana, state of Amapá, Brazil. In right corner is the smaller map of Brazil showing the localization of the State of Amapá (in red); Below map of the State of Amapá showing the study area (red circle). Satellite image of the district of Ilha de Santana with the 52 collection sites indicated according to the three collection categories (For interpretation of the references to color in this figure legend).

Anthropophily and Zoophily

These parameters were evaluated using four-hour collections carried out specific for these analyses in the peridomiciliary area and in environments with the presence of animals (extradomiciliary area). The Index of Anthropophily (I_A) was determined based on anophelines attracted by the collector seeking a blood meal, while the Index of Zoophily (I_Z) was obtained based on anophelines searching to feed on the blood of animals or resting close to them. These parameters were then compared by separately computing the species collected near humans and animals, as described by Tadei et al. (1993).

These parameters were calculated as follows: I_A= TNSD/OTSD; where: TNSD = total number of specimens collected in dwellings per species divided by OTSD = overall total number of specimens of all species collected in dwellings. I_Z = TNSS/OTSS; where: TNSS = total number of specimens collected in sheds with animals per species divided by OTSS = overall total number of specimens of all species collected at the sheds.

Human biting rate (HBR)

Twelve-hour collections were carried out to examine the attraction of mosquitoes to humans. The index of attraction as mosquito/human/hour was calculated, according to the formula: HBR = N/NC/CT, where: N = number of mosquitoes collected; NC = number of collectors; CT = collection time (Service, 1993).

Hourly biting activity

The time of highest activity and the hematophagic pattern of the species were analyzed using the data obtained from 12-hour collections.

Parity rate

All species and specimens captured during four and 12-hour collections were dissected. The abdomens were used for the dissection of ovaries for the analysis of parity rates, which were determined as nulliparous and parous based on the arrangement of tracheolar filaments, as described by Detinova (1962). After dissections, ovaries were examined under a Bel Photonics light microscope with 400x magnification. Parity rate (PR) was expressed according to the formula: PR = FP x 100/FD; where: FP = number of parous females and FD = number of dissected females.

Analysis of Plasmodium infection rate

Analysis of *Plasmodium* species infection rate was performed only for the most frequent species of mosquitoes captured during four and 12-hour collections. After identification, the heads and thoraces of females of *A. darlingi*, *Anopheles albitarsis* s.l. Lynch-Arribálzaga, 1878, *Anopheles braziliensis* (Chagas, 1907) and *Anopheles nuneztovari* s.l. Gabaldón, 1940 were removed to estimate natural infection rates of *Plasmodium* species, which was detected by amplification of fragment of ribosomal DNA. The heads and thoraces were removed to refine the results and focus on the detection of sporozoites present in salivary glands, the infectious form of the parasite.

Head and thorax were placed in microtubes containing isopropanol, in pools by species, date, hour, site and collection method and preserved in freezer at -20ºC. The pools contained up to five heads and thoraces per species. The material was then transported to the Laboratory of Population Genetics and Evolution of Malaria and Dengue Vectors of the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, where the samples were processed and analyzed.

DNA extraction was performed following the protocol described by Sambrook and Russell (2001). PCR reactions were performed following the protocol described by Snounou et al. (1993), which consisted of a semi-nested-PCR and two reactions. The second reaction, which identifies the *Plasmodium* species, was carried out only when the first reaction was positive for the *Plasmodium* genus. The primer sequences used for the detection of *Plasmodium* species as well as for *P. falciparum* Welch, 1897, *P. vivax* Grassi and Feletti, 1890, and *P. malariae* Laveran, 1881 followed Snounou et al. (1993). The PCR reactions were carried out in a VERIT thermocycler, Applied Biosystems. As a positive control, a sample confirmed for *P. vivax* was used, while the negative control consisted of DNA of *Anopheles konderi* Galvão and Damasceno, 1942 females reared in the insectary of the Laboratory of Population Genetics and Evolution of Malaria and Dengue Vectors of the INPA, first generation.

After amplification, PCR products were analyzed on a 1% agarose gel, where 8 µL of the amplified product and 2 µL of the GelRed dye were loaded onto the gel. After electrophoresis, the gel was examined under ultraviolet light and photo-documented with an imaging system coupled in the photodocumentary apparatus, Loccus Biotecnologia, model L-Pix Touch. The sizes of the obtained fragments were compared with the 100-bp ladder DNA for the first reaction and Low DNA Mass for the second reaction (Invitrogen®).

The detection rate of *Plasmodium* DNA was calculated using the minimum infection rate adapted from Forattini (2002), as MIR = N/I x 100; where: N = number of positive pools to the infection test; I = total number of tested mosquitoes of a species. The Entomological Inoculation Rate (EIR), which indicates the number of infective bites that a person can receive per unit of time, was calculated as: EIR = HBR x MIR; where: HBR = Human biting rate and MIR = minimum infection rate. For the monthly analysis of EIR, the product was multiplied by 31 (days) and for the annual analysis, the product was multiplied by 365 (days) (Williams and Pinto, 2012).

Data analysis

Means were used to compare endophily/exophily and anthropophily/zoophily with the Student's *t* test and the non-parametric substitute Mann-Whitney test, according to the data distribution.

The statistical analysis was based on the Generalized Linear Model (GLM) with the Poisson distribution. Poisson models were used to assess whether the target variable *Anopheles* abundance was influenced by the environment (intra and peridomiciliary), hematophagic activity time, and other species. Abundance was used as a response variable and environment (intra and peridomiciliary), species activity time, and species as predictor variable. GLM was also carried out to assess whether abundance was influenced by feeding preference (anthropophily and zoophily), species activity time, and other species. Considering abundance as a response variable and feeding preference (anthropophily and zoophily), species activity time and species as predictor variable. These analyzes were performed using the software R (R Core Team, 2021). The Kruskal-Wallis test was carried out to analyze HBR, hourly biting activity, and parity rate.

The most abundant species, especially the most frequent species indoor and outdoor environments were plotted in graphs with the number of specimens, by collection time intervals, combined with parity rate and number of malaria cases (data obtained from the Computerized Health System - Epidemiological Surveillance – SIVEP/MS). To test variable associations, Spearman correlations were used. Descriptive statistics were obtained with the software BioEstat version 5.0 (Ayres et al., 2007). The significance level of $\alpha = 0.05$ was used.

Results

A total of 1,330 *Anopheles* specimens were collected in the study area, distributed in nine species and two subgenera: *Nyssorhynchus* (six species) and *Anopheles* (three species). The species *A. konderi* and *Anopheles triannulatus* Neiva and Pinto, 1922 were identified by molecular methods. For specimens belonging to the Albitarsis and Nuneztovari complexes, sequences of high quality could not be obtained, making comparisons with those deposited in GenBank impossible; therefore, in this study, the specimens were denominated as *A. albitarsis* s.l. and *A. nuneztovari* s.l., respectively. The sequenced specimens of *A. braziliensis*, *A. darlingi*, and *Anopheles intermedius* (Peryassú, 1908) confirmed the occurrence of these species in the study area. Of the total, 276 (20.75%) were captured in four-hour collections (intradomiciliary and peridomiciliary), 169 (12.71%) in the 12-hour collections (only peridomiciliary), and 885 (66.54%) in four-hour collections (humans and animals), totaling 272 hours of sampling effort (Table 1).

Considering all sampling methods, *A. nuneztovari* s.l. was the most common captured species (45.26%) in the area and with 83.53% of the specimens collected when evaluating anthropophilic and zoophilic indexes. The second most abundant species was *A. darlingi* (19.10%), followed by *A. albitarsis* s.l. (18.57%), and *A. braziliensis* (10.00%) (Table 1).

Table 1
Species of *Anopheles* and absolute and relative frequency, according to the collection categories in the district of Ilha de Santana, municipality of Santana, state of Amapá (Kruskal-Wallis test [13] = 1.02; $p > 0.05$).

Species	4-h collections (Intra./Peri.)		12-h collections (Peri.)		4-h collections (Humans/Animals)		Total (%)
	nº	%	nº	%	nº	%	
Anopheles (*Ny.*) *albitarsis* s.l.	42	**17.00**	29	**11.74**	176	**71.26**	**247 (18.57)**
Anopheles (*Ny.*) *braziliensis*	47	**35.34**	20	**15.04**	66	**49.62**	**133 (10.00)**
Anopheles (*Ny.*) *darlingi*	100	**39.37**	44	**17.32**	110	**43.31**	**254 (19.10)**
Anopheles (*An.*) *intermedius*	21	**61.76**	7	**20.59**	6	**17.65**	**34 (2.56)**
Anopheles (*Ny.*) *konderi*	2	**40.00**	1	**20.00**	2	**40.00**	**5 (0.37)**
Anopheles (*An.*) *mattogrossensis*	1	**25.00**	2	**50.00**	1	**25.00**	**4 (0.30)**
Anopheles (*Ny.*) *nuneztovari* s.l.	44	**7.31**	56	**9.32**	502	**83.53**	**602 (45.26)**
Anopheles (*An.*) *peryassui*	6	**60.00**	1	**10.00**	3	**30.00**	**10 (0.75)**
Anopheles (*Ny.*) *triannulatus*	13	**36.11**	9	**25.00**	14	**38.89**	**36 (2.71)**
Anopheles spp.	-	**-**	-	-	5	**100.00**	**5 (0.38)**
Total (%)	**276**	**20.75**	**169**	**12.71**	**885**	**66.54**	**1,330 (100.00)**

Behavioral patterns

Endophily and Exophily

All species were captured more frequently in outdoor environment (76.45%). *Anopheles mattogrossensis* Lutz and Neiva, 1911, *A. konderi*, *Anopheles peryassui* Dyar and Knab, 1908 and *A. triannulatus* were collected in low frequencies (lower than 5%) (Table 2).

The levels of endophily and exophily observed for *Anopheles* species during the sampling period did not reveal significant differences between environments (Mann-Whitney U test = 24; z = -1.42; $p = 0.16$), although a predominance for the outdoor environment has been observed. Only one exception was recorded, represented by only one individual (*A. darlingi*), which was collected in the indoor environment in May/2017. In the months of January and July 2017, no individuals were collected in the indoor environment.

Fig. 2 shows the levels of endophily and exophily for *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi*, and *A. nuneztovari* s.l. Despite of predominance in outdoor environment, *A. darlingi* was the species that had the highest number of specimens collected in the indoor environment. *Anopheles albitarsis* s.l. was also captured in indoor environment in March/2017, July/2018, September/2018, and November/2018. The behavioral patterns of *A. braziliensis* and *A. nuneztovari* s.l. varied considerably during the sampling period. However, a significant difference between the two environments

Table 2
Anopheles species collected indoors (endophily) and outdoors (exophily), and peridomiciliary area (anthropophily) and in environments with the presence of animals (extradomiciliary - zoophily) in four-hour collections, in the district of Ilha de Santana, municipality of Santana, state of Amapá (Mann-Whitney U test = 24; z = -1.42; $p = 0.16$).

Species	Endophily and Exophily			Anthropophily and Zoophily				
	Intra.	Perid.	Total (%)	Human	Animals	Total (%)	I_A	I_Z
	Individuals number (%)	Individuals number (%)		Individuals number (%)	Individuals number (%)			
A. (*Ny.*) *albitarsis* s.l.	6 (14.29)	36 (85.71)	**42 (15.22)**	37 (21.02)	139 (78.98)	**176 (19.89)**	**0.31**	**0.18**
A. (*Ny.*) *braziliensis*	11 (23.40)	36 (76.60)	**47 (17.03)**	23 (34.85)	43 (65.15)	**66 (7.46)**	**0.19**	**0.06**
A. (*Ny.*) *darlingi*	26 (26.00)	74 (74.00)	**100 (36.23)**	46 (41.82)	64 (58.18)	**110 (12.43)**	**0.39**	**0.08**
A. (*An.*) *intermedius*	5 (23.81)	16 (76.19)	**21 (7.61)**	1 (16.67)	5 (83.33)	**6 (0.68)**	**0.01**	**0.01**
A. (*Ny.*) *konderi*	1 (50.00)	1 (50.00)	**2 (0.73)**	-	2 (100.00)	**2 (0.23)**		
A. (*An.*) *mattogrossensis*	-	1 (100.00)	**1 (0.36)**	1 (100.00)	-	**1 (0.11)**	**0.01**	**0.00**
A. (*Ny.*) *nuneztovari* s.l.	11 (25.00)	33 (75.00)	**44 (15.94)**	7 (1.39)	495 (98.61)	**502 (56.72)**	**0.06**	**0.65**
A. (*An.*) *peryassui*	2 (33.33)	4 (66.67)	**6 (2.17)**	3 (100.00)	-	**3 (0.34)**	**0.03**	**0.00**
A. (*Ny.*) *triannulatus*	3 (23.08)	10 (76.92)	**13 (4.71)**	1 (7.14)	13 (92.86)	**14 (1.58)**	**0.01**	**0.02**
Anopheles spp.	-	-	**-**	-	5 (100.00)	**5 (0.56)**	**-**	**-**
Total (%)	**65 (23.55)**	**211 (76.45)**	**276 (100.00)**	**119 (13.45)**	**766 (86.55)**	**885 (100.00)**		

I_A = Index of Anthropophily; I_Z = Index of Zoophily.

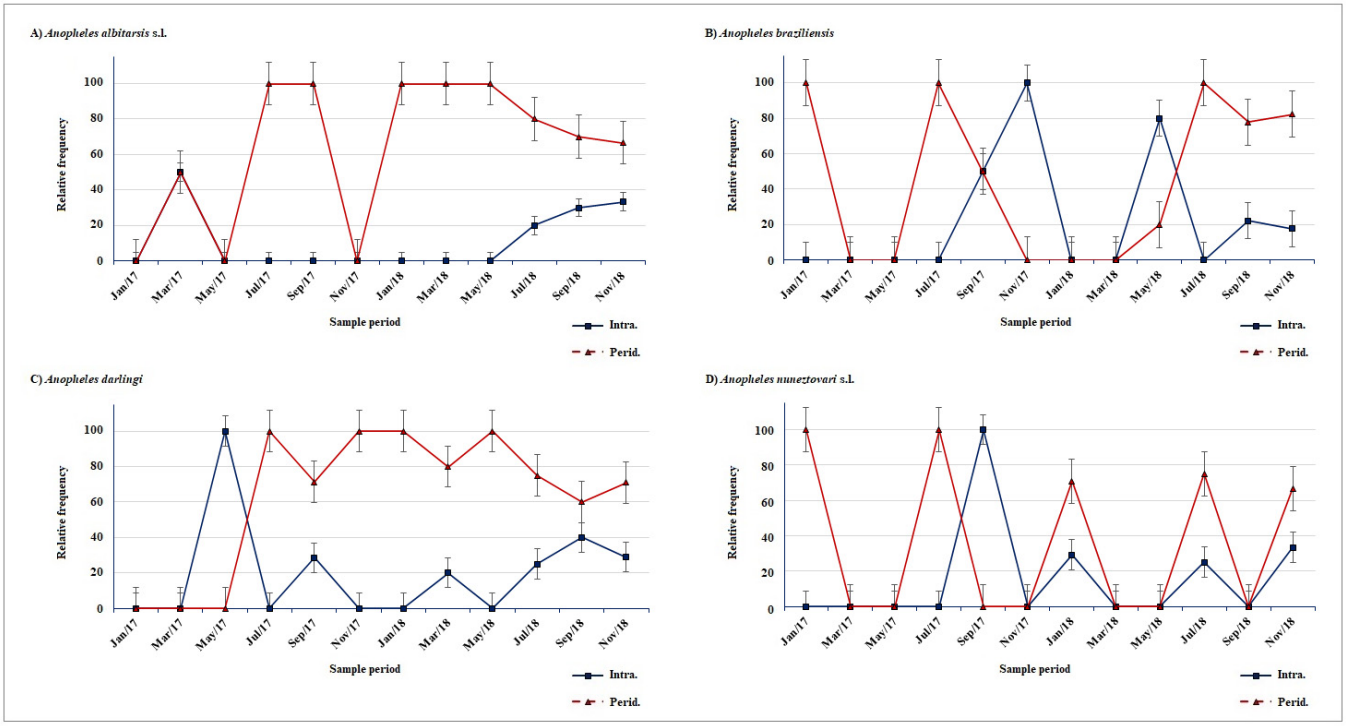

Figure 2 Species of *Anopheles* collected indoors (intradomiciliary) and outdoors (peridomiciliary) in four-hour collections, over 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Significant difference was observed between the number of individuals collected indoors and outdoors only for *A. albitarsis* s.l. (Mann-Whitney U test = 31.5; z = -2.44; $p = 0.01$). Graph with standard deviation bars.

was observed only for the abundance of *A. albitarsis* s.l. throughout the sampling period (Mann-Whitney U test = 31.5; z = -2.44; $p = 0.01$).

Regarding the collection time interval for the more frequent species collected, *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l. showed greater abundance in the second time interval (Fig. 3). A significant difference between indoor and outdoor environments was found for *A. albitarsis* s.l. and *A. braziliensis* when analyzing by time interval, indicating that the number of mosquitoes biting outdoors is significantly higher than that of those biting indoors (Student's *t* test [3] = -3.44; $p = 0.01$, Student's *t* test [3] = -2.49; $p = 0.04$; respectively).

GLM revealed that species abundance varied between intra and peridomiciliary environments, indicating the exophilic preference of *Anopheles* species, reflected in a very significant and positive relationship. Regarding abundance during collection times, a significant and negative relationship was observed, showing that anopheline activity was highest in the second collecting period. Another aspect analyzed was the general abundance of *Anopheles* species in relation to the abundance of the four most frequently collected species. A statistically significant and positive relationship was also found, showing that these species (*A. albitarsis* s.l., *A. braziliensis*, *A. darlingi,* and *A. nuneztovari* s.l.) occur in greater abundance in the district of Ilha de Santana (Table 3).

Anthropophily and Zoophily Indexes

Regarding anthropophilic and zoophilic indexes, the 885 mosquitoes collected were distributed in nine species. Highest abundance was observed in February/2017, with 346 (39.10%) individuals, followed by February/2018, with 260 (29.38%) specimens (mainly *A. nuneztovari* s.l. and *A. albitarsis* s.l.). The lowest abundance was observed in November/2017, with four (0.45%) individuals (*A. albitarsis* s.l. and *A. darlingi*). In all months, specimens were predominantly collected displaying zoophilic behavior, with the exception of November/2017 that showed two specimens (*A. albitarsis* s.l. and *A. darlingi*) collected in anthropophilic conditions and two in zoophilic conditions (*A. albitarsis* s.l.). In May/2018, 28 specimens were collected near dwellings and only one specimen (*A. intermedius*) was captured in the environment with animals. All species were more frequently collected displaying zoophilic behavior, except for *A. mattogrossensis* and *A. peryassui* with one (0.11%) and three (0.34%) individuals collected, respectively, displaying anthropophilic tendencies. *Anopheles nuneztovari* s.l. was the most abundant species, with 502 (56.72%) specimens collected predominantly in the environment with animals. Of these, only

Table 3
Parameters and *p* values estimated with a Generalized Linear Model with Poisson distribution explaining the absolute abundance of *Anopheles* obtained during two years of collections in the district of Ilha de Santana, Amapá, Brazil. The predictor variables were: environment (intra/peridomiciliary), species activity time, and the four species collected in greater abundance. A Poisson distribution GLM was also performed explaining the absolute abundance of *Anopheles*. The predictor variables were: feeding preference (anthropophily/zoophily), species activity time, and the four species collected in greater abundance.

	Estimate	*p*-value	
(Intercept)	3.5607	0.00753	**
Environment (intra/peridomiciliary)	7.6928	< 2e-16	***
Time	-0.9248	0.00570	**
Species[2]	0.8744	0.00878	**
	Estimate	*p*-value	
(Intercept)	0.24924	0.125722	
Feeding preference (anthropophily/zoophily)	1.88061	< 2e-16	***
Time	0.10241	0.000865	***
Species[2]	0.51357	< 2e-16	***

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
[2]Diference in abundance among the four main species collected: *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l.

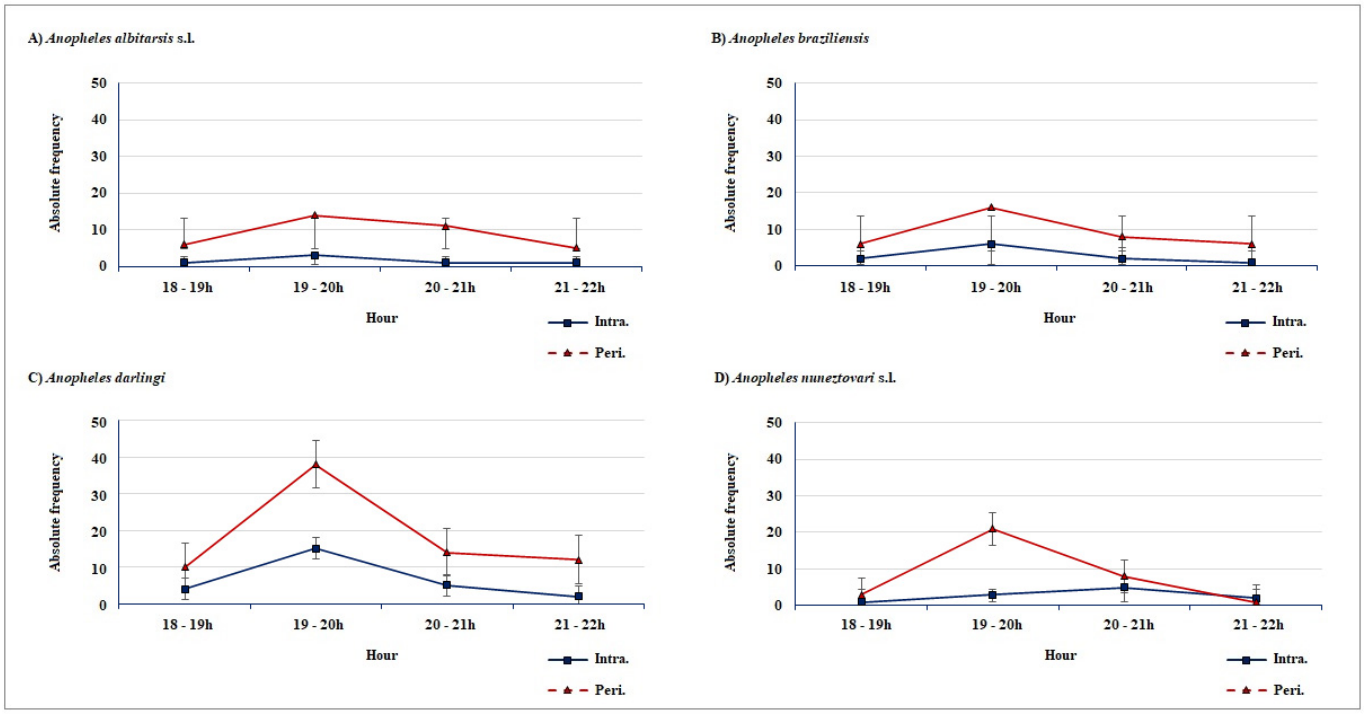

Figure 3 Species of *Anopheles* collected indoors (intradomiciliary) and outdoors (peridomiciliary) by collection time, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis;* C) *Anopheles darlingi;* D) *Anopheles nuneztovari* s.l. Significant difference between indoor and outdoor environments was found for *A. albitarsis* s.l. and *A. braziliensis* (Student's *t* test [3] = -3.44; $p = 0.01$, Student's *t* test [3] = -2.49; $p = 0.04$; respectively). Graph with standard deviation bars.

seven (1.39%) were captured near dwellings. Two peaks were observed for this species throughout the collection period, February/2017 and February/2018, with 239 (47.61%) and 231 (46.01%) specimens collected, respectively (Fig. 4). The second most abundant species was *A. albitarsis* s.l., with 176 (19.89%) and the highest abundance was observed in February/2017 with 82 (46.59%) individuals (Fig. 4). *Anopheles darlingi* was the third most frequent species with 110 (12.43%) individuals collected and showed a peak in August/2018, with 60 (55.55%) specimens captured. Of these, 32 (53.33%) were captured near dwellings and 28 (46.67%) in the environment containing animals. For this species, a simultaneous variation was observed between anthropophilic and zoophilic indexes throughout the collection period (Fig. 4). *Anopheles braziliensis* was the fourth the most abundant species, with 66 (7.46%) specimens and showed a peak of anthropophilic behavior in May/2018, represented by 17 individuals, and a peak of zoophilic behavior in November/2018, with 41 specimens collected (Fig. 4). No significant differences in abundance between anthropophilic and zoophilic behaviors were found for *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi*, and *A. nuneztovari* s.l.

Of the 885 mosquitoes collected, 119 (13.45%) individuals displayed anthropophilic behavior and 766 (86.55%) specimens showed zoophilic behavior. In environment containing animals, with the exception of the first-time interval, a high abundance was observed in all other times. The species with the highest anthropophilic index was *A. darlingi* (I_A = 0.39), followed by *A. albitarsis* s.l. (I_A = 0.31), and *A. braziliensis* (I_A = 0.19), whereas *A. nuneztovari* s.l. (Iz = 0.65) had the highest zoophilic index (Table 2; Fig. 5). *Anopheles intermedius*, *A. konderi*, *A. mattogrossesnsis*, *A. peryassui*, and *A. triannulatus* had anthropophilic and zoophilic indexes equal or lower that 0.03%. No significant differences between the anthropophilic and zoophilic indices obtained per species were observed (Mann-Whitney U test = 36.5; z = -0.31; p = 0.75).

When taking into account species distribution per time interval, the number of mosquitoes collected displaying zoophylic behavior was much higher than those captured displaying anthropophilic behavior (Fig. 6). However, only *A. albitarsis* s.l. showed significant differences between the anthropophilic and zoophilic behaviors per time interval (Student's t test [3] = -2.67; p = 0.03).

GLM was also used to determine feeding preferences (anthropophily/zoophily), time of highest hematophagic activity considering feeding preference, and ratio of general abundance to abundance of the four species collected in greater frequency. Although no significant differences were found between anthropophilic and zoophilic indices (mentioned above), GLM revealed a trend regarding feeding preference, which was as a very significant factor in the abundance of species, indicating preferentially a zoophilic behavior. Another determining factor was collection time, which showed a very significant relationship, confirming the highest zoophilic activity during the second collecting period. Finally, when analyzing the relationship between general abundance and the abundance of the four most frequently collected species, a significantly strong relationship was observed, demonstrating the predominance of the four species collected at the highest densities in the study area (Table 3).

Human biting rate (HBR)

The highest index of attraction as mosquito/human/hour was observed for *A. nuneztovari* s.l. with 3.00 in the interval 19 – 20h. *Anopheles albitarsis* s.l. had the second highest HBR, ranging from 0.08 to 0.83 at the time intervals: 01 – 02h, 04 – 05h, and 18 – 19h. The third species with the highest index was *A. darlingi* with 0.75, which was collected in almost all time intervals, except in the interval of 20 – 21h (Fig. 7). The remaining species showed HBR ranging from 0.00 to 0.33.

The analysis of HBR for 12-h collections revealed a significant difference between *A. darlingi* and the species *A. intermedius*, *A. konderi*, *A. mattogrossensis*, *A. peryassui*, and *A. triannulatus* (Kruskal-Wallis test [8] = 33.02; p < 0.05). Significant differences in HBR were found between the time intervals: 18 – 00h, 19 – 23h, 19 – 00h, 19 – 01h, 19 – 02h, 19 – 03h, 19 – 04h, and 19 – 05h (Kruskal-Wallis test [11] = 15.16; p < 0.05).

Hourly biting activity

Considering all species captured during 12-h collections, a bimodal peak between the time intervals of 21 – 22h and 02 – 03h was observed for *A. mattogrossensis,* while *A. konderi* was collected only in the

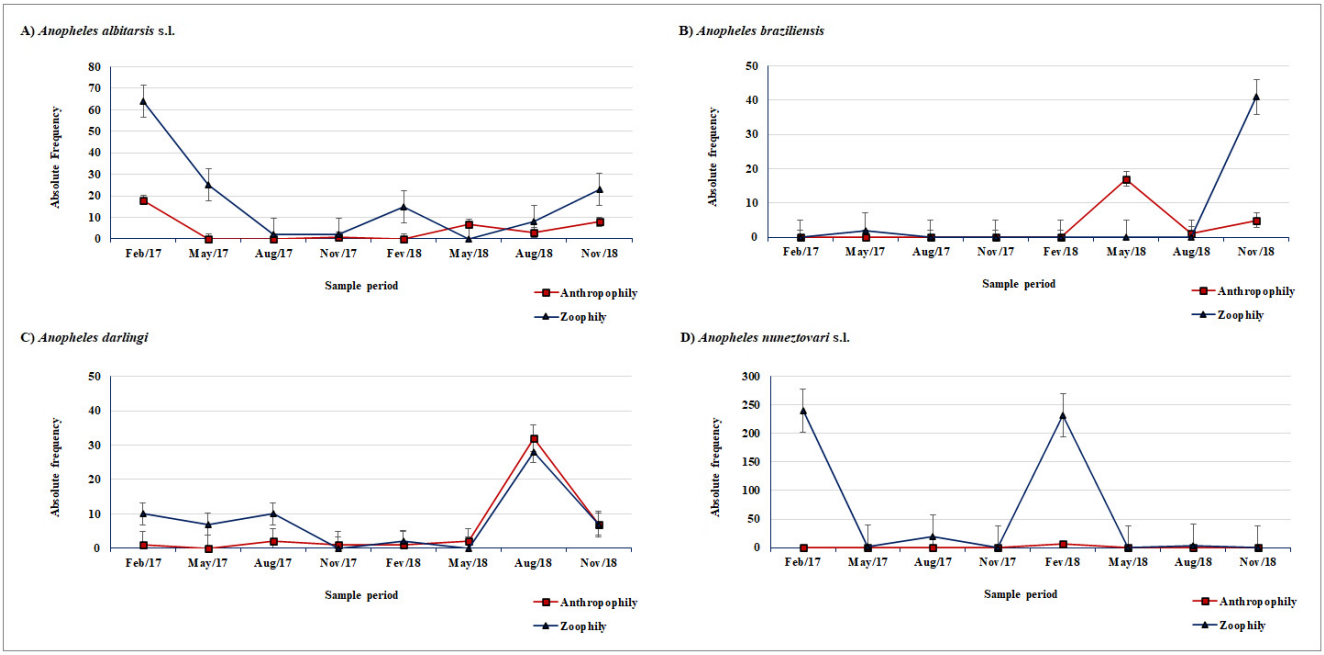

Figure 4 Species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior by month, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis;* C) *Anopheles darlingi;* D) *Anopheles nuneztovari* s.l. Graph with standard deviation bars.

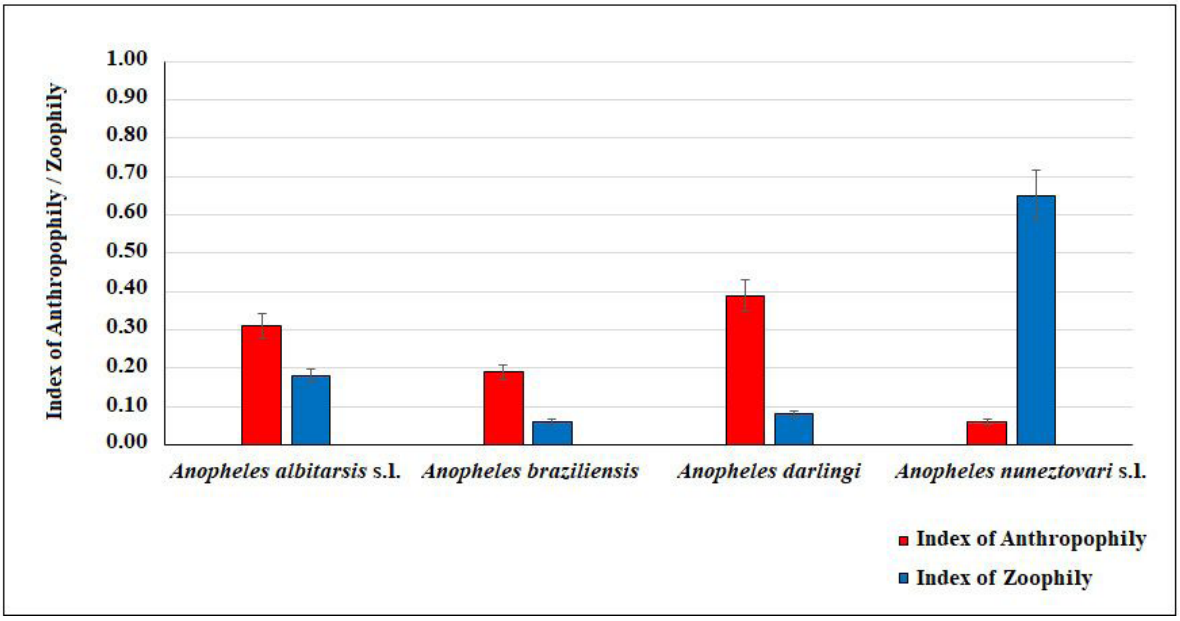

Figure 5 Species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. Graph with standard deviation bars.

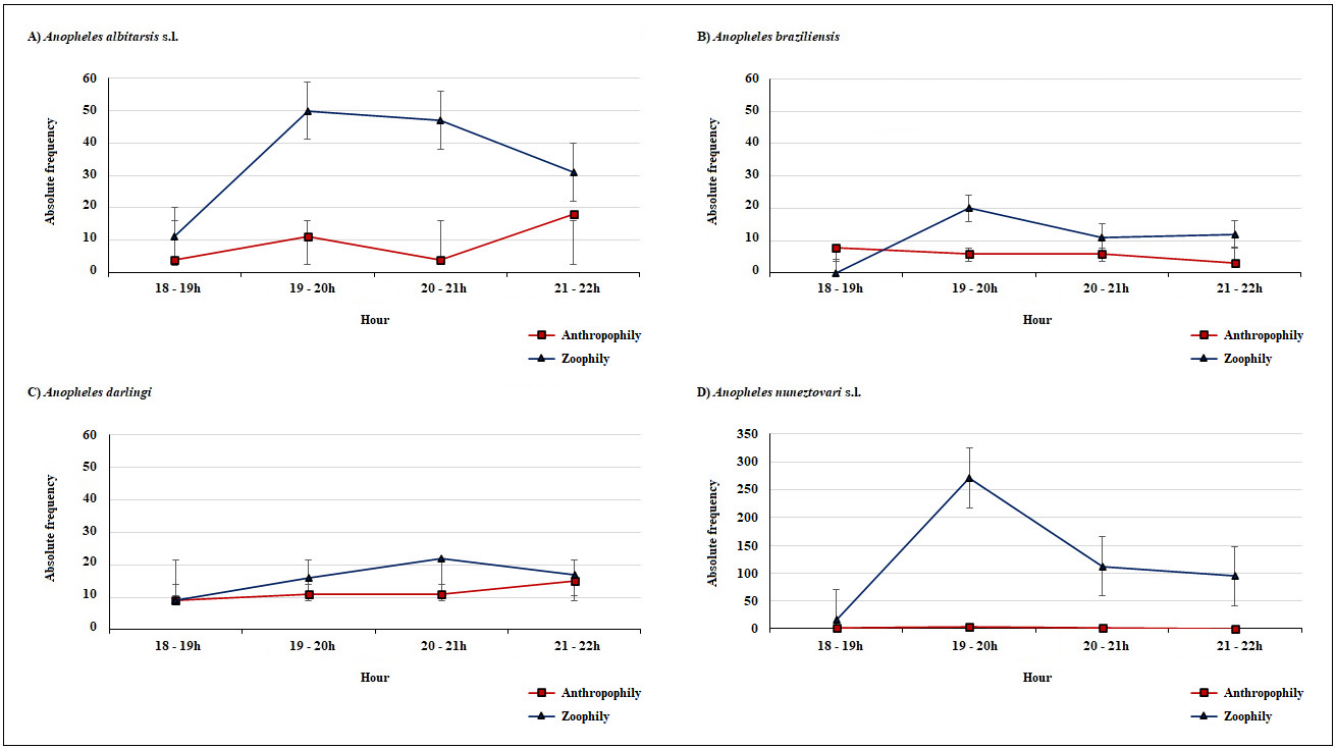

Figure 6 Absolute frequency by time of the main species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior in the district of Ilha de Santana, municipality of Santana, state of Amapá; A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Significant difference between the number of anthropophilic and zoophilic individuals was observed only for *A. albitarsis* s.l. (Student's *t* test [3] = -2.67; $p = 0.03$). Graph with standard deviation bars.

first time interval, consequently showed a unimodal peak. It should be pointed out because these species were captured in low densities. *Anopheles braziliensis* and *A. triannulatus* had multimodal peaks, although the latter was also found in low densities.

Two well-defined peaks of feeding activity (18 – 19h and 21 – 22h) were observed for *A. darlingi*, which was not collected in only one time interval (20 – 21h). On the other hand, a unimodal pattern with a peak early in the evening (19 – 20h) was found for *A. albitarsis* s.l., which was absent in the 22 – 23h; 23 – 00h; 00 – 01h; 03 – 04h; 05 – 06h time intervals.

Regarding the hourly biting activity of the main captured species, a significant difference was found between *A. darlingi* and the species *A. intermedius, A. konderi, A. mattogrossensis, A. peryassui* and *A. triannulatus*; between *A. albitarsis* s.l. and *A. konderi*; and between

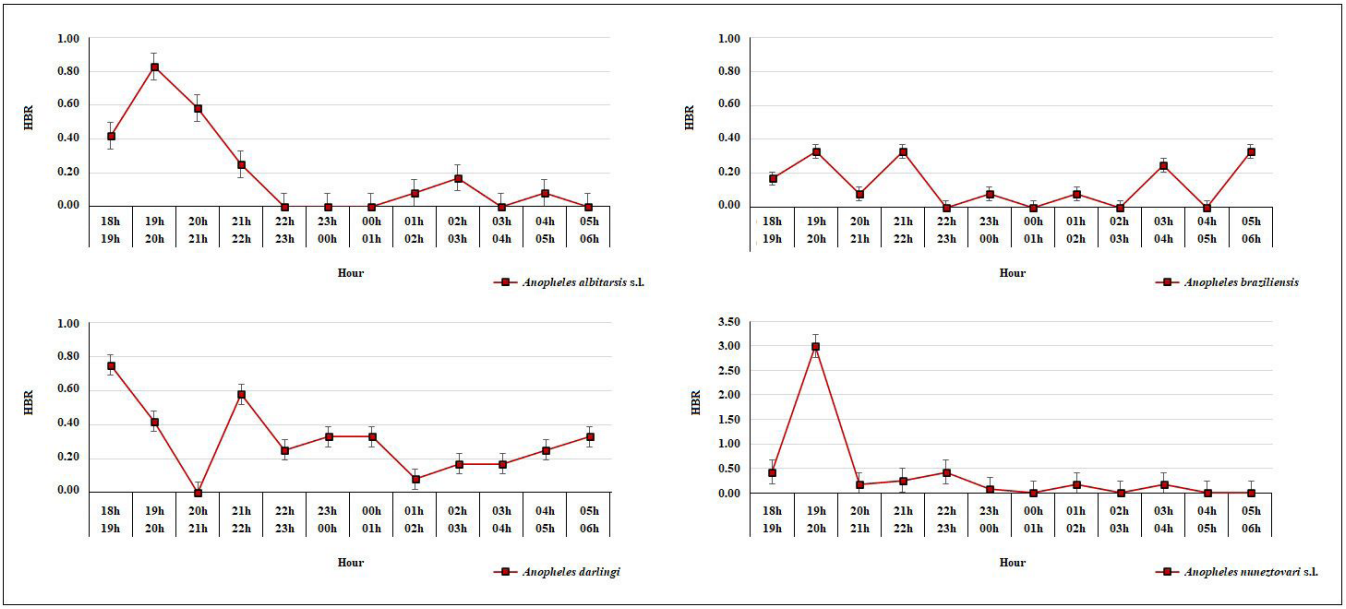

Figure 7 Human biting rate (HBR) of the most abundant species captured in the 12-hour collections in the district of Ilha de Santana, municipality of Santana, state of Amapá. Graph with standard deviation bars.

A. mattogrossensis and *A. peryassui* (Kruskal-Wallis test [11] = 18.79; $p < 0.05$). Activity was clearly higher during the 19 and 20h time interval, with a significant difference in biting activity between 18 and 00h, 19 and 23h, 19 and 00h, 19 and 01h, 19 and 02h, 19 and 03h, 19 and 04h, and 19 and 05h (Kruskal-Wallis test [8] = 40.92; $p < 0.05$).

Parity rate

Based on four-hour collections, the parity rate of *A. darlingi* ranged from 21.42 to 31.58, with the highest number of parous females (31.58) found in the third time interval (20 - 21h). The parity rates for *A. albitarsis* s.l. (42.86) and *A. braziliensis* (50.00) were highest in the first time interval (18 - 19 h). All specimens of *A. konderi* and *A. mattogrossensis* captured were nulliparous. Parous females of *A. darlingi, A. albitarsis* s.l., *A. braziliensis,* and *A. intermedius* were found at all time intervals.

No significant differences in parity rates were found among collection time intervals. However, a significant difference in parity rates was found between *A. darlingi* and *A. intermedius* and between *A. nuneztovari* s.l. and *A. intermedius* (Kruskal-Wallis test [6] = 9.65; $p < 0.05$).

In 12-h collections, all *A. darlingi* females captured at the 01 - 02h time interval were parous (100.00), with the parity rate ranging from 25.00 to 100.00. Another species with one of the highest parity rates was *A. albitarsis* s.l., varying between 30.00 and 100.00, with all parous females at the 04 – 05h time interval. Analyzing parity rates by time, a significant difference was found between the intervals 18 and 00h, 19 and 20h, 19 and 22h, 19 and 00h, 19 and 02h and 19 and 03h (Kruskal-Wallis test [11] = 11.27; $p < 0.05$). When analyzing parity rates by species, a significant difference was observed between *A. albitarsis* s.l. and *A. mattogrossensis, A. albitarsis* s.l. and *A. konderi, A. darlingi* and *A. intermedius, A. darlingi* and *A. mattogrossensis, A. darlingi* and *A. konderi,* and *A. darlingi* and *A. triannulatus* (Kruskal-Wallis test [5] = 16.08; $p < 0.05$).

In July and September 2017 there was a rise in the number of malaria cases accompanying the increase in anopheline density. For *A. darlingi,* the increase in parity rate was simultaneous with the increase in density of this species, with an overlap in variables. Also, when analyzing the increase in density and the increase in the number of malaria cases, a synchrony during the two malaria peaks also occurred (September/2017 and November/2018) (Fig. 8). The increase in density of *A. albitarsis* s.l. was followed by an increase in the number of cases malaria, with peaks in parity rate along with density (Fig. 8). The same was observed for *A. braziliensis* regarding the increase in density with the number of malaria cases (Fig. 8). For *A. nuneztovari* s.l., a simultaneous variation between density and parity was observed; however, when analyzing density and number of malaria cases, the increase in the number of individuals of this species clearly occurred only after the increase in the number of malaria cases (Fig. 8). The density and parity rate of *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l. showed a strong, positive and significant correlation. However, no correlation was found between density and number of malaria cases or between parity rate and the number of malaria cases. Despite the already mentioned synchronism observed for *A. darlingi* between density and number of malaria cases.

Minimum infection Rate (MIR) and Entomological inoculation rate (EIR)

In this study, 438 pools were analyzed for *Plasmodium* species using the PCR technique, distributed as follow: 121 for *A. darlingi*, 95 for *A. albitarsis* s.l., 54 for *A. braziliensis*, 163 for *A. nuneztovari* s.l. and five for *Anopheles* species. Of these, only one pool was positive for *P. vivax*, which contained one specimen of *A. darlingi*. This specimen was captured during 12-h collections, between 18 and 19 hours and in May/2018, when only ten mosquitoes were collected, of which five were *A. albitarsis* s.l. and five were *A. darlingi*. However, the specimens of *A. darlingi* were captured at different times. The minimum infection rate was 0.83% for *P. vivax*, while the monthly EIR was 0.11 infectious bites/human/month and the annual EIR was 1.27 infectious bites/human/year.

Discussion

In this study, a total of nine species was captured and the findings revealed that, of the four most abundant species, two (*A. darlingi* and *A. albitarsis* s.l.) have been appointed as the most important malaria vectors in the state of Amapá (Póvoa et al., 2001; Galardo et al., 2007; Barbosa et al., 2016).

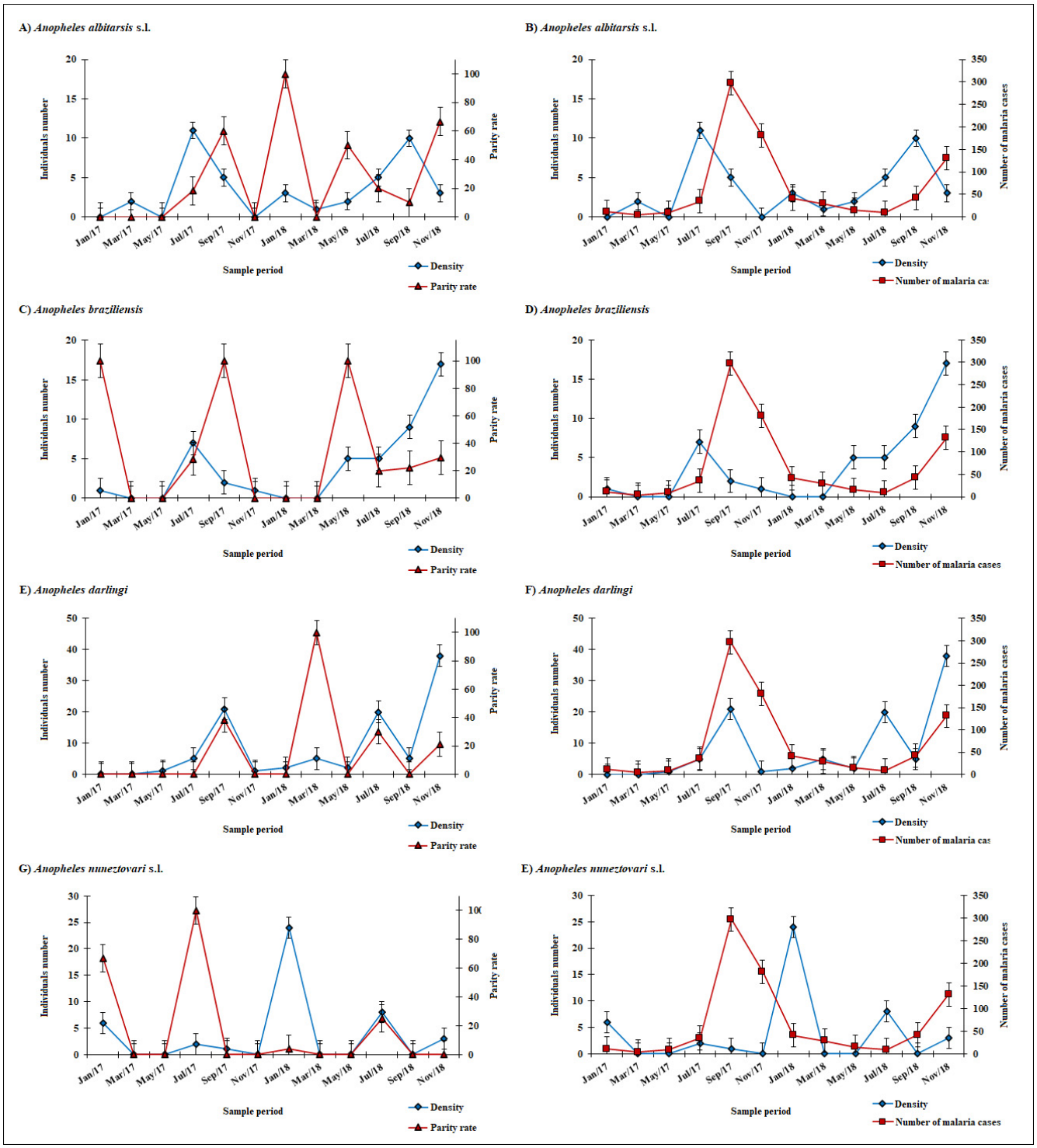

Figure 8 Density of *Anopheles* species, parity rate, and number of malaria cases by month, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A and B) *Anopheles albitarsis* s.l.; C and D) *Anopheles braziliensis*; E and F) *Anopheles darlingi*; G and H) *Anopheles nuneztovari* s.l. The Spearman correlation between density and parity rate was significant for *A. albitarsis* s.l. (rs = 0.61; $p = 0.03$), *A. braziliensis* (rs = 0.80; $p = 0.01$), *A. darlingi* (rs = 0.72; $p = 0.01$) and *A. nuneztovari* s.l. (rs = 0.74; $p = 0.01$). Graph with standard deviation bars.

The collected species were recorded in all sampling methods, revealing their behavioral plasticity. Although most individuals were collected outdoors, the main species involved in the transmission of malaria in the state of Amapá (*A. darlingi* and *A. albitarsis* s.l,) were also collected indoor environment and had the highest anthropophily index (I_A = 0.39; 0.31, respectively). *Anopheles nuneztovari* s.l. was captured in highest density in this study, but it was the most zoophilic species (I_Z = 0.65). Although *A. nuneztovari* s.l. is considered an important malaria vector in Colombia and Venezuela (Rubio-Palis et al., 1992; Schoeler et al., 2003; Turell et al., 2008; Sinka et al., 2010; Naranjo-Díaz et al., 2016), in Brazil this species is considered a secondary vector in some regions, in spite to be found frequently infected with *Plasmodium*

species (Santos et al., 2005; Galardo et al., 2007; Rezende et al., 2009). However, some studies carried out in the state of Amapá indicate that *A. nuneztovari* s.l. may contribute to malaria transmission, especially when in high densities (Galardo et al., 2007; Barbosa et al., 2016). In fact, in the Brazilian Amazon this species may consist of two or more species, as was demonstrated by Scarpassa et al. (1996, 2000).

The tools for the control of malaria vectors recommended by the World Health Organization (WHO) are long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS). However, these methodologies mainly target endophilic anophelines (Prussing et al., 2019). In this study, these control measures can become ineffective against the malaria vectors in the district of Ilha de Santana, due behavior to be predominantly outdoors. An example of unsuccessful IRS was reported in Peru, where Prussing et al. (2018) did not observe a consistent effect of spraying on the abundance of *A. darlingi*. In this case, this method was insufficient to eliminate malaria, and additional strategies were needed (Williams et al., 2018; Prussing et al., 2019).

The GML revealed a significant effect of the variable environment (indoors and outdoors) on abundance of *Anopheles* species. *Anopheles albitarsis* s.l. had the second highest index of attraction as mosquito/human/hour. Since the first studies carried out in the state of Amapá by Deane et al. (1948), *A. albitarsis* s.l. has been considered a possible secondary vector of malaria, and its epidemiological importance has been supported by Póvoa et al. (2001), Conn et al. (2002), Galardo et al. (2007) and Barbosa et al. (2016), either as primary vector at times or helping the maintenance of the disease in areas of transmission. In this study, *A. albitarsis* s.l. was found at lower densities throughout the sampling period, and may be helping the maintenance of malaria in the region and playing a role as a secondary vector of the disease.

The greater abundance of anopheline vectors collected outdoor environment allows the assessment of the level of risk of malaria transmission, given the common habit of residents be outside their residences at dusk, when anopheline activity was highest. In this study, *A. darlingi* exhibited anthropophilic and crepuscular behaviors (dawn and dusk). When analyzing the entire sampling period, however, zoophilic behavior was also observed for this species during the collections carried out in environments with animals, indicating its hematophagic plasticity. When analyzing anthropophilic and zoophilic behaviors by time interval, the anthropophily index was highest in the two species, *A. darlingi* and *A. albitarsis* s.l., demonstrating their blood-feeding preferences and epidemiological importance (Barbosa et al., 2016). Also, in 12-hour collections, the number of parous females of *A. darlingi* and *A. albitarsis* s.l. was highest in the first time intervals, confirming that these hours are when the risk for malaria transmission is highest. However, parity rate for both species peaked in the last hours of the night.

Similar results to those observed in the present study were also reported in the state of Maranhão for *A. albitarsis* s.l., *A. darlingi*, and *A. nuneztovari* s.l., regarding HBR and with peaks occurring in the first time intervals (Barros et al., 2020). A second peak in the third time interval (21 – 22 hours) was observed for *A. darlingi.* In the present study, this species had the third highest HBR and was found in almost all collection times, indicating its activity throughout the night (time plasticity). *Anopheles albitarsis* s.l. and *A. braziliensis* were captured predominantly in outdoor environment, however, they displayed anthropophilic behavior (I_A = 0.31; 0.19, respectively). These species were less anthropophilic than *A. darlingi* (I_A = 0.39). *Anopheles albitarsis* s.l. has been implicated as local vector of malaria in the states of Amapá (Conn et al., 2002; Galardo et al., 2007; Barbosa et al., 2016) and Roraima (Silva-Vasconcelos et al., 2002). Considering the findings obtained with *A. braziliensis*, further studies are needed to clarify its role in malaria transmission in this area and in other areas of Amapá.

Anopheles intermedius, *A. konderi*, *A. mattogrossensis*, *A. peryassui,* and *A. triannulatus* were found in low densities in all sampling methods, with significant differences in HBR rates compared with *A. darlingi*, supporting that these species are not epidemiologically important in malaria transmission in the studied area. However, some studies have reported *A. triannulatus* naturally infected with *Plasmodium* species in Venezuela and Colombia (Moreno et al., 2009; Rosero et al., 2013), and in some states of the Brazilian Amazon (Galardo et al., 2007; Moreno et al., 2013). Despite the wild and zoophilic behavior of these species, *A. triannulatus* can play a role as secondary vector when in high densities, behaving as an opportunistic species, depending on host availability and abundance (Galardo et al., 2007; Rosero et al., 2013). These variations in behavior may be associated with the existence of a cryptic species complex, consisting of at least three species (*Anopheles triannulatus* s.s. Neiva and Pinto, 1922; *Anopheles halophylus* Silva-do-Nascimento and Lourenço-de-Oliveira, 2002; and *Anopheles triannulatus* C Silva-do-Nascimento et al., 2006).

Although *A. nuneztovari* s.l. was found indoors, this species was abundant in environments with animals, where it displayed a zoophilic behavior throughout the sample period, confirming its feeding preference in the study area. Members of the Nuneztovari complex are widely distributed from eastern Panama to northern South America and throughout the Amazon basin, in Bolivia, Brazil, Colombia, Ecuador, Guyana, Peru, and Venezuela (Sinka et al., 2010). This complex comprises four species: *Anopheles nuneztovari* s.s. Gabaldón, 1940; *Anopheles goeldii* Rozeboom and Gabaldón, 1941; *Anopheles dunhami* Causey, 1945; and *Anopheles nuneztovari* cytotype A (Sant'Ana et al., 2015; Santos et al., 2019), with variations in behavior, vector capacity, and involvement in malaria transmission. In the Brazilian Amazon, *A. nuneztovari* s.l. may be a local vector, displaying mainly exophilic and zoophilic behaviors (Galardo et al., 2007; Barbosa et al., 2016). However, in Colombia and Venezuela, *A. nuneztovari* s.s. is endophilic, exophilic and anthropophilic (Moreno et al., 2007; Gutiérrez et al., 2009; Naranjo-Díaz et al., 2013; Naranjo-Díaz et al., 2016).

Despite a higher abundance of zoophilic individuals collected in the months of February, May and August 2017, *A. darlingi* was collected displaying both anthropophilic and zoophilic behaviors in nearly equal numbers during the entire sampling period, but with the index of anthropophily slightly higher. Regarding biting activity, although *A. darlingi* was active in almost throughout night, a bimodal pattern was observed early in the evening, between 18 and 19 hours and between 21and 22 hours.

Anopheles darlingi does not have well-defined standards of biting activity (Gil et al., 2015; Lainhart et al., 2015; Moreno et al., 2015; Tadei et al., 2017; Saavedra et al., 2019), likely due to environmental variables and very high levels of intra-population genetic variation. *Anopheles albitarsis* s.l. was more active early in the evening, with a lower peak at 02 hours. This unimodal pattern was also reported in other location from Amapá (Voorham, 2002) and in the state of Roraima (Póvoa et al., 2006), Brazil, and in western Venezuela (Rubio-Palis and Curtis, 1992).

During four-hour collections, parity rates of *A. darlingi* and *A. albitarsis* s.l. were high, with occurrence of parous females at all time intervals, indicating a greater risk of malaria transmission. The increase in the number of malaria cases coincided with the increase in *A. darlingi* density, as well as with in parity. These results, combined with a positive test for *P. vivax,* clearly indicate the involvement of this species in the malaria transmission in the district of Ilha de Santana, and confirm its role as the main malaria vector in the Amazon region. Our findings about the behavior of *A. darlingi* in the state of Amapá is in agreement with the reported by Deane et al. (1948), Lourenço-de-Oliveira et al.

(1989), Klein and Lima (1990), Osorio Quintero et al. (1996), Tadei et al. (1998), Moutinho et al. (2011) and Barbosa et al. (2016).

Anopheles darlingi was positive with *P. vivax* in the first time interval (18 – 19h), confirming the time of greatest risk of malaria infection by residents. Despite the single positive pool, the infection rate was similar to those found in Cahuide and Santa Emilia, in Peru (Prussing et al., 2018). In the Americas, the highest prevalence of malaria is caused by *P. vivax*, representing 75% of cases of infections, a milder and rarely lethal form (WHO, 2019). However, this type of malaria is responsible for most cases of relapse, as it develops latent forms in the liver cells (hypnozoites), and can remain inactive for years (White et al., 2014).

In this study, although the rate of infection with *Plasmodium* species was low, the district of Ilha de Santana is an area susceptible to malaria outbreaks, because consecutive epidemic cycles can occur even with low parasitemia (Klein et al., 1991; Alves et al., 2005). The main species responsible for the transmission of malaria were identified in the study area in high densities compared to other species. This is an important public health issue, since the migratory flow of people to the district of Ilha de Santana from other malarial areas of the Amazon may promote the local spread of the malaria parasite, triggering the disease (Barbosa et al., 2014).

Anopheles darlingi is the main vector of malaria in the Brazilian Amazon and one of the most anthropophilic anophelines, and its behavior is very heterogeneous. The district of Ilha de Santana has favorable characteristics for the development of this species, with areas of preserved vegetation (forest area, savanna, mangrove) and streams. In addition, human activity has increased in this district, as a result of deforestation for the development of agricultural activities and the construction of fish tanks, as well as intense migratory flow. These factors can favor the increase of the density of *A. darlingi* and contribute to the continuous transmission of malaria.

When analyzing the correlation between density and parity of *A. darlingi*, *A. albitarsis* s.l., *A. braziliensis*, and *A. nuneztovari* s.l., a strong correlation was observed, indicating that the higher the abundance is associated with the higher the number of parous females. In the months when the density of these species increases, *Plasmodium* circulation increases, as well as the chances of outbreaks.

In Venezuela, in riverine villages located in Bolívar, of 2,707 mosquitoes analyzed by ELISA, only two pools of *A. darlingi* were positive out of a total of 1,118 specimens analyzed (Rubio-Palis et al., 2013). In a study carried out in Colombia, the sporozoite rate obtained was 0.13%, lower than our findings, and only one positive individual of *A. darlingi* was found, also for *P. vivax* (Jiménez et al., 2014). Overall, infection rates in anophelines are very low, varying between 0.1 and 3.7% (Tucker Lima et al., 2017), supporting our findings.

In the present study, some factors may have influenced the low density of anophelines throughout the collections, such as the habit of some residents to light fires in front of homes to repel mosquitoes and the use of pesticides in plantations, a common practice in the rural area of Ilha de Santana. Indoor residual spraying in September/2017 during three consecutive days and in March and May/2018 by the Health and Surveillance Coordination, likely contributed to the low abundance of anophelines species in the studied area. Another factor that might have influenced this reduction was the intense fires, a very common practice between September and November, which are the months warmest and driest of the year in region.

Conclusion

Anopheles darlingi was the most abundant species in four-hour collections, the most frequent anopheline indoors, the most anthropophilic, and the only species positive for *P. vivax*, confirming its epidemiological importance and its involvement in the transmission of malaria in the district of Ilha de Santana. *Anopheles darlingi* also had the highest hourly biting activity and HBR in the first hours with a high parity rate, confirming the early evening as period with the highest activity of this species and coinciding with time when residents are most exposed. *Anopheles albitarsis* s.l. was also the second species more anthropophilic, consequently it may play an important role in the transmission of malaria in this district, harmoniously coexisting with *A. darlingi*. The behavior of *A. braziliensis* was a similar to that of *A. albitarsis* s.l., being found predominantly outdoors with some anthropophilic tendencies. An increase in density was observed for this species, with peaks that preceded a rise in malaria cases. Therefore, the role of *A. braziliensis* as malaria vector in the study area needs to be investigated. Although *A. nuneztovari* s.l. had highest density in this study, it was collected predominantly in outdoor environment and displayed a zoophilic behavior, with peaks after the increase in the number of malaria cases. Taken together, these findings suggest that this species is not involved in malaria transmission in the district of Ilha de Santana.

Finally, although we recognized the importance of current control methods applied in the district of Ilha de Santana, we recommend complementary control strategies tailored to local conditions, given the inherent characteristics of *A. darlingi*, its high adaptive capacity in anthropic environments as well as its behavioral plasticity (predominantly outdoors) and heterogeneity, in order to meet the goals established by WHO in the control and eradication of malaria.

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Conflicts of interest

The authors declare no conflicts of interest.

Compliance with ethical standards

This study was approved by the Research Ethics Committee of the Federal University of Amapá, registered under #78912617.9.0000.0003. The collection and transport of the target specimens was authorized by Brazilian Environmental Institute (IBAMA) through the Biodiversity Information and Authorization System (SISBIO) under #52442-1.

Author contribution statement

VM and LMCB Conceptualization and Designed the experiment. LMCB Collected and identified the specimens. LMCB Generated the data. LMCB Processed the statistical analyzes. LMCB and VMS Wrote the manuscript. VMS and LMCB: Revised and edited the manuscript. All authors reviewed and approved the manuscript.

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Supplementary material

The following online material is available for this article:

Table 1S - Details of the methodology during the study, according to the collection categories, district of Ilha de Santana, municipality of Santana, state of Amapá.