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Description of malaria vectors (Diptera: Culicidae) in two agricultural settlements in the Western Brazilian Amazon

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ABSTRACT

The majority of malaria cases in South America occur in rural areas of the Amazon region. Although these areas have a significant impact on malaria cases, few entomological studies have been carried out there. This study aimed to describe entomological parameters in settlements in Rondonia State, Brazil. Collections of anopheles were carried out using the Protected Human Attraction Technique (PHAT). The risk and the potential for malaria transmission were assessed using the human biting rate (HBR), the sporozoite rate (SR) and the entomological inoculation rate (EIR). The results confirmed that *Nyssorhynchus darlingi* is the predominant species in the two studied locations. Although settlement in the two study sites has occurred at different times, the species richness found was low, showing that environmental changes caused by anthropological actions have probably favor the adaptation of *Ny. darlingi* species. From the total of 615 anopheline mosquitoes assessed, seven (1.1%) were positive for *Plasmodium* sp. infections. The EIR revealed that *Ny. darlingi* contributes to malaria transmission in both locations, as it was responsible for 0.05 infectious bites in humans at night in the old settlement and 0.02 in the recent occupation. In the two study sites, the biting occurred more frequently at dusk. *Nyssorhynchus darlingi* was prevalent in areas of recent colonization but, even when present in a low density, this species could maintain the transmission of malaria in the older settlement. The entomological information obtained in this study is important and may aid the selection of vector control actions in these locations.

KEYWORDS: *Anopheles*. Disease transmission. Rural communities. Brazil.

INTRODUCTION

Despite the global efforts to reduce malaria, in the Americas, the incidence of malaria has been increasing since 2015, mainly due to the increment of cases in Amazon Rainforest areas that belong to Venezuela, Colombia and Brazil[1]. In 2018, Brazil accounted for 23% of malaria cases in the Americas, which were mostly caused by *Plasmodium vivax* (79.5%) and *Plasmodium falciparum* (20.5%)[2]. The majority of these cases occur in rural areas of the Brazilian Amazon, such as riverine communities, indigenous areas, mines and rural settlements[3,4].

The indigenous areas, mines and rural settlements are described by the Ministry of Health of Brazil as special areas for the control of malaria. This classification is due to the difficulty of access to these areas, but also because of the precariousness nature of the housing found in these areas, the alterations in the movement of human populations in which families settle and leave, aside from the existence of illegal activities, the differences in malaria control strategies and even because of cultural habits. These special areas in the Brazilian Amazon increase the percentage of epidemics in different States and municipalities, since they are composed of non-immune people, who live in precarious housing and working conditions. In addition, some of these areas suffer from poor access to the health system together with the lack of diagnosis, further aggravating the problem in these locations[3].

In 2019, 25% of malaria cases in the Brazilian Amazon were concentrated in indigenous areas, followed by rural settlements (6%) and mining areas (5%). Fluctuations in the number of malaria cases in these areas are common due several factors, but mainly due the difficulty of maintaining uninterrupted diagnosis and treatment. Up to 2009, the majority of malaria cases from special areas in the Amazon region were registered in rural settlements, but this situation was followed by a decrease and then a new increase in 2015[5]. Historically, rural settlement projects in the Brazilian Amazon, created by the National Institute for Colonization and Agrarian Reform (INCRA), were one of the major determinants of malaria epidemics in Brazil[6]. Currently, the Brazilian Amazon has 3,120 registered rural settlements, which are mainly concentrated in the States of Maranhao, Mato Grosso, Para, Tocantins, Rondonia and Acre[7].

The assessment of the emergence of malaria in rural settlements is known as "frontier malaria" and considers, among other factors, the "settlement time" and the "malaria incidence". The first years of occupation are marked by a rapid increase in malaria caused by ongoing anthropogenic changes in the environment, and several years after the initial settlements, a relative stability in malaria transmissions occurs, with lower malaria rates resulting from the reduction in environment changes[8,9]. Moreover, in the first years of the settlements, vector density is high due to environment changes that provide a greater number of habitats to larvae[8,10].

In several regions of the Brazilian Amazon, the main vector of malaria is the species *Nyssorhynchus darlingi*, formerly known as *Anopheles darlingi*[11]. This species is recognized as the most efficient vector of malaria due to its highly anthropophilic behavior, susceptibility to human malaria parasites, large geographic distribution and rapid adaptation to environmental changes[12-14]. Moreover, this vector has been found in high density levels and has been observed as having high contact levels with humans in the case of "frontier malaria"[15]. However, other anopheline species can be involved in malaria transmission as a secondary vector or an occasional malaria vector at local and regional levels[16]. However, these anophelines are not as well adapted as *Ny. darlingi* in anthropogenic conditions[17], even though some species can emerge as the primary vectors in specific scenarios[18,19]. In general, although there is great discussion about the "frontier malaria" hypothesis in the Brazilian Amazon, investigations regarding the patterns of *Anopheles* populations structure in human settlements are scarce and anopheline diversity continues to be underestimated and several species thought to be unknown can actually have a role in "frontier malaria"[20].

Anopheline populations dynamics, hematophagic behavior (anthropophilic level, endophagic versus exophagic and biting activity) and natural anopheline infection rates are important parameters to define efficient and sustainable strategies for malaria vector control[21,22]. According to Santos *et al.*[23], vector control for malaria can be inefficient if not used in accordance with the hematophagic activity of mosquitoes. Moreover, other entomological metrics can be used for estimating the risk and potential of malaria transmission by mosquitoes in endemic areas, such as, the human biting rate (HBR), the sporozoite rate (SR) and the entomological inoculation rate (EIR)[24].

Rondonia State is situated within the Western Brazilian Amazon Basin. In 2018, 7,685 cases of malaria were reported with 3,600 of them (46.8%) occurring in rural areas and 1,727 (22.5%) in rural settlements. The study site of this research, the municipality of Candeias do Jamari, is a risk area (annual parasite index – API > 50)[5], and even so entomological investigations in rural settlements are rare. Thus, the aim of this study was to describe the anopheline species composition, as well as the HBR, SR and EIR of anopheline mosquitoes present in the peridomicile of two agricultural settlement areas in the municipality of Candeias do Jamari, Rondonia State.

MATERIALS AND METHODS

Ethics approval and consent to participate

Ethical approval for this study was obtained from the regional committee (Research Center of Tropical Medicine – CEPEM, Nº 2976746). Mosquito collections were conducted by the authors of this manuscript after training, and following the instructions of the Brazilian Ministry of Health[25]. In addition, the study has a license from the Brazilian government: SISBIO Nº 65725-1 and SisGen Nº A948A47.

Study area

This study was performed in two rural locations in the municipality of Candeias do Jamari (08º48'35" W 63º41'44" S), located in the Northwest region of Rondonia

State, Brazil, approximately 20 km from the State capital, Porto Velho. The climate comprises a rainy season from November to April, and a dry season from May to October.

The municipality has a history of urbanization similar to other regions of the Brazilian Amazon, starting with the implementation of settlement projects by the Instituto Nacional de Colonizacao e Reforma Agraria (INCRA), whose main economic activity is related to agriculture and livestock, resulting in an increase in the fragmentation of the forest and a reduction of native vegetation.

The settlement called Acampamento Fortaleza (AF) (8°39'41.0" S 63°01'58.8" W) is situated at the end of a trail located near the highway marker 21, on State highway BR364 in the municipality of Candeias do Jamari. This settlement is considered recent, with less than five years of occupation at the time that this research has started. As the AF settlement has still not been registered by INCRA, this occupation is classified as a camp. Human dwellings are precarious and close to forest fragments (approximately 50 m) and structures do not adequately protect residents from contact with vectors. The material for the construction of houses is obtained from the forest and the cleaning of the housing area is carried out by burning. There is no electricity supply, and water is obtained from creeks. The livelihood of the local population comes from small plantations of bananas, corn, beans and cassava, from raising small animals (pigs, chickens and ducks) and from extracting wood from the forest.The second location, called Projeto de Assentamento Florestal Jequitiba (PAFJ) (8°41'00.5" S 63°11'08.8" W) is a settlement that was established more than ten years ago, in the municipality of Candeias do Jamari by INCRA, through the decree Nº 1.141/03. It is an anthropized environment with little basic infrastructure (electricity and access road). In general, human dwellings are made of wood or bricks and built about 1 km from the edge of the forest. The settlement is a reference point for farmers and other members of the local population, as well as a community meeting place for political and cultural activities. Local residents primarily depend on livestock farming (pigs and chickens), local commerce and the sale of milk and homemade cheese.

These locations were chosen according to the following criteria: i) occupation time of the area; ii) presence of malaria cases described initially by inhabitants; iii) presence of mosquitoes; iv) vegetation cover according to the occupation time; v) presence of breeding sites for anophelines; vi) human presence and vii) access to the studied locations. The locations are about 27 km apart from each other (Figure 1).

The number of cases reported for the months of sample collection and the API for 2018 and 2019 in each studied location were obtained from the Sistema de Informacao de Vigilancia Epidemiologica (SIVEP) malaria database[5].

Entomological collections

Collections of anophelines were performed 16 times, in two houses of each location (Figure 1). Eight consecutive collections were performed at the beginning of the rainy season (October and November, 2018), and other eight collections at the beginning of the dry season (May and June, 2019), being four collections at the AF location and four collections at the PAFJ location, but alternately at each location. Protected Human Attraction Technique (PHAT) was performed outside the dwellings from 6 pm to 6 am (12 h of collection).

Each capturer worked for six hours and then rested. Collection sites in human settlements were located outdoor, no more than 7 m from the selected house (Figure 1). Mosquitoes were placed in plastic containers previously

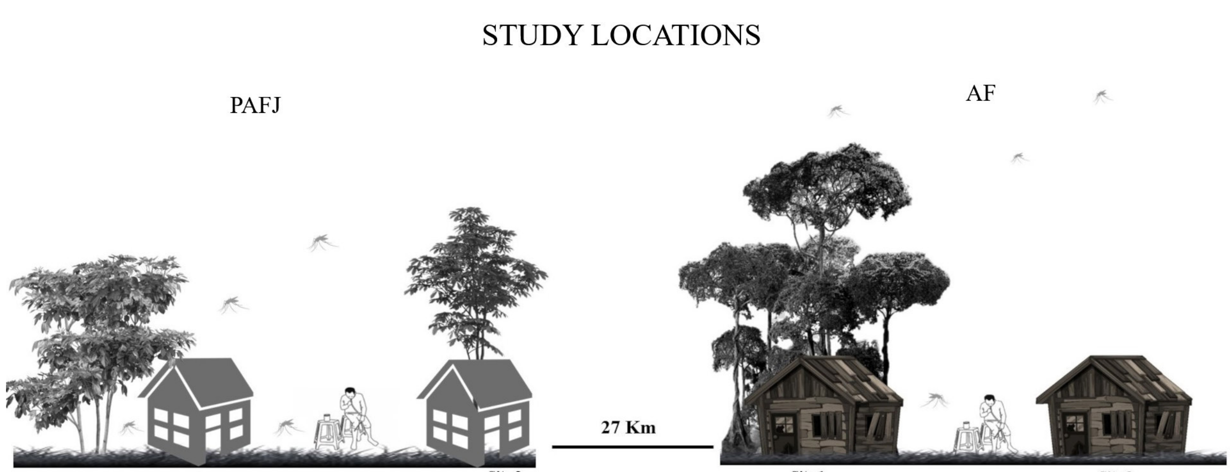

Figure 1 - Schematic representation of outdoor capture sites in typical rural settlements showing the difference between houses from the Projeto de Assentamento Florestal Jequitiba (PAFJ) and Acampamento Fortaleza (AF).

labelled with the date, time and sample location. Climate conditions, such as temperature and relative humidity were recorded for each sampling interval of one hour using a thermohygrometer (Incoterm, Porto Alegre, Rio Grande do Sul, Brazil). Data regarding habits of the human population were registered. At the end of sample collections, specimens were transported alive inside tightly closed isothermal boxes to the Entomology Laboratory located at Fundacao Oswaldo Cruz (Fiocruz - Rondonia, Porto Velho, Brazil).

The following morning, identification of anophelines was performed using the Consoli and Oliveira keys[26]. After the identification, up to 10 mosquitoes from the same species, collected at the same time and location were stored in 1.5 mL tubes with isopropanol. These tubes were kept at room temperature until the *Plasmodium* spp detection by nested PCR was performed.

Natural infection of mosquitoes by *Plasmodium* spp.

Before the molecular analysis, the heads and the initial region of the anophelines thoraces were bisected from the abdomens[27] transferred individually to new tubes. This procedure allows the detection of sporozoites from salivary glands. DNA was immediately extracted from the heads and initial region of the thoraces according to the Laporta *et al.*[18] protocol, with some adaptations. The extracted DNA was quantified using an UV-Vis spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific®, Waltham, MA, USA) and its purity was also checked.

In order to determine the malaria sporozoite infection rate, DNA from the heads and initial region of the thoraces was used as the template for *Plasmodium* sporozoite DNA detection by nested PCR. The primer sequences and nested PCR reactions have been previously described by Win *et al.*[28]. Amplifications was performed in a final volume of 15 µL, containing 1µL of genomic DNA (< 0.1 µg), 0.25 µM of each primer P1UP and P2, 0.2 mM of each dNTPs (Invitrogen®, Thermo Fisher Scientific®, Waltham, MA, USA), and 1 X 5 HotMaster primer buffer 0.1 unit of Taq DNA polymerase (5PRIME HotMaster Taq DNA Polymerase). PCR was performed in a thermocycler (Veriti®, Applied Biosystems, Foster City, USA) under the following conditions: 94 ºC for 2 min for the initial denaturation step, followed by 30 cycles at 94 ºC for 30 s, 60 ºC for 30 s and then 68 ºC for 20 s. An aliquot of 0.5 µL from the first amplification was used as the template DNA for the nested amplification of *P. vivax* (primers P1 and V1) and *P. falciparum* (primers P1 and F1), using the same master mix of reagents and cycling parameters of the first reaction, however reducing the total number of cycles from 30 to 15.

Amplification products (5 µL) were subjected to horizontal electrophoresis on 1.5% agarose gels stained with GelRed (Biotium®, Thermo Fisher Scientific®, Waltham, MA, USA) and the species-specific fragment sizes were 100 bp for *P. vivax* and *P. falciparum*. These fragments were visualized using a digital camera system (ImageQuant® TM LAS 4000, GE healthcare, Papua New Guinea).

Entomological parameters

The potencial of malaria transmission of mosquitoes was determined by estimating the human biting rates (HBR) and the entomological inoculation rates (EIR) for each location. The HBR was scored as the average hourly number of mosquitoes captured per person, per hour. The EIR was calculated by multiplying the HBR and the estimated sporozoite rates (SR). The SR is the proportion of *Plasmodium* spp.-positive mosquitoes detected by PCR.

RESULTS

Mosquito collections and human activity

A total of 615 anophelines were collected in the outdoor area of the two locations, comprising 68 anophelines collected at the PAFJ settlement and 547 anophelines at the AF settlement. *Nyssorhynchus darlingi* was the most abundant species, representing 99.68% of the total number of anophelines. Only two other anophelines species were also detected: *Nyssorhynchus triannulatus* (0.16%) and *Anopheles peryassui* (0.16%) (Table 1).

Patterns of *Ny. darlingi* biting activity by location and temperature/humidity are shown in Figure 2. Overall, a higher concentration of mosquitoes was collected in the first part of the night (6 to 11 pm) at the two locations. After that, the number of captured mosquitoes gradually decreased.

Despite the low density of mosquitoes in the PAFJ, they were captured in all hourly collections and showed a trimodal pattern, with maximum number of bites at 6 pm, 8 pm and 10 pm (Figure 2A). Human activity was observed during the whole night in this location, as bars were open, but the majority of activities were concentrated at the beginning of the night, since it is habit of the population to take a shower after 6 pm, with meetings and outdoor meals.

On the other hand, the hourly biting activity at the AF was higher, though only less than 20 mosquitoes were captured after 2 am (Figure 2B). The highest biting peak at the AF was registered at 7 pm. Human exposure to the vector at this location is higher than at the PAFJ, mainly because of the types of houses found there and of human activity, such as hunting and fishing.

Table 1 - Number of collected anophelines and entomological parameters from the Projeto de Assentamento Florestal Jequitiba (PAFJ) and the Acampamento Fortaleza (AF).

Species	Location				Total	
	PAFJ		**AF**			
	N	%	N	%	N	%
Ny. darlingi (Root, 1926)	68	100	545	99.64	613	99.68
Ny. triannulatus (Neiva and Pinto, 1922)	0	0	1	0.18	1	0.16
An. peryassui (Dyar and Knab, 1908)	0	0	1	0.18	1	0.16
Total	**68**	**100**	**547**	**100**	**615**	**100**
Entomological parameters						
Human biting rate (HBR)	0.71		5.70		3.2	
Sporozoite rate (SR)	7.35% (5/68)		0.37% (2/547)		1.13% (7/615)	
Entomological inoculation rate (EIR)	0.05		0.02		0.036	

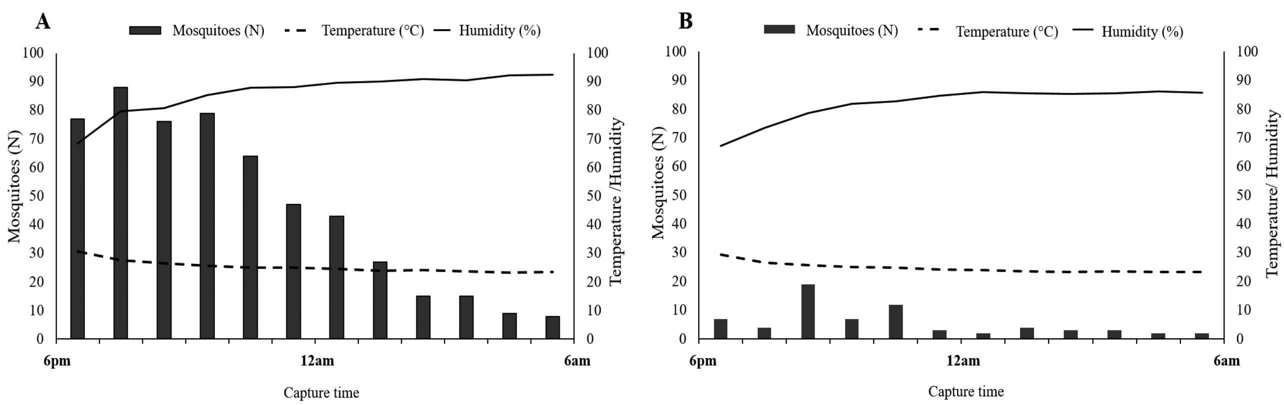

Figure 2 - Hourly hematophagic activity during the 12h collection period of anophelines, temperature and humidity variation: (A) Projeto de Assentamento Florestal Jequitiba (PAFJ) and (B) Acampamento Fortaleza (AF).

As a general observation, families from the AF have a habit of cooling off in the creek at dusk, women wash clothes, dishes and other utensils and children enjoy playing there.

During the 12 hours of sample collection, the temperature did not vary much, though there was a temperature fall after 7 pm (Figures 2A and 2B). The humidity gradually increased (Figure 2) in both locations, but the behavioral pattern of mosquitoes did not follow the pattern of temperature and humidity of both locations.

During the collection months, 16 malaria cases were reported in the PAFJ. The API was almost the same during 2018 and 2019 (API = 1,377.0 in 2018 and API = 1,344.2 in 2019). At the same time, in the AF, 132 malaria cases were reported during the same period of mosquitoes collection in PAFJ and the API there was in 2018, 1,048.6 and 524.3 in 2019.

Human-biting rates (HBRs)

The mean HBRs of all captures showed differences between the two locations. In the PAFJ, the HBR was 0.71 (SD ± 0.67), while in the AF the HBR was higher, and 5.70 bites per person, per hour were registered (SD ± 4.32). A higher HBR registered in the AF means that a person who lives or visits the AF during the anophelines' activity period is almost five times more exposed to mosquito bites than an individual who lives or visits the PAFJ.

Natural infection of mosquitoes and risk of malaria transmission

In order to assess the natural infection rates of anophelines with *Plasmodium* spp. in both locations, all collected mosquitoes were individually analyzed using nested PCR. *Plasmodium* sporozoite rates were 1.14% (7/615), being 0.81% (5/615) for *P. vivax* and 0.16% (1/615) for *P. falciparum* and 0.16% (1/615) for mixed infections (*P. vivax* and *P. falciparum*).

The greatest number of infected mosquitoes was from the PAFJ, with an SR of 7.35% (5/68), while only two mosquitoes were infected in the AF, which registered an SR of 0.37% (2/547). Both locations registered infections for *P. vivax* and *P. falciparum*; and the only mixed infection was registered in the PAFJ.

All infected mosquitoes belonged to the *Ny. darlingi* species. Mosquitoes from the PAFJ that tested positive were collected at 7 pm, 8 pm, 10 pm and 4 am, and mosquitoes that tested positive were collected from the AF at 10 pm and 12 am.

To estimate the risk of contracting malaria during the study, the EIRs were calculated for each location. The number of infective bites a person might receive at the PAFJ during our study was 0.05, while in the AF the number was lower (EIR = 0.02).

DISCUSSION

Human activity without adequate planning and infrastructure in forest areas of the Brazilian Amazon can increase contact between man and vector and, consequently, intensify malaria transmission in different locations[10,29]. Furthermore, changes in land use could completely alter the richness and composition of anopheline species, changing the dynamics of local malaria infections[30,31]. The malaria control and prevention strategies currently developed by the Ministry of Health are not effective in Brazilian Amazon areas, such as rural settlements, that suffer increasing urbanization. In addition, entomological and epidemiological knowledge regarding these locations is limited.

In the current study, *Ny. darlingi* was the dominant species in settlement areas, in the municipality of Candeias do Jamari, Rondonia State, Western Brazilian Amazon, Brazil. This observation is in accordance with the findings of other studies carried out in rural settlements in the Brazilian Amazon[10,12,32]. This mosquito species is abundant and its contact rate with humans is high in studies on the first stage of "frontier malaria"[15]. Furthermore, *Ny. darlingi* is a well-adapted species to anthropogenic changes in the environment of forests when compared to other anopheline species[13,15].

The more recent settlement investigated in the present study (AF) showed the highest density of *Ny. darlingi* in the location where other anopheline species were collected. Studies in rural settlements in Acre State, also belonging to the Western Brazilian Amazon basin, have reported a higher density of *Ny. darlingi* in sample collections carried out near a recent settlement, suggesting that a higher level of colonization decreases the presence of the vector[10]. If we consider the "frontier malaria" concept, our data regarding anophelines' density confirm what occurs in the early stages of frontier settlements. The first phase of "frontier malaria" involves changes in the natural forest landscape, which alter the abiotic characteristics and the ecology of larval habitats, leading to an increase in the abundance of the local vector[8].

The other two species of mosquitoes collected at the AF (*Ny. triannulatus* and *An. peryassui*) have already been described as vectors that inhabit the edge of forests and they usually stay away from domestic environments, but they can be associated with human activity such as deforestation in the Amazon region[33]. The houses in the AF settlement are closer to the forest, and deforestation is common because of the human colonization process in the area.

Nyssorhynchus darlingi was the only species of mosquito collected at the PAFJ and it presented a low density. This settlement has existed for more than 10 years, houses are located further from the forest and the exploration of land is no longer as intense. Some studies have demonstrated that environmental changes may affect mosquitoes populations in term of abundance and species composition[34,35].

Only a few field studies focused on the structure of anopheline populations in areas with different degrees of human activity in rural settlements in the Amazon[13,36]. However, it is a fact that *Ny. darlingi* is the main vector of malaria in both studied settlements, regardless of the length of time of land use. The main vector in different areas of Rondonia State, as well as in most of the Brazilian Amazon is *Ny. darlingi*[14,21,34]. This species is found in great abundance in the region and it is recognized as a highly anthropophilic vector[12,14]. The biting activity of *Ny. darlingi* is generally bimodal, occurring at the beginning of dusk and at dawn[37,38]. However, patterns of biting activity can be influenced by location, vector density, seasonality, presence of hosts, types of houses and the distance between dwellings and the forest[16,21,34,39]. The biting activity in our areas of study was constant during the whole night, though more activity was registered at dusk. Similar results were reported in other *Ny. darlingi* studies carried out in other areas of the Amazon basin such as some endemic areas in Rondonia State, Brazil[37] and Iquitos, Peru[40]. The habits of settlers, housing conditions and distance from the edge of forests observed at both locations may explain this pattern of anophelines activity. The settlement that showed the highest concentration of *Ny. darlingi* during the first part of the night was the recently settled area, where settlers were more exposed to the vector due to poor local housing conditions and the type of human activity found there.

Our data regarding HBR have also confirmed the effect of the "frontier malaria" concept in areas that are undergoing transformation. The human-biting rate (HBR) was higher in the AF (5.70 bites/person/hour) than in the PAFJ (0.71 bites/person/hour). Therefore, a person who lives in or visits the AF during the period of activity of *Ny. darlingi* is almost five times more exposed to mosquito bites than an individual in the PAFJ. However, for the number of infective bites represented by the EIR, our data showed that the settlers and visitors of the PAFJ are more exposed to infective bites (0.05 infective bites/person/hour) than settlers and visitors of the AF (0.02 infection bites/person/hour). The low EIR recorded at the AF was possibly determined by the low SR (0.36%) and the high vector density of the location. The PAFJ showed the highest SR (7.35%) of the two locations, although it did present a lower HBR. However, it is known that *Ny. darlingi* can maintain transmission even at very low densities[34].

The API in the PAFJ was higher (> 50) in 2018 and 2019, although, since 2012, the API in PAFJ has decreased to 295.08 in 2017. In the last stage of the "frontier malaria" concept, the infection declines after 10 years of colonization and development of the settlement, and reaches low and stable levels of transmission because of the reduction of environmental changes. However, the risk of infection in these older settlements could also be determined by behavioral factors of the population[8].

The unexpectedly low EIR at the AF probably does not truly represent the risk of malaria in this recent settlement. The API in this settlement has also been higher (> 50) in 2018 and 2019, and was higher than of the PAFJ, an expected profile for a recent settlement. In addition, the density of *Ny. darlingi* and HBR reported there was higher than that of the older settlement.

A parameter that was not assessed here, was the parity of female mosquitoes collected and this is a factor that might have interfered with the EIR. If the majority of female mosquitoes from AF were nulliparous females, they were not infected because they were getting their first blood meal[34], which may explain the low EIR in the recent settlement. Regarding the risk of contracting malaria at this location, this is most likely in the middle of the night, when the majority of infected mosquitoes were captured and when people are inside their houses, though the housing conditions do not offer much protection since they are built with holes between the wooden slats and often have unscreened windows.

Our research confirmed that *Ny. darlingi*, the main vector of malaria in Rondonia State, is the predominant anopheline species in both studied locations. The number of species recorded in the study was low, despite the two locations having different levels of human settlement. This is probably due to the number of sample collections, the sites of the sample collections (outdoors), aside from the anthropophilic behavior of *Ny. darlingi* and its adaptation to the surroundings.

The risk of contracting malaria was recorded in both, the area of recent occupation and the old settlement. Despite the AF location having registered a low SR and EIR, this area of recent occupation presented the highest HBR, and the conditions of the houses and human behavior are believed to have contributed to the greater contact between man and vector. In addition, data for EIR and malaria cases from the PAFJ confirm that *Ny. darlingi,* even at low densities, could maintain malaria transmission.

Therefore, although our study has some limitations, these entomological data may be used for planning and implementing vector control measures that are aligned with the malaria transmission dynamics in each of the settlements.

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AUTHORS' CONTRIBUTIONS

AOA conducted the sample collection, identified the insects, prepared the anopheles for molecular analysis and wrote the manuscript; NACS, RBC, ISA, ASB and FNM participated in the collection of the material; MMSR helped to designed the study; DBP and JFM performed the supervision, designed the study and helped to write the manuscript; MSA performed the formal description analysis, project administration, supervision and writing of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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