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RESEARCH

Anopheles (*Kerteszia*) *cruzii* infected by *Plasmodium* in the Atlantic Forest indicates that the malaria transmission cycle is maintained even after howler monkeys' population decline

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Abstract

The Atlantic Forests outside of the Amazon region in Brazil are low-frequency malaria hotspots. The disease behaves as a zoonosis maintained by nonhuman primates (NHPs), especially howler monkeys. Between 2016 and 2018, Brazil witnessed the largest yellow fever outbreak since 1980, resulting in massive declines in these NHP populations. However, reports of malaria cases continued in transmission areas. This scenario motivated this survey to determine the frequency of infection of the anophelines by *Plasmodium* species. Mosquitoes were captured using Shannon traps and CDC light traps and identified as to species based on morphological characters. The screening for malaria parasites targeted only *Anopheles* species belonging to the subgenus *Kerteszia*, the proven primary malaria vector. A TaqMan qPCR assay using ribosomal primers (18S rRNA gene) was performed in a Step One Plus Real-time PCR to detect *Plasmodium* species. Seven hundred sixty field-caught anophelines divided into 76 pools were examined. Out of 76 tested pools, seven (9.21%) were positive. Three pools were *Plasmodium malariae*-positive, and four were *Plasmodium vivax*-positive. The anopheline infection was expressed as the maximum infection rate (MIR), disclosing a value of 0.92%, indicative of a steady state. Such stability after the yellow fever outbreak suggests that other species of NHPs could support transmission.

Keywords *Anopheles* · Bromelia · *Plasmodium* · Mosquito vectors

Introduction

Malaria is a public health challenge in Brazil, especially in the Amazon region, where more than 99% of cases currently occur (Buery et al. 2021). The remaining 1% of the occurrences outside the Brazilian Amazon region may be imported or autochthonous. Autochthonous cases of the disease in the extra-Amazon region are found specifically in areas surrounded by the Atlantic Forest, located in the southeastern and southern parts of the country, zones previously known to be free of malaria since the 1960s. The popular name for the disease in such areas is Bromeliad malaria. It was named after bromeliads (Bromeliaceae), a native plant of the Atlantic Forest that serves as the development local of the vector. Immature forms of the transmitting mosquito *Anopheles (Kerteszia) cruzii* develop in the water that accumulates in the leaf axials of these plants (Consoli and Lourenço-de-Oliveira 1994).

The sporadic cases of Bromeliad malaria are found more frequently in rural communities, where there are

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dense fragments of preserved forest. The infected individuals usually report recent excursions into the woods for recreational (e.g., ecotourism) or professional activities (e.g., agriculture), and it is precisely the interior of the forest that *Anopheles (K.) cruzii*, the incriminated vector, inhabits (Ferreira et al. 2021). The clinical presentation is mainly one of few or no symptoms, with low parasitemia, representing an unexpected clinical pattern for the disease (Cerutti et al. 2007). *Plasmodium vivax* is the main parasite responsible for human infections, but *Plasmodium malariae* and *Plasmodium falciparum* also occur in a lower proportion (Laporta et al. 2015; Buery et al. 2017).

Bromeliad-malaria ecology also appears to involve the participation of nonhuman primates (NHPs), especially those belonging to the genus *Alouatta* (Duarte et al. 2008; Abreu et al. 2019; Monteiro et al. 2020). Epidemiological studies conducted in Atlantic Forest areas have found natural *Plasmodium* infection in NHPs, with *Plasmodium brasilianum* and *Plasmodium simium* being the species implicated as etiologic agents. Interestingly, *Plasmodium brasilianum* and *Plasmodium simium* are morphologically and genetically indistinguishable from the human parasites *Plasmodium malariae* and *Plasmodium vivax*, respectively. These last two can infect humans when they enter forested areas (Goldman et al. 1993; Tazi and Ayala 2011). Furthermore, entomological studies in Bromeliad-malaria areas have shown that *P. vivax/P. simium* and *P. malariae/P. brasilianum* frequently infect *Anopheles (K.) cruzii* (Buery et al. 2018; Demari-Silva et al. 2020. 2020). This evidence suggests that once infected, *Anopheles (K.) cruzii* can transmit *Plasmodium* species from humans to NHPs or vice versa. This hypothesized transmission dynamic is possible due to acrodendrophilic behavior and the vertical dispersion of such an incriminated vector (Deane et al. 1984; Ferreira et al. 2021). In addition, the vertical distribution associated with the close contact between NHPs and humans is possibly sufficient to enable repeated spillover and spillback events (Fig. 1) (Deane et al. 1966).

Studies carried out over the last few years in Atlantic Forest areas indicated that the predominant simian host for *P. brasilianum* and *P. simium* is *Alouatta guariba clamitans* (howler monkey), which is indigenous to South America (Deane, 1992; Duarte et al. 2008; Abreu et al. 2019; Monteiro et al. 2020).

In addition to their evident susceptibility to malaria infection, howler monkeys are also considered highly susceptible to yellow fever virus (YFV), the etiologic agent of yellow fever (YF). Yellow fever is an acute viral disease transmitted by sylvatic mosquitoes from the *Hemagogus* and *Sabethes* genera. Unlike malaria, YFV infections cause severe and often fatal illnesses for NHPs belonging to the *Alouatta* genus (Leal et al. 2016).

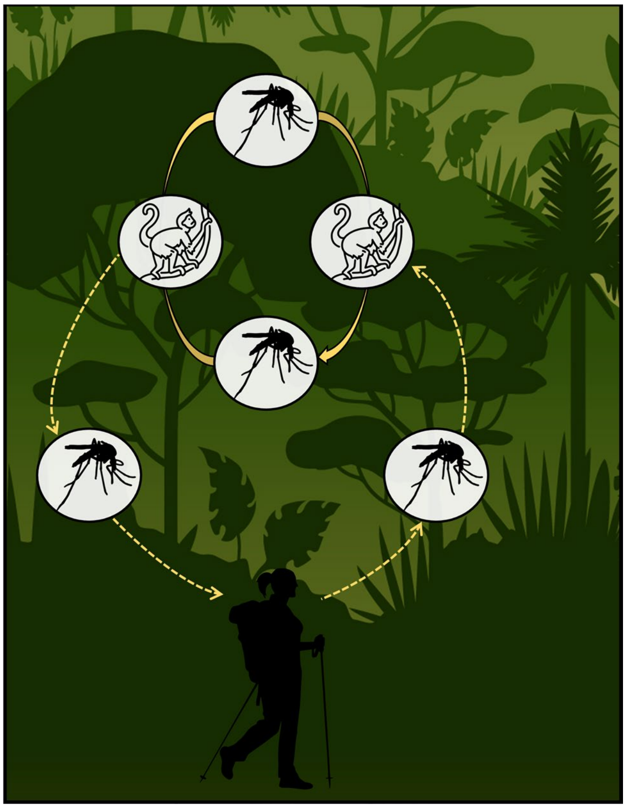

Fig. 1 The hypothetical cycle of malaria in Brazilian Atlantic Forest areas

Between 2016 and 2018, Brazil witnessed the largest YFV outbreak since 1980, with at least 2251 human YFV cases and 15,000 epizootic events (Silva et al. 2020). This outbreak caused the deaths of many howler monkeys, resulting in massive and rapid declines in these NHP populations in the Brazilian Atlantic Forest. In this context, assuming the hypothesis of howler monkeys participating in the Bromeliad-malaria transmission cycle, their population reduction would exhaust the source of infection — especially for anophelines of the subgenus *Kerteszia* — and consequently impact the malaria transmission cycle. Therefore, anophelines have become relevant biological indicators of malaria in the Atlantic Forest. Hence, analyzing anopheline infectivity while the population of the potential NHP reservoir is reduced will help to clarify the role and influence of alternative hosts/reservoirs in the disease cycle. Additionally, it will shed some light on epidemiological uncertainties regarding the transmission dynamics of bromeliad malaria.

Materials and methods

Study area

The capture of adult *Anopheles* mosquitoes occurred in Valsugana Velha (Fig. 2), a rural area of Santa Teresa municipality, located in the central mountainous region of Espírito Santo, southeastern Brazil (19°58′05.2″S, 40°34′40.8″W). The choice of this area relates to the previous detection of local autochthonous human malaria cases. Moreover, there were records of natural *Plasmodium* infection in NHPs and anopheline specimens on the same site (Cerutti et al. 2007; Rezende et al. 2009; Buery et al. 2018). Valsugana Velha is a receptive area for malaria, as it has an ecosystem that allows the cohabitation of vector mosquitoes and reservoir hosts.

Santa Teresa has a total area of 683.032 km^2, of which approximately 219.253 km^2 corresponds to the Atlantic Forest biome. Atlantic Forest is a significant rainforest in Brazil, composed of a great diversity of biotopes, which provides adequate conditions for the development of mosquitoes (Secretaria de Meio Ambiente e Recursos Hídricos 2018). According to the Köppen classification, Santa Teresa has a warm temperate climate, with an average temperature of 22 °C during summer and 18 °C during winter. The average annual rainfall for this area is 1491.6 mm, with two climatic seasons: the drier period from June to October and the rainy period from November to May (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural 2021).

Sampling, identification, and storage of Anopheles females

Anopheles female mosquitoes were sampled monthly (once a month) from May 2019 to April 2020 using two types of light traps: (1) Shannon light trap, with the help of an oral suction tube: its setting inside the forest allows the capture of anophelines for 4 h (06:00–10:00 p.m.). (2) CDC automatic (Center for Disease Control) trap with CO$_2$ baits (200 g of dry ice): the team installed two CDC traps inside

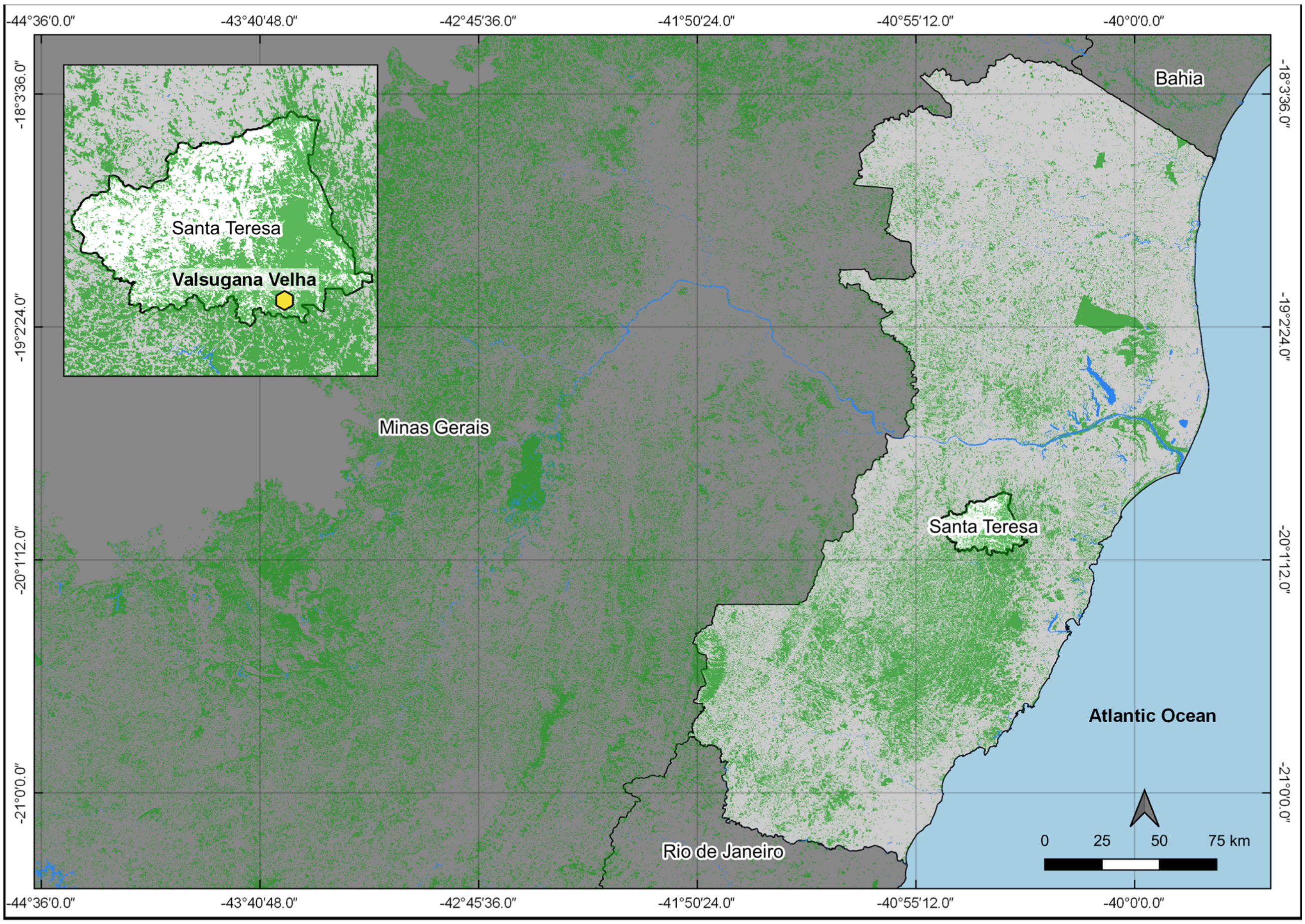

Fig. 2 Map showing the study site in Valsugana Velha, rural area of Santa Teresa municipality, Espírito Santo state, Brazil

the forest, the first at a height of 1.5 m above the ground and the second at a height of 10 m in the canopy. The CDC traps remained turned on for 12 h from the night (06:00 p.m.) until the morning (06:00 a.m.).

The procedures involving the collected mosquitoes included euthanasia by flash freezing, storage in polyethylene cages, and transportation to the Entomology and Malacology Centre of Espírito Santo (Núcleo de Entomologia e Malacologia do Espírito Santo — NEMES/ES). In NEMES/ES, the investigators identified the specimens at the species level based on their morphological characters using the identification keys of Consoli and Lourenço-de-Oliveira (1994). After the identification process, the mosquitoes were pooled into groups of up to 10 specimens based on their collection date and trap type. Each pool was placed in an Eppendorf tube and stored at – 20 °C until DNA extraction for the detection of malaria parasites.

Molecular procedures

Due to the large number of mosquitoes collected during the study, screening for malaria parasites targeted only *Anopheles* species belonging to the subgenus *Kerteszia*, considering their proven implication as the main malaria vectors in the Atlantic Forest areas.

DNA extraction and detection of natural *Plasmodium* infection

DNA extraction from each pool of mosquitoes (maximum of 10 specimens/pool) was performed using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. To detect *P. falciparum, P. malariae/P. brasilianum,* and *P. vivax/P. simium*, a TaqMan qPCR assay using ribosomal primers (18S rRNA gene) was performed in a Step One Plus Real-time PCR System (Applied Biosystems), as previously described by Bickersmith et al. (2015). The same protocol (Bickersmith et al. 2015) was applied to detect *P. malariae/P.brasilianum*, but the primers and probes used followed the description of Rougemont et al. (2004).

Each PCR used a total of 20 µl, containing 2 µl of genomic DNA, 7.5 µl of TaqMan Universal Master Mix II with UNG, 0.15 µl of the probe (10 µM), 0.45 µl of each primer, forward and reverse (10 µM), and 4.45 µl of ultrapure water. The positive control was DNA from *P. falciparum, P. malariae/P. brasilianum,* and *P. vivax/P. simium.* An aliquot of ultrapure water was the negative control. The thermocycler conditions were 50 °C for 2 min, followed by 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 60 °C for 1 min.

Table 1 Minimum infection rate (MIR) for *Plasmodium* species in *Anopheles (K.) cruzii* collected in Valsugana Velha, Espírito Santo, Brazil

Parasite species	Positive pools	Minimum infection rate (MIR)
Plasmodium falciparum	0	0
Plasmodium malariae	3	0.39
Plasmodium vivax	4	0.53
Total	**7**	**0.92**

Minimum infection rate

To evaluate the infection rate of the collected *Anopheles (K.) cruzii*, the minimum infection rate (MIR) was calculated using the following formula: MIR $= (x/n) \times 100$, where x is the number of positive pools and n is the total number of mosquitoes tested (Paiva et al. 2006). The minimum infection rate was estimated, assuming that each positive pool contained at least one infected specimen.

Ethics statement

No specific permissions were needed. The study did not involve endangered or protected species.

Results

Composition and distribution of *Anopheles (Kerteszia)* mosquitoes

The study collected 1259 *Anopheles (K.) cruzii* specimens from May 2019 to April 2020. The CDC light trap located in the canopy (approximately 10 m) collected a total of 1236 (98.17%) *Anopheles (K.) cruzii*. In turn, the Shannon light trap and CDC light trap located on the ground level collected 13 (1.03%) and 10 (0.79%) specimens of *Anopheles (K.) cruzii*, respectively.

Plasmodium species detection by qPCR

The screening involved testing mosquitoes for *P. falciparum, P. malariae/P. brasilianum*, and *P. vivax/P. simium* because in Santa Teresa, detection of these species occurred in different hosts, including humans, howler monkeys, and anophelines. A total of 760 field-caught *Anopheles (Kerteszia) cruzii* divided into 76 pools were examined. Out of 76 tested pools, seven (9.21%) were positive. Three pools of *Anopheles (K.) cruzii* were *P. malaria*-positive, and four pools were *P. vivax*-positive. *Plasmodium falciparum* was not detected in this study. The rate of *Plasmodium* species infection of *Anopheles (K.) cruzii* was expressed as MIR (Table 1). All seven *Plasmodium*-positive pools came from the CDC light trap located in the canopy. Figure 3 shows the seasonal distribution of *Plasmodium*-positive sample pools.

Minimum infection rate of *Plasmodium* species in *Anopheles (K.) cruzii*

The minimum infection rate for *Anopheles (K.) cruzii* was 0.92. In analyzing the infections individually by *Plasmodium malariae* and *Plasmodium vivax*, the MIR was 0.39 and 0.53, respectively. It was not possible to calculate the MIR of the *Anopheles (K.) cruzii* for *Plasmodium falciparum*, since no positive pools for this parasite species were found.

Discussion

Several reports have shown that *Anopheles (K.) cruzii*, incriminated as the principal vector for human and simian malaria in the Brazilian Atlantic Forest, has an acrodendrophilic habit (Deane et al. 1971; Rezende et al. 2009; Buery et al. 2018). Consistent with the literature, the present study confirmed this species' preference for tree canopies. Furthermore, the natural infection of *Anopheles (K.) cruzii* by *P. vivax/P. simium* and *P. malariae/P.brasilianum* found in this study corroborate the primary importance of this vector in the transmission of malaria in Atlantic Forest areas. Altogether, these data support the hypothesis that the specific breeding conditions of *Anopheles (K.) cruzii* favor their predominance in the canopy, where they find NHPs to be their principal blood source. The distribution of this vector in the sylvatic environment fits the hypothesized malaria transmission cycle in the Atlantic Forest, which seems to occur mainly among NHPs in the tree canopy, with humans being accidental hosts.

The nonspecificity of molecular techniques in identifying parasites of nonhuman origin is a limitation of the present study. However, several studies have suggested that *P. vivax* and *P. malariae* are the same species as *P. simium* and *P. brasilianum*, respectively, based on their genetic and morphological similarities (Goldman et al. 1993; Escalante et al. 1998; Leclerc et al. 2004; Lim et al. 2005; Tazi and Ayala 2011). Such evidence raises the possibility of the routine transfer of the parasites from one host species to another, making source identification less relevant. Moreover, the observed anopheline preference for the canopy reinforces the conclusion of a simian origin of the identified parasites.

In addition, another limitation of this study was that *Anopheles Kerteszia* identification was made only by morphological criteria. To minimize the possibility of misidentification, we improved the accuracy through evaluation by experienced entomologists. However, molecular methods in conjunction with morphological identification are the gold standard and should be recommended.

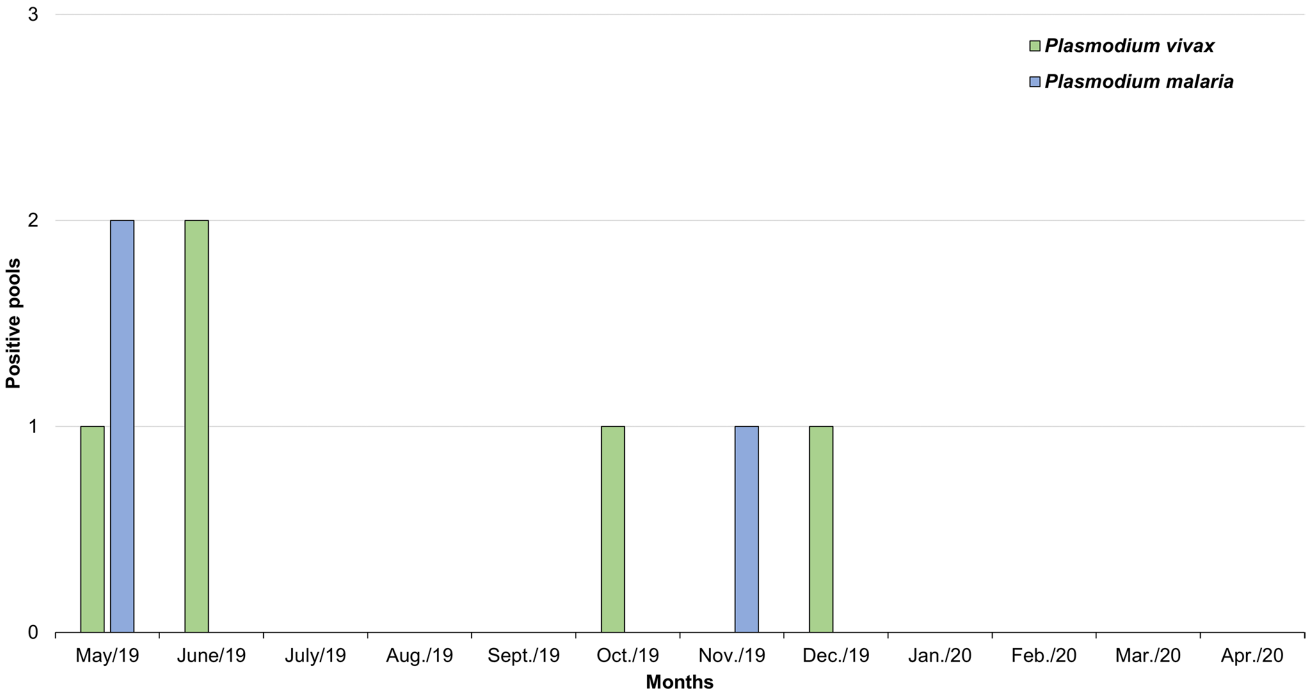

Fig. 3 Seasonal distribution of mosquito-positive pools for *Plasmodium malariae* and *Plasmodium vivax*

Valsugana Velha is a study area where human autochthonous malaria cases occur sporadically. Investigations at the site allowed the capture of a small number of mosquitoes of the species *Anopheles (K.) cruzii* at ground level. Rezende et al. (2009) and Buery et al. (2018) observed natural infection by *P. vivax/P simium* in the same area in one pool of the species *Anopheles (K.) cruzii*, captured by a CDC light trap placed on the ground. Deane et al. (1971) highlighted the versatility of the species *Anopheles (K.) cruzii* in moving between the canopy and the ground level as a risk factor for transmission of parasites from the genus *Plasmodium* between canopy-dwelling simian hosts and ground-dwelling humans.

Considering the putative transmission cycle of malaria in the Atlantic Forest, several lines of evidence suggest that NHPs may act as a source of new *Plasmodium* infections for humans and vice versa. Such a transference would depend on a competent mosquito species acting as a bridge between these different host species occupying different strata in the forest. Surveys such as those conducted by Deane et al. (1966) and Brasil et al. (2017) reported transfers of *Plasmodium* from NHPs to humans or from humans to NHPs. However, the possible species of NHPs involved in this shared transmission cycle are the object of speculation. Recent evidence suggests that NHPs of the genus *Alouatta* sp. should be the principal potential reservoir of malaria in the Atlantic Forest. The surveillance of simian malaria performed over the last few years in the Brazilian Atlantic Forest disclosed in *Alouatta* sp. the highest prevalence of infection by *P. brasilianum* and *P. simium*, species indistinguishable from the human *Plasmodium* species *P. malariae* and *P. vivax*, respectively (Duarte et al. 2006, 2008; Yamasaki et al. 2011; Abreu et al. 2019; Monteiro et al. 2020; Assis et al. 2021).

The epidemiological relevance of the *Alouatta* sp. genus for malaria in Atlantic Forest areas became the object of scrutiny after their drastic population reduction promoted by the sylvatic yellow fever outbreak, which spread in the Atlantic Forest areas of southern and southeastern Brazil in 2016. As postulated by Abreu et al. (2019), the expectation was that the population decrease of the supposed principal reservoir of malaria in the Atlantic Forest would affect its transmission dynamics, resulting in a decreased infection rate of the predominant vector *Anopheles (K.) cruzii*. However, such an infection rate reduction did not occur, mainly in Espírito Santo state. The stability of the vector infection rate is evident in the present study, as four pools of *Anopheles (K.) cruzii* had infection by *Plasmodium vivax*, giving an infection rate of 0.53. Such a rate is similar to that of Rezende et al. (2009) in a study conducted in Valsugana Velha (MIR = 0.5), the same area of this study. The overall infection rate for malaria parasites found in the examined mosquito pools was 0.92. Buery et al. (2018) found a similar minimum infection rate in *Anopheles (K.) cruzii* in the same study area (MIR = 0.96). However, the MIR in the *Anopheles (K.) cruzii* from Atlantic Forest areas of São Paulo is distinctly different. Duarte et al. (2013) and Neves et al. (2013) found an MIR for the *Anopheles (K.) cruzii* of 0.09 and 0.24, respectively.

The stability of *Plasmodium* infection rates in *Anopheles (K.) cruzii* after the yellow fever outbreak suggests that other species of NHPs could support the transmission cycle in areas of the Atlantic Forest. This would be particularly relevant during the reduction in the population of *Alouatta* specimens. In Rio de Janeiro state, an area of malaria autochthonous cases, the studies disclosed natural infection in NHPs of the *Sapajus* genus by *P. vivax/P. simium* and *P. malariae/P. brasilianum* (de Alvarenga et al. 2015). More recently, in the municipality of Santa Teresa — the setting of this study — researchers identified the presence of *P. falciparum* and *P. malariae/P. brasilianum* in the feces of *Sapajus nigritus* (manuscript in preparation). These results further support the hypothesis that a variety of NHPs can act as reservoirs for malaria in Atlantic Forest areas, thus contributing to the persistence of the parasite in the environment.

Despite these results, questions remain. Therefore, investigative studies exploring potential reservoirs in the Atlantic Forest areas are necessary and should be encouraged. However, such studies should not focus only on the diagnosis of infection. They should mainly seek to understand the infection course and profile in the putative wild hosts.

Author contribution Conceptualization: Lucas Mendes Ferreira and Crispim Cerutti Júnior; performed the research: Lucas Mendes Ferreira and Helder Ricas Rezende; performed the data analysis: Lucas Mendes Ferreira and Ana Maria Ribeiro de Castro Duarte; writing — original draft preparation: Lucas Mendes Ferreira and Crispim Cerutti Júnior; writing — review and editing: Julyana Cerqueira Buery, Crispim Cerutti Júnior, Filomena Euridice Carvalho de Alencar, Ana Maria Ribeiro de Castro Duarte, Blima Fux and Ana Carolina Loss.; figure production: Lucas Mendes Ferreira.

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Data availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All the authors have read and agreed to the published version of the manuscript.

Conflict of interest The authors declare no competing interests.

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