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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Residual malaria of Atlantic Forest systems and the influence of anopheline fauna

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Abstract

In Brazil, the Amazon region comprises 99.5% of the reported malaria cases. However, another hotspot of the disease is the Atlantic Forest regions, with the sporadic occurrence of autochthonous human cases. In such context, this study sought to investigate the role of anopheline mosquitoes (Diptera: Culicidae) in the residual malaria transmission in Atlantic Forest areas. Two rural areas in the Espírito Santo state were the surveyed sites. Mosquitoes were captured using Shannon trap and CDC light traps and identified into species based on morphological characters. Ecological indexes (Shannon–Wiener diversity, Simpson's dominance, Pielou equability, and Sorensen similarity) were the tools used in the anopheline fauna characterization and comparison along with the two explored areas. The assessment of the sampling adequacy in the studied areas was possible through the generation of a species accumulation curve. A correlation test verified the influence of climatic variables on the anopheline species abundance. A total of 1471 female anopheline mosquitoes were collected from May 2019 to April 2020, representing 13 species. The species richness was higher in Valsugana Velha (hypo-endemic) than in Alto Caparaó (non-endemic). There was a significant variation in the species abundance between Valsugana Velha (n = 1438) and Alto Caparaó (n = 33). The most abundant species was *Anopheles (Kerteszia) cruzii* complex Dyar and Knab, 1908 representing 87% of the total anophelines collected. These results suggest that the *Plasmodium* spp. circulation in Brazilian Atlantic Forest areas occurs mainly due to the high frequency of *Anopheles (K.) cruzii* complex, considered the principal vector of simian and human malaria in the region.

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

Introduction

Mosquitoes pose a threat to public health worldwide. Vector-borne illnesses currently account for approximately 17% of all infectious diseases (WHO 2017). One of the most relevant mosquito-borne diseases is malaria, caused by the protozoan parasite *Plasmodium* spp. and transmitted by female *Anopheles* mosquitoes infected (Ashley et al. 2018). According to World Health Organization (WHO 2020) estimation, there were 229 million malaria cases in 2019 worldwide. Despite substantial progress in the fight to eliminate malaria, the disease still is a significant health problem, causing economic impacts in many countries in the region of the Americas, where the health authorities reported more than 800,000 cases in 2019, with Brazil accounting for approximately 20% of all occurrences.

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Engaged in the fight against malaria since the beginning of the twentieth century, Brazil has made several attempts to control the disease over time (Deane 1988). The estimates for the epidemiologic scenario in 1940 indicated that almost all country regions were receptive and reported malaria cases (Pinotti 1951; Deane 1986). Malaria elimination from some states in 1965 was the consequence of the global eradication campaign proposed by the WHO and adopted by the Brazilian Ministry of Health, a challenging effort given the high magnitude of the disease in the previous years. At that time, dichlorodiphenyltrichloroethane (DDT) and chloroquine were the cornerstones for the control activities. Dichlorodiphenyltrichloroethane was the residual insecticide useful for vector control, and chloroquine was the drug in general use to treat the infection (Loiola et al. 2002). The actions adopted by the Brazilian authorities were enough to control and eliminate the disease from several regions of Brazil, including the Northeast, Southeast, South, and Central-West. However, due to eco-epidemiological factors, such measures failed to interrupt the transmission in some Atlantic Forest areas and mostly, in the Amazon region, still classified as an endemic area (Carlos et al. 2019; Buery et al. 2021).

Although autochthonous malaria transmission being under control in most extra-Amazonian regions, residual foci persist along with the Brazilian Atlantic Forest areas of southern and southeastern states. In such spots, howler monkeys, anophelines mosquitoes, and humans use to be infected naturally by *Plasmodium vivax/Plasmodium simium*, *Plasmodium malariae/Plasmodium brasilianum*, and, more recently, *Plasmodium falciparum* (Laporta et al. 2015; Buery et al. 2017; Demari-Silva et al. 2020). Some research studies have indicated that non-human primates (NHP) may participate in the human malaria transmission cycle in the Atlantic Forest (Brasil et al. 2017). The clinical aspects and spatial–temporal dynamics of the malaria incidence in these areas associated with vector behavior reinforce such a hypothesis.

The Brazilian Atlantic Forest is considered one of the most distinguished biodiversity repositories of the world. The region has geographic and climatic characteristics that benefit the development of insects, mainly mosquitoes (Ribeiro et al. 2009). Such insects belonging to the Culicidae family of the Diptera order can play a relevant epidemiological role in arboviruses and parasite transmission (WHO 2017). In this context, the Brazilian Atlantic Forest can become a hotspot for malaria, putting the disease elimination plan at risk. The present study aimed to elucidate the possible contribution of the dynamics of anopheline populations in sustaining residual malaria transmission.

Methods

Study areas

Malaria vector surveillance occurred in Valsugana Velha (19°58′05.2″S, 40°34′40.8″W) and Alto Caparaó (9°44′12.7″S, 40°58′33.1″W) (Fig. 1), two inland rural communities situated at high elevation points of Santa Teresa (hypo-endemic) and Itaguaçu (non-endemic), respectively. Both sites have proximity to Atlantic Forest fragments, potential breeding sites for *Anopheles* species.

Santa Teresa (altitude of 100–1050 m) and Itaguaçu (altitude of 70–1320 m) are municipalities of the central mountainous region of Espírito Santo State, southeastern Brazil (IJSN 2020). While Santa Teresa has an area of 683,032 km^2 with 32.1% covered by Atlantic Forest, Itaguaçu, located 53 km away from Santa Teresa, has 535,021 km^2, of which 17.7% have forest cover (SEAMA 2018; IBGE 2019).

According to the Capixaba Institute for Research, Technical Assistance and Rural Extension (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural—INCAPER), the area of Valsugana Velha and Alto Caparaó experiences cold and humid weather conditions (INCAPER 2020). There is one rainy season between January and April, a dry or less rainy season between May and September, and another rainy season between October and December. The minimum and maximum average temperatures of the coldest and warmest months range from 7.3–9.4 °C and 25.3–27.8 °C, respectively. These areas receive annual precipitation of 1332 mm (Valsugana Velha) and 1066 mm (Alto Caparaó) (INCAPER 2020). Cases of residual malaria from Atlantic Forest systems occur primarily in Santa Teresa, where there are findings of infected *Anopheles (K.) cruzii* complex specimens (Cerutti Junior et al. 2007; Buery et al. 2018).

Adult mosquito sampling and identification of *Anopheles* species

During 1 year, from May 2019 to April 2020, there has been a sampling of *Anopheles* mosquitoes twice a month (one site per night), from 06:00 pm to 06:00 am the next morning. The setting of two types of traps was the strategy chosen for the capture of adult mosquitoes: (1) Captures performed in a Shannon light trap, with the help of an oral suction tube: the setting inside the forest allowed the capture of anophelines for 4 h (06:00–10:00 pm). (2) Captures performed by a CDC automatic (Center for Disease Control) trap with CO$_2$ baits (200 g of dry ice): the team installed two CDC traps inside the forest, the first at 1 m above the ground, and the second at 10 m height, in the canopy. CDC traps remained turned on for 12 h, from the night (06:00 pm) until the morning (06:00 am).

Every morning after the collection, the team removed the mosquitoes from the traps. The researchers transferred the insects to polyethylene cages, transporting them to the Entomology and Malacology Centre of Espírito

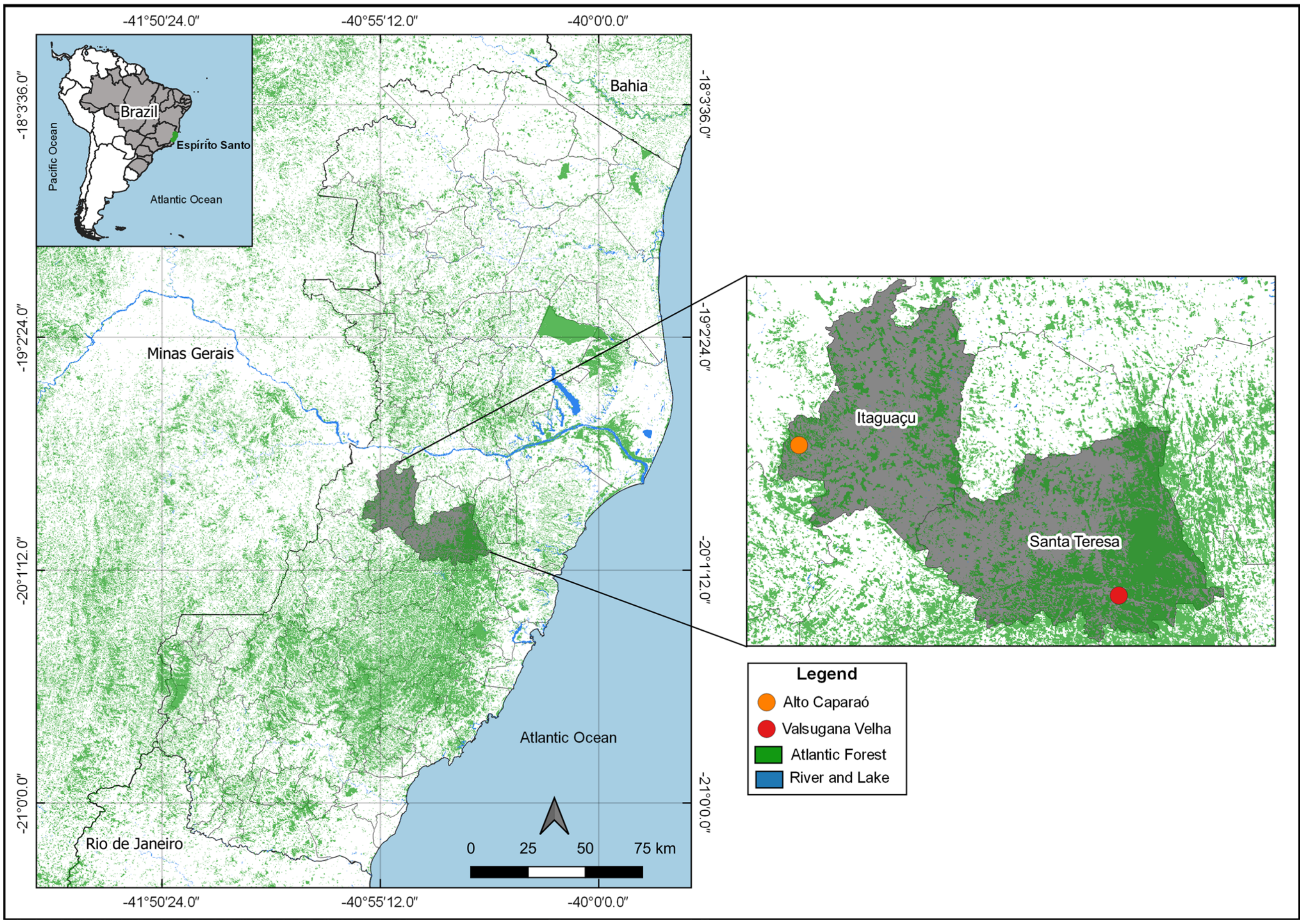

Fig. 1 Map showing *Anopheles* collection sites. Itaguaçu (Alto Caparaó) and Santa Teresa (Valsugana Velha), municipalities of Espírito Santo state, Southeast Brazil

Santo (Núcleo de Entomologia e Malacologia do Espírito Santo—NEMES/ES), where they performed the identification of the anophelines. After the insects' killing in a freezer, the researchers identified the adult female anophelines through the key developed by Consoli and Lourenço-de-Oliveira (1994).

Climatic variables

Santa Teresa Station of the National Meteorology Institute of Brazil provided the meteorological data. Data used included the temperature (mean of the day of the capture) and the precipitation (mean from seven days before the capture event).

Data analysis

The statistical software for data analyses was the *vegan* package in R Statistical Software (version 1.3.1093) and PAST software (version 4.03). The indexes used were the Shannon–Wiener diversity index (H'), Simpson's dominance index (C), and the Pielou equability index (J) for *Anopheles* species diversity, dominance, and evenness, respectively. The Sorensen index (SI) allowed the assessment of the species composition similarity among the sampled areas. The Hutcheson *t*-test ($\alpha = 0.05$) disclosed the occurrence of statistically significant differences in the Shannon–Wiener diversity index (H') and Simpson's dominance index (C) between the two studied areas: Valsugana Velha and Alto Caparaó.

To assess the sampling sufficiency, a species accumulation curve was generated through the EstimateS program (Colwell 2006). The species accumulation curve considered the estimator's Sobs (Mao Tao), which represents observed species richness, and Jackknife 1, which indicates the expected species richness to be sampled. Five-hundred randomizations were used, with a confidence interval of 95%.

Data analyses included a correlation testing between climatic variables (mean temperature; rainfall) and the abundance of species in both areas. After checking the data normality through the Shapiro–Wilk test since there was no normality in the data distribution, the investigators performed a non-parametric Spearman's correlation procedure ($\alpha = 0.05$).

Results

Species composition and abundance

The sampling comprised 1471 *Anopheles* mosquitoes, belonging to 13 different species and three subgenera (Table 1). The three most abundant species were *Anopheles (K.) cruzii* complex (87%), *Anopheles (Nyssorhynchus) strodei* Root, 1926 (6%), and *Anopheles (Nyssorhynchus) evansae* Brethes, 1926 (4.21%). These three species comprised 97.2% of all collected specimens.

Valsugana Velha had the highest abundance and richness of anophelines, with 1438 (97.7%) specimens distributed in 10 species. In contrast, in Alto Caparaó, there was a collection of only 33 (2.24%) anophelines, belonging to seven different species. The sample-based species accumulation curves in both areas were close to the asymptote (Fig. 3). A total of 10 species resulted from the Valsugana Velha survey and seven species from Alto Caparaó, which gave a sampling efficiency of 78.46% (Jackknife 1 = 12.75 ± 3.86) and 71.79% (Jackknife 1 = 9.75 ± 3.86), respectively.

Anopheles (K.) cruzii complex, the primary vector of human and simian malaria in the Atlantic Forest, was the dominant and most abundant species in the two sites, constituting 87% of all captured *Anopheles* mosquitoes. Regarding the distribution, Valsugana Velha yielded 1259/1438 specimens (87.5%) and Alto Caparaó 21/33 (63.6%) of them.

Anopheles species diversity

Table 1 discloses the richness, abundance, and diversity of *Anopheles* species in each area. Four of the species (31%) were similar between the sites: *Anopheles (K.) cruzii* complex, *Anopheles (N.) strodei*, *Anopheles (N.) evansae*, and *Anopheles (Nyssorhynchus) lutzii* Cruz, 1901. The value estimated with Sorensen's index indicated a low similarity among the areas (SI = 0.47).

Despite the trapping of the lowest numbers of species and specimens in Alto Caparaó, this area showed the highest diversity index value (H' = 1.2912). On the other hand, Valsugana Velha, accounting for the highest richness and abundance, showed lower diversity (H' = 0.53514). According to the Hutcheson *t*-test, the Shannon–Wiener diversity index was significantly different between Alto Caparaó and Valsugana Velha ($p < 0.01$). The Simpson dominance index was higher in Valsugana Velha than in Alto Caparaó (C = 0.77508 versus C = 0.42883, $p < 0.01$). The values of both the Shannon diversity index and the Simpson dominance index suffered the influence of the evident abundance and dominance of the *Anopheles (K.) cruzii* complex, which represented 87.5% and 63.3% of the *Anopheles* population of Valsugana Velha and Alto Caparaó, respectively. This observation has corroboration of the values of equability. The Pielou equability index was J = 0.6635 for Alto Caparaó and J = 0.2324 for Valsugana Velha.

Comparing the ecological indexes of diversity and dominance by trap (Table 2) for Valsugana Velha and Alto Caparaó, we found that there was no significant difference in Simpson dominance index (C = 0.2849 versus

Table 1 *Anopheles* species abundance per trap in Valsugana Velha and Alto Caparaó, rural areas in the Atlantic Forest, Espírito Santo, Brazil

Mosquito species	Total number (%)	Valsugana Velha			Alto Caparaó		
		Shannon	CDC ground	CDC canopy	Shannon	CDC ground	CDC canopy
Anopheles (K.) cruzii complex	1280 (87)	13	10	1236	0	1	20
Anopheles (N.) strodei	89 (6)	80	7	0	2	0	0
Anopheles (N.) evansae	62 (4.21)	43	15	1	2	1	0
Anopheles (K.) homunculus	14 (0.95)	0	0	14	0	0	0
Anopheles (N.) galvaoi	7 (0.47)	6	1	0	0	0	0
Anopheles (A.) mediopunctatus	4 (0.27)	3	0	1	0	0	0
Anopheles (N.) lutzii	3 (0.2)	1	1	0	1	0	0
Anopheles (N.) triannulatus	3 (0.2)	2	1	0	0	0	0
Anopheles (N.) lanei	2 (0.13)	1	1	0	0	0	0
Anopheles (N.) oswaldoi	2 (0.13)	0	0	0	2	0	0
Anopheles (A.) fluminensis	2 (0.13)	0	0	0	1	1	0
Anopheles (A.) punctimacula	2 (0.13)	0	0	0	1	1	0
Anopheles (N.) argyritarsis	1 (0.06)	1	0	0	0	0	0
Total	1471	150	36	1252	9	4	20

A., *Anopheles; K.*, *Kerteszia; N.*, *Nyssorhynchus*

Table 2 Diversity and dominance indexes of *Anopheles* mosquitoes per trap. Values of Shannon–Wiener diversity index and Simpson's dominance index for three trap types in two areas, Valsugana Velha and Alto Caparaó

Index	Trap	Valsugana Velha	Alto Carapaó	p-value
Shannon's diversity	Shannon	1.2702	1.7351	0.06
Simpson's dominance	Shannon	0.37902	0.18519	< 0.05*
Shannon's diversity	CDC canopy	0.074337	0	< 0.01*
Simpson's dominance	CDC canopy	0.97473	1	< 0.05*
Shannon's diversity	CDC ground	1.4527	1.3863	0.85
Simpson's dominance	CDC ground	0.2849	0.25	0.85

*Significant differences

C = 1.3863, p = 0.85) and species diversity (H' = 1.4527 versus H' = 1.3863, p = 0.85) for CDC trap placed on the ground. However, Simpson dominance index (C = 0.97473 versus C = 1, p < 0.05) and Shannon–Wiener diversity index (H' = 0.074337 versus H' = 0, p < 0.01) were significantly different for CDC traps placed in the canopy. The same observation applies to the Simpson dominance index (C = 0.37902 versus 0.18519, p < 0.05) for the Shannon light trap.

Exploration of seasonal trend in Valsugana Velha

The temporal distribution for the three most common species captured in Valsugana Velha revealed a multimodal pattern for *Anopheles (K.) cruzii* complex and a unimodal for *Anopheles (N.) strodei* and *Anopheles (N.) evansae* (Fig. 2). *Anopheles (K.) cruzii* complex was the dominant species, present throughout the year with two relevant peaks, one in May 2019 (dry season) and the other in November 2019 (rainy season). *Anopheles (N.) strodei* and *Anopheles (N.) evansae* presented only one peak in October 2019 (rainy season). There was no significant correlation between climatic variables and *Anopheles* abundance.

Exploration of seasonal trend in Alto Caparaó

Anopheles (K.) cruzii complex had a unimodal distribution pattern, with a higher peak in February 2020 (rainy season) (Fig. 2). There was no significant correlation between climatic variables and *Anopheles (K.) cruzii* complex abundance.

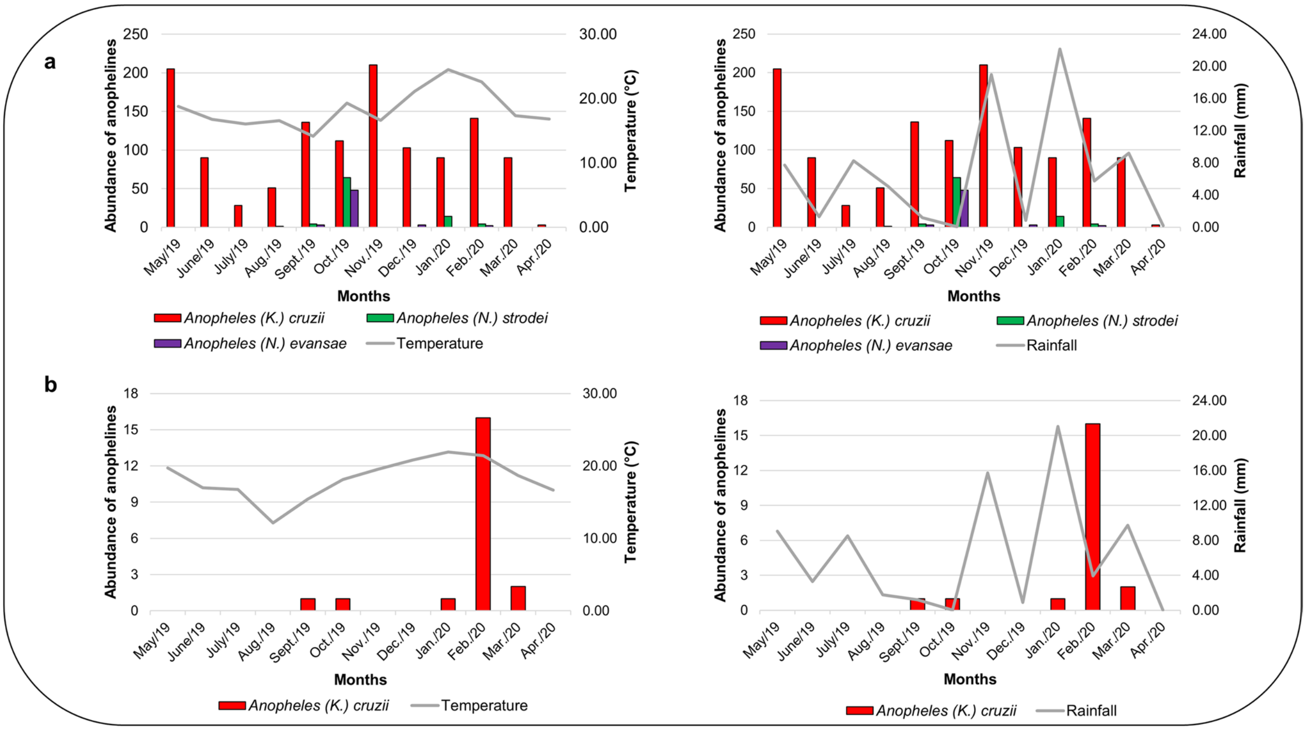

Fig. 2 Frequency of *Anopheles* (K.) *cruzii* complex, *Anopheles (N.) strodei*, and *Anopheles (N.) evansae* in relation to the climatic variables (temperature and rainfall). **A** Valsugana Velha. **B** Alto Caparaó

Discussion

This study provides updated information on the distribution and diversity of *Anopheles* species in the Atlantic Forest regions of Brazil. In the present study, 13 *Anopheles* species were found. *Anopheles (K.) cruzii* complex was the most common species in both areas. Although found in a distinct amount, this species represented 63.6% and 87.5% of the anopheline fauna of Alto Caparaó and Valsugana Velha, respectively. This is consistent with the existing literature. *Anopheles (K.) cruzii* complex has been described as the dominant species in the Brazilian Atlantic forest (Ueno et al. 2007; Reis et al. 2010; Guedes and Navarro-Silva 2014; Ceretti-Junior et al. 2020). Studies performed in Espírito Santo, Rio de Janeiro, and São Paulo have incriminated *Anopheles (K.) cruzii* complex as the primary malaria vector. In Espírito Santo, this vector was found naturally infected by *P. vivax/P. simium* on two occasions (Rezende et al. 2009; Buery et al. 2018). In 2015–2016, the state of Rio de Janeiro reported 49 autochthonous malaria cases, all from the Atlantic Forest area, a place with high *Anopheles (K.) cruzii* density (Brasil et al. 2017). Furthermore, specimens of *Anopheles (K.) cruzii* complex were found naturally infected with *P. vivax/P. simium*, *P. malariae/P. brasilianum*, and most recently *P. falciparum* in the Atlantic Forest areas of São Paulo (Duarte et al. 2013; Laporta et al. 2015; Demari-Silva et al. 2020).

The abundance of mosquitoes and the species richness were different between the two areas. Although Alto Caparaó and Valsugana Velha have the same biome, there was a substantial difference in the number of specimens collected in one and the other. Some hypotheses proposed to explain these differences are the forest cover and the anthropogenic environmental disturbance (e.g., deforestation, agriculture). The expressive abundance and dominance of *Anopheles (K.) cruzii* complex, found in Valsugana Velha, and the small number of specimens of this species in Alto Caparaó probably indicate that the first area has a lower level of environmental degradation than the second. According to Dorvillé (1996), anophelines from the subgenus *Kerteszia* are excellent bioindicators of anthropogenic activity. Ribeiro et al. (2012) and Chaves et al. (2016) revealed differences in the *Anopheles (K.) cruzii* complex abundance between preserved and degraded Atlantic Forest environment. Recently, Medeiros-Sousa et al. (2019) also observed the influence of anthropogenic landscape changes on the abundance of this vector.

Climatic factors such as temperature and precipitation can also influence the composition and abundance of anophelines added to the specific causes mentioned above. Studies conducted in areas of Atlantic Forest in São Paulo state indicated that the abundance of *Anopheles (K.) cruzii* complex was associated with temperature and rainfall (Guimarães et al. 2000, 2001). However, like the study of Rezende et al. (2009) developed in Atlantic Forest areas of Espírito Santo, our study also found no correlation between climate variables and the abundance of this vector. This lack of correlation might result from the low frequency of the collected specimens, especially in Alto Caparaó. Alternatively, the complex topography of the Atlantic Forest can generate environmental and microclimate conditions capable of influencing the abundance of some anophelines (Marques et al. 2012). For example, mosquitoes of the subgenus *Kerteszia*, mainly *Anopheles (K.) cruzii* complex, reproduce in waters that accumulate inside of bromeliads, a plant located under the shade of the treetops, protected from the sun's rays, which prevents the rapid evaporation of the water contained therein (Consoli and Lourenço-de-Oliveira 1994). This specific condition of reproducing inside bromeliads can generate particular microclimate conditions (e.g., temperature and humidity) that become indistinguishable when analyzing only data from macroclimate.

The differences in *Anopheles* species richness and abundance observed in the present study can influence the differential epidemiological scenario of malaria observed in rural areas of Itaguaçu (non-endemic) and Santa Teresa (hypo-endemic). In general, these findings agree with those from previous entomological studies conducted in areas of malaria transmission in the Brazilian Atlantic Forest. According to Rezende et al. (2013), species richness, density of anophelines species, and dominance of the *Anopheles (K.) cruzii* complex are factors that can influence the dynamics of malaria transmission in the Atlantic Forest.

In the present study, the Shannon–Wiener diversity index (H'), Simpson's dominance index (C), and the Pielou equability index (J) were used to measure the species diversity, species dominance, and species evenness. Although the Valsugana Velha area has presented higher richness, it was also where the highest species dominance was verified. In the Alto Caparaó area, the Shannon–Wiener diversity index (1.2912), Pielou equability index (0.6635), and Simpson's dominance index (0.42883) indicated higher diversity and equitability and lower dominance compared to the Valsugana Velha area. In this sense, it is noted that the highest richness observed does not always reflect the higher existing diversity in the area since diversity is determined by both richness and equability (Melo 2008). The equitability index refers to the distribution of the number of specimens among species. It is proportional to diversity and inversely proportional to dominance (Pielou 1975). Therefore, the expressive abundance of the species *Anopheles (K.) cruzii* complex in Valsugana Velha gave a greater dominance and lower equitability to the area compared to Alto Caparaó, which showed greater diversity probably due to the greater equitability of the number of specimens among the species.

Considering that the species accumulation curves (Fig. 3) showed a tendency to an asymptote but did not stabilize and that the results of the Jackknife 1 showed that there may be more additional species to the ones collected, new species of genus *Anopheles* can be sampled in both areas with the continuation of sampling.

As expected, Simpson's dominance index (C) for both study sites was higher in the CDC trap placed in the tree canopy (C = 1 for Alto Caparaó and C = 0.97473 for Valsugana Velha). This result reflects the high proportions of *Anopheles (K.) cruzii* complex collected using this method. Regarding the vertical distribution of the *Anopheles (K.) cruzii* complex, this species represented 60.6% and 86% of the anopheline community in the canopy strata of Alto Caparaó and Valsugana Velha, respectively. According to Forattini et al. (1968), there is evidence of the preference of this species for inhabiting the tree canopies compared with ground level, when they observed the presence of 83.3% of the specimens at 15 m in height in an area located in the state of São Paulo. This event was observed in Espírito Santo by Deane et al. (1971) and Rezende et al. (2009) and also by Guimarães et al. (1985) in Rio de Janeiro, where this species was much more numerous in the canopy than at the ground level.

Altogether, these data support the hypothesis that the specific breeding conditions of the *Anopheles (K.) cruzii* complex favor their predominance in the canopy, where they find, as the principal blood source, the non-human primates. On the other hand, different breeding sites occur at the ground level, favoring more species diversity. The distribution of the *Anopheles (K.) cruzii* complex fits the proposed malaria transmission cycle, which would take place mainly among the non-human primates, being human dwellers, accidental hosts.

In this study, other anopheline species, including *Anopheles (N.) strodei*, *Anopheles (N.) evansae*, and *Anopheles*

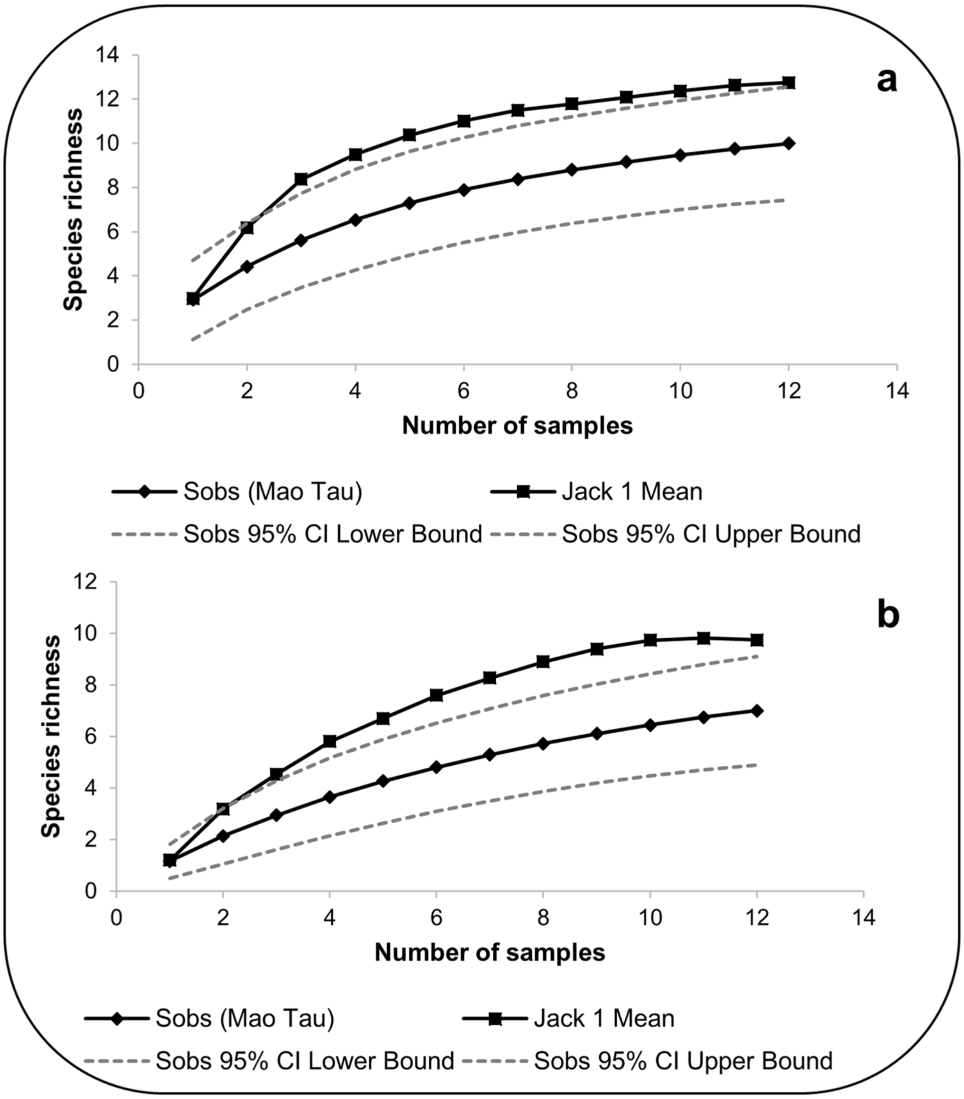

Fig. 3 Sample-based species accumulation curve for mosquito species richness observed (Mao Tao) and estimated (Jackknife 1). Dotted lines represent the confidence interval (95%). **A** Valsugana Velha. **B** Alto Caparaó

(Nyssorhynchus) triannulatus Neiva and Pinto, 1922, were found in low frequency. However, it is relevant to document their occurrence, as they are also able to transmit malaria. Different authors have reported their susceptibility to infection by *Plasmodium* parasites in Atlantic Forest areas (Duarte et al. 2013; Laporta et al. 2015). According to Consoli and Lourenço-de-Oliveira (1994), these species possibly are secondary vectors and can participate in the transmission cycle when present at high densities.

Finally, this study reinforces that *Anopheles (K.) cruzii* complex is the principal vector involved in malaria transmission in the Brazilian Atlantic Forest. The high frequency of this vector found in Valsugana Velha might explain the difference observed in the epidemiological scenario of malaria between Santa Teresa (hypo-endemic) and Itaguaçu (non-endemic).

Author contributions L.M.F. and C.C.J. conceptualized the article; L.M.F., H.R.R., and L.S.d.S. performed the research; L.M.F. performed the data analysis; L.M.F. and C.C.J. drafted the article; A.M.R.d.C.D., B.F., C.C.J., H.R.R., J.C.B., and T.C.C.F. critically revised the article; all authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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