

## PUBLIC HEALTH

# Entomological Characterization and Natural Infection of Anophelines in an Area of the Atlantic Forest with Autochthonous Malaria Cases in Mountainous Region of Espírito Santo State, Brazil

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Edited by Álvaro Eiras – UFMG

*Neotropical Entomology* 38(2):272-280 (2009)

### Caracterização Entomológica e Infecção Natural de Anofelinos em Área de Mata Atlântica, com Casos Autóctones de Malária, em Regiões Montanhosas do Espírito Santo

**RESUMO** - No Espírito Santo, os casos de malária autóctone estão distribuídos na região serrana próximo aos fragmentos de Mata Atlântica. Uma vez que alguns aspectos da doença são obscuros, a detecção das possíveis espécies de vetores pode auxiliar na elucidação de incertezas epidemiológicas. Estudos entomológicos e de infecção natural foram realizados com anofelinos (Diptera: Culicidae) capturados no município de Santa Tereza, ES. Capturas mensais foram realizadas de março de 2004 a fevereiro de 2006. Armadilhas CDC-CO<sub>2</sub> foram utilizadas do crepúsculo (18:00h) ao amanhecer (6:00h), para capturar anofelinos nos seguintes habitats: próximo ao domicílio e área aberta (solo), margem e interior da mata (solo e copa). Armadilhas Shannon também foram utilizadas nos mesmos locais que as de CDC-CO<sub>2</sub>. Capturou-se o total de 2.290 anofelinos distribuídos em 10 espécies. A maior frequência relativa foi de *Anopheles (Kerteszia) cruzii* Dyar & Knab / *A. (K.) homunculus* Komp, sendo a maioria capturada em CDC-CO<sub>2</sub> instalada na copa da mata. A principal espécie capturada em armadilha Shannon foi *A. (Nyssorhynchus) strodei* Root. O maior número de anofelinos foi capturado entre julho e setembro das 18:00h às 22:00h. Provavelmente *A. (K.) cruzii* é responsável pela transmissão da malária dentro ou próximo aos fragmentos de Mata Atlântica. Entretanto, a participação de outras espécies não pode ser ignorada, visto que 53% da amostragem foi constituída pelo subgênero *Nyssorhynchus*. A detecção de *Plasmodium vivax* no tórax de *A. cruzii*, *A. parvus* (Chagas) e *A. galvaoi* Causey, Deane & Deane por meio de PCR reforça esse argumento.

**PALAVRAS-CHAVE:** *Anopheles cruzii*, malária autóctone, Plasmodium, PCR, ecologia

**ABSTRACT** - Autochthonous malaria cases in the state of Espírito Santo, Brazil, are distributed in mountainous regions surrounded by the Atlantic Forest. While some aspects of this disease are unclear, detection of possible vector species can help to elucidate epidemiological uncertainties. Entomological and natural infection studies were carried out using anophelines (Diptera: Culicidae) captured in the municipality of Santa Tereza, ES. Monthly captures were made from March 2004 to February 2006. CDC-CO<sub>2</sub> traps were used from dusk (6:00 P.M.) to dawn (6:00 A.M.) to capture anophelines in the following habitats: near the houses, in open areas (at ground level) and inside, and at the margins of the forest (canopy and ground level). Shannon light traps were also used at the same locations of the CDC-CO<sub>2</sub> traps. A total of 2,290 anophelines within 10 species were captured. The relative frequency of *Anopheles (Kerteszia) cruzii* Dyar & Knab / *A. (K.) homunculus* Komp was the highest, with the majority captured in CDC-CO<sub>2</sub> traps installed in the forest canopy. The main species captured in Shannon traps was *A. (Nyssorhynchus) strodei* Root. The largest number of anophelines was captured from July to September and from 6:00 P.M. to 10:00 P.M. *Anopheles (K.) cruzii* is the probable vector for malaria transmission inside or near the Atlantic Forest fragments, but the role of other species cannot be ignored, as 53% of the sampled anophelines belonged to the subgenus *Nyssorhynchus*. The natural infection of *A. cruzii*, *A. parvus* (Chagas) and *A. galvaoi* Causey, Deane & Deane by *Plasmodium vivax*

detected by PCR from DNA extracted from their thoraxes supports this view.

KEY WORDS: *Anopheles cruzii*, autochthonous malaria, Plasmodium, PCR, ecology

In general, the spatial distribution of anophelines (Diptera: Culicidae) follows that of malaria, as their species play an important role in this major health problem. The majority of cases notified in Brazil are reported in the Amazon region (576,963) (Ministry of Health 2005), but residual cases have also been notified in areas outside the Amazon, such as Southern and Southeastern Brazil. These areas have their own particular ecosystems and are covered by the Atlantic Forest (Pinotti 1951). Indigenous malaria in these regions is known as bromeliad malaria (Downs & Pittendrigh 1946).

The possible effect of the coexistence of anophelines, non-human primates and human beings on malaria transmission in these regions is not well understood, and little is known on the real dynamics of this infection in such ecosystem (Curado *et al* 1997, Duarte *et al* 2006, Cerutti *et al* 2007).

Extra-Amazonian autochthonous malaria cases are distributed in mountainous regions and are associated with agricultural activities near the forest (Cerutti *et al* 2007). Cases are restricted to these areas and there is no evidence of outbreaks that could affect either the tourist or agricultural potential.

Studies to identify possible vector species in the state of Espírito Santo are important for the public health system, as they can provide the basis for future preventive measures, and the results obtained can be extrapolated to other regions covered by Atlantic Forest in other states in Brazil.

Anophelines preliminary identification data and the nature of the landscape suggest that *Plasmodium* transmission in these regions is different from that in the Amazon (Espírito Santo State Secretary of Health, unpublished observations), due

to the absence of *Anopheles darlingi* Root and *A. aquasalis* Curry, the small number of specimens of *A. albitarsis* s.l. Linch Arribáizaga, *A. cruzii* Dyar & Knab and *A. bellator* Dyar & Knab, and the presence of *A. argyritarsis* Robineau-Desvoidy, *A. evansae* (Brèthes), *A. strodei* Root and *A. lutzi* Cruz outdoors. The question as to whether these species can participate in the transmission of autochthonous malaria also remains to be answered. In here, we endeavor (a) to determine by means of an entomological survey whether *Plasmodium* transmission in a mountainous region of the state of Espírito Santo correlates with the existence of a dominant species and (b) to identify by means of molecular techniques which species of anophelines are infected and by which parasite species.

## Material and Methods

**Area description.** Two strategies were used to capture anophelines. The first one, here referred to as "fixed point", was used at a site located at Valsugana Velha (19°57'58.4" S, 40°34'45.2" W and 790 m), in the municipality of Santa Tereza, where autochthonous malaria cases had been reported (Fig 1). This region is characterized by abundant rainfall and low temperatures. The second, referred to as "mobile points", was used at sites spread over an extensive region that includes eleven municipalities (about 5,000 km<sup>2</sup>) where autochthonous malaria cases were reported during this study. Twenty-four captures were made at the fixed point and 17 at mobile points.

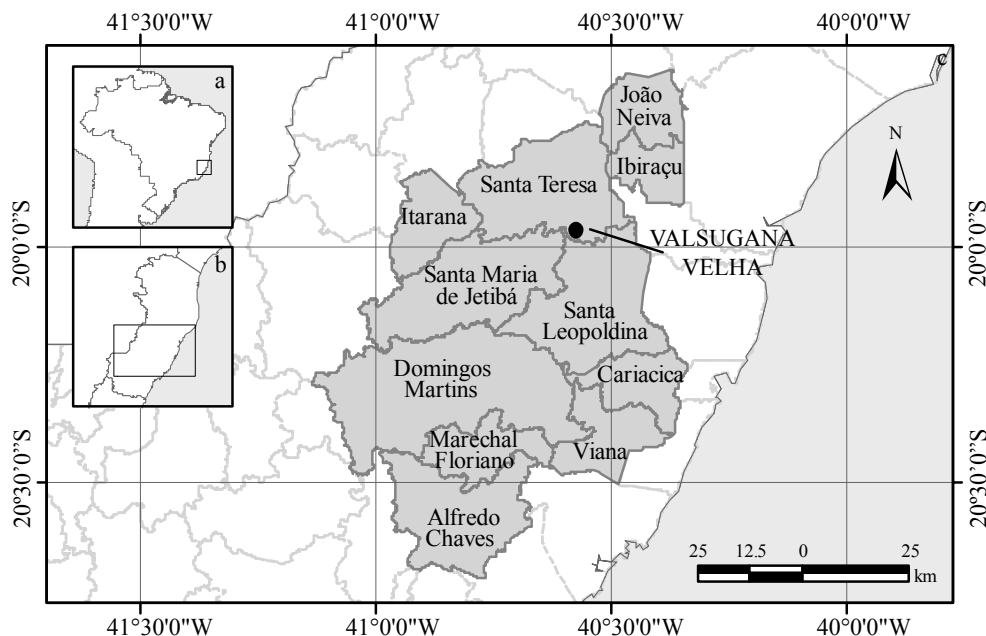


Fig 1 Geographic location of the permanent station in Valsugana Velha, Santa Tereza Municipality, and of the mobile points, at Espírito Santo State, where CDC-CO<sub>2</sub> light traps and Shannon traps had been set from March 2004 to February 2006.

**Capture methods and periodicity.** Two methods were used: (1) CDC-300gCO<sub>2</sub> light traps installed close to the houses in open areas (at floor level), at the margin of the forest (canopy and ground) and inside the forest (also canopy and ground); and (2) Shannon traps close to the houses (mobile points) and at the margin of the forest (fixed point).

The six CDC-CO<sub>2</sub> traps were installed simultaneously, with two of them being placed at a height of 10 m in the canopy (at the margin and inside the forest). The source of CO<sub>2</sub> used in the CDC traps was dry ice (approximately 300 g by trap). Three members of the team carried out the captures at the Shannon traps one at a time, at a rotation of 4h per member, giving a total of 12h of captures, which followed a monthly schedule at the fixed point and a demand-based schedule (related to the number of malaria cases that occurred) at the mobile points. The traps were set from 6:00 P.M. to 6:00 A.M., and captures were performed from March 2004 to February 2006. Rainfall and relative-humidity data were obtained from a meteorological station in the municipality of Santa Tereza. Temperature data were estimated according to Feitoza *et al* (2001).

**Specimens storage and identification.** Captured specimens were kept in silica gel tubes or isopropanol and identified according to Consoli & Lourenço-de-Oliveira keys (1994). In spite of the researchers' expertise in identifying anophelines, the presence of sibling species led to initial misclassification of some specimens of *A. homunculus* as *A. cruzii*. To avoid confusion, in this report we refer to them as *A. cruzii* / *homunculus* specimens.

**Molecular techniques.** Anopheline mosquitoes captured at the various locations were assayed by PCR. DNA from the thoraxes

and abdomens was extracted according to the method described by Oskam *et al* (1996) with modifications. The extractions from thoraxes and abdomens were carried out independently to separate mosquitoes that were carrying sporozoites in their salivary glands, and could be considered potential vectors, from those that were carrying oocysts in their abdomen. The supernatant was precipitated by adding two volumes of ice-cold absolute ethanol in the presence of 0.3 M sodium acetate (NaAc). After centrifugation, the DNA was rinsed with 70% ethanol, dried and resuspended in 50 µl of tris-EDTA buffer (TE). The PCR protocol was performed using primers and methodology described by Kimura *et al* (1997) and modified by Win *et al* (2002). The products were electrophoresed in 2% agarose gel and visualized under UV-light.

**Statistical analysis.** Spearman's rank correlation coefficient was used to test for a correlation between the number of anophelines and the weather variables (temperature, humidity and rainfall). The Kruskal-Wallis non-parametric test and the Berger-Parker dominance index were used to compare the number of anophelines captured at different periods and at different locations in the same period ( $P \leq 0,05$ ). Data analysis was conducted using the BioStat 4.0 software.

## Results

A total of 2,290 specimens within 10 species were captured, with *A. cruzii* being the most frequent (Table 1).

**Variations in weather conditions** (Figs 2, 3). Highest capture frequencies were observed in the colder, drier months of July and September, when the mean temperature

Table 1 Number of anopheline species captured in CDC-CO<sub>2</sub> and Shannon traps from 6:00 P.M. to 6:00 A.M. from March 2004 to February 2006 in a mountainous region of the State of Espírito Santo.

Species	Valsugana Velha fixed point		Mobile points		Total	
	(n°)	%	(n°)	%	(n°)	%
<i>Anopheles cruzii</i> Dyar & Knab / <i>A. homunculus</i> Komp	1,021	61.2	53	8.5	1,074	46.9
<i>A. strodei</i> Root	251	15	66	10.6	317	13.8
<i>A. evansae</i> (Brethes)	169	10.1	128	20.6	297	12.9
<i>A. lutzi</i> Cruz	57	3.4	174	27.9	231	10.0
<i>A. albitarsis</i> l.s. Lynch Arribálzaga	66	3.9	75	12	141	6.1
<i>A. argyritarsis</i> Robineau-Desvoidy	30	1.8	60	9.6	90	3.9
<i>A. parvus</i> (Chagas)	19	1.1	39	6.3	58	2.5
<i>A. galvaoi</i> Causey, Deane & Deane	24	1.4	12	1.9	36	1.5
<i>A. mediopunctatus</i> (Theobald)	24	1.4	10	1.6	34	1.4
<i>A. lanei</i> Galvão & Amaral	7	0.4	5	0.8	12	0,5
Total	1,668	100	622	100	2,290	100

\* Mobile points: Cabeceira do Rio Saltinho, Encantado, Penha and Córrego Seco (Santa Teresa Municipality); Chapéu, Nova Almeida, Parajú and Urânia (Domingos Martins Municipality); Garrafão (Santa Maria do Jetibá Municipality); Rio das Farinhas (Santa Leopoldina Municipality); Costa Pereira (Marechal Floriano Municipality); Rio Lampê (João Neiva Municipality); Ribeirão do Cristo (Alfredo Chaves Municipality); Morro Alto (Ibiraçu Municipality); Jatibocas (Itarana Municipality); Biriricas (Viana Municipality) and Pau Amarelo (Cariacica Municipality).

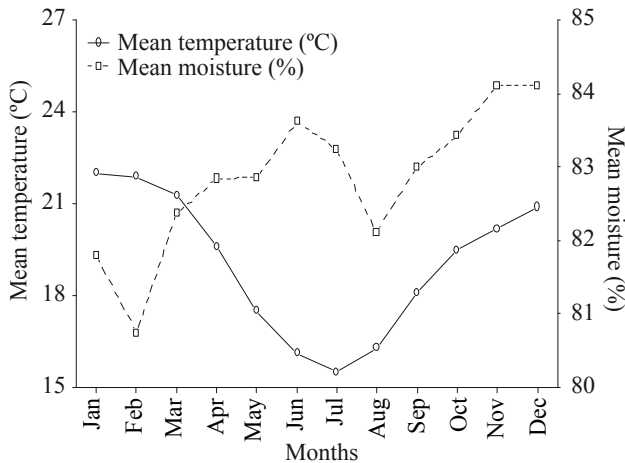


Fig 2 Monthly average of temperature and moisture estimated for Valsugana Velha, Santa Tereza Municipality, ES, from 1996 to 2004.

and rainfall were 15.5°C/29.3 mm and 18.1°C/77.5 mm, respectively. The only month in which all ten species were captured simultaneously was September. *A. cruzii*, *A. strodei*, *A. evansae* and *A. lutzi* were present all year round, and *A. albitarsis s.l.* was only absent in January. Of all the anophelines captured, *A. cruzii* was the predominant species, except in September, and like *A. lutzi*, the highest capture frequency for this species was observed in July. The lowest frequency for *A. cruzii* was registered in May, when *A. albitarsis s.l.* achieved its higher frequency. *Anopheles evansae* and *A. strodei* were predominant in September (Fig 3). Although a negative trend was observed between the number of anopheline specimens, temperature ( $r = -0,11$ ;  $P = 0,7$ ), rainfall ( $r = -0,08$ ;  $P = 0,8$ ) and humidity ( $r = -0,09$ ;  $P = 0,7$ ), and a positive trend observed between the number of *A. cruzii* specimens, temperature ( $r = 0,3$ ;  $p = 0,3$ ) and

rainfall ( $r = 0,11$ ;  $P = 0,7$ ), neither were significant.

**Spatial distribution.** *Anopheles evansae*, followed by *A. strodei* and *A. albitarsis s.l.*, were the most frequently captured species in Shannon traps, whereas *A. cruzii* was the most frequent in CDC CO<sub>2</sub> traps (Figs 4, 5). The higher frequency of *A. cruzii* in CDC-CO<sub>2</sub> traps can be explained by their location in the canopy, where most of the specimens prefer to feed. The Parker dominance test revealed indices (d) of 0.9 for *A. cruzii* inside the forest and 0.8 at the margin, both recorded in the canopy (Fig 4). *Anopheles strodei* was the most frequent species captured in open areas (d = 0.4) and near the houses (d = 0.2), followed by *A. evansae*. *Anopheles lutzi* was frequent in Shannon traps near the houses and in CDC-CO<sub>2</sub> traps in the forest canopy (d = 0.2). The same ten species captured in the forest were also captured in the open area and near the houses.

**Hourly frequency.** The samples obtained from 6:00 P.M. to 10:00 P.M. (64.8% of the specimens) was more representative than those obtained from 10:00 P.M. to 2:00 A.M. or 2:00 A.M. to 6:00 A.M. ( $P = 0.002$ ; Kruskal-Wallis test). The smallest sample size was that obtained from midnight to 1:00 A.M. (1.9% of the specimens). However, some species, such as *A. albitarsis s.l.*, *A. lutzi*, *A. evansae* and *A. strodei* were active throughout the night (Fig 5).

**Natural infection** (Table 2). Only one pool of *A. cruzii* / *A. homunculus* was captured at ground level in the forest. All the anopheline specimens from the subgenus *Nyssorhynchus* infected by *P. vivax* were captured at the margins of the forest.

### Discussion

Neotropical anophelines are classified into five subgenera: *Nyssorhynchus*, *Kerteszia*, *Stethomyia*, *Lophopodomysia*

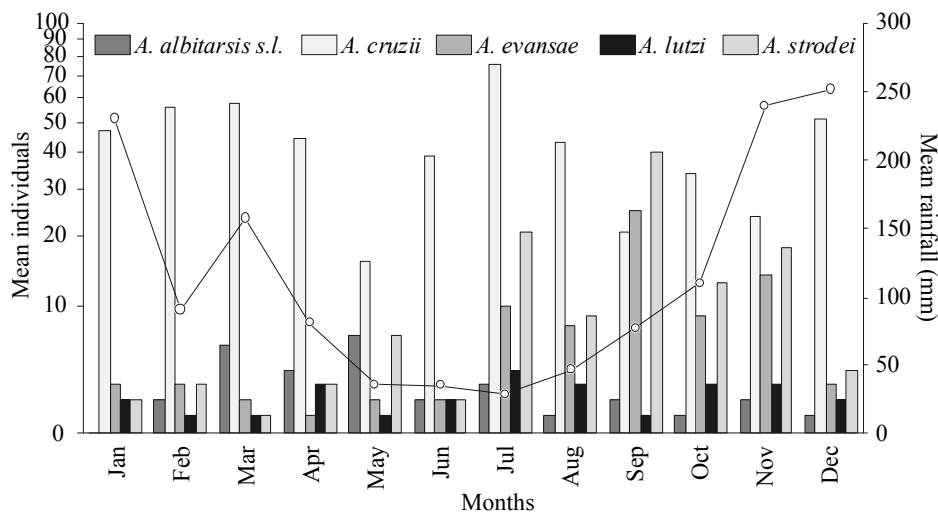


Fig 3 Monthly average of rainfall and monthly frequency of the species most frequently captured in CDC-CO<sub>2</sub> light traps and Shannon traps, from 6:00 P.M. to 6:00 A.M., in Valsugana Velha, Santa Tereza Municipality, ES, from March 2004 to February 2006.

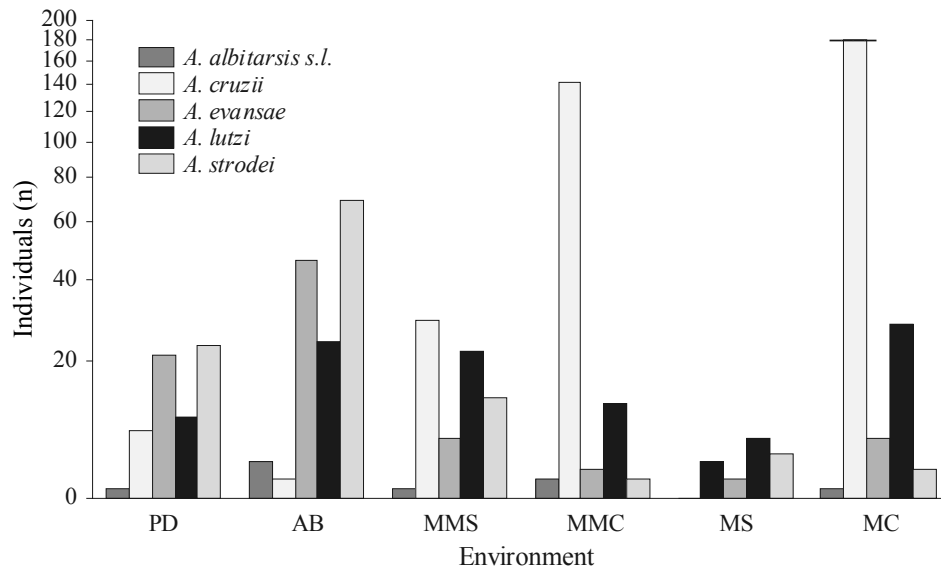


Fig 4 Spatial distribution of the anopheline species most frequently captured in CDC-CO<sub>2</sub> light traps, in the mountainous region of Espírito Santo State from 6:00 P.M. to 6:00 A.M., from March 2004 to February 2006. PD: Peridomicile, AB: Open area, MMS: Ground at the margin of the forest, MMC: Canopy at the margin of the forest, MS: Ground inside the forest, MC: Canopy inside the forest.

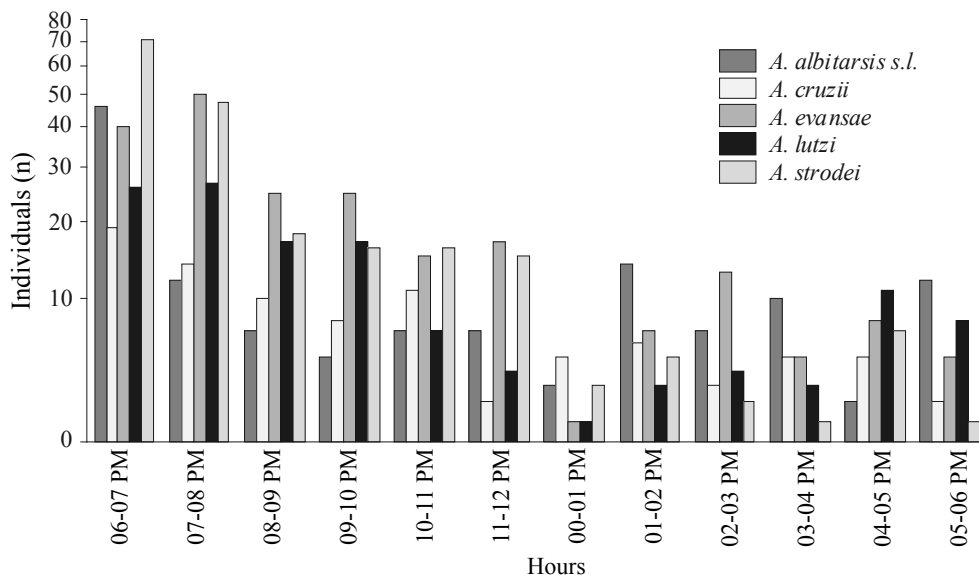


Fig 5 Hourly frequency of the species most frequently captured in Shannon traps in the mountainous region of Espírito Santo State, from 6:00 P.M. to 6:00 A.M., from March 2004 to February 2006.

and *Anopheles* (Faran & Linthicum 1981, Sallum *et al* 1999, Wilkerson & Sallum 1999). In South America, species reported as vectors of human malaria belong to the subgenera *Nyssorhynchus*, *Anopheles* and *Kerteszia* (Deane 1986, Consoli & Lourenço-de-Oliveira 1994). While *Nyssorhynchus* mosquitoes, and *Anopheles* (*N. darlingi* Root in particular, are the main malaria vectors in the Brazilian Amazon (Forattini 1962, Deane 1988, Lourenço-de-Oliveira *et al* 1989), a few species of the subgenera *Anopheles* [*A. mattogrossensis* Lutz & Neiva, *A. peryassui* Dyar & Knab and *A. mediopunctatus s.l.* (Theobald)] are considered less

important vectors (Tadei & Thatcher 2000). *Kerteszia* is a small neotropical subgenus composed of only 12 species (Zavortink 1973) and its geographic distribution extends from Southern Mexico to Southern Brazil (Aragão 1964). Because of the association between *Kerteszia* mosquitoes and the bromeliads that serve as their breeding sites, Down & Pittendrigh (1946) created the term “bromeliad malaria” to describe malaria transmission vectored by *Kerteszia* mosquitoes, thus differentiating it from forest malaria, transmitted by anophelines of the subgenera *Nyssorhynchus* and *Anopheles*, which breed in other water collections.

Table 2 *Plasmodium vivax* specie detected by PCR in the abdomen and thorax of anophelines. The table shows the species, trap type, habitat and time the anophelines were collected in the Valsugana area (fixed point).

Species	Trap/habitat	Hour	Abdomen	Thorax
<i>Anopheles albitarsis</i>	Shanonn/margins of the forest	6:00-7:00 P.M.	X	
<i>A. strodei</i>	Shanonn/margins of the forest	7:00-8:00 P.M.	X	
<i>A. lutzii</i>	Shanonn/margins of the forest	9:00-10:00 P.M.	X	
<i>A. galvaoui</i>	Shanonn/margins of the forest	10:00-11:00 P.M.	X	
<i>A. galvaoui</i>	Shannon/margins of the forest	6:00-7:00 P. M		X
<i>A. parvus</i>	Shannon/margins of the forest	6:00-7:00 P. M.		X
<i>A. cruzii/A. homunculus</i>	Shanonn/margins of the forest	12:00 P.M-1:00 A.M.	X	
<i>A. cruzii/A. homunculus</i>	CDC/canopy at the margin of the forest	6:00 P.M-6:00 A.M.	X	X
<i>A. cruzii/A. homunculus</i>	CDC/canopy inside the forest	6:00 P.M-6:00 A.M.	X	X
<i>A. cruzii/A. homunculus</i>	CDC/ground inside the forest	6:00 P.M.-6:00 A.M.		X

Bromeliad malaria was a public health problem in Brazil during the nineteenth and early twentieth centuries (Gadelha 1994). During that period, the close contact between humans and the Atlantic Forest habitat as a result of population growth, the expansion of urban centers and the construction of railways provided conditions for extensive exposure of humans to mosquito bites and bromeliad-malaria transmission. The introduction of control measures to reduce the number of mosquito breeding sites through deforestation and the elimination of the Bromeliaceae population, together with the use of chemical insecticides and anti-malarial drugs over the years, resulted in a significant reduction in the number of malaria cases, from 40,000 in 1940 to only 71 in 1982 (Deane 1988).

Despite these control measures, malaria has never been eradicated from the states of São Paulo or Espírito Santo in Southeastern Brazil (Ministry of Health, 2005) or Santa Catarina (Machado *et al* 2003) in Southern Brazil.

The results of this entomological survey in Espírito Santo in an area covered by Atlantic Forest with a very low level of transmission of autochthonous malaria revealed a different profile from that of other Brazilian regions covered by the Atlantic Forest and do not support the assumption that *A. (Ker.) cruzii* / *A. (Ker.) homunculus* by itself is responsible for the maintenance of human malaria in these regions.

Given the presence of *A. strodei*, *A. parvus*, *A. evansae* and *A. galvaoui* in mobile capture points, one cannot disregard the co-participation of the subgenus *Nyssorhynchus* in malaria transmission. These species may be involved in malaria transmission in locations where they can be found in higher frequency and where their presence is probably related to environmental modifications that have reduced the number of sources of domestic and wild blood. However, it can be argued that the fact that these species were found in areas of low endemicity or that malaria cases were not as frequent in Espírito Santo as in the Amazon region constitutes evidence against their role in the transmission of the disease. Our finding of *P. vivax* in the thorax of *A. parvus* and *A. galvaoui*, however, reinforces the argument that they are, in fact, acting as vectors in these locations. It is possible that

these *Nyssorhynchus* specimens could have become infected by *Plasmodium* when biting human beings, but the absence of malaria outbreaks leads us to believe that human-to-human transmission is very low.

Several authors have reported *A. cruzii* in low frequency in this region of Espírito Santo (Deane *et al* 1968, Rezende *et al* 2005). We believe that we were successful in capturing this species because CDC-CO<sub>2</sub> traps were used. *A. cruzii* was captured with higher frequency in colder, drier months. This finding could be explained by its adaptation to mountain habitats, like other species in the subgenus *Kerteszia* (Zavortink 1973). The majority, however, were captured inside the forest using CDC-CO<sub>2</sub> traps or during the crepuscular peak using the Shannon trap. Inside the forest, the temperature is milder and there is less variation in weather conditions.

A relationship between rainfall and the maintenance of breeding sites was observed in Valsugana Velha. At this location, one stream close to the margin of the forest dried up in the drier months, limiting the availability of breeding sites for anophelines of the *Nyssorhynchus* and *Anopheles* subgenera in the forest environment. Near the houses, on the contrary, the level of water in the breeding sites remained constant, favoring the persistence of these subgenera close to the human population. A different picture was observed for breeding sites for *A. cruzii* / *A. homunculus*, as the bromeliads continued to hold water despite the dry season.

The diversity of the species captured in Valsugana Velha (fixed point) and in the mobile points was similar, although there were differences in the number and frequency of each species captured. While *A. cruzii* / *A. homunculus* predominated in Valsugana Velha, *A. lutzii* was the most abundant species in the mobile points. CDC-CO<sub>2</sub> traps showed better results in Valsugana Velha than in mobile points, where Shannon traps were more efficient. The different traps were found to influence the species captured, with the best results for *A. cruzii/A. homunculus* being obtained with CDC-CO<sub>2</sub> traps.

A higher species diversity was expected inside the forest (Guimarães *et al* 2000), but the diversity was in fact lower

than that observed near the houses, where there are more human beings, probably because of the availability of food, shelter and breeding sites. Unlike the findings of previous studies in Southeastern Brazil (Deane et al 1984, Deane 1992, Tubaki et al 1993), our results showed a decrease in frequency of *A. cruzii* / *A. homunculus* as the distance from the forest and canopy increased, reflecting their limited domiciliary habits and vertical dispersal. However, other species, such as *A. strodei*, were found to display different behavior, with their frequency increasing near houses and decreasing inside the forest, indicating possible replacement of *A. cruzii* by other species.

*Anopheles cruzii* is frequently considered an acrodendrophilic species (Deane et al 1968, Deane 1992), as it breeds in bromeliads in the canopy and prefers to feed on simian and bird species. It appears that the other species, unlike *A. cruzii* / *A. homunculus*, can adapt more easily and live in areas modified and inhabited by human beings (Deane et al 1984, Guimarães et al 2000, 2003, Forattini 2002). Based on this assumption, our findings may demonstrate the replacement of *A. cruzii* / *A. homunculus* by *A. strodei*, *A. albitarsis* s.l. and *A. evansae*, as the environment is progressively transformed by humans. Nevertheless, as we were still able to find these wild species (*A. cruzii* / *A. homunculus*) in Valsugana Velha and in the mobile points, we conclude that this transformation is not complete. Interestingly, almost 80% of bromeliad-malaria cases in Espírito Santo occur in males (Cerutti et al 2007). In such a scenario, *A. cruzii* could be maintaining malaria transmission in human males, as women do not normally enter the forest. If *A. cruzii* is considered the probable vector in this region, malaria is probably being transmitted as a zoonosis, as this species prefers to remain in the canopy feeding on non-human primates.

The majority of anophelines were captured from 6:00 P.M. to 10:00 P.M., and the nycthemeral activity of *A. albitarsis* s.l., *A. cruzii*, *A. evansae*, *A. luzzi* and *A. strodei* was observed all night long. The risk of contact between vectors and humans increases at the margin of the forest from 6:00 P.M. to 10:00 P.M. during the month of July.

Serological studies revealed a high frequency of antibodies against peptides of the circumsporozoite protein corresponding to *Plasmodium vivax* variants (VK210 and VK247), *P. malariae* / *P. brasilianum* and human *P. vivax*-like / *P. simiovale* in local human populations and in different wild monkey species. Also, PCR analysis revealed that one inhabitant was infected by *P. malariae*, suggesting, once more, transmission in the context of a zoonosis, with monkeys acting as malaria reservoirs (Curado et al 1997, 2006, Duarte et al 2006).

The nycthemeral activity of *A. cruzii* captured in Espírito Santo was different from that of *A. cruzii* reported in other areas. This observation reinforces the hypothesis proposed by several authors that *A. cruzii* is, in fact, a complex of sibling species instead of a monotypical (Ramirez & Dessen 2000, Carvalho-Pinto & Lourenço-de-Oliveira 2004, Malafronte et al 2007). Such possibility was also supported by the initial misclassification of *A. homunculus* as *A. cruzii* in this study (Zavortink 1973, Rosa-Freitas 1998, Forattini 2002).

The few studies that deal with the natural infection of anophelines used different techniques to detect *Plasmodium* species in Brazilian mosquitoes, including immunoassays (Arruda et al 1986, Branquinho et al 1997) and comparison between PCR and immunoassay techniques (Moreno et al 2004). *Anopheles cruzii* is still involved in human and simian malaria transmission in valleys in the Atlantic Forest in the states of São Paulo and Santa Catarina (Carvalho et al 1988, Branquinho et al 1997, Curado et al 1997), and when tested by ELISA were found to be infected with *P. vivax* and one of its variants, *P. vivax* VK247 (Branquinho et al 1997).

In this study, the PCR technique yielded interesting results. In order to detect potential vectors and those that are carrying sporozoites in their salivary glands (and could consequently be considered vectors), the mosquitoes were split into two parts - the thorax and the abdomen. The positive results obtained with tests performed on the thoraxes indicate that *A. cruzii* is not the only species involved in malaria transmission, but that the subgenus *Nyssorhynchus* may also play a role. In fact, it would appear more likely that *A. cruzii* is involved in the transmission of simian malaria, as the majority of the *P. vivax* positive specimens were captured in CDC-CO<sub>2</sub> traps in the canopy (minimum infection rate = 0.5%), whereas *Nyssorhynchus* species were captured in Shannon traps at the margins of the forest around human dwellings. Because there is evidence of identity between *P. vivax* and *P. simium* (Li et al 2001), such a possibility deserves further study.

*A. cruzii* / *A. homunculus* specimens were found infected by *P. falciparum* (data not shown) in this study and it was surprising, as bromeliad malaria has traditionally been related to *P. vivax* infections (Carvalho et al 1988, Curado et al 1997). The fact that these specimens were not captured in the CDC-CO<sub>2</sub> traps in the canopy means that the relationship between *P. falciparum* and simian malaria is less clear-cut. We cannot dismiss the possibility of false positive PCR results, but *P. falciparum* infections were reported in inhabitants of others Atlantic Forest regions of Brazil (Curado et al 2006). The question also arises as to whether this positive result for *P. falciparum* could represent evidence of a modified strain, as *P. falciparum* is always transmitted by anophelines of the *Nyssorhynchus* subgenus. It is possible that transmission by another subgenus could impose some modification on strain behavior (Li et al 2001). Our findings of *P. falciparum* in asymptomatic local inhabitants add to such a possibility, which has been associated by others with the antigenic variability present in several isolates of the parasite (Rich et al 2000, Awadalla et al 2001). These preliminary results point to the need for further studies to help on understanding the transmission cycle of extra-Amazonian malaria infection.

### Acknowledgments

To Agenor, Edmar, Edson, Gustavo, João, Jorge and Mauro for technical support; to Anselmo and Lúcia, who live in the study area; and to FAPESP (grant number 2003/07631-4).

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Received 07/XII/07. Accepted 22/I/09.