

1 **Running Title: Malaria vectors in the Atlantic Forest**

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3 **Ecological characterization and infection of Anophelines (Diptera: Culicidae) of**
4 **the Atlantic Forest in the southeast of Brazil over a 10 year period: Has the**
5 **behaviour of the autochthonous malaria vector changed?**

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26 **ABSTRACT**

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28 In the south and southeast of Brazil, autochthonous malaria cases can be found near
29 Atlantic Forest fragments. The transmission is not totally clarified; thus, the behaviour
30 of the possible vectors in those regions must be observed. An entomological and natural
31 infection study was performed on anophelines (Diptera: Culicidae) captured in the
32 municipalities of the mountainous region of Espírito Santo state in 2004-2005.

33 Similarly, between the years 2014 and 2015, 12 monthly collections were performed at
34 the permanent trapping station of the study mentioned above (Valsugana Velha, Santa
35 Teresa, ES). Light traps with CO₂ (CO₂-baited Center for Disease Control [CDC] traps)
36 were set in open areas, at the edge of the forest (canopy and ground) and inside the
37 forest (canopy and ground), whereas Shannon traps were set on the edge of the forest. A
38 total of 1,414 anophelines were collected from 13 species. *Anopheles (Kerteszia) cruzii*
39 Dyar and Knab remained the most captured species in the CO₂-baited CDC traps set in
40 the forest canopy and was also the vector with the highest prevalence of *Plasmodium*
41 *vivax* infection according to molecular PCR techniques. Regarding mosquitoes of the
42 subgenus *Nyssorhynchus*, *P. vivax* was found only in abdomens, weakening the
43 hypothesis that this subgenus also plays a role in malaria transmission in this specific
44 region.

45 **Keywords:** Malaria, *Plasmodium vivax*, *Anopheles*.

46

47 **Sponsorship:** Espírito Santo Research Foundation (Fundação de Amparo à Pesquisa e
48 Inovação do Espírito Santo – FAPES).

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51 **INTRODUCTION**

52 Despite being a highly prevalent disease in the Amazon region, malaria remains residual
53 in Atlantic Forests of the south and southeast regions of Brazil. In this region, the
54 disease is known as bromeliad-malaria since the vectors of the genus *Anopheles*
55 reproduce in the whorls of *Bromeliaceae*, which are typical plants of this biome (Downs
56 & Pittendrigh, 1946). The presence of a few autochthonous (sometimes asymptomatic)
57 human cases, the spatial distance between the cases and the low parasitemia by
58 microscopy weaken the possibility of the traditional transmission chain. It is believed
59 that the cycle of *Plasmodium spp.* is maintained by reservoirs of the parasite, in the
60 forest or in the rural population, represented by both apes and humans, asymptomatic or
61 not. Studies have been conducted to clarify these beliefs (Curado et al., 1997; Duarte et
62 al., 2006; Cerutti et al., 2007; Rezende et al., 2009; Meneguzzi et al., 2009). Regarding
63 the vector, in the state of Espírito Santo, Brazil, 26 species of *Anopheles sp.* have been
64 identified, indicating an abundance of anophelines (Coutinho, 1947; Andrade&Brandão,
65 1957; Ferreira et al., 1964; Natal, 2007; Sallum, 2008; Rezende et al. 2009; Meneguzzi
66 et al. 2009; Silva et al., 2013). The vectors of greater vectorial capacity and competence
67 belong to the subgenus *Kerteszia*, mainly *Anopheles cruzii*, in the south and southeast
68 Brazilian states (Meneguzzi et al., 2009). However, species of anophelines involved in
69 the dynamics of malaria transmission outside the Amazon region vary according to
70 environmental and epidemiological conditions (Pina-Costa et al., 2014). The
71 autochthonous cases of extra-Amazonian malaria are found in mild mountainous
72 regions and are associated with agricultural activities near the forest that are performed
73 by young men (Cerutti Jr et al., 2007). States such as São Paulo, Santa Catarina and
74 Espírito Santo are covered by dense Atlantic Forest regions (IESB, 2007). Among them,
75 Espírito Santo has recorded the largest number of bromeliad-malaria cases in recent

76 years. The biome in question is very humid, with abundant rainfall and vegetation that
77 favours the reproduction of the anophelines. In addition, bromeliad-malaria is beginning
78 to be used as a biological marker of human activities within these forests (Gomes, 1985;
79 Rezende et al., 2013). Rezende et al. (2009) found anopheline behaviour similar to that
80 expected, based on the literature, in Valsugana Velha, Santa Teresa municipality: a high
81 prevalence of *A. cruzii* was reported in a mountainous region of the Atlantic Forest, in
82 an endemic area of the disease in the state of Espírito Santo. However, considering the
83 hypothesis of a typical transmission model of zoonosis in this scenario, this model
84 would require a stable and constant vectorial behaviour. Such behaviour, therefore, must
85 be re-evaluated to establish transmission characteristics over the years.

86

87 MATERIALS AND METHODS

88 **Description of the study site.** The study was performed in the rural area of the
89 municipality of Santa Teresa, which is located approximately 78 km from Vitória, in the
90 state of Espírito Santo (ES), Brazil. The permanent trapping station is in Valsugana
91 Velha ($19^{\circ}57'58.4\text{''S}$, $40^{\circ}34'45.2\text{''W}$), where cases of residual malaria from Atlantic
92 Forest systems were recorded in the study by Cerutti Jr. et al. (2007) (Figure 1).

93

94 [Figure 1. Political map of the municipality of Santa Teresa, highlighting the rural
95 community where the collections were performed. Source: Instituto Jones dos Santos
96 Neves, 2013.]

97

98 **Collection of anophelines.** Hourly collections of anophelines were conducted at a
99 permanent trapping station located in the bromeliad-malaria transmission area. These

100 collections were performed one day each month for one year, from June 2014 to May
101 2015, totalling 12 collections. Two capture methods were used:

- 102 • Center for Disease Control (CDC) light traps with CO₂ (Gomes, 1985) that were
103 set in open areas (peridomestic environment = PD), at the edge of the forest
104 (canopy and ground) and inside the forest (canopy and ground) and
- 105 • Shannon traps (Bustamante, 1951), set at the edge of the forest.

106 The five CDC traps were set simultaneously, two of them at 15 metres from the ground
107 in the canopy (on the edge and inside the forest), two at one metre from the ground (at
108 the edge and inside the forest), and one at the border between the forest and the area
109 close to the dwellings. The collections lasted for 12 hours, with the traps placed at night
110 (6:00 PM) and removed in the morning (6:00 AM). For the Shannon traps, the insects
111 were captured during the first 4 hours after sunset (6:00 PM to 10:00 PM) each month.

112 **Storage and identification of insects.** The specimens were stored in tubes containing
113 isopropanol and later identified using the identification keys proposed by
114 Consoli&Lourenço-de-Oliveira (1994). The identification was made by a team from the
115 Entomology and Malacology Centre of Espírito Santo (Núcleo de Entomologia e
116 Malacologia do Espírito Santo - NEMES/ES).

117 **Molecular techniques.** The DNA for the detection of *Plasmodium* sp. was obtained
118 from the thorax, abdomen or entire mosquito of the mosquito groupings in pools
119 (maximum of 10 samples/pool), depending on the subgenus of the specimens. Those of
120 the subgenus *Nyssorhynchus* were sectioned, whereas those of the subgenus *Kerteszia*
121 were processed in whole. The same extraction kit (DNeasy Blood & Tissue Kit, Qiagen,
122 Germany) was used, following the manufacturer's instructions. Each pool included
123 females of the same species, collected in the same type of trap, on the same date. The

124 presence of *P. vivax* or *P. malariae* in the pools was detected using the nested-PCR
125 protocol described by Kimura et al. (1997) and modified by Win et al. (2002). The
126 target was the gene encoding the 18S ribosomal subunit. The amplification products
127 were analysed by electrophoresis in 2% agarose gel under ultraviolet light. In positive
128 cases, 100-bp fragments were amplified.

129 **Statistical analysis.** To determine the importance and distribution of the various
130 *Anopheles* species, analyses of diversity, dominance and abundance were performed
131 using Shannon's diversity index (H') and Simpson's dominance index (D). In the
132 various comparisons, the level of significance was set at 5%. Bivariate Spearman
133 correlation calculations were used to determine the relationship between anopheline
134 capture, temperature and rainfall (data provided by the Capixaba Institute of Research,
135 Technical Assistance and Rural Extension [Instituto Capixaba de Pesquisa, Assistência
136 Técnica e Extensão Rural - INCAPER]), also at a level of 5%.

137

138 ETHICAL STANDARDS

139 During the collection process, no harm was inflicted to the environment. The team
140 members wore long clothing, gloves and hats with head nets to avoid anopheline
141 mosquito bites. Boots were also used to prevent bites by venomous animals. A license
142 to capture arthropod insects was obtained via the SISBIO/Chico Mendes Institute
143 (ICMBio/IBAMA/MMA) under number 19227-1.

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145 RESULTS

146 A total of 1,414 specimens were captured, resulting in a set of 13 species. The largest
147 number of specimens was collected in April 2015 (341) and the smallest number of

148 specimens in March 2015 (05). In all collections, there was a predominance of
149 *Anopheles (K.) cruzii*, totalling 1,044 of the 1,414 mosquitoes captured (Table 1). The
150 trap in which most mosquitoes were captured was the CO₂-baited CDC trap placed in
151 the canopy, in which *A. cruzii* was also predominant (Graph 1).

152

153 [Graph 1. Percentage of species captured per trap, from June 2014 to May 2015, at the
154 permanent trapping station in Santa Teresa, ES.]

155

156 **Climate.** The months of November 2014, September 2014 and April 2015 showed the
157 highest capture frequencies (Graph 2). There was no simultaneous capture of all species
158 in the monthly collections, and all species were absent for at least 1 month during the
159 total collection period. *A. cruzii* was not captured only in June 2014 and March 2015
160 (Graph 2). In fact, those were the only months in which *A. cruzii* ceased to be the
161 predominant species and was replaced with *A. strolei* and *A. triannulatus*, respectively.

162

163 [Graph 2. Species captured each collection month, from June 2014 to May 2015, at the
164 permanent trapping station of Santa Teresa, ES.]

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166 A negative trend was observed for the correlation between the number of anophelines of
167 the subgenus *Nyssorhynchus* and the temperature, although without statistical
168 significance ($r = -0.04$, $p = 0.89$). Additionally, a positive trend was observed for the
169 correlation between the number of anophelines and rainfall ($r = 0.17$, $p = 0.59$) but also
170 with no statistical significance. For the subgenus *Kerteszia*, the correlation between
171 capture frequency and temperature ($r = -0.004$, $p = 0.99$) or rainfall ($r = -0.13$, $p = 0.68$)

172 revealed negative coefficients but with no statistical significance. The monthly average
173 temperature and the rainfall in Valsugana Velha are shown in Graph 3.

174

175 [Graph 3. Temperature and rainfall data in Santa Teresa, ES, between June 2014 and
176 May 2015.]

177

178 **Spatial distribution.** *A. strolei* and *A. triannulatus* were more frequently captured in
179 the Shannon traps, and *A. cruzii* was captured more frequently in the CO₂-baited CDC
180 traps (Graph 1) located in the tree canopy. Simpson's dominance index (D) reveals that
181 dominance in the Shannon trap (D1 = 0.227) was greater than the dominance of
182 individuals collected in the CO₂-baited CDC trap (D2 = 0.172) (p < 0.02), both at the
183 forest edge.

184 Based on Shannon's diversity index (H'), the diversity of anophelines collected in the
185 Shannon trap (H'2 = 1.866) was higher compared to that of the anophelines collected in
186 the CDC trap at the forest edge, near the dwellings (H'1 = 1.734) (p = 0.004). *Anopheles*
187 *strolei* was captured in larger numbers at forest edge areas, followed by *A. triannulatus*.
188 *A. lanei* and *A. rangeli* were captured only in the CO₂-baited CDC trap located near the
189 dwellings, whereas *A. homunculus* was only captured in the CDC traps set in the tree
190 canopy.

191 **Natural infection rates** (Table 2). Of the total pool of specimens investigated, 13 were
192 positive for *Plasmodium vivax* (Figure 2). Of these, 10 belonged to the species *A. cruzii*,
193 and three were abdomens that belonged to specimens of the subgenus *Nyssorhynchus*.
194 As shown in Table 2, most of the infected mosquitoes were *A. (K.) cruzii*, captured in
195 CO₂-baited CDC traps located in the tree canopy. Graph 4 shows the monthly

196 distribution of captured mosquitoes in which *P. vivax* was found. *P. malariae* infection
197 was not detected.

198

199 [Figure 2. Ethidium bromide-stained agarose gel, photographed using the Alpha Imager
200 software, with 13 samples of pools of *P. vivax*-positive anopheline females. Source:
201 Buery JC and Natal L, 2016.]

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203 [Graph 4. Chronological distribution of *P. vivax*-infected anopheline females captured
204 in the permanent trapping station in Santa Teresa, ES.]

205

206 DISCUSSION

207 Bromeliad malaria became more evident in the Brazilian south and southeast Atlantic
208 forest regions after control measures implemented by the government eliminated
209 transmission in flat areas exploited by humans (Ministério da Saúde (BR), 2015; Barata,
210 1998). As residual foci remained in the eradication areas, bromeliad-malaria or malaria
211 of Atlantic Forest systems began to be studied with more intensity in the 1980s and
212 1990s (Duarte et al., 2013). With a more consistent understanding of vector ecology and
213 outbreak epidemiology, more evidence has emerged in favour of behaviour consistent
214 with a zoonosis. The zoonosis hypothesis presupposes the concomitant existence of
215 simian malaria. Such an existence has been proven in the past (Duarte et al., 2008), but
216 its prospective evaluation demands extremely complex logistics. Therefore, the
217 evaluation of vector behaviour favourable to enzootic transmission is more feasible and
218 may provide indirect evidence of the presence of the infection among monkeys and of
219 its source of occurrence in human cases. Given the need for stable and constant vector
220 behaviour for the maintenance of simian malaria, follow-up studies of the endemic

221 regions are necessary. In addition, according to Marrelli et al. (2007), the entire Atlantic
222 Forest territory that survived deforestation must be carefully monitored due to
223 environmental changes and the possibility of maintaining the infection cycle given the
224 high number of asymptomatic individuals who can act as reservoirs of the parasite in
225 those regions.

226 In the present study, *A. cruzii* still prevailed as the main vector found at the Valsugana
227 Velha trapping station. Between 2004 and 2005 (Rezende et al. 2009), 61.2% of the
228 2,290 mosquitoes collected belonged to this species. In 2014 and 2015, 73.8% of the
229 1,414 anophelines captured were *A. cruzii*. Therefore, an increase in the proportion of
230 the main vector of bromeliad malaria was observed in the local fauna. Older studies
231 such as those of Deane (1988) and more recent studies such as those of Duarte et al.
232 (2013), Neves et al. (2013) and Kirchgatter et al. (2014) have previously demonstrated
233 the magnitude of the presence of this species in Atlantic Forest regions with preserved
234 native forest.

235 The distribution of *A. cruzii* when moving from the inside to the edge of the forest has
236 also remained the same over time. As Rezende et al. (2009) described in 2004 and 2005,
237 the anophelines of this species appeared mostly in the tree canopy inside the forest,
238 whereas the numbers decreased drastically in the traps that were set closer to the forest
239 edge and the dwellings. The anopheline fauna was dominated by species such as *A.*
240 *strophei* and *A. triannulatus* closer to human-occupied areas. In São Paulo, between 2009
241 and 2011, this trend was also detected when comparing anthropic and wild areas in the
242 town of Parelheiros. There, 438 *A. cruzii* were recorded in a certain anthropic area and
243 4,832 in the wild area (Duarte et al., 2013).

244 The absolute predominance of *A. cruzii* in the canopy, observed in the present study,
245 indicates a non-synanthropic behaviour (Forattini et al., 1990) and reinforces its role in
246 the transmission of simian malaria. However, despite being present mostly in the
247 canopy, some specimens were found in the CO₂-baited CDC traps set in the ground and
248 in the Shannon traps, which points to the possibility of these vectors descending from
249 their preferential site in the canopy, at which time they could incidentally transmit the
250 parasites to humans. The presence of these specimens reinforces the hypothesis that
251 bromeliad-malaria is maintained in the region by means of simian infection. The fact
252 that humans are in the forest and that *A. cruzii* comes down from the canopy to feed
253 creates the conditions for *Plasmodium spp.* originating from the monkeys in the canopy
254 to infect humans.

255 The morphometric diagnosis studies of Lorenz et al. (2012) showed a differentiation
256 between *A. cruzii* and *A. homunculus* not predicted in the study of Rezende et al. (2009).
257 However, only 39 *A. homunculus* were captured during the collection period between
258 2014 and 2015, against 1,045 duly identified *A. cruzii*.

259 Unexpectedly, the season during which most mosquitoes were captured was not the
260 summer. Interestingly, the seasons with milder weather had the largest capture rates. For
261 example, the most successful captures occurred in the months of September 2014 and
262 April 2015. This result corroborates the study of Rezende et al. (2009), who suggested
263 the adaptation of anophelines to mild environments. There was also a high number of
264 specimens collected in November 2014. The mean rainfall at that time was the highest
265 of that year (mean of 207.6 mm/month), and the temperature was mild on collection day
266 (ranging from 19.7 to 21.3 °C). That month, the "rain" factor may have triggered an
267 increase in the mosquito population.

268 In this study, 13 pools of mosquitoes were positive for *P. vivax*. In 2004 and 2005, 10
269 pools of anophelines infected by the same species of the parasite were obtained
270 (Rezende et al., 2009). However, unlike 10 years ago, the PCR reactions for the 2014
271 and 2015 collections did not show infection in the thorax of mosquitoes of the subgenus
272 *Nyssorhynchus*. The infective form should reach the salivary gland of the anopheline for
273 infection to occur. Thus, the separation into thorax and abdomen during the experiments
274 reinforces evidence of the participation of other vector species in the transmission chain.
275 Because *Kerteszia* was a subgenus of a known vector, there was no separation of the
276 body to perform the experiments, as occurred in mosquitoes of the subgenus
277 *Nyssorhynchus*, to assess the possibility that these mosquitoes participate in the
278 transmission chain. Since no infection was detected in the *Nyssorhynchus* thoraxes,
279 unlike 10 years ago, *Nyssorhynchus* may have stopped playing the role of secondary
280 vector and *A. cruzii* may currently be the only bromeliad-malaria vector in this area. The
281 progressive exploitation of the rural and forest environment by the local inhabitants may
282 have led to greater spatial distances between the transmission events and the anthropic
283 environment, where *Nyssorhynchus* has greater dominance. In these conditions, given
284 the *A. cruzii* dominance, *Nyssorhynchus* does not have the opportunity to become
285 infected. Humans venture into the forest to clean the river springs or to gather firewood
286 and thus acquire the disease. Once they return to their homes, they likely infect the
287 *Nyssorhynchus* near their dwellings.

288 Infected *A. cruzii* were collected in CO₂-baited CDC traps in the tree canopy inside the
289 forest and in one CO₂-baited CDC trap located near the ground, at a height of one metre.
290 This fact reinforces the idea that infections can occur in both habitats as a result of the
291 acrodendrophilic behaviour with eventual descent to lower heights, where they can feed

292 on non-usual hosts. This fact also reinforces the possibility of the disease being a
293 zoonosis in regions such as Valsugana Velha, in Santa Teresa, ES.

294 Regarding the Shannon's dominance index (H'), there was greater dominance in the
295 Shannon trap than in the CO₂-baited CDC trap, both at the forest edge.

296 Regarding Simpson's diversity index (D), the diversity of anophelines collected with
297 the Shannon trap was also higher than that observed in the CO₂-baited CDC trap in the
298 same habitat. The placement of the trap near the body of water, where there were
299 *Nyssorhynchus* breeding sites, would justify the greater diversity.

300 These data corroborate a study conducted in the same region and published in 2013
301 (Rezende et al., 2013), when greater dominance and diversity of anophelines were
302 observed in an anthropic environment with the presence of malaria. These findings
303 reinforce the role of human occupation in the determination of both anopheline
304 distribution and behaviour since both indices show higher values in collections
305 performed in an environment closer to human dwellings.

306 The study shows that there was little change in vector behaviour in the region studied.
307 *A. cruzii* remains the most infected anopheline in Valsugana Velha, and mosquitoes of
308 the subgenus *Nyssorhynchus* do not appear to participate in the transmission chain. The
309 acrodendrophilic behaviour of *A. cruzii*, particularly those infected, reinforces the
310 hypothesis that the presence of *P. vivax* in these specimens arises from blood feeding in
311 animals that live in the tree canopy, such as simians.
312

313 **ACKNOWLEDGEMENTS**

314 We would like to thank the State Health Department of Espírito Santo and the
315 Entomology and Malacology Centre of Espírito Santo/SESA for the logistical support

316 and supply of equipment in the field; Claudiney Biral dos Santos for technical support;
317 Filomena E C Aguiar, Creuza Rachel Vicente, Marcelo Urbano Ferreira, Priscila
318 Thihara Rodrigues and Lais Camoese Salla for the critical reading of the manuscript;
319 the owners of the Recanto da Preguiça farm for allowing sampling at that site; and the
320 Capixaba Institute of Research, Technical Assistance, and Rural Extension
321 (INCAPER/ES) for providing the rainfall and temperature data.

322

323 **FINANCIAL SUPPORT**

324 This work was supported by the Espírito Santo Research Foundation (Fundação de
325 Amparo à Pesquisa e Inovação do Espírito Santo - FAPES) and by the Research
326 Program for Unified Health System of Brazilian Ministry of Health (Programa de
327 Pesquisa para o Sistema Único de Saúde do Ministério da Saúde) (Grant number
328 65834119/2014).

329

330 **CONFLICTS OF INTEREST**

331 None.

332

333 **REFERENCES**

334

335 Andrade RM, Brandão H. Contribuição para o conhecimento da fauna de anofelinos
336 do estado do Espírito Santo: área de distribuição e incidência das espécies por
337 cidades, vilas e povoados. Rev Bras Malariol Doenças Trop. 1957; 9: 391-403.

338

339 Barata RB. Malária e seu controle. São Paulo: Editora Hucitec; 1998.

340

- 341 Bustamante FM. Shannon dawn trap: its use in the verification of the durability of
342 residual toxic effects of insecticides. Folha Med . 1951; 32(8):53-55.
- 343
- 344 Cerutti Jr C, Boulos M, Coutinho AF, Hatab M do CLD, Falqueto A, Rezende HR,
345 et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low
346 incidence in Brazil. Malar J. 2007; 6:33.
- 347
- 348 Consoli RAGB, Oliveira RL. Principais mosquitos de importância sanitária no
349 Brasil. Cad Saúde Pública. 1994; 11: 228 p.
- 350
- 351 Coutinho JO. Contribuição para o estudo da distribuição geográfica dos anofelinos
352 do Brasil (dissertação). São Paulo, SP, Brasil: Faculdade de Medicina da
353 Universidade de São Paulo; 1947.
- 354
- 355 Curado I, Duarte AMRC, Lal AA, Oliveira SG, Kloetzel JK. Antibodies anti
356 Bloodstream and Circumsporozoite Antigens (*Plasmodium vivax* and *Plasmodium*
357 *malariae/P. brasiliense*) in Areas of Very Low Malaria Endemicity in Brazil. Mem
358 Inst Oswaldo Cruz. 1997; 92(2):235–43.
- 359
- 360 Deane LM. Malaria studies and control in Brazil. Am J Trop Med Hyg. 1988;
361 38(2):223-230.
- 362 Downs WG, Pittendrigh CS. Bromelian malaria in Trinidad, British West Indies;
363 Am J Trop Med. 1946; 26: 47-66.
- 364

- 365 Duarte AMRC, Porto MAL, Curado I, Malafronte RS, Hoffman EHE, Oliveira SG
366 *et al.* Widespread occurrence of antibodies against Circumsporozoite protein and
367 against blood forms of *Plasmodium vivax*, *P. falciparum* and *P. malariae* in
368 Brazilian wild monkeys. Int J Primatol. 2006; 35: 87-96.
369
370 Duarte AM, Malafronte RS, Cerutti C Jr, Curado I, Paiva BR, Maeda AY et al.
371 Natural Plasmodium infections in Brazilian wild monkeys: reservoirs for human
372 infections? Acta Trop. 2008; 07:179–85.
373
374 Duarte AM, Pereira DM, de Paula MB, Fernandes A, Urbinatti PR, Ribeiro AF, et
375 al. Natural infection in anopheline species and its implications for autochthonous
376 malaria in the Atlantic Forest in Brazil. Parasit Vectors. 2013; 6:58
377
378 Ferreira E. Distribuição geográfica dos anofelinos no Brasil e sua relação com o
379 estado atual da erradicação da malária. Rev Bras Malariol Doenças Trop. 1964; 16:
380 329-48.
381
382 Forattini OP, Kakitani I, Massad E, Marucc D. Studies on mosquitoes (Diptera:
383 Culicidae) and anthropic environment. 11 - Biting activity and blood-seeking parity
384 of Anopheles (Kerteszia) in South-Eastern Brazil. Rev Saude Publica. 1990; 24(2):
385 107-14.

386 Gomes AC, Rabello EX, Natal D. A new collecting chamber for a CDC-miniature
387 trap. Rev Saude Publica. 1985; 19(2):190-91.

388 Instituto de Estudos Socioambientais do Sul da Bahia (IESB). Projeto de
389 conservação e utilização sustentável da diversidade biológica brasileira – PROBIO.
390 Levantamento da Cobertura Vegetal Nativa do Bioma Mata Atlântica. Rio de
391 Janeiro: Instituto de Geociências da Universidade Federal do Rio de Janeiro, 2007.
392
393 Kimura M, Kaneko O, Liuc Q, Zhouc M, Kawamotoc F, Watayad Y, et al.
394 Identification of the four species of human malaria parasites by nested PCR that
395 targets variant sequences in the small subunit rRNA gene. Parasitol Int. 1997;
396 46:91-95.

397 Kirchgatter K, Tubaki RM, Malafronte RM, Alves IC, Lima GFMC, Guimarães LO
398 et al. *Anopheles (Kerteszia) cruzii* (Diptera: Culicidae) in peridomiciliary area
399 during asymptomatic malaria transmission in the Atlantic Forest: molecular
400 identification of blood-meal sources indicates humans as primary intermediate
401 hosts. Rev Inst Med Trop São Paulo. 2014; 56(5):403-09.

402 Lorenz C, Marques T, Sallum MA, Suesdek L. Morphometrical diagnosis of the
403 malaria vectors *Anopheles cruzii*, *An. homunculus* and *An. bellator*. Parasit Vectors.
404 2012; 5(1):257.

405 Marrelli MT, Malafronte RS, Sallum MAM, Natal D. Kerteszia subgenus of
406 Anopheles associated with the Brazilian Atlantic rainforest: current knowledge and
407 future challenges. Malar J .2007; 6:127.
408

- 409 Meneguzzi VC, Santos CB, Pinto IS, Feitoza LR, Feitoza HN, Falqueto A. Use of
410 geoprocessing to determine malaria risk areas and evaluation of the vectorial
411 importance of anopheline mosquitoes (Diptera: Culicidae) in Espírito Santo, Brazil.
412 Mem Inst Oswaldo Cruz. 2009; 104: 570-75.
- 413
- 414 Ministério da Saúde (BR). Ministério da Saúde lança Plano de Eliminação da
415 malária no Brasil. Brasília: Ministério da Saúde, 2015.
- 416
- 417 Natal D. First record of *Anopheles (Anopheles) costai* Fonseca & Ramos, 1939 in
418 Espírito Santo State. Rev Inst Med Trop São Paulo. 2007; 49(5):323-26.
- 419
- 420 Neves A, Urbinatti PR, Malafronte RS, Fernandes A, Paganini WS, Natal D.
421 Malaria outside the Amazon region: Natural Plasmodium infection in anophelines
422 collected near an indigenous village in the Vale do Rio Branco, Itanhaém, SP,
423 Brazil. Acta Trop. 2013; 125: 102-06.
- 424
- 425 Pina-Costa A, Brasil P, Santi S MD, de Araujo MP, Suárez-Mutis MC, Santelli AC,
426 et al. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem
427 Inst Oswaldo Cruz. 2014; 109(5):618-33.
- 428
- 429 Rezende HR, Soares RM, Cerutti C, Alves IC, Natal D, Urbinatti PR, et al.
430 Entomological characterization and natural infection of anophelines in an area of the
431 Atlantic Forest with autochthonous malaria cases in mountainous region of Espírito
432 Santo State, Brazil. Neotrop Entomol. 2009; 38(2):272–80.
- 433

- 434 Rezende HR, Falqueto A, Urbinatti PR, De Menezes RM, Natal D, Cerutti C Jr.
- 435 Comparative study of distribution of anopheline vectors (Diptera: Culicidae) in
- 436 areas with and without malaria transmission in the highlands of an extra-Amazonian
- 437 region in Brazil. J Med Entomol. 2013; 50(3):598-602.
- 438
- 439 Sallum MAM. Primeiro registro de *Anopheles (Kerteszia) homunculus* Komp
- 440 (Diptera, Culicidae) no Estado do Espírito Santo, Brasil. Rev Bras Entomol. 2008;
- 441 52: 671-73.
- 442
- 443 Silva KS, Pinto IS, Leite GR, Virgens TM, Santos CB, Falqueto A. Ecology of
- 444 Anopheline Mosquitoes (Diptera: Culicidae) in the Central Atlantic Forest
- 445 Biodiversity Corridor, Southeastern Brazil. J Med Entomol. 2013; 50(1):24-30.
- 446
- 447 Win TT, Lin K, Mizuno S, Zhou M, Liu Q, Ferreira MU, et al. Wide distribution of
- 448 *Plasmodium ovale* in Myanmar. Trop Med Int Health. 2002; 7(3):231–39.
- 449
- 450
- 451
- 452
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454 **TABLES**

455 **Table 1.** Percentage of anopheline species found between June 2014 and May 2015 at
456 the permanent trapping station in Santa Teresa, ES.

Species	Number	[%]
<i>A. (K) cruzii</i>	1044	73.8
<i>A. (N) strodei</i>	103	7.3
<i>A. (N) triannulatus</i>	84	6.0
<i>A. (N) evansae</i>	52	3.7
<i>A. (K) homunculus</i>	39	2.7
<i>A. (N) galvaoi</i>	29	2.0
<i>A. (N) lutzii</i>	21	1.5
<i>A. (N) albitarsis</i>	17	1.2
<i>A. (N) argyritarsis</i>	09	0.6
<i>A. (N) parvus</i>	07	0.5
<i>A. (A). mediopunctatus</i>	05	0.4
<i>A. (N) rangeli</i>	03	0.2
<i>A. (N) lanei</i>	01	0.1
TOTAL	1414	100

457 **Table 2.** Species, traps and collection dates for *P. vivax*-positive mosquitoes at the
458 permanent trapping season in Santa Teresa, ES.

Sample	Species	Trap	Date
260a*	<i>A. (N.) lutzii</i>	Shannon	September 2014
279	<i>A. (K.) cruzii</i>	CDC canopy	August 2014
343	<i>A. (K.) cruzii</i>	CDC canopy	August 2014
479	<i>A. (K.) cruzii</i>	CDC canopy	October 2014
485	<i>A. (K.) cruzii</i>	CDC canopy	October 2014
494a*	<i>A. (N.) evansae</i>	Shannon	October 2014
632	<i>A. (K.) cruzii</i>	CDC ground	November 2014
633	<i>A. (K.) cruzii</i>	CDC canopy	November 2014
868	<i>A. (K.) cruzii</i>	CDC canopy	February 2015
1142	<i>A. (K.) cruzii</i>	CDC canopy	April 2015
1144	<i>A. (K.) cruzii</i>	CDC canopy	April 2015
1248	<i>A. (K.) cruzii</i>	CDC canopy	April 2015
1294a*	<i>A. (N.) strolei</i>	CDC near dwellings	May 2015

459 *a = abdomen.

460

461 **FIGURES**

462 **Figure 1.** Political map of the municipality of Santa Teresa, highlighting the rural
463 community where the collections were performed. Source: Instituto Jones dos Santos
464 Neves, 2013.

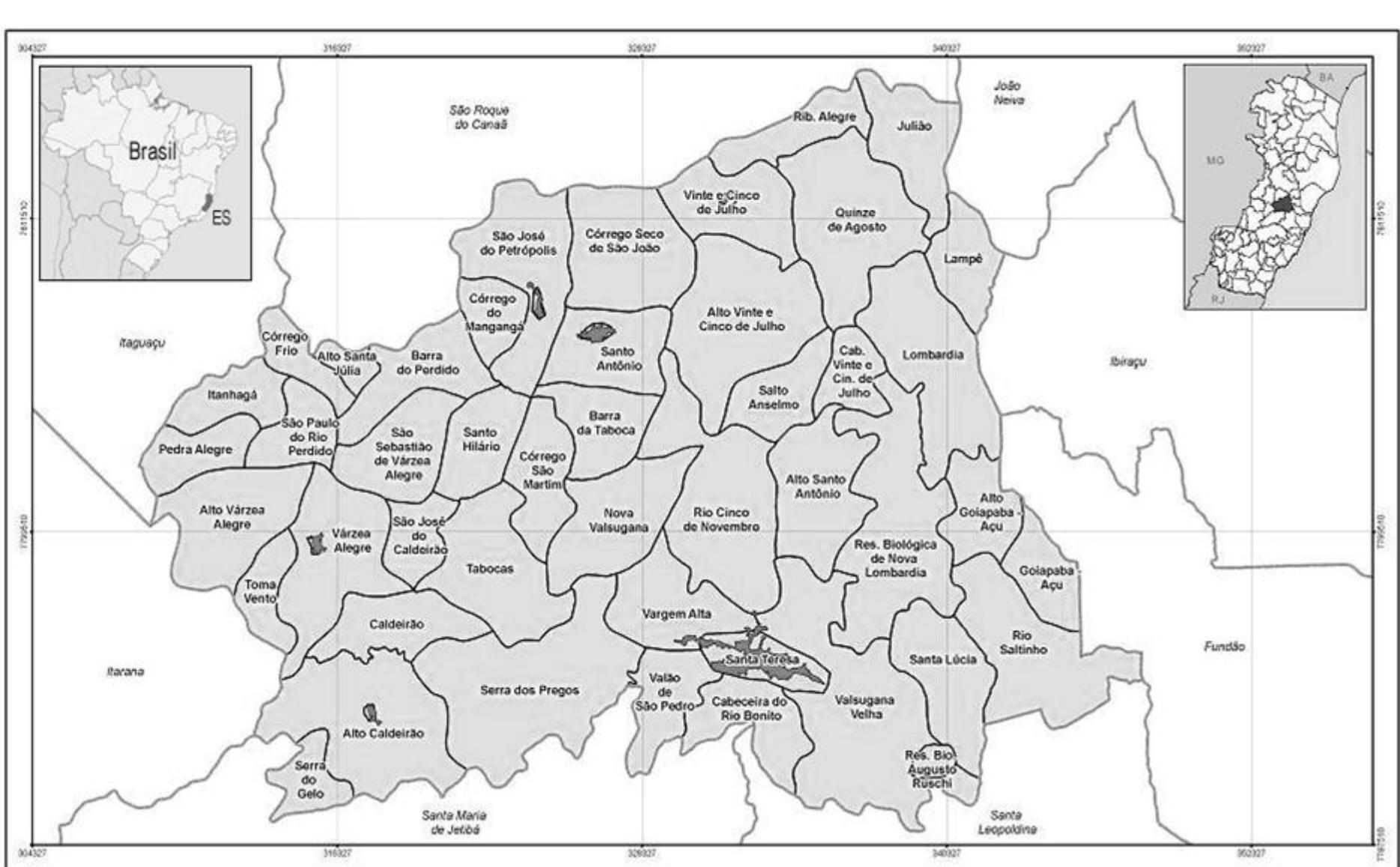
465 **Figure 2.** Ethidium bromide-stained agarose gel, photographed using the Alpha Imager
466 software, with 13 samples of pools of *P. vivax*-positive anopheline females. Source:
467 Buery JC and Natal L, 2016.

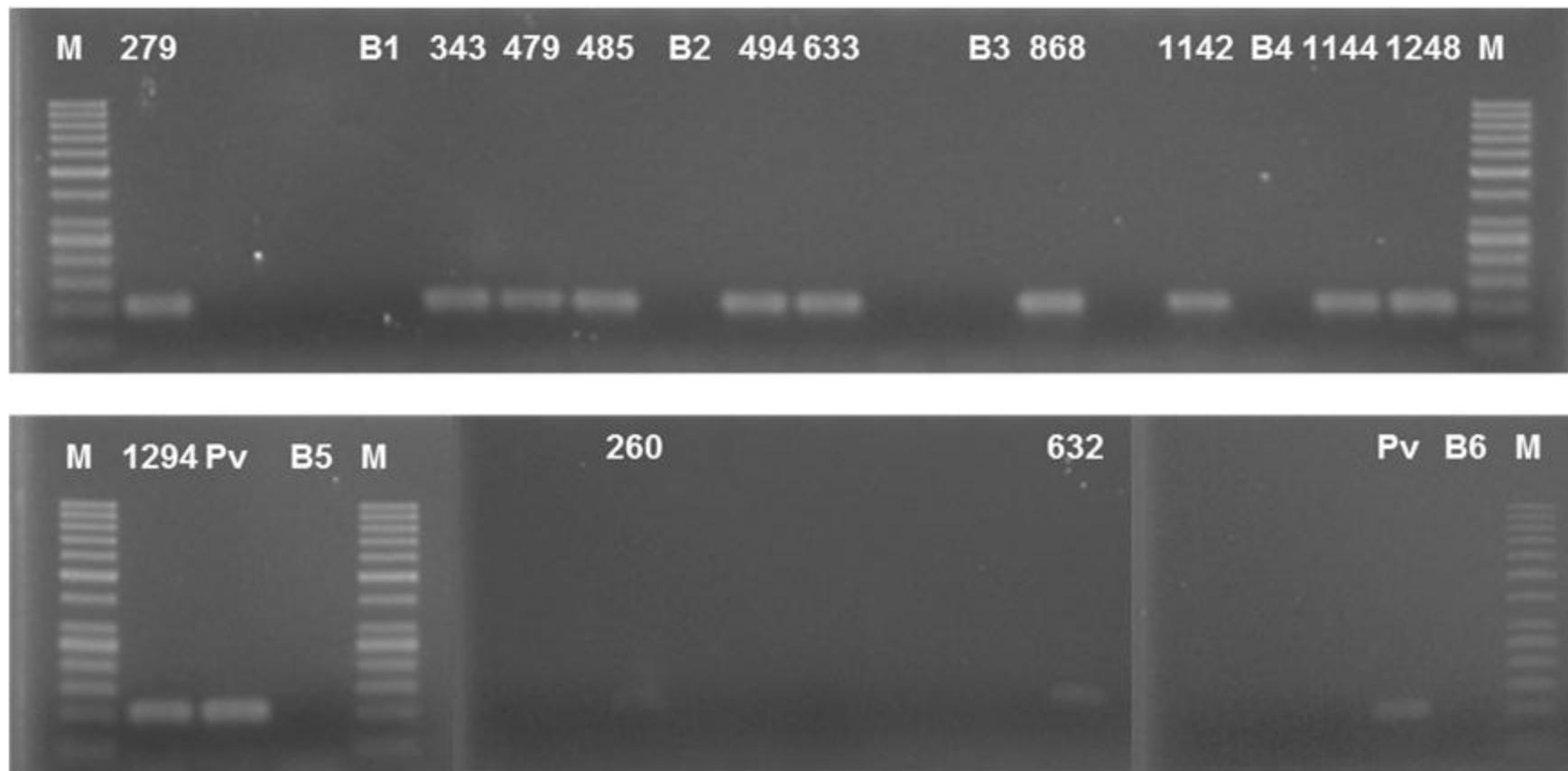
468 **Graph 1.** Percentage of species captured per trap, from June 2014 to May 2015, at the
469 permanent trapping station in Santa Teresa, ES.

470 **Graph 2.** Species captured each collection month, from June 2014 to May 2015, at the
471 permanent trapping station of Santa Teresa, ES.

472 **Graph 3.** Temperature and rainfall data in Santa Teresa, ES, between June 2014 and
473 May 2015.

474 **Graph 4.** Chronological distribution of *P. vivax*-infected anopheline females captured
475 in the permanent trapping station in Santa Teresa, ES.





M: 50 bp molecular weight markers; **B1, B2, B3, B4, B5 and B6:** negative controls (blanks); **Pv:** positive controls for *Plasmodium vivax* (100 bp bands)

