Parasites & Vectors

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# Ecological aspects of Phlebotomines (Diptera: Psychodidae) and the transmission of American cutaneous leishmaniasis agents in an Amazonian/Guianan bordering area

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## Abstract

**Background:** An entomological study was conducted in the municipality of Oiapoque (lower Oyapock River Basin) in the Brazilian side bordering French Guiana to gain information on the transmission pattern of American cutaneous leishmaniasis (ACL) in that region, presumed to reflect the classical Amazonian/Guianan enzootic scenario.

**Methods:** Three ecologically isolated forested areas near urban environments were surveyed during the rainy and dry seasons of 2015 and 2016, using a multi-trapping approach comprising ground-level and canopy light traps, black and white colored cloth Shannon traps and manual aspiration on tree bases. Female phlebotomines were dissected to find infections and isolate flagellates from *Leishmania* spp. The strains were characterized by restriction fragment length polymorphism analysis and compared with those of local ACL cases and World Health Organization reference strains.

**Results:** *Nyssomyia umbratilis*, *Trichopygomyia trichopyga* and *Evandromyia infraspinosa* were the most frequently found species. Findings on relative abundance, spatiotemporal vector/ACL congruence, natural infections and anthropophilic insights strengthened the Guianan classical transmission of *Leishmania* (*Viannia*) *guyanensis* by *Ny. umbratilis* and suggested further investigations for *Ev. infraspinosa*. *Nyssomyia umbratilis* showed an eclectic feeding habit, including bird blood. Ecological data and literature reports also included *Psychodopygus squamiventris maripaensis* and *Bichromomyia flaviscutellata* on the list of suspected vectors.

**Conclusions:** These findings contributed to understanding ACL ecoepidemiology in the Amazonian/Guianan scenario. Local studies are required to better comprehend the *Leishmania* spp. enzootic mosaic in specific ecotopes.

**Keywords:** *Leishmania guyanensis*, Disease transmission, Vector ecology, State of Amapá, Brazil

## Background

Phlebotomine sand flies (Diptera: Psychodidae) play determinant roles in transmitting Leishmaniinae (Kinetoplastida: Trypanosomatidae) parasites, the causative agents of leishmaniasis [1–5]. In American cutaneous leishmaniasis (ACL), biologically compatible vector/parasite/reservoir arrangements can be driven naturally or triggered by ecological/human-made pressures, resulting in highly diverse and countless natural transmission cycles [2, 6–8]. Such diversity is reflected in the emergence of a wide and worrisome clinical-immunological spectrum, since some phlebotomine species can carry ACL agents that cause life-threatening and debilitating disease forms such as the anergic diffuse and mucosal forms [9].

In the Amazonian/Guianan region, the major *Leishmania*/vector-recognized transmission cycle involves

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*Leishmania* (*Viannia*) *guyanensis* and the phlebotomine *Nyssomyia umbratilis* [10–13]. However, in this region, an emerging ACL pattern is currently being considered, and at least four dermotropic *Leishmania* species have been reported: *L.* (*V.*) *braziliensis*, *L.* (*Leishmania*) *amazonensis*, *L.* (*V.*) *lainsoni* and *L.* (*V.*) *naiffi* [14]. Within that region, in the Oyapock River Basin, *L.* (*V.*) *guyanensis* accounts for 81% of ACL etiology, followed by *L.* (*V.*) *braziliensis* (17%) and *L.* (*V.*) *lainsoni* (2%) [15]. However, in this region, ACL foci are assumed to be concentrated in the upper basin, where gold mining represents a high-risk factor for exposure [16]. Underreported outbreaks associated with periurban forested environments should be surveyed.

The present study assessed potential ACL transmission cycles in the lower Oyapock River Basin to promote knowledge on phlebotomine ecology, mainly focusing on species composition, multi-trapping stratification, blood-source investigation and natural *Leishmania* spp. infections. The isolates obtained were also compared with human isolates.

## Methods

### Study area

The municipality of Oiapoque (03°49'29"N, 51°49'05"W) is in the Oyapock River Basin, a border region between Brazil and the Ultramarine Department of French Guiana. It is the northernmost municipality of the Brazilian State of Amapá (AP) and is limited by the AP municipalities of Calçoene, Serra do Navio and Pedra Branca do Amapari to the south, by Laranjal do Jari to the west, by the Atlantic Ocean to the east, and by the French-Guianan communes of Camopi and Saint Georges de l'Oyapock to the north. Oiapoque has a population of approximately 24,263 distributed over 22,625 $km^2$ [17]. During 2008–2017, a total of 1299 new ACL cases were registered by the health services in Oiapoque (average of 118 cases/year), with 560 shown to be autochthonous for that municipality (average of 50 cases/year). Because of Oiapoque's border characteristics, ACL epidemiology is a mosaic of "binational" infections, as half of ACL-notified cases were autochthonous from Brazil and half were likely imported from French Guiana [18].

### Sampling sites

Located in the lower Oyapock River Basin, the urban area of Oiapoque is surrounded by different forested environments with slightly distinct ecological characteristics. Thus, three "terra-firme" (dry-land) forested sites, approximately 7 km apart, were selected for sampling as follows (Fig. 1):

(i) Vila Vitória Road (03°51'28.1"N, 51°48'41.3"W): a recently opened road that provides eastern access from Oiapoque to Vila Vitória. The sampling site shows minimal evidence of human activity and is considered well preserved.

(ii) Highway BR156-Km6 (03°49'21.0"N, 51°45'59.6"W): an impacted area in southern urban Oiapoque with evidence of human activities, such as wood extraction.

(iii) Clevelândia do Norte Road (3°49'4.14"N, 51° 51'6.35"W): an old colonized area on the western side of urban Oiapoque, where the original vegetation was partially suppressed and replaced by secondary forest. It is currently an environmentally protected area by the Brazilian Army.

### Captures

Systematic field expeditions were initially performed during 2015–2016 to provide information on the predominantly rainy (February-May) and dry (September-November) Guianan/Amazonian seasons of the three sampling sites. Phlebotomines were obtained by surveying a horizontal transect from the edge inside each sampling site's forested area, using a multi-trapping approach described elsewhere [8]. The approach comprised captures using CDC light traps (*John* W. *Hock Company*, Gainesville, USA) set up from 6:00 h to 18:00 h at 1.5 m (ground level; $n$ = 8 traps/night) and at 20 m (canopy level; $n$ = 2 traps/night), captures from 6:00 h to 20:00 h with modified Shannon black and white colored cloth [19], and manual aspiration on tree bases from 6:00 h to 20:00 h.

### Processing and identification of phlebotomines

Phlebotomines were immediately processed in the field laboratory. Females were dissected under sterile conditions [20]. Flagellate infection was semi-quantified (in a cross '+' scale) according to Freitas et al. [21], and parasite development was classified by Lainson & Shaw [22]. The guts of infected females were triturated and inoculated into $Difco^{B45}$ culture media (Becton, Dickinson and Company, Franklin Lakes, USA) to isolate the parasites. Phlebotomine species were identified under fresh conditions using morphological characteristics. Unidentified specimens were processed for mounting on glass slides using Canada balsam. Morphology and taxonomic criteria, terminology and generic abbreviations were adopted following Galati [23] and Galati et al. [24].

### Investigation of phlebotomine blood sources

Intestines dissected from engorged females were macerated in PBS (pH 7.2, 0.001 M) and stored at -20 °C until processing by *Enzyme*-Linked Immunosorbent Assay according to Afonso et al. [25]. Based on local observation and the antisera available for testing, the panel chosen comprised bird, armadillo, opossum, dog, rodent and human antisera, obtained from the Immunodiagnostics

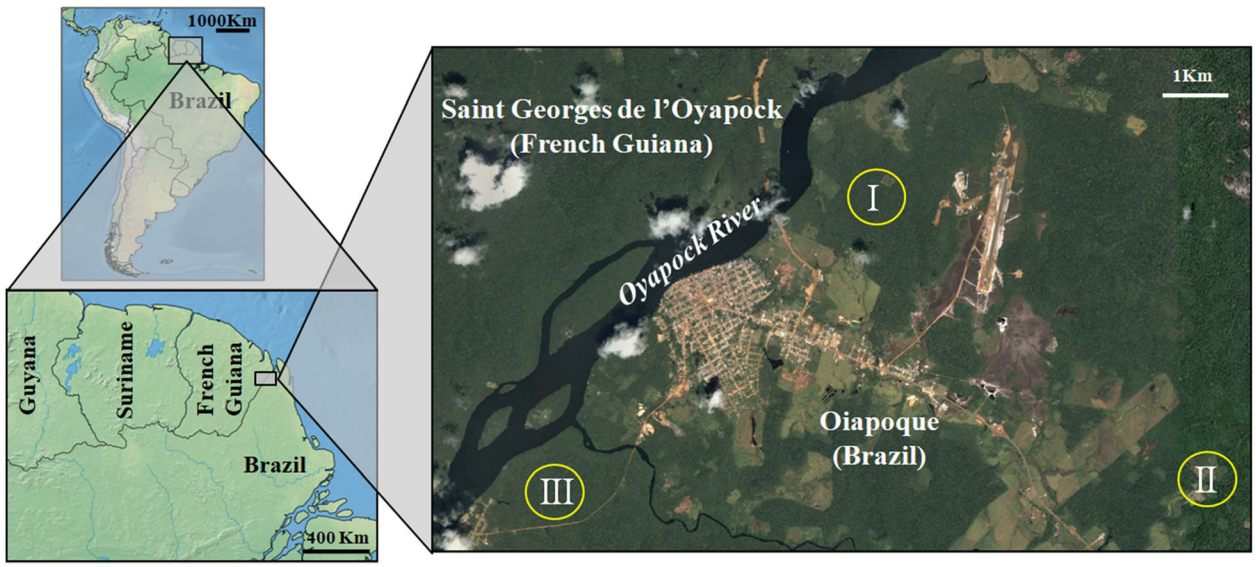


**Fig. 1** Study area on the outskirts of the Oiapoque urban area (lower Oyapock River Basin), Amapá State, Brazil, bordering French Guiana. *Abbreviations*: I, Vila Vitória Road; II, Highway BR156-Km6; III, Clevelândia do Norte Road

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### Investigation of ACL cases

Patients residing the study area who required diagnosis in the field laboratory (Oiapoque) or in the Ralph Lainson Leishmaniasis Laboratory, Instituto Evandro Chagas (Ananindeua, Brazil), were investigated according to the standard procedures of the Programa de Vigilância e Controle da Leishmaniose Tegumentar Americana (ACL Surveillance and Control Programme, Brazil). When clinically epidemiologically suggestive, patients were diagnosed by parasitological demonstration (Giemsa-stained smears of exudates from ACL lesions), the Montenegro skin test (inactivated promastigotes of *L.* (*V.*) *braziliensis* - MHOM/BR/M17323 - $1 \times 10^7$ parasites/ml) and parasite isolation (inoculating exudates from ACL lesions in Difco$^{B45}$ media) [2, 26].

### *Leishmania* spp. characterization

In both cases (phlebotomines and ACL patients), *Leishmania* DNA was obtained from successfully isolated strains. If no growth or contamination occurred, parasites were recovered from the remaining dissection slide contents (phlebotomines) or Giemsa-stained slides (ACL patients) using the DNeasy Tissue and Blood Kit (Qiagen, Hilden, Germany). Species were characterized by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) of a 615 bp region of the *RNA polymerase II* gene amplified using the primers RPOF2 (5'-AGA ACA TGG GCG GCC-3') and RPOR2 (5'-CGA GGG TCA CGT TCT TG-3') and digested with *Tps*RI and *Hga*I endonucleases following previously established and validated methodology [27]. Digestion profiles were compared with those of the WHO *Leishmania* reference strains occurring in the Guiana Shield.

### Environmental assessment and data analysis

Forest cover degree was estimated using digital hemispherical images captured 1.6 m above ground, pointing directly upward in the CDC-ground trap sites (8 images per sampling site). Canopy coverage in each image was determined using CAN-EYE v.6.314 hemispherical image analysis software. Microclimate parameters (temperature and humidity) were measured using data loggers placed in the CDC traps. Species composition at the three sampling sites was analyzed using the Shannon-Wiener diversity index (*H'*) using PAST version 2.12 software [28]. All comparisons were performed using Student's t-test to determine significance level ($P \leq 0.05$). Sampling effort, species infection rate (SIR) and number of phlebotomines per hour were calculated according to Souza et al. [8].

## Results

### Phlebotomine composition/Environmental assessment

From the three sample areas, 9119 phlebotomines were captured during 2015–2016, belonging to 48 species. Among the 15 genera identified, *Psychodopygus* and *Psathyromyia* had the highest number of species (10 spp. and 9 spp., respectively). The composition was dominated (74.8%) by *Nyssomyia umbratilis* (29.65%), *Trichopygomyia trichopyga* (28.50%) and *Evandromyia infraspinosa* (16.36%) (Table 1). Area I had more species

[40 spp.; specimens captured (*n*) = 3905; Shannon's diversity index (*H*) = 2.251] than II (33 spp.; *n* = 2377; *H* = 1.857) and III (38 spp.; *n* = 2837; *H* = 1.662). The t-test for diversity was significant for all comparisons between sample sites ($t_{(3)}$ = 11.1, *P* = 0.008).

Estimation of canopy cover differed significantly between areas I and II ($t_{(2)}$ = 11.83, *P* = 0.004) and between I and III ($t_{(2)}$ = 21.46, *P* = 0.002) (Fig. 2). Average temperature (°C) and humidity (%) registered in the tree sampled sites were, respectively: I (27.4 °C; 82.9%); II (26.6 °C; 77.1%); III (25.0 °C; 85.7%). However, no statistically significant differences were found between average temperature ($t_{(3)}$ = 1.77, *P* = 0.63) or humidity ($t_{(3)}$ = 1.95, *P* = 0.21).

Based on 4608 h of CDC ground, 1152 h of CDC canopy trap, 48 h of white Shannon, 48 h of black Shannon, and 24 h of aspiration on tree bases (total sampling effort: 5880 h), white Shannon traps rendered 5.6 phlebotomines per hour. The greatest number of phlebotomines found per hour for these capture methods included *Ny. umbratilis* in the CDC canopy trap (0.96 ♀♀), white Shannon trap (0.93 ♀♀), and on aspiration on tree bases (1.16 ♀♀; 1.62 ♂♂). *Evandromyia infraspinosa* was also found on the black Shannon trap (0.97 ♀♀), and *Ps. s. maripaensis* was found on the white Shannon trap (2.29 ♀♀). These three species attempted to bite the professionals during capture (Table 2).

### Parasite infections and *Leishmania* typing

Forty-eight parasite infections were found, representing 40 flagellates, five nematodes (Nemathelminthes) and three gregarines (Apicomplexa). Flagellate infections were detected in *Nyssomyia umbratilis* (*n* = 28), *Evandromyia infraspinosa* (*n* = 3), *Migonemyia migonei* (*n* = 2), *Sciopemyia fluviatillis* (*n* = 2) *Viannamyia furcata* (*n* = 2), *Psathyromyia dendrophyla* (*n* = 1), *Sciopemyia sordellii* (*n* = 1) and *Pintomyia damascenoi* (*n* = 1). Nematode infections occurred in *Evandromyia williamsi* (*n* = 2), *Psathyromyia aragaoi* (*n* = 1), *Evandromyia monstruosa* (*n* = 1) and *Ps. s. maripaensis* (*n* = 1). Gregarines were found in *Bi. flaviscutellata* (3).

Twelve flagellate strains were successfully isolated (Table 3), all without visible blood meals, and with infections varying from ++ to ++++. Isolates occurred in 10 *Ny. umbratilis* at sites I (*n* = 3), II (*n* = 6) and II (*n* = 1) and in one *Sc. fluviatilis* (site I) and one *Vi. furcata* (site I).

All isolates from *Ny. umbratilis* exhibited a PCR-RFLP profile identical to that of the *L.* (*V.*) *guyanensis* WHO reference strain (MHOM/BR/1975/M4147) (Fig. 3), while those from *Sc. fluviatilis* and *Vi. furcata* were inconclusive.

Twenty-eight infections were not isolated. The PCR-RFLP for the remaining DNA fixed on the glass dissection slides allowed characterizing *L.* (*V.*) *guyanensis* from one *Ny. umbratilis* and two *Ev. infraspinosa* specimens (Fig 4).

### Blood sources

One hundred thirty-eight guts were tested, and 20 (14.4%) reacted to at least one antiserum on the available panel (Table 4). Positive species comprised *Ny. umbratilis* (*n* = 12), *Ps. s. maripaensis* (*n* = 4), *Ps. claustrei* (*n* = 2), *Bichromomyia flaviscutellata* (*n* = 1) and *Ev. infraspinosa* (*n* = 1). The sampling site with the most engorged flies was I (*n* = 16). The engorged specimens were mostly found in tree bases (*n* = 7), followed by the CDC ground trap (*n* = 4) and lastly the Shannon trap (*n* = 1).

Species with the greatest numbers of identified blood sources included *Ny. umbratilis* (bird, dog, armadillo, opossum and human), followed by *Ps. claustrei* (dog, opossum, rodent and armadillo). The other positive species had only one blood source: *Bichromomyia flaviscutellata* (bird); *Ps. s. maripaensis* (armadillo); and *Ev. infraspinosa* (armadillo).

Species with more than one blood source in the same specimen included *Ny. umbratilis* (sample 13: dog and opossum; sample 16: bird, dog and armadillo) and *Ps. claustrei* (sample 3: dog, opossum and rodent). One *Ny. umbratilis* naturally infected with flagellates morphologically compatible with *Leishmania* spp. was positive for bird antiserum.

### American cutaneous leishmaniasis cases

During 2015–2016, ten patients belonging to the Guiana Shield asked for ACL diagnostics in our laboratory, and eight believed that they had been infected on the Brazilian-French Guianan/Oyapock border (six from gold mining and two from agricultural settlements) (Table 5). All *Leishmania* strains were isolated and characterized as *L.* (*V.*) *guyanensis*. A Brazilian agricultural settlement with *L.* (*V.*) *guyanensis* isolated from an ACL patient (MHOM/BR/2017/M32218) is located in site I, near the forested area subjected to the captures.

Figure 3 shows a PCR-RFLP analysis of *Leishmania* isolates from infected phlebotomines and a human ACL case compared against the WHO *L.* (*V.*) *guyanensis* reference strain.

## Discussion

Few studies have been conducted on ACL ecology in the lower Oyapock basin. The first available information on that region (the French Guianan side) came from entomological studies conducted in the 1940s and 1950s, which provided much information on phlebotomine taxonomy and ecology [29]. The available commented checklist including that region can be found elsewhere [30] as a metabarcoding-based local inventory [31]. Evidence on ACL etiology was only provided recently [15].

**Table 1** Phlebotomine species compositions at three sampling sites surveyed during four 12-day field expeditions in Oiapoque, Amapá, Brazil (2015–2016). Species found infected by flagellates are shown in bold, with the number of specimens found infected with flagellates in parentheses

| No. | Species | I | | II | | III | | Total | | SIR |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ♀♀ | ♂♂ | ♀♀ | ♂♂ | ♀♀ | ♂♂ | *N* | % | |
| 1 | ***Nyssomyia umbratilis*** (28) | 482 (19) | 426 | 725 (8) | 479 | 355 (1) | 237 | 2704 | 29.65 | 1.79 |
| 2 | *Trichopygomyia trichopyga* | 456 | 522 | 128 | 124 | 655 | 714 | 2599 | 28.50 | – |
| 3 | ***Evandromyia infraspinosa*** (3) | 344 (2) | 352 | 233 (1) | 122 | 255 | 186 | 1492 | 16.36 | 0.36 |
| 4 | *Trichophoromyia ininii* | 172 | 158 | 27 | 34 | 13 | 24 | 428 | 4.69 | – |
| 5 | *Psathyromyia aragaoi* | 108 | 121 | 16 | 9 | 39 | 47 | 340 | 3.73 | – |
| 6 | *Psyshodopygus maripaensis* | 68 | 11 | 130 | 9 | 2 | – | 220 | 2.41 | – |
| 7 | ***Sciopemyia sordellii*** (1) | 24 (1) | 14 | 18 | 19 | 45 | 15 | 135 | 1.48 | 1.14 |
| 8 | *Micropygomyia rorotaensis* | 67 | 36 | 6 | 3 | 19 | 2 | 133 | 1.46 | – |
| 9 | *Psyshodopygus ayrozai* | 59 | 25 | 29 | 15 | 4 | – | 132 | 1.45 | – |
| 10 | *Bichromomyia flaviscutellata* | 69 | 30 | 1 | 3 | 7 | 1 | 111 | 1.22 | – |
| 11 | *Nyssomyia anduzei* | 4 | 1 | 4 | – | 64 | 18 | 91 | 1.00 | – |
| 12 | *Evandromyia williamsi* | 17 | 5 | 7 | 36 | 5 | 1 | 71 | 0.78 | – |
| 13 | *Evandromyia brachyphalla* | 41 | 14 | 5 | – | 4 | 5 | 69 | 0.76 | – |
| 14 | ***Viannamyia furcata*** (2) | 17 (1) | 10 | 18 (1) | 11 | 11 | 1 | 68 | 0.75 | 4.34 |
| 15 | *Viannamyia tuberculata* | 19 | 3 | 26 | 3 | 5 | – | 56 | 0.61 | – |
| 16/17 | *Pressatia choti/ Pr. trispinosa* | 12 | 8/21 | 8 | –/1 | 2 | –/1 | 53 | 0.58 | – |
| 18 | *Evandromyia* sp. of Baduel | 11 | 19 | 8 | 10 | – | – | 48 | 0.53 | – |
| 19 | *Nyssomyia pajoti* | 9 | 2 | 3 | – | 15 | 8 | 37 | 0.41 | – |
| 20 | ***Sciopemyia fluviatilis*** (2) | 4 (2) | 16 | 4 | 3 | 4 | – | 31 | 0.34 | 16.6 |
| 21 | *Psathyromyia inflata* | 8 | 13 | 4 | 6 | – | – | 31 | 0.34 | – |
| 22 | *Lutzomyia spathotrichia* | 2 | 1 | 17 | 11 | – | – | 31 | 0.34 | – |
| 23 | *Evandromyia monstruosa* | 13 | 3 | 10 | – | 2 | 2 | 30 | 0.33 | – |
| 24 | ***Migonemyia migonei*** (2) | 14 (2) | 8 | 1 | – | 3 | 1 | 27 | 0.30 | 11.1 |
| 25 | *Psyshodopygus hirsutus* | 6 | 2 | 8 | 6 | 2 | 1 | 25 | 0.27 | – |
| 26 | ***Pintomyia damascenoi*** (1) | 4 (1) | – | 7 | 6 | 2 | – | 19 | 0.21 | – |
| 27 | *Psyshodopygus davisi* | 4 | 4 | 1 | 2 | 4 | – | 15 | 0.16 | – |
| 28 | ***Psathyromyia dendrophyla*** (1) | 3 (1) | 4 | 3 | 1 | 4 | – | 15 | 0.16 | 10 |
| 29 | *Psychodopygus claustrei* | 4 | 7 | – | – | 2 | 1 | 14 | 0.15 | – |
| 30 | *Pschodopygus corrosoniensis* | 3 | 1 | – | – | 8 | 2 | 14 | 0.15 | – |
| 31 | *Psathyromyia bigeniculata* | 4 | 1 | 5 | 1 | 1 | 1 | 13 | 0.14 | – |
| 32 | *Psathyromyia dreisbachi* | – | – | – | – | 9 | 4 | 13 | 0.14 | – |
| 33 | *Trichophoromyia ubiquitalis* | – | – | – | – | 5 | 8 | 13 | 0.14 | – |
| 34/35 | *Brumptomuyia travassosi/Br. pentacantha* | 4 | 1/1 | 1 | – | 2 | 2/– | 7 | 0.07 | – |
| 36 | *Psyschodopygus amazonensis* | – | – | 2 | 2 | 1 | – | 5 | 0.05 | – |
| 37 | *Psychodopygus paraensis* | 3 | – | – | – | – | 1 | 4 | 0.04 | – |
| 38 | *Psathyromyia punctigeniculata* | – | – | – | 3 | 1 | – | 4 | 0.04 | – |
| 39 | *Trichopygomyia dasypodogeton* | 3 | – | – | – | – | – | 3 | 0.03 | – |
| 40 | *Pintomyia pacae* | 1 | – | – | – | 1 | – | 2 | 0.02 | – |
| 41 | *Pintomyia serrana* | 1 | – | – | – | – | 1 | 2 | 0.02 | – |
| 42 | *Psathyromyia lutziana* | 2 | – | – | – | – | – | 2 | 0.02 | – |
| 43 | *Psathyromyia abonnenci* | 1 | 1 | – | – | – | – | 2 | 0.02 | – |
| 44 | *Psathyromyia barrettoi barrettoi* | – | – | 1 | 1 | – | – | 2 | 0.02 | – |

**Table 1** Phlebotomine species compositions at three sampling sites surveyed during four 12-day field expeditions in Oiapoque, Amapá, Brazil (2015–2016). Species found infected by flagellates are shown in bold, with the number of specimens found infected with flagellates in parentheses *(Continued)*

| No. | Species | I | | II | | III | | Total | | SIR |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ♀♀ | ♂♂ | ♀♀ | ♂♂ | ♀♀ | ♂♂ | *N* | % | |
| 45 | *Evandromyia begonae* | – | – | – | – | – | 1 | 1 | 0.01 | – |
| 46 | *Micropygomyia* (Pilosa Series) | 1 | – | – | – | – | – | 1 | 0.01 | – |
| 47 | *Psychodopygus bispinosus* | – | – | – | – | 1 | – | 1 | 0.01 | – |
| 48 | *Psychodopygus carrerai* | – | – | 1 | – | – | – | 1 | 0.01 | – |
| | Total | 2064 | 1841 | 1457 | 920 | 1552 | 1,285 | 9119 | 100.00 | – |
| | | 3905 | | 2377 | | 2837 | | | | 0.78 |
| | Taxa (*S*) | 40 | | 33 | | 38 | | – | – | – |
| | Shannon's *H*[a] | 2.251 | | 1.857 | | 1.662 | | – | – | – |

*Abbreviations*: *I* Vila Vitória Road, *II* Highway BR156-Km6, *III* Clevelândia do Norte Road, ♀♀ females, ♂♂ males, *SIR* species infection rate (flagellates), *n/n* number of males, while indistinguishable females, *N* total number

[a]The t-test for diversity was significant for all comparisons between sample sites ($t_{(3)} = 11.1$, $P = 0.008$)

With reasonable sampling efforts (5880 h) for the multi-trapping approach, the present findings showed high species diversity (48 spp.). We preferred to use different capture methods because, although light-baited suction traps are one of the most widely used tools for vector surveillance, they have biases and limitations in terms of their effect on collection efficiency, population data, and pathogen detection [32]. Multi-trapping approaches with large samplings may offer a broader picture on the surveyed fauna, as shown by Souza et al. [33] in the Lower Amazon Basin (68 spp.) and by Freitas et al. [21] (46 spp.) and Souza et al. [8] (63 spp.) in Amapá. However, the use of CDC light traps in long term surveys and/or with strategical placement (i.e. with spatial stratification biasing to find feeding/resting sites) may, in part, supply some of these limitations. The present results are also reasonably compatible with 'CDC trap-based' surveys recently conducted in the

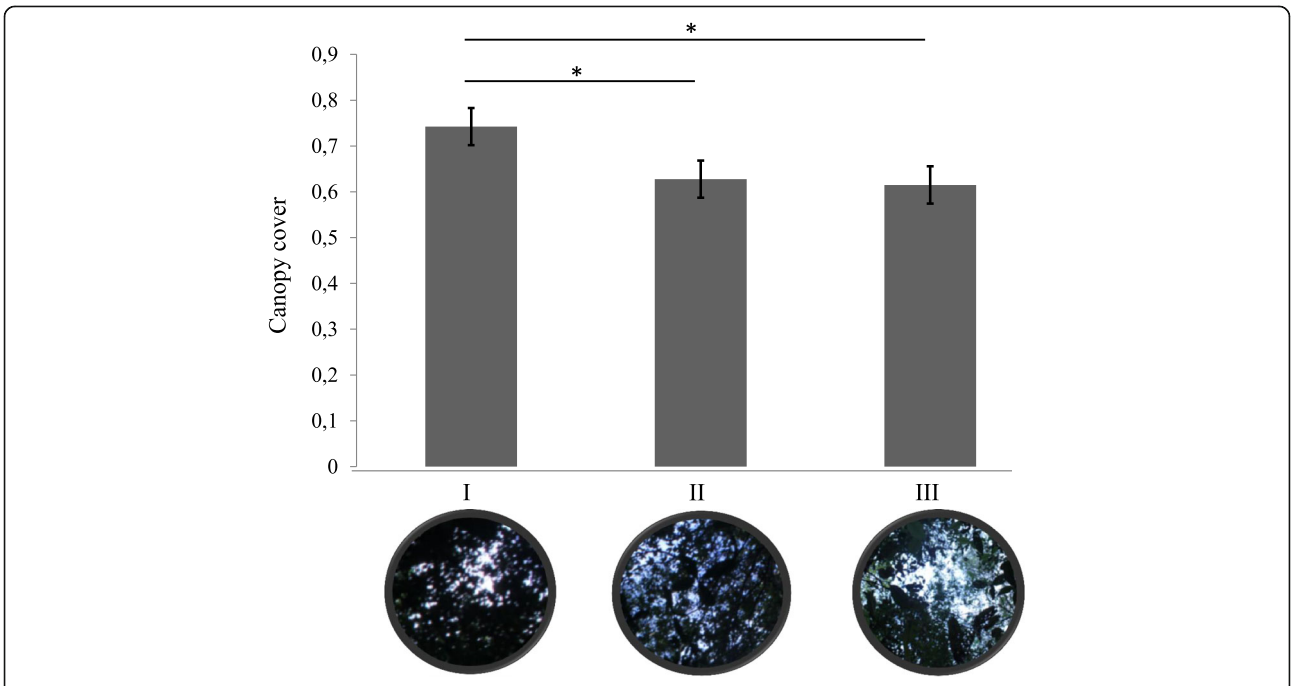


**Fig. 2** Estimation of canopy cover degree at the three surveyed ecotopes (forested areas) on the outskirts of the Oiapoque urban area (lower Oyapock River Basin), Amapá State, Brazil, bordering French Guiana. Asterisk indicates significant differences ($P \leq 0.05$). *Abbreviations*: I, Vila Vitória Road; II, Highway BR156-Km6; III, Clevelândia do Norte Road

**Table 2** Averages for the ten most frequently captured species per hour compiled for the three sampling sites surveyed during four 12-day field expeditions in Oiapoque, Amapá, Brazil (2015–2016). Highest individual values found (above 0.9) are shown in bold

| No. | Species | Capture method[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CDC ground | | CDC canopy | | White Shannon | | Black Shannon | | Tree bases | | Total[b] |
| | | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | |
| 1 | *Nyssomyia umbratilis* | 0.08 | 0.05 | **0.96**[c] | 0.74 | **0.93**[c] | 0.1 | 0.04 | – | **1.16** | **1.62** | 0.46 |
| 2 | *Trichopygomyia trichopyga* | 0.23 | 0.25 | 0.11 | 0.15 | 0.08 | 0.08 | 0.12 | 0.06 | – | – | 0.44 |
| 3 | *Evandromyia infraspinosa* | 0.13 | 0.10 | 0.13 | 0.11 | 0.5[c] | 0.29 | **0.97**[c] | 0.54 | 0.04 | 0.08 | 0.25 |
| 4 | *Trichophoromyia ininii* | 0.04 | 0.04 | 0.00 | 0.01 | 0.04 | – | 0.08 | – | – | – | 0.07 |
| 5 | *Psathyromyia aragaoi* | 0.02 | 0.02 | 0.04 | 0.02 | – | – | 0.08 | – | – | – | 0.05 |
| 6 | *Psychodopygus squamiventris maripaensis* | 0.00 | 0.00 | 0.02 | 0.00 | **2.29**[c] | 0.1 | 0.68[c] | 0.12 | 0.04 | – | 0.03 |
| 7 | *Sciopemyia sordellii* | 0.02 | 0.00 | 0.02 | 0.01 | 0.04 | 0.04 | 0.12 | 0.04 | 0.08 | – | 0.03 |
| 8 | *Psychodopygus ayrozai* | 0.00 | 0.00 | 0.04 | 0.02 | 0.22[c] | – | 0.22[c] | 0.02 | – | – | 0.02 |
| 9 | *Micropygomyia rorotaensis* | 0.01 | 0.00 | 0.02 | 0.01 | 0.1 | – | 0.06 | – | 0.16 | 0.16 | 0.02 |
| 10 | *Bicrhomomyia flaviscutellata* | 0.01 | 0.00 | 0.00 | 0.00 | 0.14 | 0.08 | 0.1 | – | – | – | 0.01 |
| | Other species (11–48) | 0.05 | 0.03 | 0.15 | 0.09 | 0.35 | 0.1 | 0.22 | 0.06 | 0.12 | 0.29 | 0.12 |
| | Total | 0.62 | 0.54 | 1.54 | 1.21 | 4.7 | 0.81 | 2.75 | 0.05 | 1.62 | 2.16 | 1.55 |
| | Total (♀ + ♂) | 1.17 | | 2.76 | | 5.6 | | 3.6 | | 3.79 | | – |

[a]Based on 4608 h CDC ground; 1152 h CDC canopy; 48 h white Shannon; 48 h black Shannon; and 24 h tree bases
[b]Total sampling effort: 5880 h
[c]Specimens found attempting to bite

Guiana Shield by Rotureau et al. [34] (46 spp.) and Fouque et al. [35] (38 spp.) in French Guiana, as well as by Furtado et al. [36] (45 spp.) in Amapá. Compiled information shows that approximately 84 species are registered in Amapá, and *Brumptomyia pentacantha* was a newly recorded species for that state. In Brazil, this species was recorded only in Pará, Acre, Rondônia and Mato Grosso states [8, 36–45].

Despite high overall species diversity, numerical domination (74.8%) of only three species was expected. Studies on forested environments have shown a phlebotomine fauna generally composed of a few dominant species and many species with few specimens [46, 47].

Differences in the degree of canopy cover between the three sampling sites were congruent with their respective Shannon indices (*H*), suggesting forest cover as an eligible variable for maintaining species diversity, although deforestation associated with human settlements can also produce environmental conditions suitable for maintaining

**Table 3** Strains of *Leishmania* and other flagellates isolated *in vitro* from naturally infected phlebotomine specimens captured at the three sampled sites in Oiapoque, Amapá, Brazil (2015–2016)

| No. | Species | IEC code | Capture site | Capture method | Infection[a] | PCR-RFLP result - WHO code |
|---|---|---|---|---|---|---|
| 1 | *Nyssomyia umbratilis* | M31681 | II | CDC ground | +++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2015/M31681 |
| 2 | *Nyssomyia umbratilis* | M32146 | I | CDC ground | +++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32146 |
| 3 | *Nyssomyia umbratilis* | M32149 | I | Tree bases | ++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32149 |
| 4 | *Nyssomyia umbratilis* | M32152 | I | CDC canopy | ++++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32152 |
| 5 | *Nyssomyia umbratilis* | M32154 | II | CDC ground | +++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32154 |
| 6 | *Nyssomyia umbratilis* | M32156 | II | CDC canopy | ++++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32156 |
| 7 | *Nyssomyia umbratilis* | M32157 | II | CDC canopy | ++++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32157 |
| 8 | *Nyssomyia umbratilis* | M32158 | II | CDC canopy | ++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32158 |
| 9 | *Nyssomyia umbratilis* | M32159 | II | CDC canopy | ++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32159 |
| 10 | *Nyssomyia umbratilis* | M32160 | III | CDC ground | +++ | *L.* (*V.*) *guyanensis* - IUMB/BR/2016/M32160 |
| 11 | *Sciopemyia fluviatilis* | M32316 | I | CDC ground | ++ | Unconclusive - IFLU/BR/2016/M32316 |
| 12 | *Viannamyia furcata* | M32652 | I | CDC ground | +++ | Unconclusive - IFUR/BR/2016/M32652 |

*Abbreviations*: *I* Vila Vitória Road, *II* Highway BR156-Km6, *III* Clevelândia do Norte Road
[a]Parasites per field (×40 objective): ++, 6–20; +++, 21–40; ++++, > 40

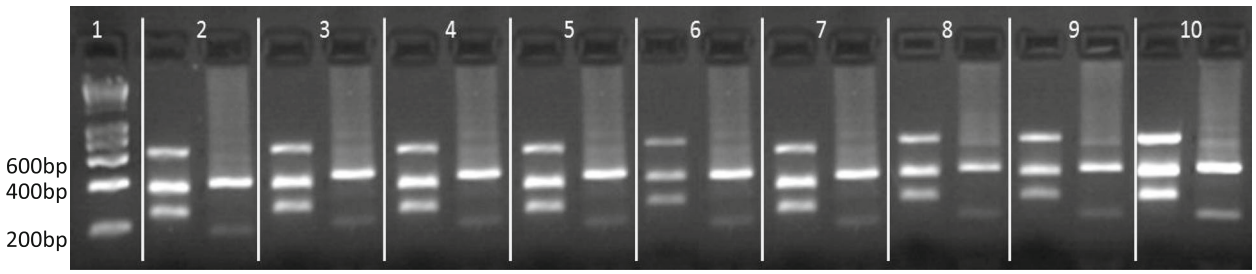


**Fig. 3** PCR-RFLP analysis (primers RPOF2/RPOR2, restriction enzymes *TspR*I/ *Hga*I) of *Leishmania* (*Viannia*) *guyanensis* isolates from phlebotomine species and an ACL case from the outskirts of the Oiapoque urban area (lower Oyapock River Basin), Amapá State, Brazil, bordering French Guiana, compared with that of the WHO reference strain. The two columns for each sample represent the digestion products of *TspR*I and *Hga*I, respectively. Lane 1: molecular weight marker Smart Ladder; Lane 2: IUMB/BR/2015/M31681; Lane 3: IUMB/BR/2016/M32146; Lane 4: IUMB/BR/2016/M32149; Lane 5: IUMB/BR/2016/M32156; Lane 6: IUMB/BR/2016/M32158; Lane 7: IUMB/BR/2016/M321597; Lane 8: IUMB/BR/2016/M32160; Lane 9: MHOM/BR/2017/M32218; Lane 10: *L.* (*V.*) *guyanensis* WHO reference strain (MHOM/BR/1975/M4147)

the life-cycles of several sand fly species that are adaptable to these environments [48, 49]. Conversely, high SIR was found at sites with high degrees of canopy cover. Dense and humid substrate provided by a well-covered canopy may contribute to vector/host availability. In addition, low light penetration in the denser forest can provide suitable conditions for vector/host movements, as observed with the inverse correlation of phlebotomine density on light traps *versus* moonlight [50]. However, a minimum light is important for these insects to fly [51]; thus, vector-host interactions may result from equilibrium between these factors.

Numerous nematodes were found in the body cavity of five phlebotomine species. Although these flies were captured at different sampling sites and different vertical strata (ground/canopy level), some entomopathogenic nematode species infect phlebotomines on the ground, during the larval stage [52], suggesting that these infected flies may share the same breeding site.

Gregarines found in *Bi. flaviscutellata* (three specimens) were morphologically similar to *Psychodiella* sp. oocysts, although only molecular sequencing could confirm the species. Insect-host specificity between *Bi. flaviscutellata* and the gregarine species supports the hypothesis of a long, strong coevolutionary association between them [53].

Only 14.4% of blood-fed phlebotomines tested were positive by ELISA. This result could be attributed to the low blood content in the specimens as well as the blood recuperation procedure for the dissected slides, which may have contributed to the loss of material. Another possibility is the presence of animal blood; this was not accounted for by the available test panel since anteaters and sloths, for example, are presumably present in the Guianan ecosystem, acting as potential reservoir hosts of

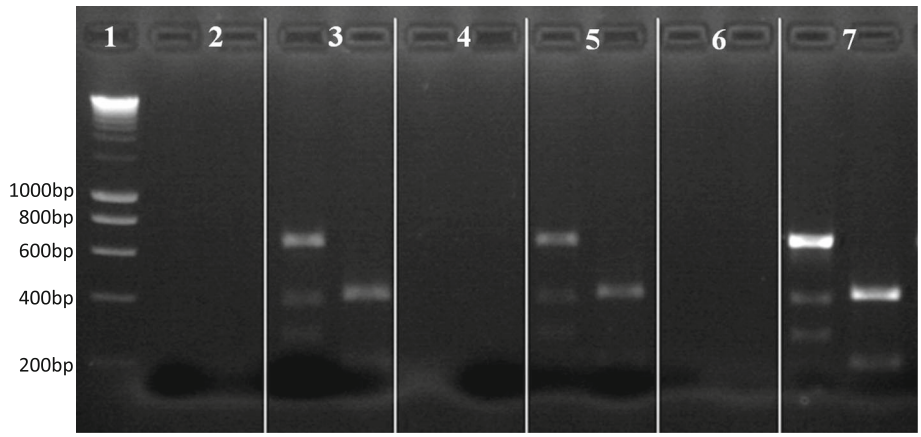


**Fig. 4** PCR-RFLP analysis (primers RPOF2/RPOR2, restriction enzymes *TspR*I/ *Hga*I) of a 615 bp amplified fragment of the *RNA polymerase II* gene from *Leishmania* (*Viannia*) *guyanensis* DNA from material remaining on glass dissection slides of infected phlebotomine specimens captured in Oiapoque, Amapá Brazil, compared with that of the WHO reference strain. The two columns for each sample represent the digestion products of *TspR*I and *Hga*I, respectively. Lane 1: molecular weight marker Smart Ladder; 1 kb; Lane 2: negative sample; Lane 3: *Ny. umbratilis*-M32153; Lane 4: negative sample; Lane 5: *Ev. infraspinosa*-M32155; Lane 6: negative control; Lane 7: WHO reference strain (MHOM/BR/1975/M4147)

**Table 4** Phlebotomine specimens captured at three sampling sites in Oiapoque, Amapá, Brazil (2015–2016), tested by ELISA for blood sources and found positive for at least one antiserum from the available panel

| No. | Species | Site | Method | Blood source[a] |
|---|---|---|---|---|
| 1 | *Nyssomyia umbratilis* | III | CDC canopy | Bird |
| 2 | *Nyssomyia umbratilis* | III | CDC canopy | Bird |
| 3 | *Psychodopygus claustrei* | I | CDC ground | Dog, opossum, rodent |
| 4 | *Psychodopygus claustrei* | I | CDC ground | Armadillo |
| 5 | *Bichromomyia flaviscutellata* | I | CDC canopy | Bird |
| 6 | *Psychodopygus squamiventris maripaensis* | I | CDC canopy | Armadillo |
| 7 | *Psychodopygus squamiventris maripaensis* | II | CDC canopy | Armadillo |
| 8 | *Psychodopygus squamiventris maripaensis* | II | CDC canopy | Armadillo |
| 9 | *Evandromyia infraspinosa* | I | CDC ground | Armadillo |
| 10 | *Nyssomyia umbratilis* | I | Tree bases | Bird |
| 11 | *Nyssomyia umbratilis* | I | Tree bases | Bird |
| 12 | *Nyssomyia umbratilis* | I | Tree bases | Bird |
| 13 | *Nyssomyia umbratilis* | I | Tree bases | Dog, opossum |
| 14 | *Psychodopygus squamiventris maripaensis* | I | Shannon | Armadillo |
| 15 | *Nyssomyia umbratilis* | I | Tree bases | Bird |
| 16 | *Nyssomyia umbratilis* | I | Tree bases | Bird, dog, armadillo |
| 17 | *Nyssomyia umbratilis* | I | CDC canopy | Bird |
| 18 | *Nyssomyia umbratilis* | I | CDC ground | Man |
| 19 | *Nyssomyia umbratilis* | I | Tree bases | Armadillo |
| 20 | *Nyssomyia umbratilis*[b] | I | CDC canopy | Bird |

*Abbreviations*: *I* Vila: Vitória Road, *II* Highway BR156-Km6, *III* Clevelândia do Norte Road
[a]Antisera panel: dog, bird, opossum, man, armadillo and rodent
[b]Positive sample with flagellates morphologically compatible with *Leishmania* sp.

*L.* (*V.*) *guyanensis* [54, 55]. These results provided a better understanding of the biology of five phlebotomine species.

Undoubtedly, *Ny. umbratilis* is closely associated with *L.* (*V.*) *guyanensis* and has been consequently implicated as the main ACL vector in Oyapock based on data consistent with its well-recognized regional importance in the Guiana Shield [14] and in the wide Amazonian region [7]. Infection rates for this fly vary greatly in the literature, with some being consistent with the present findings [8, 56, 57]. Higher rates are usually biased by captures performed in the dry season [58] or supporting the dissection of fed and gravid females [21]. Infected specimens were captured in both levels of CDC traps and tree bases; however, they may have been infected at other sites. A natural vertical migration of these flies is well documented [13] and may explain dissociation movements between infection/resting sites as supposed for *L.* (*V.*) *naiffi* in the Lower Amazonian basin, where a canopy of *Ps. davisi* was found infected by that parasite, whose only recognized

**Table 5** *Leishmania* (*Viannia*) *guyanensis* strains isolated from cutaneous lesions of patients treated at the Ralph Lainson Leishmaniasis Laboratory (IEC/SVS/MS) (2015–2017) who declared the Brazil-French Guiana border as the probable place of infection

| Mnemonic | Infection site | No. of lesions (location) | IDRM (mm) | WHO code |
|---|---|---|---|---|
| FCF | Vila Vitória (BR) | 2 (face/neck) | 12 × 12 | MHOM/BR/2017/M32218 |
| HMLR | Gold mining (FG) | 1 (foot) | 12 × 12 | MHOM/BR/2016/M31987 |
| MRP | Gold mining (FG) | 2 (hand/leg) | 7 × 7 | MHOM/BR/2016/M32048 |
| ARSN | Gold mining (FG) | 1 (arm) | 8 × 8 | MHOM/BR/2015/M31041 |
| PSLS | Gold mining (FG) | Disseminated | 10 × 10 | MHOM/BR/2015/M31157 |
| HS | Gold mining (FG) | 1 (thorax) | 12 × 12 | MHOM/BR/2015/M31498 |
| OSM | Régina (FG) | 1 (leg) | 10 × 10 | MHOM/BR/2015/M32273 |
| LMSJr | Gold mining (FG) | 6 (leg (4), arm (1), neck (1)) | 17 × 17 | MHOM/BR/2015/M32382 |

*Abbreviations*: *BR* Brazil, *FG* French Guiana

potential reservoir host is the terrestrial armadillo, *Dasypus novemcintus* [32].

Blood contents from *Ny. umbratilis* reacted mainly with bird antisera (9/12). The role of birds in the population dynamics of phlebotomine species has been discussed [59–62]. *Nyssomyia umbratilis* being found with bird blood and concomitantly with a flagellate (likely leishmanine parasites) infection could be an occasional finding or suggests that this blood source provided suitable conditions for *L.* (*V.*) *guyanensis* development, as has been demonstrated in experiments between *Gallus gallus* blood and *L.* (*L.*) *mexicana* [63]. The findings on the eclectic food habits of *Ny. umbratilis* are consistent with those of other studies [64, 65]. Rodents, for example, appear to be alternative blood sources in disturbed environments [66], contributing to a possible groundward vertical migration of this traditionally canopy-loving phlebotomine species, as presumed to occur in a hydroelectric system-affected area of Jari River Basin [36].

Three infections found in *Ev. infraspinosa* were not isolated, but two were successfully characterized as *L.* (*V.*) *guyanensis* from the DNA content on the dissection slides. Considerable infections (++) and absence of blood observed with these peripylaric parasites suggest the necessity to continuously investigate this fly's possible involvement in the *L.* (*V.*) *guyanensis* enzootics. In addition, this species was frequent in Shannon captures (mainly in the black cloth, 0.97 females/h), with some specimens attempting to bite the professionals. It was impossible to determine whether *Ev. infraspinosa* could feed on potential *L.* (*V.*) *guyanensis* reservoirs. The present results indicate that this phlebotomine can feed on armadillos. The rodent *Dasyprocta leporina* is the only known blood source for this species [65] although anuran trypanosomatid isolated from this species from the western Brazilian Amazon suggests it feeds on cold-blooded rather than warm-blooded vertebrates [67].

In addition to *Ny. umbratilis* and *Ev. infraspinosa*, flagellate infections have been found in *Mi. migonei*, *Sc. fluviatilis*, *Sc. sordellii* and *Vi. furcata*. Negative PCR-RFLP for the other infected specimens suggested low DNA for *Leishmania*-typing or that they were other trypanosomatids. The apparent high infection rates of some of these species may have been biased by the low number of dissected females and thus cannot yield conclusive findings.

Other phlebotomine species were found with flagellates. The trypanosomatid isolated from *Sc. fluviatilis* will be further characterized. Parasites found in the blood of *Pa. dendrophyla* should be cautiously interpreted; this species shares the same ecotope as *Ny. umbratilis*, and some specimens likely receive occasional parasite ingestions, as observed by Freitas et al. [21]. These considerations can be extended to *Vi. furcata* and *Pi. damascenoi* [13].

Although no *Leishmania* infections were found for the following two fly species, they should still be discussed as putative vectors in the lower Oyapock River Basin based on the current Guianan/Amazonian ACL epidemiological background:

(i) *Psychodopygus s. maripaensis* has been included on the long list of possible vectors of *L.* (*V.*) *naiffi* based on infection findings in Régina, French Guiana [35] and Serra do Navio (AP) [8], extending its epidemiological relevance in other Brazilian/Guianan regions [36]. In addition, the *Ps. s. maripaensis* specimens tested for blood sources reacted positively to antiserum from an armadillo, the recognized potential reservoir of *L.* (*V.*) *naiffi*. Interestingly, DNA from *L.* (*V.*) *braziliensis* was detected in a pooled sample of *Ps. s. maripaensis* [referred to as *P. squamiventris* (*s.l.*)] in Sabajo Heuvels, Suriname, suggesting an additional putative vector role [68]. However, *L.* (*V.*) *braziliensis* transmission in Oyapock remains unclear. The most females found per hour in our Shannon captures (2.29; white colored cloth), with some attempting to bite the professionals, demonstrates the aggressive behavior of this species.

(ii) *Bichromomyia flaviscutellata* is the vector of *L.* (*L.*) *amazonensis* [7]. The presence of this sand fly in the surveyed sites, mainly site I, is noteworthy because of the pathological spectrum of its associated parasite [9] despite only the cutaneous form being documented in French Guiana [14, 15]. The synanthropic behavior of the Guianan population of *Bi. flaviscutellata*, which appears to adapt to environments under ecological pressures and human-made modifications [36, 69], has been documented. One *Bi. flaviscutellata* from a CDC canopy trap was positive for bird antiserum, raising the hypothesis that this species could migrate vertically to search for alternative blood sources. Domestic birds, such as chickens, may be attractive for this phlebotomine species, triggering it to adapt to modified environments. Our preliminary results from a peridomiciliary-forest stratification study have shown that some *Bi. flaviscutellata* specimens are captured outdoors, where animal shelters can stimulate phlebotomines to cross a 200 m gradient between the forest border and households (Vasconcelos dos Santos, unpublished data).

Most ACL isolates (6/8) were from patients infected while gold mining, showing that *L.* (*V.*) *guyanensis* ACL hotspots may be concentrated in these environments (upper Oyapock River Basin) [14, 15, 18]. Conversely, the present entomological results showed considerable infection rates of enzootics near urban cities, in which less economically attractive periurban forests (absence of gold mining) may reflect less human exposure to the disease (and consequently few ACL cases) in that area.

## Conclusions

Our findings show that ACL transmission in the Oyapock River Basin reflects the Guianan/Amazonian

classical ecosystem, where *Ny. umbratilis* remains the main vector. A putative alternative transmission by *Ev. infraspinosa* is possible, but circumstantial parasite ingestion is also likely, as seen with other biologically compatible phlebotomine species cohabiting the same potential *L.* (*V.*) *guyanensis* reservoir ecotopes. Conversely, epidemiological relevance of these putative alternative transmission cycles cannot be estimated with certainty. Literature-based evidence indicates that others fly species, such as *Ps. s. maripaensis* and *Bi. flaviscutellata* are also epidemiologically relevant, and we included them on the priority list for vector surveillance in the lower Oyapock basin. Local studies on ACL enzootics should be encouraged, since each an ecological mosaic is unique. The ACL etiology shows that the transmission pattern in the upper Oyapock may differ slightly from the lower basin, but only further surveys of the former environment can confirm this hypothesis.

#### Abbreviations

ACL: American cutaneous leishmaniasis; WHO: World Health Organization; CDC: Center of Diseases Control

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#### Availability of data and materials

All data supporting the conclusions of this article are included within the article. The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

#### Authors' contributions

Study design: TVS, MMP and EFR. Data acquisition: TVS, GP, MG, RD and FTS. Resources: GP, MG, RD and FTS. Data analysis: TVS, GP, MG, RD, FTS, MMP and EFR. Manuscript, original draft: TVS and EFR; final version: TVS, GP, MG, RD, FTS, MMP and EFR. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Procedures involving humans were submitted and approved by the Comitê de Ética em Pesquisa - CEP (Ethics in Research Committee), under protocol CAAE: 57710416.2.0000.0019. Capturing and processing invertebrate fauna (phlebotomines) were authorized by the Sistema de Autorização e Informação em Biodiversidade - SISBIO (Biodiversity Authorization and Information System), under protocol No. 44524.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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