Phlebotomines (Diptera: Psychodidae) from a Urban Park of Belém, Pará State, Northern Brazil and Potential Implications in the Transmission of American Cutaneous Leishmaniasis

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Abstract

In urban ecotourism parks, the life cycle of American cutaneous leishmaniasis (ACL) agents can remain established, where phlebotomines may comprise potential risks for visitors. The present study aimed to survey the phlebotomine fauna of a forest park ‘Bosque Rodrigues Alves-Jardim Botânico da Amazônia’ (BRAJBA), in the urban area of Belém, Brazil. The park was monthly surveyed in 2018 using CDC light traps placed in ground and canopy strata. Leishmania spp. isolated from dissected females were characterized by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) analysis. Fluctuations in specimen capture were correlated with rainfall. Nyssomyia antunesi (Coutinho, 1939) was predominant for all surveyed ecotopes and capture methods in both areas. Females of Ny. antunesi resting on tree bases were observed attempting to bite researchers during early morning. One Bichromomyia flaviscutellata (Mangabeira, 1942) and one Trichophoromyia brachipyga (Mangabeira, 1942) were found naturally infected by flagellates. Only the strain from Th. brachipyga was isolated and characterized as Leishmania (Viannia) lainsoni Silveira, Shaw, Braga and Ishikawa, 1987. Monthly fluctuations of the three most abundant species, Ny. antunesi, Trichophoromyia ubiquitalis (Mangabeira, 1942) and Th. brachipyga, had statistically significant negative correlations with rainfall. The present study provided further information to better understand ACL ecology in the Belém urban area, where the urban parks surveyed appeared to offer potential risk of contracting the disease, thus requiring environmental management. These observations highlighted the need for including Ny. antunesi, Bi. flaviscutellata, Th. ubiquitalis, and Th. brachipyga in the priority list for continuous entomological surveillance.

Key words: Phlebotomine, American cutaneous leishmaniasis, Urban park, Belém - Pará - Brazil.

Background

Phlebotomines comprise a group of medically important insects due their vector role in the transmission of several pathogens, particularly leishmaniasis agents (Ready 2013). In nature, a complex mosaic between mammals acting as reservoir hosts and phlebotomines is responsible for the maintenance of enzootic Leishmania parasites (Lainson and Shaw 2010). Affecting man, some species of Leishmania parasites may give rise to American cutaneous leishmaniasis (ACL), which, in some cases, comprises a clinically worrisome disease, with a spectrum ranging from self-limiting to mutilating forms (Silveira et al. 2013, 2018).

In Brazil, ACL presents three epidemiological transmission patterns: (i) sylvatic, (ii) occupational/recreational, and (iii) rural/peri-urban in colonization areas (Brasil 2017). Of these, pattern (ii) has an important impact on ecotourism. Forest parks opened to the public may offer conditions conducive to Leishmania spp. life cycles and consequently potential risk for ACL infection, requiring
specialized ecological management and entomological surveillance in these areas.

In the Belém Metropolitan Region (BMR), in Pará State, northern Brazil, ACL epidemiology assumes an occasional characteristic attributed to four Leishmania species: L. (Viannia) braziliensis Vianna 1911, L. (Leishmania) amazonensis Lainson and Shaw 1972, L. (V.) lainsoni Silveira, Shaw, Braga and Ishikawa, 1987 and L. (V.) lindenbergi Silveira, Ishikawa, Souza and Lainson 2002 (P.K.S. Ramos, unpublished data). Infection foci are mainly associated with forest fragments, where phlebotomines, reservoir hosts, and synanthropic fauna can survive environmental pressures and are able to establish ecological niches for Leishmania spp. maintenance (Ferreira et al. 2014).

In this sense, in the center of the urban area of Belém, the capital of Pará State, there are important urban parks that are worthy of attention as potential sources of ACL transmission because they are located in forest fragments with over populated captive mammal and free/semifree fauna. In the context of ACL eco-epidemiology, however, these parks are likely understudied.

Thus, the present work aimed to investigate, in an urban park of Belém, the phlebotomine sand fly fauna, associated or not associated with Leishmania spp. infections, spatiotemporal fluctuation patterns (monthly frequency and vertical stratification), and potential implications of these findings in the transmission of ACL agents.

Methods

Study Area

Belém is the capital and the biggest city of the Brazilian State of Pará, with an estimated population of 1,485,732 inhabitants. Climate is equatorial (average of 85–95% of humidity and 3,084 mm of yearly precipitation), directly influenced by the Amazon rainforest. In the urban area, ACL epidemiology is considered occasional, with only seven autochthonous cases registered in the last 5 yr (2014–2018). Taking into consideration the possibility of ecotourism parks currently open for visitation comprise risk areas for transmission of ACL agents, the following site was selected in the urban area of Belém (Fig. 1) as follows:

Bosque Rodrigues Alves-Jardim Botânico da Amazônia (BRAJBA) 1° 25' 48.2" S; 48° 27' 24.9" W. A 15 ha area of remaining preserved primary forest (80% of green area), with an estimated flora of 10,000 trees from 300 species and a fauna of 345 animals, with 29 species living in captivity and 29 others in free/semifree conditions.

Captures of Phlebotomines

Captures were performed in forested sites of the park described above, with the presence of trees with an average of 20 m height, to sample the canopy environment. Both sampling strata were systematically surveyed from January to December 2018 using Center of Diseases Control (CDC) light traps, placed in ground ($n = 2$ CDC at 1.5 m above ground level) and in canopy strata ($n = 2$ CDC at 20 m a.g.l), operating from 06:00 p.m. to 06:00 a.m., during four nights per month.

As an effort to make certain that there were not species present that did not respond to the light traps, Shannon captures, with manual aspiration made by two professionals were also performed from 06:00 p.m. to 08:00 p.m. during three intercalated nights of November 2018. Meanwhile, aspirations were performed on tree bases, with aid of an electric aspirator (an adapted CDC trap), from 06:00 a.m. to 08:00 a.m. during three intercalated mornings of October 2018. In both methods, specimens captured while attempting to bite researchers were preserved individually for identification.

Processing and Identification of Phlebotomines

Captured specimens were transported and immediately processed in Ralph Lainson’s Leishmaniasis Laboratory, Instituto Evandro Chagas (IEC). Females were prioritized for dissection under sterile conditions for species identification and to find parasites, following the method of Ryan et al. (1987). In the case of flagellate infection, parasitosis was semiquantified according to Freitas et al. (2002), and the pattern of parasite development was classified with the methods of Lainson and Shaw (1987). The gut contents of infected females were triturated, suspended in 0.2 ml of dissection solution, and inoculated in Novy-McNeal, and Nicolle culture media to attempt isolation. Males were identified under fresh conditions and/or stored in 70% ethanol to be later processed for mounting in Berlese or Canada balsam. Species identification followed the taxonomic criteria of Galati (2018).

Environmental Assessment

Climatic data were assessed to find correlations with phlebotomine fluctuations. The data were obtained from the ‘Instituto Nacional...
de Meteorologia-INMET\textsuperscript{*} database of the Belém Automatic Weather Station (code: 82191) [www.inmet.gov.br].

Data Analysis

The sampling effort was calculated by multiplying the number of traps installed by the number of hours and by the number of nights of capture for each park. To correct differences in sample effort, we calculated the average number of specimens captured per trap/hour (Rebelo et al. 2009, Vasconcelos dos Santos et al. 2018). The Shannon-Wiener (H) diversity index was estimated and evaluated using the \( t \) test for Shannon diversity. The Wilcoxon test was used to evaluate differences between the vertical strata. Rarefaction curves were constructed and species richness was estimated with the nonparametric estimators, Chao 2, Jackknife 1, Jackknife 2, and Bootstrap, using Past software version 1.64 (Hammer et al. 2001). Confidence interval was set at 95\%.

Additionally, to evaluate differences between abundance and richness, the Mann–Whitney \( U \) test using the BioEstat 5.3 program was used. The species infection rate (SIR) was calculated as follows: SIR = number of infected females of a given species divided by the number of dissected females of a given species and multiplied by 100 (Paiva et al. 2007). To define the association between the monthly frequency of captured phlebotomines and rainfall, a univariate Pearson (r) correlation was performed with the aid of Past software (Hammer et al. 2001). Thus, the calculated value indicates the following: > 0.7 to 1 is a strong correlation; 0.3–0.7 is a moderate correlation; 0.1–0.3 is a weak correlation; and < 0.1 is an insignificant correlation.

**Leishmania** spp. Characterization

*Leishmania* spp. were characterized by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) following previously established and validated methodology routinely applied in our lab (P.K.S. Ramos, unpublished data) (Simon et al. 2010). Digestion profiles of the amplicons were compared with those of the WHO *Leishmania* reference strains.

Environmental Issues

Following Brazilian legislation, procedures for capturing and processing of sylvatic invertebrate fauna for the present work were authorized by the 'Sistema de Autorização e Informação em Biodiversidade/Instituto Chico Mendes/ Ministério do Meio Ambiente' under protocol number 61923. In addition, permissions were requested and granted within the park.

Results

With 2,304 h of sampling with CDC trap, a total of 25,594 phlebotomines were captured and identified, belonging to 19 species; the most frequent species included *Nyssomyia antunesi* (Coutinho, 1939) (16,516; 64.56\%), *Trichophoromyia ubiquitalis* (Mangabeira, 1942) (3,789; 14.76\%) and *Th. brachipyga* (Mangabeira, 1942) (2,669; 10.40\%), accounting for 90.72\% of the total specimens captured (Table 1). Females (15,705) predominated over males (9,889) (ratio 1: 1.6).

With respect to vertical stratification, the most predominant species in the canopy was *Ny. antunesi*, representing 98\% (5,817 ð♀, 2,090 ð♂). Of the abundance, whereas on the ground, the predominant species were *Ny. antunesi* 49\% (5,551 ð♀, 3,058 ð♂), *Tr. ubiquitalis* 21\% (1,852 ð♀, 1,919 ð♂), and *Th. brachipyga* 15\% (1,427 ð♀, 1,223 ð♂) (Table 1).

Rarefaction curves for phlebotomines captured in canopy and ground level showed stabilization of species richness observed, considering the sample effort applied, and were in accordance with the values of all nonparametric estimators tested (Fig. 2).

**Table 1.** Species composition and vertical stratification of phlebotomines captured in the Bosque Rodrigues Alves - Jardim Botânico da Amazônia with CDC light traps, Belém, Brazil (Jan. to Dec. 2018)

| Species                      | Vertical strata | Ground | Canopy | Total | %     |
|------------------------------|-----------------|--------|--------|-------|-------|
|                              | ð♀ | ð♂ | ð♀+ð♂ | ð♀ | ð♂ | ð♀+ð♂ | Canopy | Total |       |
| *Nyssomyia antunesi*         | 5,551          | 3,058 | 8,609   | 5,817 | 2,090 | 7,907 | 16,516 | 64.5  |
| *Trichophoromyia ubiquitalis*| 1,852          | 1,919 | 3,771   | 10    | 8     | 18    | 3,789  | 14.8  |
| *Trichophoromyia brachipyga* | 1,427(1)       | 1,446 | 2,650   | 13    | 6     | 19    | 2,669  | 10.4  |
| *Presatia choti*             | 166            | 823   | 989     | 5     | 5     | 10    | 999    | 3.9   |
| *Bichromomyia flaviscutellata*| 424(1)         | 326   | 750     | 17    | 24    | 41    | 791    | 3.1   |
| *Beamontomyia avellari*      | 300            | 323   | 623     | 8     | 5     | 13    | 636    | 2.5   |
| *Viannamyia furcarta*        | 34             | 4     | 38      | 17    | 22    | 39    | 77     | 0.3   |
| *Scyphomyia sordellia*       | 16             | 12    | 28      | -     | -     | -     | 28     | 0.1   |
| *Esandromyia brachyphalla*   | 10             | 16    | 26      | -     | -     | -     | 26     | 0.1   |
| *Esandromyia monstrosa*      | 9              | 9     | 18      | -     | -     | -     | 18     | 0.1   |
| *Psathyromyia bigeniculata*  | 7              | 7     | 14      | -     | -     | -     | 14     | 0.1   |
| *Viannamyia tuberculata*     | 6              | 1     | 7       | -     | -     | -     | 7      | 0.0   |
| *Esandromyia infraspinosa*   | 1              | 5     | 6       | -     | -     | -     | 6      | 0.0   |
| *Lutzomyia gomezi*           | 5              | -     | 5       | -     | -     | -     | 5      | 0.0   |
| *Psathyromyia barrettoi barrettoi* | 3       | 2     | 5       | -     | -     | -     | 5      | 0.0   |
| *Micropygomyia rotaenaensis* | 3              | -     | 3       | -     | -     | -     | 3      | 0.0   |
| *Psathyromyia aragaoi*       | 1              | 1     | 2       | -     | -     | -     | 2      | 0.0   |
| *Micropygomyia trimidadiensis*| 2              | -     | 2       | -     | -     | -     | 2      | 0.0   |
| *Psychodopygus ayrozai*      | -              | -     | 1       | -     | -     | -     | 1      | 0.0   |
| **Total**                    | 9,817          | 7,729 | 17,546  | 5,888 | 2,160 | 8,048 | 25,594 |       |

\(n\): number of specimens found infected by flagellates; ð♀: females; ð♂: males.

The Shannon diversity index was: canopy \( H = 0.1174 \), ground \( H = 1.4402 \) \( t = 119.13, P = 0 \).
In relation to the phlebotomines captured per hour, with a total sampling effort of 2,316 h, an average of 15.1 specimens/hour, were obtained with CDC ground sampling (1,152 h of sampling), 6.96 specimens/h with CDC canopy sampling (1,152 h), 34.17 specimens/h with Shannon traps (6 h), and 616.17 specimens/h with aspiration on tree bases. *Nyssomyia antunesi* was the species with the highest average specimens/hour for all capture methods: CDC ground (4.82 ♀♀/h; 2.65 ♂♂/h), CDC canopy (5.05 ♀♀/h; 1.81 ♂♂/h), Shannon trap (12.33 ♀♀/h; 11.50 ♂♂/h), and aspiration on tree bases (238.67 ♀♀/h; 376.50 ♂♂/h). The other species that showed a high capture frequency were *Th. ubiquitalis* (1.61 ♀♀/h; 1.67 ♂♂/h) and *Th. brachipyga* (1.24 ♀♀/h; 1.06 ♂♂/h) for the CDC ground sampling. Specimens of *Ny. antunesi* were captured attempting to bite the professionals during aspiration on the tree bases (Table 2).

Monthly fluctuations of the three most frequent species, *Ny antunesi*, *Th. ubiquitalis*, and *Th. brachipyga*, were tested against precipitation. For all these phlebotomines, moderate but statistically significant negative correlations were observed (Fig. 3).

Parasite infections (gregarines and flagellates) were observed on four occasions for specimens captured in CDC ground. Two females of *Bichromomyia flaviscutellata* (Mangabeira, 1942) were found with gregarines and a female of *Bi. flaviscutellata* out of 441 dissected females (SIR: 0.22) and a female of *Th. brachipyga* out of 1,440 dissected females (SIR: 0.06) were found harboring flagellates, with estimated parasitosis and development patterns of (+++/suprapylarian) and (++/peripylarian), respectively. Only the parasite of *Th. brachipyga* was successfully isolated in culture, being cryopreserved in the cryobank of Ralph Lainson’s Leishmaniasis Laboratory under the WHO code IBRA/BR/2018/M33013. DNA characterization showed a PCR-RFLP profile identical to that of the *L. (V.) lainsoni* WHO reference strain (Fig. 4).

**Discussion**

In Belém, although the BRAJBA is an important source of ecological data, with a well-studied inventory of fauna and flora, ACL ecology in this environment seems to be understudied. Thus, the present work provided, for the first time, with an entomological survey,
information on phlebotomines and their potential implications in the transmission of ACL agents.

Past studies accounted for 62 phlebotomine species from Belém and its outskirts (Ryan 1986). However, over the years, vegetal suppression and environmental pressure, mainly due to the urbanization process, contributed to an apparent reduction of local species diversity. A more recent inventory of seven forest fragments accounted for 22 species (Ferreira et al. 2014), numerically proportional to our present results (19 spp.).

Great species diversity, as well abundance, was found in the ground level. Canopy and ground strata can be seen as distinct habitats, with physical components and biological diversity. Canopy is a primary site of flowering and fruiting, a particular ecological subsystem suitable to housing arboreal frugivorous/herbivorous vertebrates (e.g., sloths and primates), while the ground hosts low-flying insects attracted to terrestrial animals, that generally comprise the greatest composition of phlebotomine fauna in the tropical rainforest. The vertical distribution of Phlebotomines is probably a response to physical and biological conditions of microhabitats, such as temperature, relative humidity, light intensity and spectral composition, air movement, and CO2 (Chaniotis et al. 1971). It should not be neglected that the other trapping methods (Shannon traps × tree bases) were performed in different months (with different environmental conditions, like rainfall and wind), which strongly impacts the comparison between very different results.

Species infection rate for flagellates was estimated in 0.06 for Th. brachipyga and 0.22 for Bi. flaviscutellata. Dissection-based infection rates are usually lower than those provided by PCR-based methods, but also can vary greatly in the literature, with high values usually biased by captures performed in the dry season (Le Pont and...
birds may also serve as alternative blood sources to maintain the population dynamics (Vasconcelos dos Santos et al. 2018).

Still in regard of vertical stratification of Ny. antunesi, this species represented 98% and 49% of the phlebotomine community in the canopy and ground strata, respectively. These results contrast with those of Silveira et al. (2002), who surveyed, in the late 1990s, an ACL focus, a military forest fragment approximately 2 km from the BRAJBA. In that occasion, Ny. antunesi was regarded as a predominantly terrestrial phlebotomine species. Conversely, present results lead us to speculate that there is an attractive feeding source for Ny. antunesi in the canopy strata and, if it is the vector of L. (V.) lindenbergi as originally supposed, it is reasonable to suspect of an arboreal mammal, as potential reservoir host. Attempting to fill this eco-epidemiological gap, our future steps in the studied area may include searching for feeding sources of phlebotomines, in particular for that species.

Bichromomomyia flaviscutellata has been found to be infected by gregarines and flagellates. Gregarines are relative common in this phlebotomine species, and despite a lack of molecular methods to perform species identification of this protozoan, our experience allows us to speculate that they were Psychodiella sp. oocysts (most likely Psychodiella chagasi (Adler and Mayrink, 1961) (Vasconcelos dos Santos et al. 2018)). With respect to flagellate infection, the suprapylarian behavior and luxuriant development of large promastigotes also allows us to speculate that they belonged to L. (L.) amazonensis. Belém is the type-locality of that leishmanian parasite, and natural infections are well documented in other forest fragments of the region where Bi. flaviscutellata is the only known vector on the local scale (Lainson and Shaw 1968).

The medical importance of Trichophoromyia has thus far gained attention. In Brazil, there are 23 described species, with some of them potentially implicated in the transmission of ACL agents (Vasconcelos dos Santos et al. 2019). In the present work, two species, Th. ubiquitalis and Th. brachipyga accounted for 14.76% and 10.40% of the captured specimens, respectively. Based on naturally infected specimens and anthropophilic behavior under lab conditions, Th. ubiquitalis has been implicated in the vector transmission of L. (V.) lainsoni (Silveira et al. 1991, Lainson et al. 1992). Conversely, one female of Th. brachipyga was found naturally infected with L. (V.) lainsoni in the present work. Trichophoromyia specimens captured in an hydroelectric system affected area with sympatric occurrence of indistinguishable females of Th. brachipyga and Th. adelsonsouzaei (Vasconcelos dos Santos, Silva, Barata, Andrade & Galati, 2014) were found housing peripylarian flagellates morphologically suggestive to be metacyclic stages of L. (V.) lainsoni (Silveira et al. 2002). It is not clear whether these apparently isolated facts may represent a occasional finding or an underreported alternative vector role of this phlebotomine in ACL epidemiology due to L. (V.) lainsoni. If Th. brachipyga truly shares a vector role with Th. ubiquitalis, present finding is worrisome because this species is very frequently occurring in some forest environments, such as the Parque Estadual do Utinga, the largest forested park of Belém (Feirera et al. 2014). Life cycle of L. (V.) lainsoni is quite possible to be maintained in the BRAJBA, since the mammal inventory of that park accounts several specimens of the rodent Cuniculus paca (Linnaeus, 1766), the potential reservoir host of that parasite. Also worth of note, both Trichophoromyia species were found predominantly at ground level, spatially congruent with the ecotope of C. paca.

No specimen of Trichophoromyia, distinct from Th. brachipyga and Th. ubiquitalis, was found in the BRAJBA. According to the phlebotomine inventory of Pará State presented in Ryan (1986), Th. Pajot 1980) or favoring the dissection of fed/gravid females (Freitas et al. 2002).

As expected, Ny. antunesi was the most frequently collected species for all environments and capture methods. These findings corroborate our past experience of entomological surveillance while searching presumed ACL infection sites in Belém and outskirts (Vasconcelos dos Santos, personal observation). In addition, based on its high frequency and anthropophilic behavior, this species has drawn attention for the suspected transmission of L. (V.) lindenbergi, a parasite species with geographical distribution apparently restricted to Belém and its outskirts (Silveira et al. 2002), but recently extended to the Brazilian western Amazon region (Cantanhêde et al. 2019). Surprisingly, in the present work, a markedly high number of specimens were captured on tree bases during the morning, including females attempting to bite the professionals, most probably reacting due disturbance on their resting sites. This result highlights the potentially aggressive behavior of this species during a time when visitors may be exposed in these parks. Similar biting behavior on tree bases, over the night it is essentially arboreal, climbing to the treetops to bit sloths and anteaters (Ready et al. 1986). In same circumstances,
Conclusions

In summary, the present study provided further elements to better understand ACL ecology in Belém urban areas. The urban park surveyed may offer potential risks of disease transmission for which environmental management and continuous entomological surveillance are required. The results also highlighted the medical importance of *Trichophoromyia* species, mainly due to the observations of a possible role of *Th. brachiipygna* in the transmission of *L. (V.) lainsoni*. Present and past data also note *Bi. flaviscutellata* as an important vector in Belém. On the other hand, *Ny. antunesi* remains outstanding among the list of potential *L. (V.)* *lindenbergi* vectors, especially with regard to its observed high frequency and potentially aggressive behavior of females resting on tree bases during the early morning. The monthly fluctuations of these species, however, do not seem to be positively correlated with rainfall.

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Availability of Data and Materials

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

Author Contributions

Study design: T.V.S., F.T.S., M.M.P.; data acquisition: Y.d.V.S.U., T.V.S.; resources: F.T.S., M.M.P.; data analysis: Y.d.V.S.U., T.V.S., F.T.S., E.J.M.S., P.K.S.R., M.M.P.; manuscript—original draft: Y.d.V.S.U., T.V.S.; manuscript—final version: Y.d.V.S.U., T.V.S., F.T.S., E.J.M.S., P.K.S.R., M.M.P. All authors read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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