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Breeding sites of phlebotomine sand flies (Diptera: Psychodidae) and efficiency of extraction techniques for immature stages in terra-firme forest in Amazonas State, Brazil

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ABSTRACT

Information on natural breeding sites of phlebotomine sand flies is scanty, due to the difficulties of isolation of immatures from the soil where they occur. The present study investigated breeding sites in several microhabitats in a "terra-firme" forest in Pitinga, Amazonas State, Brazil. Results on the efficacy of different extraction techniques used for isolating sand flies, and the temperature and the pH of the samples collected, are presented. Samples of soil and organic matter from different microhabitats, processed by floatation-sieving, direct examination, Berlese–Tullgren, and emergence cages, revealed, for the first time in Amazonas, breeding sites in five microhabitats (tree bases, unsheltered forest floor, soil from under fallen logs, soil from under roots, and palm-tree bases). Overall, 138 immatures and 29 newly emerged adults were recovered from these microhabitats. The abundance of immatures in samples close to tree bases was significantly higher than in more open sites not adjacent to tree bases. Floatation-sieving and direct examination were the most effective techniques for immature extraction and survival, respectively. Eleven species of the genus *Lutzomyia* s.l. were identified, with *Lutzomyia monstruosa* (Floch & Abonnenc) and *Lutzomyia georgii* Freitas & Barrett being the most abundant. Differences in the specific composition and relative abundance of the immature and adult sand flies on tree bases suggest that breeding sites may be distant from resting or aggregation sites of adults. The pH, which revealed a slightly acidic soil, as well as the temperature, did not show any significant correlation with the number of immature sand flies collected.

1. Introduction

Just over a century since the first report of larval Phlebotominae in the field (Grassi, 1907), the pre-imaginal ecology and breeding sites remain unknown for the vast majority of species of these medically important insects. In contrast to some other Psychodidae, no aquatic larvae are known for this subfamily, but considerable effort has been devoted to the extraction of phlebotomines from soil and other organic substrates in diverse habitats, often with disappointing yields (in the Old World: Marett (1910), McCombie-Young et al. (1926), and Ghosh and Bhattacharya (1991) and in the Neotropical region: Ferreira et al. (1938), Pifano (1941), Coutinho and Barretto (1941), Rutledge and Mosser (1972), Rutledge and Ellenwood (1975a,b,c), Ferro et al. (1997), Casanova (2001), and Alencar (2007). Among the more successful efforts is that of Hanson (1961), who extracted close to 2200 immature sand flies, mainly from soil around the bases of trees with buttress roots in Panama. This research was the first to note the lack of correlation between the species composition of the larval population and the associated adult dendrobatic community (associated with trunk and canopy trees). This success obtained by Hanson (1961) was due to the employment of the extraction technique of immature sand flies, named "floatation-sieving". This technique was used for the first time with great success by McCombie-Young et al. (1926) in India. Beside this method, some other techniques are also used to detect breeding sites of phlebotomine sand flies. Recently, Feliciangeli (2004) revised the most useful methods. Unfortunately, little is known about their relative yields and effect on survival of individuals extracted.

Studies on the larval biology of phlebotomines have been justified on the grounds of possible applications to the control of vector species. More pragmatically, in the case of rainforest sand flies, information on their larval habitats could contribute to the successful establishment of closed laboratory colonies. Most rainforest species, including important vectors of cutaneous leishmaniasis,

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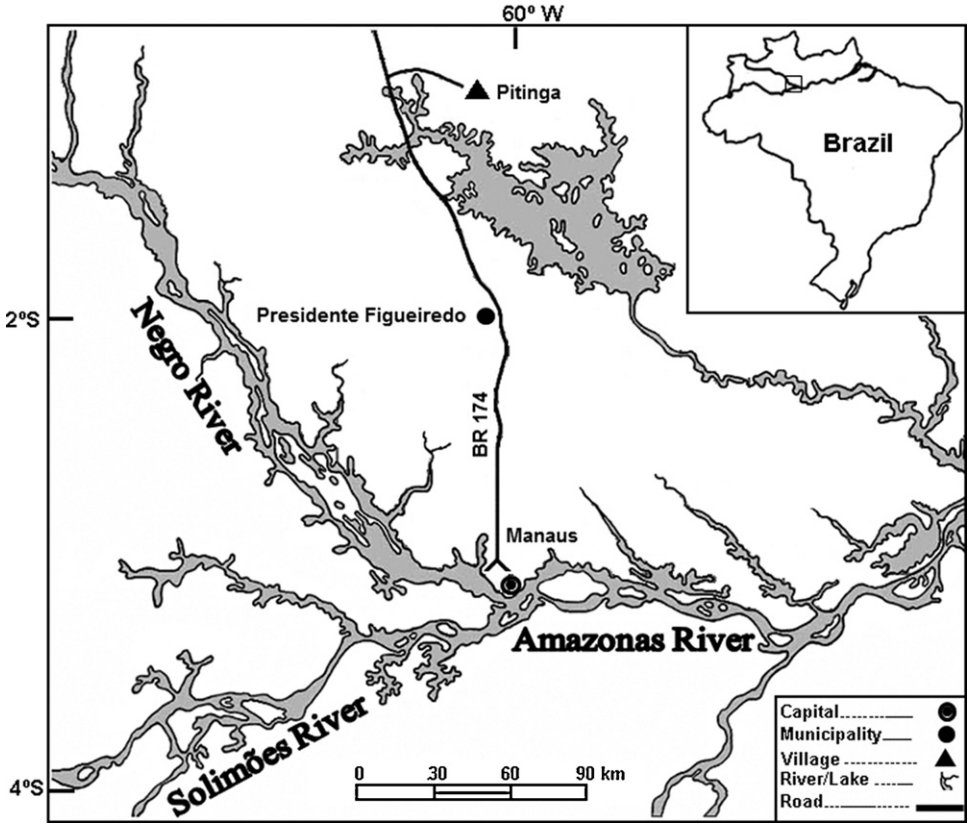

Fig. 1. Geographic location of the Pitinga study area.

appear to be very specialized and highly fastidious with regard to the exact biotic and abiotic parameters of the larval environment, and the reproductive biology of many vectors is completely unknown. Sustainable colonies of such species could make an important contribution to experimental parasitology and medical entomology.

In this report we present the results of a search for breeding places of phlebotomines in an area of primary "terra-firme" forest. The species composition, temperature and pH of samples from different microhabitats and the yields of four different extraction techniques are compared.

2. Materials and methods

2.1. Study area

Pitinga Village (00°47′S, 60°04′W) is an isolated industrial mining town established for the extraction of tin ore and rare minerals, 280 km north of Manaus in the municipality of Presidente Figueiredo, Amazonas State, Brazil. The town is 55 km east, by road, from the BR-174 federal highway (Fig. 1). The geomorphology is dissected and dominated by a plateaux approximately 280 m a.s.l. (Mineração Taboca, 2001). The entire region, with the exception of the urban nucleus and associated areas cleared for mining, is tropical rainforest with a high canopy (35–40 m) and occasional emergent trees (>45 m). Mean annual precipitation is 2432 mm, without a pronounced dry season. Temperature deviates little from the average of 28 °C.

2.2. Sampling

Samples of topsoil and organic debris were collected in March and monthly from June to December 2002, using a trowel and machete, and placed in 1-L plastic pots, which were then maintained as close as possible to 27 °C until processing. Material from tree-forks and holes near canopy level [36.1 L] was obtained using the appropriate climbing gear. Samples from ground level were discriminated according to composition (mature soil/soil with abundant small fragments of litter/mainly litter) and according to location as follows: tree bases between buttress roots (Fig. 2) and base of trees without buttress roots [197 L], forest floor not sheltered – dead leaves (5–10 m from the sampled tree bases) [101 L], soil from under fallen logs [9.7 L], soil from under surface tree-roots [5 L], base of palm-tree stems [1 L], animal burrows [4.1 L], hollow roots [0.6 L], hollow base of standing trees [2 L], soil from under lianas [0.8 L], soil over surface tree-roots [1 L], loose bark [5.8 L], and abandoned termite nests [7 L].

In order to make a quantitative comparison between "tree base" and "unsheltered forest floor", 20 trees observed to harbor adult phlebotomines in daylight were selected. "Tree base" was defined as samples collected within a 1 m radius from the trunk. "Unsheltered forest floor" was defined as samples collected 5–10 m from the same trees, in relatively exposed spots. A single 1-L sample was collected every month from August to December, from each of the 20 trees and each of the associated open areas, avoiding repetition of the exact sampling points.

2.3. Processing of soil samples

Three techniques for extracting immature stages (floatation-sieving, Berlese–Tullgren funnels, and direct examination), and one for recovering emerging adults (incubation cages), were used.

The floatation-sieving method was first used on phlebotomine sand flies in India (McCombie-Young et al., 1926) and later applied and described in detail by Hanson (1961) while working in Panama. The samples are suspended in saturated sucrose solution at ambi-

Fig. 2. Typical tree with buttress root found in Pitinga. Photo: Monte.

ent temperature and allowed to decant. The floating material is then analyzed. This is the least labor-intensive method and the one used for most of the samples.

In the Berlese–Tullgren method (Lincoln and Sheals, 1979), the larvae migrating away from the heat of the light to the base of the apparatus fall into pots from which they are transferred to the rearing vessels.

For direct examination, a part of each sample was carefully examined under a dissecting microscope with the aid of fine paint brushes, dissecting needles and forceps.

For recovery of emerging adults, samples with a layer of about 2.5 cm deep were incubated at 27 °C and 95% relative humidity on plastic trays in 20 cm × 20 cm × 20 cm emergence cages for a period of two months.

The relative yield of the different extraction techniques was evaluated using each of the 1-L samples from tree bases (100 samples) and forest floor not adjacent to tree bases (100 samples). For each of the 200 samples, 500 ml was used for floatation and approximately 167 ml for each of the remaining three techniques. Results were quantified as total individuals extracted, individuals extracted per unit volume, and proportion of extracted immatures surviving to adult stage.

All immature sand flies extracted alive were reared separately according to the origin of the sample and the extraction method. The larval diet was prepared from a mixture of rabbit feces, commercial hamster ration and a small quantity of the original substrate.

Statistical analysis was carried out with EXEL (WINDOWS 98) and SYSTAT 9.0 software.

3. Results

A total of approximately 370 L of soil and associated organic matter were processed. The total volume of the positive samples was 59 L, originating from five categories of microhabitat: tree bases (45 L), open forest floor (8 L), soil from under roots (3 L), soil from under fallen logs (2 L), and bases of palm trees (1 L).

A total of 167 phlebotomines were obtained, consisting of 126 larvae, 12 pupae and 29 emergent adults. The majority (147 or 88.0%) of these individuals were from soil adjacent to tree bases (with or without buttress roots), while far fewer individuals were collected from open forest floor (8 or 4.8%), and soil under fallen logs, soil from under roots, and palm tree bases (4 or 2.4%, each). Surprisingly, not a single specimen was found in the 36 L of substrate collected in tree-forks and cavities at canopy level.

Of all immatures extracted, 41 (29.7%) were in the third larval instar, 38 (27.5%) in the fourth, 36 (26.1%) in the second, four (2.9%) in the first, and 12 (8.7%) were pupae. Seven larvae did not have its stages determined (5.1%). Eighty-nine (64.0%) of the 138 immature sand flies were successfully reared to adult stage. The total number of adults, including those obtained from the incubation cages, was 118, and consisted of 11 species of *Lutzomyia* s.l. (Table 1).

In the standardized comparison between tree base and open forest floor samples, the number of individuals recovered from these two microhabitats was significantly different: 79 and 7, respectively (Mann–Whitney *U*-test, $P = 0.001$) (Fig. 3).

Of the individuals recovered from soil at tree bases, 48 (60.0%) were from ten trees with buttress roots and 31 (40.0%) from five trees lacking buttress roots. Of the 20 trees sampled, two individual trees contributed over half the total: *Dinizia excelsa*

Table 1
Species of the immature stages of *Lutzomyia* found in soil/organic matter in terra-firme forest in Pitinga Village – AM.

Subgenus or species-group	Phlebotomines for microhabitat					Sex		Total	%
	BT	FF	FT	SR	BP	♂	♀		
L. (*Evandromyia*) *monstruosa*	39	2	0	1	0	17	25	42	35.59
L. (*E.*) *georgii*	29	1	3	2	0	0	35	35	29.66
Lutzomyia (*E.*) sp. of Pitinga[a]	2	0	0	0	0	2	0	2	1.69
Lutzomyia (*Nyssomyia*) *umbratilis* Ward & Fraiha	3	1	0	0	0	2	2	4	3.39
Lutzomyia (*N.*) *anduzei* (Rozeboom)	0	1	0	0	0	0	1	1	0.85
Lutzomyia (*N.*) *flaviscutellata* (Mangabeira)	0	0	0	0	1	1	0	1	0.85
Lutzomyia sp. of Baduel (Floch & Abonnenc) [Migonei group]	12	0	0	0	0	7	5	12	10.17
Lutzomyia sericea (Floch & Abonnenc) [Migonei group]	8	0	0	0	0	5	3	8	6.78
Lutzomyia saulensis (Floch & Abonnenc) [Saulensis group]	2	1	1	0	2	2	4	6	5.08
Lutzomyia (*Sciopemyia*) *sordellii* (Shannon & Del Ponte)	5	0	0	0	0	1	4	5	4.24
Lutzomyia (*Trichopygomyia*) *trichopyga* (Floch & Abonnenc)	1	1	0	0	0	0	2	2	1.69
	101	7	4	3	3	37	81	118	100.00

BT, base of tree; FF, forest floor; FT, soil under fallen trunk; SR, soil under root; BP, soil at base of palm tree.

[a] New species in description process.

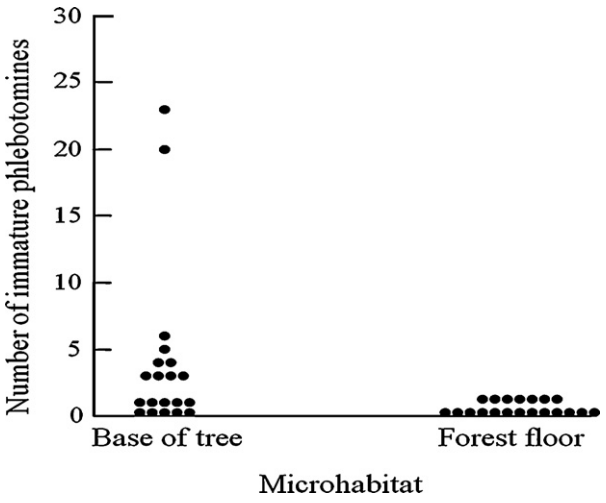

Fig. 3. Distribution of immature phlebotomine sand flies at tree bases and open forest floor, for each selected tree of the systematic portion of the study.

Table 2
Phlebotomine sand flies (immature/adult) extracted from soil/organic matter samples collected around trees bases in the period August to December/2002 in terra-firme forest in Pitinga Village – AM.

Tree		Samples[a]		Ind
Species	Family	+	–	
Dinizia excelsa Ducke[c]	Mimosaceae	3	2	23
Sclerolobium chrysophyllum Poepp.[b]	Caesalpiniaceae	2	3	20
Manilkara cavalcantei Pires & Rodrigues[c]	Sapotaceae	1	4	6
Aspidosperma spruceanum Benth[c]	Apocynaceae	4	1	5
Goupia glabra Aublet[b]	Celastraceae	2	3	4
Erisma bicolor Ducke[c]	Vochysiaceae	2	3	4
Micropholis casiquiarensis Aubrév.[c]	Sapotaceae	2	3	3
Simarouba amara Aubl.[b]	Simaroubaceae	1	4	3
Hymenolobium heterocarpum Ducke[b]	Fabaceae	1	4	3
Sextonia rubra (Mez)[c]	Lauraceae	2	3	3
Buchenavia parvifolia Ducke[c]	Combretaceae	1	4	1
Buchenavia parvifolia Ducke[c]	Combretaceae	1	4	1
Manilkara cavalcantei Pires & Rodrigues[c]	Sapotaceae	1	4	1
Eschweilera atropetiolata Mori[b]	Lecythidaceae	1	4	1
Dinizia excelsa Ducke[c]	Mimosaceae	1	4	1
Tachigali mymercophyla (Leg. – Pap.)[b]	Caesalpiniaceae	0	5	0
Sextonia rubra (Mez)[c]	Lauraceae	0	5	0
Eschweilera atropetiolata Mori[b]	Lecythidaceae	0	5	0
Lecythis zabucajo Aubl.[c]	Lecythidaceae	0	5	0
Pouteria caimito (Ruiz & Pav.)[c]	Sapotaceae	0	5	0
		25	75	79

Ind, number of phlebotomines (immature/adult) extracted.
[a] Each sample consisted of 1 L soil/organic matter; +, samples with phlebotomine sand flies; –, samples without phlebotomine sand flies.
[b] Without buttress.
[c] With buttress.

(with buttresses, 23 phlebotomines) and *Sclerolobium chrysophyllum* (without buttresses, 20 phlebotomines) (Table 2).

Very few dead larvae were extracted. Survival of live immature insects to adult stage was over 50% for all extraction methods, with direct examination associated with the lowest mortality (Fig. 4).

Temperature (24.9–25.9 °C) and pH (3.9–4.7) varied little between samples.

4. Discussion

In the Neotropical region, tree bases, mainly those with buttress roots, have been suggested as potential natural breeding sites for phlebotomine sand flies (Ferreira et al., 1938; Coutinho and Barretto, 1941; Hanson, 1961; Rutledge and Mosser, 1972). The accumulation of organic matter and protection from flooding, rainfall, direct light and wind are among the factors that allow the survival and/or development of immature sand flies in these sites (Rutledge and Ellenwood, 1975c). On the other hand, the forest floor usually presents a slightly shallower organic matter layer (rarely surpassing 5 cm in Pitinga), which is more exposed to sunlight, desiccation, flooding and erosion (Rutledge and Ellenwood, 1975a).

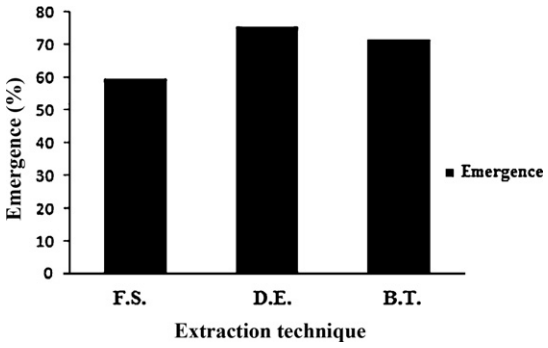

Fig. 4. Percent survival to adult emergence of immature phlebotomines extracted from soil by different methods. F.S., floatation-sieving; D.E., direct examination; B.T., Berlese–Tullgren method.

In Pitinga, almost 90% of the immature sand flies found were recovered from tree bases (with or without buttress roots). This was similar to the one found by Hanson (1961), in Panama, while studying mainly tree bases with buttress roots.

The results of the comparative studies carried out in Pitinga and presented here showed that tree bases seem to provide better conditions for the development of immature sand flies of some species (Figs. 2 and 3). However, even for this microhabitat, it was relatively common to recover immature sand flies on sample sites adjacent to others in which no immature sand fly was found. In addition, samples from the bases of the same tree species [e.g. *D. excelsa* Ducke, *Manilkara cavalcantei* Pires & Rodrigues, *Sextonia rubra* (Mez) and *Eschweilera atropetiolata* Mori] did not yield the same numbers of immature sand flies (Table 2), suggesting that breeding sites may be focal within the tree-base area. This might be the result of a preference by gravid females for laying their eggs in specific spots of breeding sites influenced by oviposition pheromone (Elnaiem and Ward, 1990, 1991; Elnaiem et al., 1991) and/or stimulus or attraction by animal feces (Schlein et al., 1989; Elnaiem and Ward, 1992). Since more than half of immature sand flies extracted in Pitinga were second and third instar larvae, it is possible to infer that some were probably in the egg stage at collecting time, as it took between one and four weeks to process all samples.

Substrates found under fallen tree logs, surface tree roots or palm tree bases yielded more immature sand flies per processed sample volume compared to samples from tree bases (not palm trees) and open forest floor. However, the reduced sample size from these sites precludes statistical comparison with tree bases.

In Pitinga, the upper and middle layers of the canopy accounted for the third largest volume of sample processed, but no immature sand flies were found. Canopy sampling was only done during the dry season. In Panama, Thatcher (1968) recovered 12 immature sand flies from the canopy during the rainy season and observed that breeding site availability in these forest strata decreased during the period of lower rainfall.

Studies on phlebotomine breeding sites normally use floatation-sieving techniques with saturated sucrose solution as the main way to extract the immature sand flies. In the present study, this technique was in fact, the most efficient in terms of the absolute number of extracted immature sand flies. However, it was also the technique which processed the largest volume of substrate. When considering the number of individuals by volume of processed sample, the incubation cage was the most efficient technique. Recently

in India, good results were found using this latter technique after recovering 70 adults (Singh et al., 2008). This technique has the advantage of adult stage phlebotomine recovery, saving time and effort, when compared to extraction and rearing immature sand flies to adult stage.

Direct examination was the most efficient technique for larval survival, followed by the Berlese–Tullgren method and then floatation-sieving. The low adult count from emergence samples extracted by the floatation-sieving method could be due to damage caused to the larvae and pupae during the sample processing, while direct examination, although slow and tedious, is less aggressive.

In general, the laboratory breeding protocol used in this study presented itself as very efficient, considering that about 65% of immature sand flies reached the adult stage. In comparison, Hanson (1961) reared 600 immatures to adult stage, which represented only 28% of the total of immatures (2123) extracted.

Of all the species represented by the immature sand flies, six of them were extracted from only one type of microhabitat (Table 1). *Lutzomyia mostruosa* and *Lutzomyia georgii*, species with higher numbers of adults recovered, were collected in more than one microhabitat, and most of the immatures recovered were from tree base samples. The resting sites of adults for those species are unknown. The concentration of immature sand flies could be the result, as already mentioned, of gravid females' preference for specific niches for oviposition in a forest environment, not necessarily located close to their resting sites. For example, adults of *Lutzomyia umbratilis*, the main vector of *Leishmania guyanensis* in the Amazon, are predominantly found on tree-trunks (dendrobatic behavior) during the day in forest areas located at the east side of the Negro River and north side of Amazonas River (Barrett et al., 1991; Cabanillas and Castellón, 1999). However, few adults of this species emerged from tree base larval samples. Similar lack of correspondence between larval and adult habitats was found by Deane and Deane (1957) studying *Lutzomyia longipalpis* and Hanson (1961) in Panamá studying *Lutzomyia trinidadensis*, *Lutzomyia yelephiletor* and *Lutzomyia shannoni*.

Hanson (1961) pointed out that forest floor could be a possible breeding site for *Lutzomyia trapidoi*, a species from the same subgenus (*Nyssomyia*) as *L. umbratilis* and *Lutzomyia anduzei*.

Immature sand flies of other dendrobatic species (*Lutzomyia dendrophila*, *Lutzomyia scaffi*, and *L. shannoni*) were not found at tree bases or elsewhere. On the other hand, adults of species rarely found on tree-trunks, like *Lutzomyia monstruosa*, *L. georgii*, *Lutzomyia* sp. of Baduel, *Lutzomyia sericea* and *Lutzomyia sordelli* were well represented by their immature stages in tree base samples (Table 1).

Hanson (1961) found most immatures in a superficial layer of soil collected at most 5 cm deep. He stated that immature sand flies of species whose adults are mainly found in the base of the trees are more prone to bury deeper in the soil, this behavior being responsible for the smaller recovery of immature stages of these dendrobatic species. However, *Brumptomyia hamata* was abundantly collected from superficial soil samples and showed preference for deeper areas of the substrate when reared in the laboratory.

Species identification of most phlebotomines is based on the adult, and extraction techniques for the study of larval habitats should therefore take into account the yield of imagos from the collected immatures.

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