Phlebotomine fauna, natural infection rate and feeding habits of *Lutzomyia cruzi* in Jaciara, state of Mato Grosso, Brazil

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Visceral leishmaniasis (VL) in Brazil is transmitted by the phlebotomine Lutzomyia longipalpis *and in some midwestern regions by* Lutzomyia cruzi. *Studies of the phlebotomine fauna, feeding habits and natural infection rate by* Leishmania *contribute to increased understanding of the epidemiological chain of leishmaniases and their vectorial capacity. Collections were performed in Jaciara, state of Mato Grosso from 2010-2013, during which time 2,011 phlebotomines (23 species) were captured (68.70%* Lu. cruzi *and 20.52%* Lutzomyia whitmani). Lu. cruzi *females were identified by observing the shapes of the cibarium (a portion of the mouthpart) and spermatheca, from which samples were obtained for polymerase chain reaction to determine the rates of natural infection. Engorged phlebotomines were assessed to identify the blood-meal host by ELISA. A moderate correlation was discovered between the number of* Lu. cruzi *and the temperature and the minimum rate of infection was 6.10%. Twenty-two females were reactive to the antisera of bird (28%), dog (3.30%) and skunk (1.60%). We conclude that* Lu. cruzi *and* Lu. whitmani *have adapted to the urban environment in this region and that* Lu. cruzi *is the most likely vector of VL in Jaciara. Moreover, maintenance of* Leishmania *in the environment is likely aided by the presence of birds and domestic and synanthropic animals.*

Key words: visceral leishmaniasis - *Lutzomyia* sp. - PCR - ELISA

Leishmaniases are diseases caused by parasites of the genus *Leishmania*, which are protozoans transmitted by phlebotomines that manifest clinically in three forms (cutaneous, mucocutaneous and visceral), depending on the parasite species. Visceral leishmaniasis (VL) is the most severe form, resulting in high mortality if it is not treated early, and it is caused by *Leishmania infantum chagasi* in the Americas (Harhay et al. 2011).

In Brazil, two species have been related to the transmission of VL, including *Lutzomyia longipalpis* and *Lutzomyia cruzi*, the former of which is considered the main vector and the latter of which appears in only some municipalities, where it most likely participates in the transmission cycle (Almeida et al. 2010). The species *Lu. cruzi* is morphologically related to *Lu. longipalpis* because the females of both are identical and the males can only be distinguished by small differences in genitalia (Young & Duncan 1994). *Lu. cruzi* has been detected in 24 municipalities in the state of Mato Grosso (MT) (Missawa & Lima 2006).

Identifying the natural infection rates of phlebotomines by *Leishmania* represents an important component of epidemiological studies of leishmaniases and their vectorial competence (Paiva et al. 2007). One technique involving dissection of the digestive system of the vector for direct research of the parasite has been commonly employed for this purpose; however, this method is laborious and time-consuming because it involves searching for the parasite *in loco*. Furthermore, in positive cases, the infection requires confirmation by in vitro *Leishmania* cultures, which are frequently prone to contamination or by the inoculation of laboratory animals because other flagellates are commonly found in the digestive tracts of these insect vectors (Michalsky et al. 2002). The development of molecular biology techniques used in association with polymerase chain reaction (PCR) has made the identification of even the smallest quantities of parasite genetic material possible, regardless of its stage and location within the digestive system of the vector insect (Oliveira-Pereira et al. 2006).

The eating habits of phlebotomines provide information on the identification of hosts, revealing the potential reservoirs of leishmaniases (Missawa et al. 2008), which may include rodents, marsupials, edentulous and canids in the wild (Ashford 2000) and domestic dogs in urban settings (Dantas-Torres 2007). The attraction that various wild and domestic animals exercise over the phlebotomines as food sources is an important factor contributing to the understanding of host-vector relationships in various environments, especially in dealing with the transmission of leishmaniases (Marassá et al. 2006).

Currently, 21 of the 27 states of Brazil, which are situated in all five Regions of the country, register the autochthonous transmission of VL (Tonini et al. 2012). According to information provided by the Informatics Department of the Unified Health Service (dtr2004.saude.gov.br/sinanweb/tabnet/dh?sinannet/leishvi/bases/

leishvbrnet.def.), Brazil registered 3,348 cases of VL in 2012. In MT, 56 autochthonous cases were registered, with Rondonópolis and Jaciara together accounting for approximately 43% of these cases. In Jaciara, the first human case of VL occurred in 2003 and, between that date and 2013, 19 autochthonous cases with one death in 2011 were reported. In accordance with the Environmental Health Surveillance Department of the State Health Secretariat, surveys of canine serum have indicated high positive levels of contamination and entomological data have revealed the absence of *Lu. longipalpis* and frequent presence of *Lu. cruzi* in areas including those with registered cases of both human and canine VL.

The objective of this study was to investigate phlebotomine fauna and their natural infection rate and identify the animals that serve as feeding sources for the *Lu. cruzi* vector in the municipality of Jaciara.

MATERIALS AND METHODS

Study area - Jaciara, which is located 145 km from the state capital of Cuiabá, is situated in the southern region of the state at the coordinates 15°57'55''S 54°58'06''W at an altitude of 367 m above sea level. Its estimated population in 2012 was 25,927, with inhabitants living in an area of 1,654 km^2 in size. This municipality lies within a savanna biome and has a hot, tropical, sub-humid climate with a four-month dry season occurring from May-August [Brazilian Institute of Geography and Statistic (ibge.gov.br/cidadesat/topwindow.htm?1)].

Phlebotomine captures - Captures were performed in accordance with the Manual for the Surveillance and Control of VL (MS/SVS 2006) using CDC-type light traps (Sudia & Chamberlain 1962) installed in peridomiciles at dusk (05:00 pm). These captures were conducted in the morning (06:00 am) on three consecutive days. Traps were installed in 10 dwellings in four suburbs (2 traps in São Sebastião, Jardim Aurora and Planalto and four traps in Santo Antônio) selected according to the register of human and canine cases from previous years. The collections were performed from July 2010-June 2011, from August-December 2011, in October and December 2012 and in February and March 2013 for a total of 63 captures. The captures carried out after the first 12 months were necessary to increase the number of engorged females. All males and females that were not *Lu. cruzi* were clarified and mounted on slides in Berlese liquid and identified in accordance with Young and Duncan's (1994) taxonomic key, the shapes of the female cibarium (a region of the mouthpart) and spermatheca and the different structures forming the genitalia in males. The specific identification of *Lu. cruzi* was based on the characteristics of the male genitalia [4 foliaceous setae at the inner base of the coxite, not 6 as described by Mangabeira (1938)] because female *Lu. longipalpis* and *Lu. cruzi* are indistinguishable (Young & Duncan 1994). The abbreviations of the phlebotomine genera were made in accordance with Marcondes (2007).

Molecular tests - DNA extractions were performed according to Michalsky et al. (2002) and Souza et al. (2004) with the phenol/chloroform method and samples of up to 10 females from the same trap were pooled.

For the detection of constitutive sandfly genes (cacophony) we used a specific primer pair targeting the IVS6 region of sandflies of the genus *Lutzomyia* as follows: 5Llcac, 5'-GTGGCCGAACATAATGTTAG-3' and 3Llcac, 5'-CCACGAACAAGTTCAACATC-3,' as described by Michalsky et al. (2011).

PCR was performed using the primers 150 (sense), 5'-GGG(G/T)AGGGGCGTTCT(C/G)CGAA-3' and 152 (antisense), 5'-(C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTACACCAACCCC-3', as described by Degrave et al. (1994), which amplified a 120-bp DNA fragment of a conserved region of the kDNA minicircle found in all species of *Leishmania*. The temperature and time conditions for amplification were as follows: initial denaturation at 94ºC for 4 min, followed by 30 cycles of 94ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s and a final extension of 72ºC for 10 min. Next, the amplified product was dyed with GelRed and analysed by electrophoresis in 2% agar gel using a transilluminator (ultraviolet 300 nm). Strains of *Leishmania braziliensis* (MHOM/BR/75/M2903) were used as positive controls and master mix lacking DNA was used as a negative control according to Michalsky et al. (2011).

The pooled DNA samples positive for the genus *Leishmania* were submitted to PCR in accordance with Lachaud et al. (2002), using the primers RV1, 5'-CTTTTCTGGTCCCGCGGGTAGG-3' and RV2, 5'-CCACCTGGCTATTTT ACACCA-3', which amplified a 145-bp fragment specific to *L. infantum chagasi*. The time and temperature of amplification were the same for all *Leishmania* sp. For all tests, *L. infantum chagasi* (MHOM/BR/1974/PP75) reference samples were used as positive controls and master mix without DNA was employed as the negative control.

Because they were assessed in pools, the minimum infection rate of the phlebotomines was calculated using the following formula: minimum rate (MR) = number of positive pools x 100/total number of insects (Paiva et al. 2007).

Analysis of food source - Sixty-one *Lu. cruzi* females that were either engorged or contained some residual blood were dissected to expose their digestive tubes and were macerated in accordance with the ELISA protocol described by Burkot et al. (1981), as modified by Duarte (1997), using antisera from a bird, dog, skunk, primate and rodent. The choice of antisera was based on the animals observed in the environments where the collections had been made. Samples were diluted to 1:20 in carbonate-bicarbonate buffer (pH 9.6, 0.05 M; Sigma Chemical Co, USA) and added to a 96-well polystyrene microplate (NuncC, 442404, Maxisorp, Denmark). After incubation (at 37°C for 2 h), the plates were washed in phosphate buffered saline (PBS)/Tween 20 (0.05%) (Sigma Chemical Co). The next steps involved the addition of antisera (PBS/Tween 20 plus 1% skim milk; Molico-Nestle, Brazil) to the wells and the incubation of the microplate at 37°C for 30 min (goat anti-rabbit serum peroxidase conjugate; Sigma Chemical Co). Following a recommended wash step, the 1:20,000-diluted conjugate was added and after an additional incubation and wash, a developing buffer [citrate/phosphate, pH 5.0,

0.05 M hydrogen peroxide, 30 vol. (Merck Diagnostica, Brazil) and o-phenylenediamine (Sigma Immunochemical Co, USA)] was added. The reaction was stopped after 15 min by adding 50 µL of 1N sulphuric acid solution and measurements were obtained with an ELISA plate reader (USA) using 490-nm filters.

Statistical analysis - The accumulated frequency and percentage were calculated for each species (those with > 50% were classified as constant, those with between 10-49% as common and those with < 10% as rare). For the calculation of species diversity, Shannon-Weiner's diversity index (H') was used according to the following equation: H' = -□pi(log pi). The relationship between the climatic data [National Notifiable Diseases Surveillance System (sisam.cptec.inpe.br.)] and the density of *Lu. cruzi* was calculated using the model adopted by Vilela et al. (2011) *via* Pearson's correlation index (*r*) and only the data collected regularly between July 2010-June 2011 were used. All analyses were conducted using Microsoft Excel 2010 and are described in the Tables and Figures.

RESULTS

At the end of the collections carried out in 21 states, 2,011 phlebotomines belonging to 23 species had been captured. A total of 1,354 of these were male and 657 were female. *Lu. cruzi* predominated, representing 68.70% of the specimens collected, followed by *Lu.*

whitmani, comprising 20.52%, and *Lutzomyia sordellii*, amounting to 5.16% (Table I). These species, together with *Brumptomyia brumpti*, were considered constant because their absolute frequency was above 50%. The majority of the species (11) were classified as rare, as they were only registered for one or two of the captures. Among the species identified, with the exception of *Lu. cruzi*, the observed leishmaniasis vectors included *Lutzomyia flaviscutellata*, *Lu. whitmani* and *Lutzomyia antunesi*, which were involved in the transmission of *Leishmania* causing cutaneous leishmaniasis.

Monthly captures were performed regularly for one year (from July 2010-June 2011), resulting in 1,240 phlebotomines captured, 919 of which (74%) were identified as *Lu. cruzi*. Analysis of the monthly distribution of *Lu. cruzi* demonstrated an absence of any correlation with the variables of accumulated rainfall (*r* = 0.07) and relative humidity (*r* = 0.08) and a moderate correlation with average temperature (*r* = 0.4) (Fig. 1).

Of the four districts investigated, Santo Antônio registered the greatest number of specimens, with 1,028 individuals; however, despite having the greatest species richness, it exhibited the lowest H' (Table II).

A total of 229 *Lu. cruzi* females, which were divided into 105 pools, were submitted to PCR testing for *Leishmania*. Fourteen pools were observed to possess the characteristic DNA band of *Leishmania* (120 bp), which was later

TABLE I
Species of phlebotomines captured in an urban area of the municipality of Jaciara, state of Mato Grosso, 2010-2013

| Species	♂	♀	Total	Accumulated frequency	%
Brumptomyia brumpti	19	30	49	61.90	2.43
Lutzomyia acanthopharynx	0	2	2	9.52	0.10
Lutzomyia antunesi	2	1	3	14.28	0.15
Lutzomyia carrerai carrerai	0	2	2	9.52	0.10
Lutzomyia christenseni	0	1	1	4.70	0.05
Lutzomyia cruzi	976	406	1,382	100	68.70
Lutzomyia davisi	0	1	1	4.70	0.05
Lutzomyia evandroi	0	4	4	19.40	0.20
Lutzomyia flaviscutellata	0	1	1	4.70	0.05
Lutzomyia hermanlenti	3	1	4	19.04	0.20
Lutzomyia lenti	1	4	5	14.28	0.25
Lutzomyia longipennis	1	0	1	4.70	0.05
Lutzomyia microps	0	1	1	4.70	0.05
Lutzomyia punctigeniculata	1	4	5	19.40	0.25
Lutzomyia quinquifer	1	0	1	4.70	0.05
Lutzomyia sallesi	8	6	14	38.09	0.70
Lutzomyia saulensis	2	1	3	14.28	0.15
Lutzomyia sordellii	42	62	104	90.40	5.16
Lutzomyia teratodes	1	5	6	23.80	0.30
Lutzomyia termitophila	2	5	7	28.50	0.34
Lutzomyia walkeri	1	0	1	4.70	0.05
Lutzomyia whitmani	294	119	413	90.40	20.52
Lutzomyia yuillii yuillii	0	1	1	4.70	0.05
Total	1,354	657	2,011	-	100
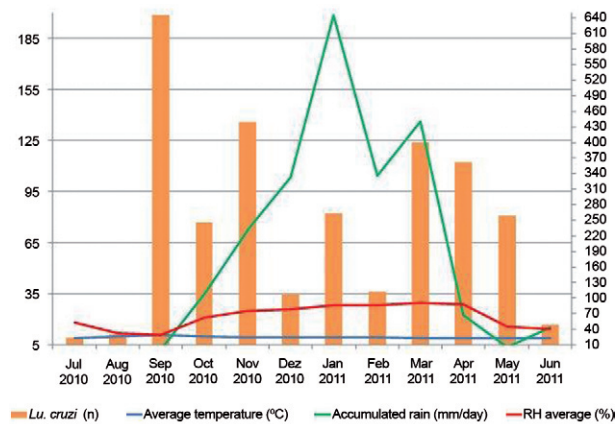
Fig. 1: number of *Lutzomyia cruzi* accumulated rainfall, average relative humidity (RH) and the average monthly average temperature, municipality of Jaciara, state of Mato Grosso, from July 2010-June 2011.

TABLE II

Number and diversity index (H') of species by suburb investigated in the municipality of Jaciara, state of Mato Grosso, 2010-2013

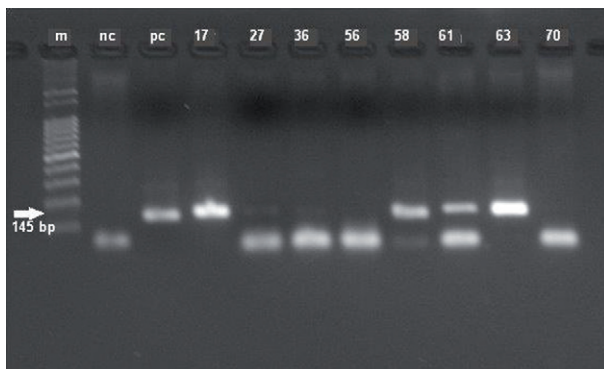| Suburb	Species (n)	Phlebotomines (n)	H'
São Sebastião	14	708	1.30
Santo Antônio	17	1,028	0.75
Jardim Aurora	7	172	0.85
Planalto	11	104	1.58

Fig. 2: products of amplification for *Leishmania chagasi* undertaken in females of *Lutzomyia cruzi*. m: marker of 100 bp; nc: negative control; pc: positive control (MHOM/BR/1974/PP75); 17, 27, 36, 58, 61, 63: positive samples of phlebotomines.

confirmed to be *L. infantum chagasi* (145 bp) (Fig. 2), indicating the natural infection of *Lu. cruzi* at a MR of 6.1%. The districts of Santo Antônio and São Sebastião presented with the greatest numbers of positive samples (6 each).

Analysis of blood obtained from the digestive tubes of the 61 engorged females assessed by ELISA indicated that only 22 were reactive, including 17 to bird antiserum, two for dog and one for skunk. Mixed profiles were observed

TABLE III

Feeding habits by ELISA test in engorged females of *Lutzomyia cruzi* collected in the municipality of Jaciara, state of Mato Grosso, from 2011-2013

Antisera	Absolute (n)	Relative (%)
Bird	17	27.90
Dog	2	3.30
Skunk	1	1.60
Human	0	0
Rodent	0	0
Bird and skunk	1	1.60
Dog and skunk	1	1.60
Not reactive	39	64
Total	61	100

in two samples (bird and skunk; dog and skunk), suggesting that those females had fed on both hosts. There was no reaction to primate or rodent antiserum (Table III).

DISCUSSION

The proximity of the city of Jaciara to the vegetation of its natural surroundings may explain the diversity of the phlebotomines found in this study despite the predominance of only two species, *Lu. cruzi* and *Lu. whitmani*, which together accounted for approximately 90% of the phlebotomines collected. The predominance of *Lu. cruzi* in the areas investigated demonstrates its adaptation to these environments, corroborating the findings of Missawa and Lima (2006), who discovered a greater predominance of *Lu. cruzi* in municipalities containing areas of marsh and savanna, the latter being their preferred environment.

Studies have suggested that *Lu. whitmani* is the most important vector of anthroponotic cutaneous leishmaniasis in Brazil in association with *L. braziliensis*. This species is present in various Brazilian biomes and has adapted to different climatic conditions. In addition, this species can survive in the intra and peridomiciliary environments of impacted areas (Vilela et al. 2011). Despite the high density of *Lu. whitmani*, according to the Environmental Surveillance of Jaciara, there is no record of the transmission of cutaneous leishmaniasis in urban areas, which has consistently been reported in rural, forested areas.

Despite the finding that the greatest number of phlebotomines was present in Santo Antônio, it cannot be confirmed that this location contains the greatest density of these insects because twice as many traps were placed there compared to other locations. This greater number of traps was due to the larger size of the district, the existence of various residences with an appearance suggestive of the presence of the vector and the various cases of canine VL registered there in the years immediately prior to this research project. Considering the number of phlebotomines in relation to the number of traps installed, São Sebastião (2 traps) presented with the greatest number of individuals.

Michalsky et al. (2009) reported that many authors have shown a clear relationship between abiotic factors and phlebotomine population density due to the interference of these factors with their biological cycles and associated modifications of their locations of reproduction. Variable population densities of *Lu. longipalpis* were observed in Montes Claros, state of Minas Gerais, which exhibited an increase in density every other month independent of season, probably due to the peculiar weather conditions of this municipality, where the climate variables followed an almost constant seasonal cycle.

Although Fig. 1 shows an increase in the population density of *Lu. cruzi* before and after the rainy season, in this study, only temperature exhibited a moderate correlation with the density of *Lu. cruzi*. Specifically, a tendency towards the abundance of these insects during periods of high temperature was observed. For a more precise analysis, a larger amount of data should be assessed, requiring testing over longer periods of time.

The comparison between species richness and diversity, such as that performed among the suburbs investigated in Jaciara, showed that the Santo Antônio presented a greater richness (number of species), but a lower diversity, which was due to the great disparity between species' frequencies with an absolute predominance of *Lu. cruzi*.

One important step towards the verification of a particular *Leishmania* vector is the determination of the occurrence of naturally infected phlebotomines (Killick-Kendrick & Ward 1981). Studies of the rate of natural infection in phlebotomines in various regions of the country have revealed low values, even in areas of widespread transmission. Studies of different populations of *Lu. longipalpis* performed using distinct techniques have found the following minimal rates of infection: 2.6% in Antônio João, state of Mato Grosso do Sul (MS) (Nascimento et al. 2007), 1.1% in Teresina, state of Piauí (PI) (Silva et al. 2007), 1.25% in São Luís, state of Maranhão, in an area of ancient colonisation and 0.25% in an area of recent colonisation (Soares et al. 2010), and 3.5% in Misiones, Argentina (Acardi et al. 2010).

Few studies have assessed *Lu. cruzi*, although it has been suggested that this species is a vector of *L. infantum chagasi*. For example, the study by Santos et al. (1998) reported a MR of infection of 0.4% in Corumbá and Ladário, MS and in 2008 Pita-Pereira et al. reported a 1.5% rate of infection of *Lu. cruzi* at that same locality. The infection rate of 6.1% obtained in the present study was higher than that which has been previously reported; however, it is in accordance with that reported by Missawa et al. (2011), who found a positive reaction in one of three pools of *Lu. cruzi* females collected from the same municipality.

Studies of food sources have demonstrated that phlebotomines are very diverse with regard to their choice of host. Analyses of *Luyzomyia intermedia* females carried out in Mesquita, state of Rio de Janeiro using the precipitin technique have found that 39.8% are reactive for rodents, followed by 23.7% for birds, 20.4% for dogs and 16% for humans (Afonso et al. 2005). In Várzea Grande, *Lu. longipalpis* was shown to react to the antisera of birds (30.8%), rodents (21.2%), humans (13.5%) and dogs (4.8%) using the precipitin technique (Missawa et al. 2008). In the municipality of Teresina, PI, out of 58 *Lu. longipalpis* females analysed using PCR/

FTA, 41 had fed on chicken and two on dogs as a food source and the food sources of 15 could not be identified (Sant'Anna et al. 2008). In a study of *Lu. longipalpis* obtained from Jequié, state of Bahia, Sobral and Massapé, state of Ceará and Teresina, PI, Afonso et al. (2012) detected positive reactions in various animals, predominantly birds (36% in Jequié, 67% in Teresina, 29% in Sobral and 51% in Massapê), using the ELISA method. Chagas et al. (2007) studied the haematophagous behaviour of *Lu. cruzi* bred in the laboratory and found that the females fed more easily on and preferred humans to hamsters when both sources of food were offered simultaneously. A previous study of the eating habits of *Lu. cruzi* in Jaciara revealed that of 22 positive females, 18 used birds as a blood source. Although chickens do not act as reservoirs of *Leishmania*, they can be important in the maintenance of populations of *Lutzomyia* by attracting reservoir mammals or by acting as sentinels for these insects in the area investigated (Alexander et al. 2002, Soares et al. 2013).

The reaction to skunk antiserum observed in this study indicates that synanthropic animals can act as a link between domestic and wild transmission cycles, thus increasing the risk of canine infection by 2.6-fold, which has previously been reported by Missawa et al. (2008). Although this municipality registered high levels of positive reactions to canine antiserum, the presence of dog blood was detected in few females, which is probably due to competition with birds (chickens), which are normally bred in larger numbers than other domestic animals when present in peridomiciles.

Despite the low number of reactive samples, our findings suggest that there is a preference with regard to the type of host; however, further studies should be designed to study a greater number of specimens. These results may have been due to the advanced digestion of the blood ingested, which was possibly a result of the time lapse between the capture and separation of the females, the presence of a small quantity of blood or the feeding on a host that was not evaluated in this study.

The high densities and frequencies of *Lu. cruzi* and *Lu. whitmani* collected from the urban zone of Jaciara support the adaptation of these species to anthropic environments and suggest that both are present in urban regions of Jaciara. Moreover, the high rate of infection associated with the presence of synanthropic animals as blood sources for these insects in the peridomicile indicates the participation of *Lu. cruzi* in the transmission of VL in Jaciara.

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