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Phlebotomine Sand Flies (Diptera: Psychodidae) and *Leishmania* Infection in Gafanhoto Park, Divinópolis, Brazil

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ABSTRACT The potential of Gafanhoto Park as an American cutaneous leishmaniasis (ACL) focus was evaluated by examination of sand fly vectors of the *Leishmania* parasite. This forest remnant is located in a periurban area of Divinópolis, Brazil, where autochthonous cases of ACL have been reported. Sand fly populations were monitored over a 2-yr period (2006–2008) by using light traps (HP and Shannon). During systematic collections with HP traps, 824 specimens in total (342 males and 482 females) of 21 species were captured. Most prevalent species were as follows: *Brumptomyia brumpti* (Larrouse), *Lutzomyia aragaoi* (Costa Lima), *Lutzomyia lutziana* (Costa Lima), *Lutzomyia sordellii* (Shannon & Del Ponte), and *Lutzomyia whitmani* (Antunes & Coutinho). Using Shannon traps, 257 specimens representing 15 species were collected (159 females and 98 males), with a high prevalence of *L. whitmani* and *Lutzomyia neivai* (Pinto), both vectors of *Leishmania braziliensis* (Vianna). To ascertain the level of natural infection, a sample of females captured in Shannon traps was assayed for the presence of *Leishmania* by using polymerase chain reaction-restriction fragment length polymorphism, where 39% of insects were positive. The most infected species was *L. whitmani* (29 sand flies; 18.2%), followed by *L. neivai* (21; 13.2%), *Lutzomyia christenseni* (Young & Duncan) (five; 3.1%), *Lutzomyia pessoai* (Coutinho & Barreto) (three; 1.9%), *L. aragaoi* (one; 0.6%), *Lutzomyia fischeri* (Pinto) (one; 0.6%), *Lutzomyia lenti* (Mangabeira) (one; 0.6%), *L. lutziana* (one; 0.6%), and *Lutzomyia monticula* (Costa Lima) (one; 0.6%). The finding of potential and incriminated vectors naturally infected with *Leishmania* reinforces the need of epidemiologic surveillance in the area.

KEY WORDS Phlebotominae, leishmaniasis, epidemiology, *Lutzomyia*, *Leishmania*

Leishmaniasis is a vector-borne disease caused by ≈21 species of *Leishmania* that are transmitted through the bite of phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) of the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World (Young and Duncan 1994). In humans, the disease has a large spectrum of clinical manifestations ranging from mild self-healing cutaneous lesions to the severe lethal visceral form (VL) (Herwaldt 1999). In Brazil, the disease is highly prevalent, with increasing incidence reported in many areas, especially in urban areas of the southeast, east central, and northeast (Margonari et al. 2006, Neto et al. 2009). More recently, expansion of the disease has been observed in the Amazon basin as a result of human activities such as deforestation, establishment of field plantations, mining, and new settlements (Silva-Nunes et al. 2008). After an expanded interest of entomologists looking for sand flies, many reports have detected the presence of potential and suspected vectors such as *Lutzomyia shannoni* (Dyar) and *Lutzomyia vexator* (Coquillett) in rural areas of the United States (Minter et al. 2009). Also, adaptation of proven vectors such as *Lutzomyia longipalpis* (Lutz & Neiva), *Lutzomyia intermedia* (Lutz & Neiva), and *Lutzomyia whitmani* (Antunes & Coutinho) to urban areas has been reported in many cities of Brazil (Margonari et al. 2004, Barata et al. 2005, Gontijo et al. 2005).

Divinópolis was founded in 1911 and has ≈210,000 people in a total area of 711 km^2 (IBGE 2007). In the 1990s, 135 human cases of American cutaneous leishmaniasis (ACL) were reported by health authorities, followed by 30 cases between 2000 and 2006 (DEDCH 2006). The disorderly growth of Divinópolis favors proximity to periurban forested areas such as Gafanhoto Park. However, the potential of this park as an ACL focus is unknown, and the lack of knowledge about leishmaniasis in the city hinders development of preventive measures and epidemiologic surveillance.

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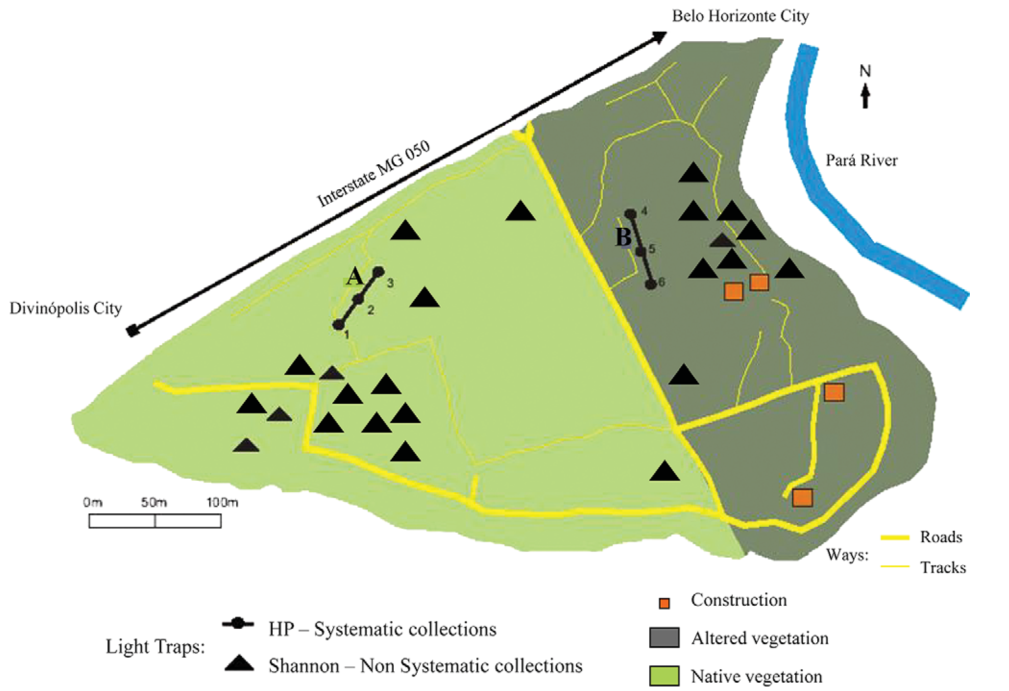

Fig. 1. Gafanhoto Park area with collection sites for HP traps (transects A and B) (circles) and Shannon traps (triangles). Vegetation types, scales, roads, and tracks are indicated in the legends.

Because alterations in the environment followed by deforestation are the main causes of insect adaptation (Chaves et al. 2008), this scenario strongly favors an outbreak of ACL. To better understand the epidemiology of leishmaniasis in Divinópolis, this work evaluated the phlebotomine sand fly fauna and associated *Leishmania* infections in the Gafanhoto Park.

Materials and Methods

Study Area and Systematic Collections. Gafanhoto Park is a primary forest remnant (150,000 m^2) located in the peri-urban area of Divinópolis (20° 8′21″ S and 44° 53′17″ W), MG, Brazil. Six light traps (HP) (Pugedo et al. 2005) were set in two transects (A and B), and overnight collections were performed monthly for 2 yr (October 2006–September 2008). Transect A was located in a preserved area of the Park, whereas transect B was in an altered area with introduced vegetation (palm trees [Arecaceae]; bamboo [Poaceae]; mango, *Mangifera indica* L., trees; and eucalyptus [Myrtaceae]) and construction (Fig. 1). Collected insects were stored at −20°C in 70% ethanol before taxonomic identification using the keys of Young and Duncan (1994).

Nonsystematic Collections. In total, 24 captures (one per month, 2006–2008) in areas randomly selected were performed using Shannon traps from 5:30 p.m. to 2:00 a.m. (Fig. 1). Insects were captured from the illuminated surface of the trap with mouth aspirators and transferred to a small holding container until they could be frozen at −20°C. After sex determination, females were subjected to DNA extraction to facilitate *Leishmania* detection by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) (Margonari et al. 2004).

PCR-RFLP. PCR reactions and thermal profile followed the procedure of Degrave et al. (1994). Amplified PCR products were digested with HaeIII (1 U; 3 h at 37°C) and visualized on polyacrylamide gels (8%) (Volpini et al. 2004, Andrade et al. 2006). This process was repeated three times to exclude false positives.

Statistical Analysis. Data on sand fly collections were analyzed using Pearson's coefficient and correlation (parametric analysis). Graphs were plotted using Prism 4.0 software (GraphPad Software, San Diego, CA).

Results

Systematic Collections. Using HP traps, 824 specimens (58.5% females and 41.5% males) from 21 species were captured (Table 1). The most prevalent species were *Lutzomyia aragaoi* (Costa Lima) (41.7%), *Brumptomyia brumpti* (Larrouse) (18.7%), *Lutzomyia lutziana* (Costa Lima) (11.7%), *Lutzomyia sordellii* (Shannon & Del Ponte) (5.8%) and *L. whitmani* (5.3%). The numbers of insects captured in transects A and B were 371 and 453, respectively. *L. aragaoi* was the most abundant species at both sites. Most of species involved in the transmission of *Leishmania braziliensis* (Vianna), including *Lutzomyia neivai* (Pinto) and *L. whitmani* were captured in both transects (70

Table 1. Sand fly numbers by species and sex captured using HP light traps between 2006 and 2008 in two transects of Gafanhoto Park, Divinópolis, MG, Brazil

Species	Area		Sex		Total (%)
	Transect A	Transect B	Male	Female	
B. brumpti	50	103	82	71	153 (18.7)
B. pintoi	8	11	10	9	19 (2.3)
L. amarali	1	0	0	1	1 (0.1)
L. aragaoi	209	134	141	202	343 (41.7)
L. bacula	2	1	0	3	3 (0.4)
L. brasiliensis	2	9	5	6	11 (1.3)
L. christenseni	15	3	0	18	18 (2.2)
L. cortelezzi	1	0	1	0	1 (0.1)
L. evandroi	0	1	1	0	1 (0.1)
L. fischeri	0	2	0	2	2 (0.2)
L. lenti	2	2	2	2	4 (0.5)
L. lutziana	39	57	55	41	96 (11.7)
L. mamedei	1	0	0	1	1 (0.1)
L. monticola	16	13	1	28	29 (3.5)
L. neivai	1	20	8	13	21 (2.5)
L. pessoai	1	2	0	3	3 (0.4)
L. sallesi	1	15	3	13	16 (1.9)
L. sordellii	12	36	13	35	48 (5.8)
L. teratodes	1	0	0	1	1 (0.1)
L. termitophila	1	8	4	5	9 (1.1)
L. whitmani	8	36	16	28	44 (5.3)
Total (%)	371 (45.0)	453 (55.0)	342 (41.5)	482 (58.5)	824 (100.00)

insects). However, 85.7% of them were collected in transect A (Table 2). Environmental conditions seemed not to affect sand fly densities (Fig. 2). An increase in the number of sand flies captured was noted especially during drier periods, although this was not statistically significant. From 2007 to 2008, there was a 112.7% increase in the number of sand flies collected.

Nonsystematic Collections. Using Shannon traps, 257 sand flies (61.9% females and 38.1% males) of 15 species were captured (Table 2), with prevalence rates of 56.4% and 21.0% for *L. whitmani* and *L. neivai*, respectively.

Natural Sand Fly Infection. Of 159 females, 63 (39.6%) were positive for *Leishmania*, exhibiting a 120-bp–sized band as expected after PCR-RFLP (Degrave et al. 1994). This *Leishmania*-specific DNA fragment was observed from the respective sand flies as follows: one specimen of *L. aragaoi*, *Lutzomyia fischeri* (Pinto), *Lutzomyia lenti* (Mangabeira), *L. lutziana*, *Lutzomyia monticola* (Costa Lima), three *Lutzomyia pessoai* (Coutinho & Barreto), five *Lutzomyia christenseni* (Young & Duncan), 21 *L. neivai*, and 29 *L. whitmani*. Figure 3A and B displays a representation of the results of three different experiments. The 120-bp restriction DNA fragment was subjected to digestion with HaeIII enabling the identification of the particular *Leishmania* species. One specimen of *L. whitmani* was positive for *Leishmania chagasi* [syn. *Leishmania infantum* (Nicolle)]. One specimen each of *L. monticola*, *L. lutziana*, *L. christenseni*, and *L. lenti* was positive for *L. braziliensis*. Controls were represented by World Health Organization reference strains *L. chagasi* (MHOM/BR/74/PP75) and *L. braziliensis* (MHOM/BR/75/M2903), with expected band profiles of 120, 80, 60, and 40 bp and 80 and 40 bp, respectively (Volpini et al. 2004, Andrade et al., 2006) (Fig. 4).

Table 2. Sand fly numbers by species and sex and PCR-positive females ($F+$) for *Leishmania* captured with Shannon traps in Gafanhoto Park, Divinópolis, MG, Brazil

Species	Sex		PCR $F+$	Total (%)
	Male	Female		
B. brumpti	3	2	0	5 (1.9)
B. pintoi	0	1	0	1 (0.4)
L. aragaoi	3	4	1	7 (2.7)
L. brasiliensis	1	0	0	1 (0.4)
L. christenseni	0	7	5	7 (2.7)
L. fischeri	0	1	1	1 (0.4)
L. lenti	0	1	1	1 (0.4)
L. lutziana	2	3	1	5 (1.9)
L. monticola	1	12	1	13 (5.1)
L. neivai	17	37	21	54 (21.0)
L. pessoai	8	5	3	13 (5.1)
L. sallesi	0	2	0	2 (0.8)
L. sordellii	0	1	0	1 (0.4)
L. whitmani	63	82	29	145 (56.4)
L. (*Pressatia*) sp.	0	1	0	1 (0.4)
Total (%)	98 (38.1)	159 (61.9)	63 (39.6)	257

Discussion

Sand Fly Collections. Sand flies were collected every month over a 2-yr period with HP and Shannon traps, indicating a high prevalence in Divinópolis. They seemed not to be affected by local climatic conditions (humidity, temperature, and precipitation), because there was an increase in the total sand fly population from 2007 to 2008. However, an increase in the number of sand flies was observed during drier months. Climatic factors may have an influence on sand fly densities, being commonly found during the hot and humid months (Aguiar and Soucasaux 1984, Salomón et al. 2002, Souza et al. 2004, Margonari et al.

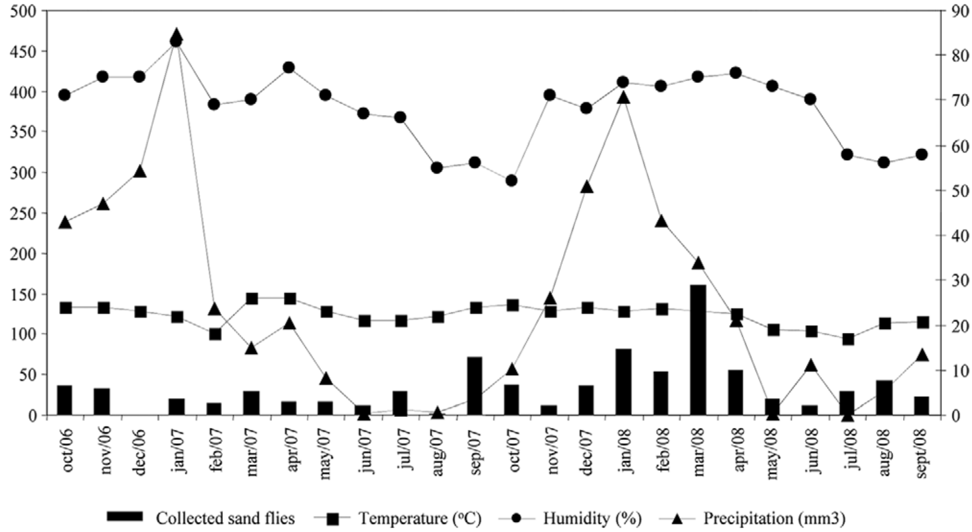

Fig. 2. Temperature, relative humidity (percentage), precipitation (millimeters), and sand flies captured with HP traps between October 2006 and September 2008 in the Gafanhoto Park, Divinópolis, MG, Brazil.

2006, Ready 2008). Our data suggest that from an epidemiological perspective, continuous monitoring of the vector populations in the neighboring vicinities of Gafanhoto Park is warranted. During systematic sand fly collections, more females than males were captured, which could be a result of light attraction and a blood source close to the traps (Loiola et al. 2007). However, other workers (Aguiar et al. 1985, Alves 2007, Oliveira et al. 2007) detected a higher number of males, so this seems not to be a general rule. Males tend to form "leks" close to the host to attract females for feeding and copulation. In this study, most of the sand flies captured in Shannon traps are proven vectors of ACL, including *L. neivai* and *L. whitmani* (Souza et al. 2004, Andrade-Filho et al. 2007, Costa et al. 2007). Together with these species, *L. fischeri* and *L. pessoai* also were captured and that was already reported (Camargo-Neves et al. 2002, Muniz et al. 2006). Barreto (1943) considered *L. fischeri* as a secondary vector due its high anthropophily and common occurrence in areas of *L. braziliensis* transmission. Interestingly, the Shannon trap was employed in the areas of the park that were altered, degraded, or both (transect B in Fig. 1). Appearance of human ACL cases is usually associated with deforestation, increasing the chances of human–vector contact and promoting adaptation of vectors to human environments (Souza et al. 2004, das Virgens et al. 2008). Similarly, in altered transect B, the possibility of contact between visitors and sand flies may increase the chances of ACL transmission after hours.

Natural *Leishmania* Infection. At our collection sites, sinanthropic and wild animals (*Rattus rattus, Oryzomys subflavus*, and *Didelphis albiventris*) are of-

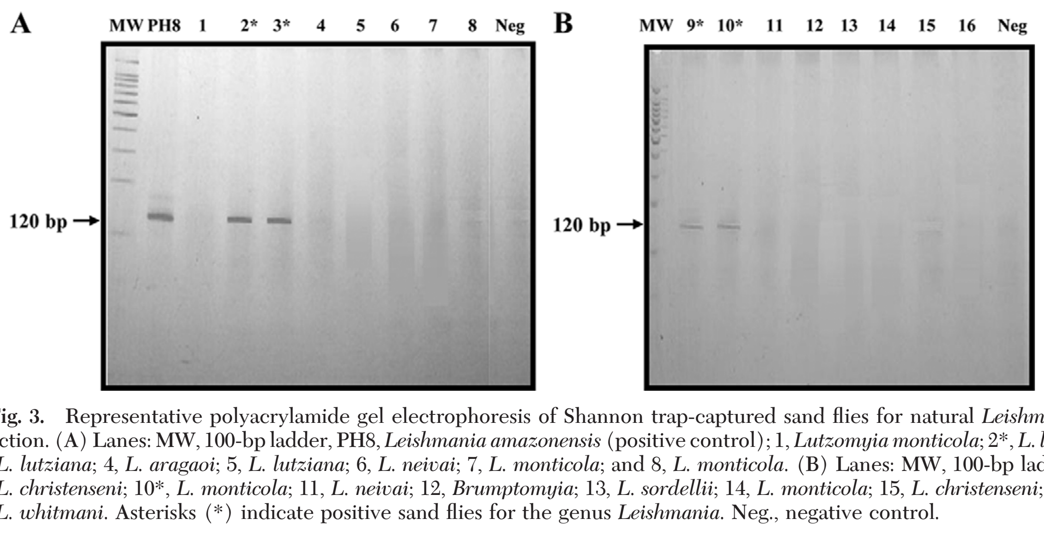

Fig. 3. Representative polyacrylamide gel electrophoresis of Shannon trap-captured sand flies for natural *Leishmania* infection. (A) Lanes: MW, 100-bp ladder, PH8, *Leishmania amazonensis* (positive control); 1, *Lutzomyia monticola*; 2*, *L. lenti*; 3*, *L. lutziana*; 4, *L. aragaoi*; 5, *L. lutziana*; 6, *L. neivai*; 7, *L. monticola*; and 8, *L. monticola*. (B) Lanes: MW, 100-bp ladder; 9*, *L. christenseni*; 10*, *L. monticola*; 11, *L. neivai*; 12, *Brumptomyia*; 13, *L. sordellii*; 14, *L. monticola*; 15, *L. christenseni*; and 16, *L. whitmani*. Asterisks (*) indicate positive sand flies for the genus *Leishmania*. Neg., negative control.

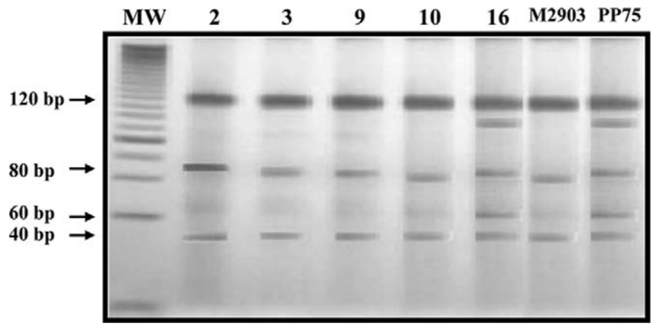

Fig. 4. Polyacrylamide gel electrophoresis of PCR-RFPL fragment after enzymatic digestion with HaeIII. Lanes: MW, 25-bp ladder; 2, *Lutzomyia lenti*; 3, *L. lutziana*; 9, *L. christenseni*; 10, *L. monticola* (infected with *L. braziliensis*); 16, *L. whitmani* (infected with *L. chagasi*); M2903 (control strain of *L. braziliensis*); and PP75 (control strain of *L. chagasi*).

ten observed. These animals were reported as potential reservoirs for *Leishmania* (Sherlock 1996, Brandão-Filho et al. 2003, Santiago et al. 2007). *D. albiventris* is an important wild and urban reservoir in the city of Belo Horizonte, having been found infected with *L. chagasi* and *L. braziliensis* (Sherlock et al. 1984, Schallig et al. 2007). In another city (Araçuaí), 62 rodents were found infected with *Leishmania mexicana* (Biagi), *L. braziliensis*, and *Leishmania donovani* (Laveran & Mesnil) (Oliveira et al. 2005). These potential reservoirs should be investigated further, because the high *Leishmania* infection rates in sand flies (39.6%) observed in this study provide strong evidence that they may play an important role in the sylvatic *Leishmania* transmission cycle. Among the infected species, *L. whitmani* (18.2%), followed by *L. neivai* (13.2%), proven vectors of *L. braziliensis*, were the most prevalent. Other species included secondary or suspected such as *L. christenseni* (3.1%), *L. pessoai* (1.9%), *L. aragaoi* (0.6%), *L. fischeri* (0.6%), *L. lenti* (0.6%), *L. lutziana* (0.6%), and *L. monticula* (0.6%). It is interesting to notice the higher infection rates in the proven vectors compared with the others. Although *L. fischeri* and *L. pessoai* are commonly captured together with *L. whitmani*, their infections rates were very low. According to Coutinho and Barreto (1940) *L. fischeri* had never been found infected with *Leishmania*, contrasting with our data that detected its presence for the first time. Rangel and Lainson (2003) suggested that *L. fischeri* would have the potential to adapt to altered environments and ability to transmit *L. braziliensis* in the wild cycle. In our work, this species (two sand flies) was found in transect B, which was closer to altered areas. However, its role as a potential vector still remains to be understood.

The high infection rates found here strongly contrast with previous observations in other places, where natural *Leishmania* infections were low (0.2–2%) even in high transmission areas (Rodriguez et al. 1999, Miranda et al. 2002, Gontijo et al. 2005, Silva et al. 2007).

Vector Biology. Phlebotomine sand fly diversity in Gafanhoto Park revealed the presence of at least 21 species. *L. aragaoi* and *B. brumpti* were the most common species captured. They are usually associated to armadillo holes (Dasipodidae) frequently seen in the area (Oliveira et al. 2003). In spite of its wide distribution in the Americas, the genus *Brumptomyia* does not have any importance in public health (Damasceno et al. 1949). *Lutzomyia lutziana*, a nonurban sand fly, was the third most common species. Importantly, this species is now reported for the first time to be naturally infected with *L. braziliensis*, warranting additional attention to establish its real status as a vector of ACL. *L. whitmani* was the fourth most common species captured and is considered an important vector of ACL in Brazil (Costa et al. 2007). Several specimens, captured with Shannon traps were infected with *L. braziliensis*, and one specimen was infected with *L. chagasi*. The biological interactions of *L. whitmani* infected with *L. chagasi* should be explored to determine the possible role of this vector in the transmission of this parasite. Similarly, other sand fly species were reported as possible hosts for *L. chagasi*, including *Lutzomyia cortelezzii* (Brethés) (Carvalho et al. 2008), *Lutzomyia cruzi* (Mangabeira) (Pita-Pereira et al. 2008), and *Lutzomyia evansi* (França) (Montoya-Lerma et al. 2003). However, the finding of an infected sand fly is not the only condition for its incrimination as a vector. In addition to this condition, the distribution of the suspected vector sand fly must be coincident with the distribution of human disease; the insect must be found infected in peridomestic or domestic areas and it has to feed avidly on humans and many hosts (Killick-Kendrick 1999).

L. neivai also is involved in the transmission of *L. braziliensis* in Brazil (Andrade-Filho et al. 2007), and recent reports suggest its expansion southward (Marcondes et al. 2009, Pita-Pereira et al. 2009, Saraiva et al. 2009). In this study, four sand flies were found naturally infected with *Leishmania.* The fly species *L. monticola* and *L. sordellii* are essentially sylvatic and *L. monticola* is highly anthropophilic and susceptible to infection with *Leishmania* (Souza et al. 2001, 2004). In Belo Horizonte, *L. monticola* was found in small forests close to the city, under conditions similar to those in Gafanhoto Park, suggesting adaptation to altered areas. Another interesting result is that this species, together with *L. lenti* and *L. christenseni*, was found for the first time naturally infected with *L. braziliensis. L.*

lenti had been reported to be refractory to *Leishmania* infection in the laboratory (Brazil et al. 1997). However, Sherlock (1996) observed natural infections in this species in Bahia and suspected it played a role in *L. chagasi* transmission to dogs. *L. christenseni* was captured in Gafanhoto Park in a Malaise trap (Andrade-Filho et al. 2008) but was found negative for *Leishmania* infection. In addition, *L. pessoai* could be involved in the transmission of *L. braziliensis* in southeastern Brazil judging from its high densities, considerable anthropophily and endophily (Rangel and Lainson 2003). In our study, one insect (*L. pessoai*) was found positive for *Leishmania* sp. Together, our data show the possibility of *Leishmania* infections in several sand fly species, including some that have never been found before with natural infections (*L. lenti, L. lutziana, L. monticola,* and *L. christenseni*), underscoring the need for further studies to establish their real role as vectors of *Leishmania.*

Gafanhoto Park, a Periurban Focus. Based on our data, there is strong evidence that the Gafanhoto Park area represents an ACL focus. It was observed a great biodiversity in the sand fly fauna in a relatively small area. Our previous experience in a larger area covering most of the regions of Belo Horizonte city did not achieved the same biodiversity (Souza et al. 2004). However, to establish a real epidemiological importance of this area, information on the reservoirs and their infection rates still awaits further investigation. The high rates of *Leishmania* infection, presence of reservoirs, and high diversity of potential and proven vectors would support a recommendation of restricted accessibility to the park during hours of darkness. Although *L. chagasi* was detected in wild-caught sand flies, the presence of its main natural vector, *L. longipalpis* was not observed. Our unpublished data from a pilot survey of canine VL prevalence in Divinópolis showed that in 77 dogs, 30% were positive (C.M., unpublished data). These preliminary data reinforce the need for a more detailed study of VL epidemiology in the city.

In this work, important epidemiologic aspects of a periurban forested area in Divinópolis, Brazil, were investigated. Human cases of ACL have been recently reported in the city, highlighting the need for epidemiologic surveillance and control measures.

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