Veterinary Parasitology

Transmissibility of *Leishmania infantum* from maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) to *Lutzomyia longipalpis*

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ABSTRACT

Leishmania (Leishmania) infantum is the cause of visceral leishmaniasis in the Americas. The disease is transmitted mostly through the bite of the invertebrate vector, the phlebotomine *Lutzomyia longipalpis* in the New World. Although the domestic dog is considered the most important reservoir of the disease, other mammalian, including wildlife, are susceptible to infection. The goal of this study was to perform xenodiagnosis to evaluate the capacity of naturally infected maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) to transmit *Leishmania infantum* to female sand flies (*L. longipalpis*). Xenodiagnoses were performed in February and August, 2013, when 77.7% (three maned wolves and four bush dogs) or 100% of the animals were positive, respectively. However, parasite loads in the engorged sand flies was low (<200 promastigotes and <150.2 parasites/μg of DNA). No statistically significant differences were observed between the two species or the two time points (February and August). In conclusion, this study demonstrated that maned wolves (*C. brachyurus*) and bush dogs (*S. venaticus*) asymptomatically infected with *L. infantum* are capable of transmitting *L. infantum* to the invertebrate host *L. longipalpis*, although the parasite loads in engorged phlebotomines exposed to these animals were very low.

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1. Introduction

Visceral leishmaniasis (VL) is an anthropozoonosis that is extremely relevant for public health. VL is caused by protozoa of the genus *Leishmania*. In the Americas, VL is caused by *Leishmania* (*Leishmania*) *infantum* (synonym *Leishmania chagasi*), which belong to the *Leishmania donovani* complex, Order Kinetoplastidae, and Family Trypanosomatidae (Lukes et al., 2007; Baneth et al., 2008). Although *L. infantum* may be transmitted in the absence of the invertebrate vector (Turchetti et al., 2014) in most of the cases it is transmitted through the bite of sand flies of the genus *Lutzomyia* in the New World (particularly *L. longipalpis*). Humans often

develop an asymptomatic infection, while some individuals may develop a chronic systemic disease, which affects specially children or immunosuppressed patients such as HIV coinfected patients. (Caldas et al., 2001; Guerin et al., 2002; Desjeux and Alvar, 2003; Gontijo and Melo, 2004).

Although *L. longipalpis* feeds on several animal species, the domestic dog (*Canis familiaris*) is considered the most important reservoir for human VL in urban areas (Diniz et al., 2008; Quinnell and Courtenay, 2009). However, *L. infantum* has also been detected in other wild mammals that are present in urban areas, including rodents (Oliveira et al., 2005), marsupials (Schallig et al., 2007; Santiago et al., 2007), as well as in captive or free ranging wildlife including canids (Figueiredo et al., 2008; Luppi et al., 2008; Souza et al., 2010; Muñoz-Madrid et al., 2013; Del Río et al., 2014; Lombardi et al., 2014), primates (Malta et al., 2010; Tenório et al., 2011; Lombardi et al., 2014), felids (Sobrino et al., 2008; Dahroug et al., 2010; Libert et al., 2012), mustelids (Muñoz-Madrid et al., 2013; Del Río

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et al., 2014), chiropterans (Lima et al., 2008), lagomorphs (Molina et al., 2012), xenarthrans (Araújo et al., 2013), and wild rodents (Papadogiannakis et al., 2010). Nevertheless, not much is known about the potential of wild species for transmitting VL. It is thought that most of wild animals exposed to *Leishmania* spp. are resistant and capable of eliminating the pathogen. However, certain wild species may be susceptible to infection and multiplication of *Leishmania* sp., without developing the disease, but allowing infection of *L. longipalpis*, thus potentially contributing for transmission of VL.

L. infantum infection has been reported in several wild species (Souza et al., 2014). Among wild canids, infection or exposure to *L. infantum* have been described in gray wolves (*Canis lupus*) (Mohebali et al., 2005; Sastre et al., 2008; Sobrino et al., 2008), crab-eating foxes (*Cerdocyon thous*) (Courtenay et al., 1994; Curi et al., 2006), hoary foxes (*Lycalopex vetulus*) (Deane and Deane, 1954), red foxes (*Vulpes vulpes*) (Mancianti et al., 1994; Mohebali et al., 2005; Dipineto et al., 2007; Sobrino et al., 2008; Davoust et al., 2014), gray foxes (*Urocyon cinereoargenteus*) (Rosypal et al., 2010), and golden jackals (*Canis aureus*) (Shamir et al., 2001; Mohebali et al., 2005; Bessad et al., 2012). In Brazil, there have been several reports of *Leishmania* sp. infection in captive or free ranging wild canids, including hoary foxes (*L. vetulus*) (Luppi et al., 2008), crab-eating foxes (*C. thous*) (Curi et al., 2006; Luppi et al., 2008; Souza et al., 2010; Jusi et al., 2011), bush dogs (*Speothos venaticus*) (Souza et al., 2010; Jusi et al., 2011), and maned wolves (*Chrysocyon brachyurus*) (Curi et al., 2006, 2012; Luppi et al., 2008; Jusi et al., 2011). The role of these species as reservoirs of VL is still unclear, although the maned wolf and the bush dog are susceptible to the development of clinical disease (Luppi et al., 2008; Malta et al., 2010).

A key step in transmission of vector born diseases is the capacity of the vertebrate host to transmit the pathogen to the invertebrate biological vector. Xenodiagnosis is a tool that allows to evaluate whether a given infected individual is infectious for sand fly vectors (Sadlova et al., 2015). Most of the studies demonstrate that the capacity of domestic dogs to transmit the parasite to the invertebrate vector correlates with clinical signs, although asymptomatic dogs can also transmit (Courtenay et al., 2002; Costa-Val et al., 2007; Michalsky et al., 2007; Amorim et al., 2011; Soares et al., 2011; Laurenti et al., 2013). In contrast, the potential of transmission of VL from wild animals, particularly canids, is not well established. Therefore, considering the lack of information regarding the potential of wild canids as reservoirs of VL, the distribution of some of these animals in areas endemic for VL with an abundant population of *L. longipalpis* (Resende et al., 2006), and the proximity of captive wild animals with the human population in zoological gardens in endemic urban areas (Diniz et al., 2008), the aim of this study was to perform xenodiagnosis in wild canids, namely bush dogs (*S. venaticus*) and maned wolves (*C. brachyurus*), kept at the Fundação Zoo-Botânica in Belo Horizonte (Minas Gerais, Brazil).

2. Material and methods

2.1. Animals

Five bush dogs (*S. venaticus*) and four maned wolves (*C. brachyurus*) kept at the Fundação Zoo-Botânica de Belo Horizonte (Belo Horizonte, Brazil) were included in this study. Detailed information about these animals is provided in Table 1. This experimental protocol has been approved by the Institutional Ethics Committee for Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol number 94/2013).

Xenodiagnosis was performed on all animals twice, in February and August, 2013, which corresponds to the time of the year with the highest and lowest populations of *L. longipalpis* in Belo Hor-

izonte, respectively (Resende et al., 2006). At both time points (February and August) serum samples were obtained for serologic exam.

2.2. Enzyme linked immunosorbent assay (ELISA)

Serum samples were obtained for serological evaluation by ELISA using the rK39 antigen and a commercial kit (Biomanguinhos/FIOCRUZ, Rio de Janeiro). ELISA was performed using 96-well plates coated with rK39 in carbonate buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). After coating, plates were washed four times with PBS containing 0.05% Tween-20. Unspecific reactions were inhibited by incubating with 2% casein in PBS. Serum samples (1:80 dilution) were added to the well, and incubated at 4 °C for 12 h. Plates were then washed and incubated with the secondary antibody (anti-canine IgG; 1:5000 dilution) for 40 min, followed by another wash, and incubation for 10 min in citrate buffer (0.1 M citric acid, 0.2 M bibasic sodium phosphate, pH 5.0) containing 0.02 g of *o*-phenylenediamine (OPD) and 16 μL de H_2O_2. Optical density (OD) was measured at 490 nm, and cut off was established at two standard deviations above the average OD of the negative controls. The commercial ELISA kit (Biomanguinhos/FIOCRUZ) was used according to the manufacturer's instructions.

2.3. Xenodiagnosis

In order to assess whether the animals were infectious for sand fly vectors (*L. longipalpis*), xenodiagnosis was performed as previously described (Silva et al., 2010) with modifications. Bush dogs were subjected to general anesthesia using a combination of xylazine (2 mg/kg) (União Química Farmacêutica, Brazil) and ketamine (11 mg/kg) (União Química Farmacêutica, Brazil) by the intramuscular route. Maned wolves were physically contained according to the standard protocols of the Fundação Zoo-Botânica de Belo Horizonte, initially using a metallic support with a thick rope placed around the thorax, which was followed by manual containment by two animal handlers.

From 40 to 50 female 4-day-old phlebotomine sand flies (*L. longipalpis*), placed in a FleboContainer (Costa-Val et al., 2007), feed directly on a previously shaved left ear for 30 min. Phlebotomines were then feed with 50% sucrose in distilled water and kept at 28 °C for 5 days.

At the first time point (February) ten randomly selected engorged female phlebotomines were dissected in a drop of PBS, and the midgut was examined under light microscopy for detecting promastigotes, and determining the infection index. Semi-quantitative measurement of the number of promastigotes was determined as follows: (−) absence of promastigotes, (+) 1–50 promastigotes; (++) 51–200 promastigotes, and (+++) more than 200 promastigotes (Travi et al., 2002).

2.4. Quantitative PCR

Average numbers of parasites/μg of DNA were compared between time points (February and August) for both maned wolves and bush dogs to evaluate possible seasonal effects on transmissibility of *L. infantum*. Parasite loads in engorged sand flies were measured at both time points (February and August) by quantitative PCR (qPCR). For DNA extraction, ten randomly selected engorged female *L. longipalpis* were separately homogenized in 100 μL of TE buffer pH 8, and then 500 μL of GES (5 M guanidium thiocyanate, 100 mM EDTA, pH 8.0, and 0.5% v/v Sarkosyl) were added. Tubes were gently moved for homogenizing the suspension, incubated at room temperature for 10 min, followed by incubation on ice for 5 min. Then, 250 μL of 7 M ammonium acetate at −20 °C

Table 1
Characterization of maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) included in this study.

Animal	Species	Age[a]	Sex[b]	Origin[c]
1	*C. brachyurus*	11	F	FZB-BH
2	*C. brachyurus*	16	M	Zoo (Araxá, State of Minas Gerais)
3	*C. brachyurus*	2	M	Zoo (Brasília, Federal District)
4	*C. brachyurus*	11	F	Zoo (Goiânia, State of Goiás)
5	*S. venaticus*	5	M	FZB-BH
6	*S. venaticus*	5	F	FZB-BH
7	*S. venaticus*	6	M	FZB-BH
8	*S. venaticus*	5	M	FZB-BH
9	*S. venaticus*	5	M	FZB-BH

[a] Age in years.
[b] Sex: male (M), female (F).
[c] Origin: animals that were born at the Fundação Zoo-Botânica de Belo Horizonte (FZB-BH); or city and state of zoos where some of the animals came from are indicated.

Table 2
Primers used in this study for amplification of *Leishmania infantum* genes.

Gene	Primers	Product (base pairs)
DNA polymerase	TGTCGCTTGCAGACCAGATG	90
	GCATCGCAGGTGTGAGCAC	
β-actin	CTTCTACAACGAGCTGCGCG	305
	TCATGAGGTAGTCGGTCAGG	

(without agitation) was added and incubated on ice for 10 min, followed by addition of 500 μL of chloroform-2-pentanol at −20 °C, and vigorous agitation. The mixture was centrifuged at $21,000 \times g$ for 10 min, and the supernatant was transferred to another tube, 405 μL of isopropanol at −20 °C were added, gently mixed for 1 min, and centrifuged at $21,000 \times g$ for 5 min. The supernatant was then discarded and the pellet was washed twice with 500 μL of 70% ethanol, followed by centrifugation at $21,000 \times g$ for 5 min, and dissolved in 30 μL of ultra-pure water.

Primers targeting *L. infantum* DNA polymerase and β-actin were used in this study (Table 2). qPCR was performed in a final volume of 10 μL, with 2 μM of each primer, 5 μL of 1X SYBR Green PCR master-mix (Applied Biosystems, USA), and 4 μL of template DNA (5 ng/μL), under the following parameters: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Standard curves were established using serial dilutions of the 90 pb of the DNA polymerase gene of *L. infantum* cloned into the pGEM-T *Easy Vector System Plasmids* (Promega, USA). The same procedure was performed for the β-actin gene, which was amplified to ensure integrity of samples and for normalization DNA concentrations. Reactions were performed using a ABI Prism 7500 (Applied Biosystems, USA). Male and female non engorged sand flies were used as negative controls.

2.5. Statistical analysis

The Mann–Whitney test was used to analyze the frequency of promastigote-containing female sand flies exposed to maned wolves or bush dogs. The means of parasites/μg DNA were compared by the Student's *t* test. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Serological assessment of Leishmania sp. infection

Six out of nine canids were serologically positive (66.6%) for *Leishmania* sp. (Table 3). Serologically positive animals included three maned wolves (3/4, 75%), and three bush dogs (3/5, 60%). There was 100% coincidence in the results of both ELISA protocols,

Table 3
Serological results for detection of *Leishmania* sp. antibodies in maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*).[a]

| Animal	Species	ELISA biomanguinhos	ELISA rK39
1	*C. brachyurus*	−	−
2	*C. brachyurus*	+	+
3	*C. brachyurus*	+	+
4	*C. brachyurus*	+	+
5	*S. venaticus*	−	−
6	*S. venaticus*	+	+
7	*S. venaticus*	−	−
8	*S. venaticus*	+	+
9	*S. venaticus*	+	+

[a] Results were completely coincident between the two time points (February and August).

and serological results were the same at both time points (February and August).

3.2. Xenodiagnosis

Ten phlebotomine sand flies (engorged females) previously exposed to each animal were randomly selected for searching promastigotes in the medium intestine. Fig. 1 demonstrates the number of promastigote-containing phlebotomine sand flies. At the first time point (February) only two animals (one maned wolf and one bush dog) were negative by xenodiagnosis. These two animals were also serologically negative. However, none of the animals had all female sand flies containing promastigotes, and the animal with the highest number of positive sand flies was a bush dog that had eight out of ten promastigote-containing sand flies (Fig. 1A). At the second time point, in August (during the dry season) all animals had at least 3 out of ten engorged sand flies that contained *Leishmania* sp. promastigotes—ranging from 30% to 90% of promastigote-containing sand flies (Fig. 1B). There were no significant differences between the frequency of promastigote-containing female sand flies exposed to maned wolves or bush dogs both in February ($P = 0.3873$) and August ($P = 0.8024$). There was no statistically significant difference in the frequency of positive sand flies when both time points (February and August) were compared ($P = 0.1088$).

Parasite load is another important parameter that was evaluated. At the first time point (in February), morphological assessment of promastigotes in the midgut of sand flies was performed in addition to qPCR. Among maned wolves, all 10 phlebotomines examined from each positive animal had up to 50 parasites, which was associated with relatively low frequencies of positive sand flies (Fig. 1, Table 4). Similarly, bush dogs also had low parasite loads, with all phlebotomines with up to 50 parasites, except for one sand fly that had between 51 and 200 promastigotes (Table 4). Together these results indicate that although maned
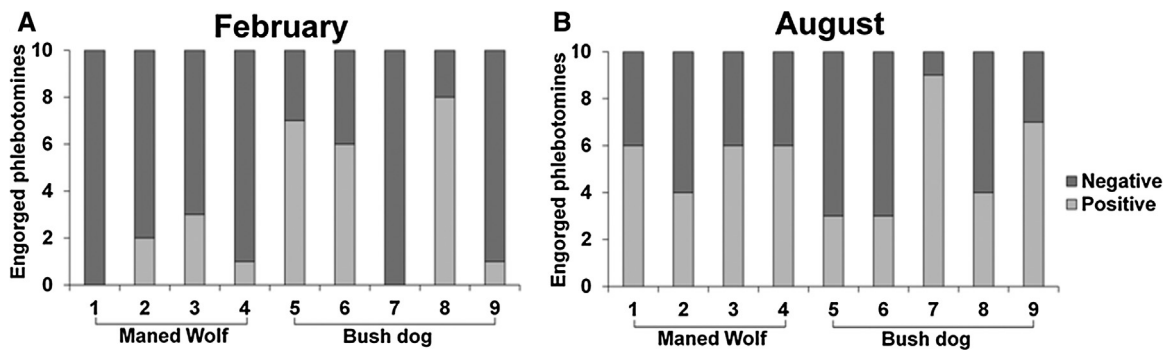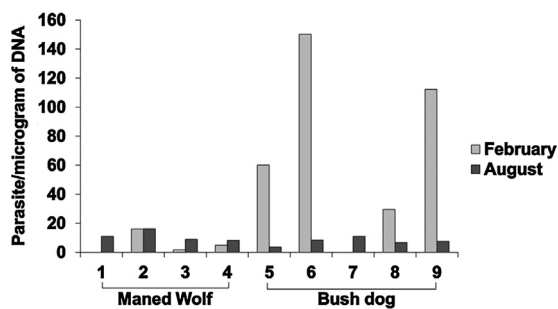
Fig. 1. Transmission of *Leishmania infantum* from maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) to *Lutzomyia longipalpis*. Ten engorged female sand flies (*L. longipalpis*) exposed by a xenodiagnosis protocol to each vertebrate host were analyzed for detection of promastigotes. The sand flies were dissected in a drop of PBS and examined under light microscopy. Animals were exposed in (A) February and again in (B) August.

Table 4

Results of xenodiagnosis based on microscopic assessment of the number of promastigotes in the midgut of engorged female sand flies (*Lutzomyia longipalpis*) exposed to maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*).[a]

Female phlebotomine	Vertebrate host species								
	Chrysocyon brachyurus				*Speothos venaticus*				
	1	2	3	4	5	6	7	8	9
1	−	−	−	−	−	−	−	+	−
2	−	+	+	−	+	+	−	+	−
3	−	−	+	−	−	+	−	−	−
4	−	−	−	+	+	−	−	++	+
5	−	−	−	−	+	−	−	−	−
6	−	−	+	−	+	+	−	+	−
7	−	+	−	−	+	−	−	+	−
8	−	−	−	−	−	+	−	+	−−
9	−	−	−	−	+	+	−	+	−
10	−	−	−	−	+	+	−	+	−

[a] (−) absence of promastigotes; (+) 1-50 promastigotes; (++) 51-200 promastigotes; and (+++) >201 promastigotes. Morphological evaluation of promastigotes in the midgut of engorged female sand flies was performed only at the first time point (February).

Fig. 2. Semi-quantitative analysis of parasite loads in engorged female sand flies (*Lutzomyia longipalpis*) exposed by a xenodiagnosis protocol to four maned wolves (*Chrysocyon brachyurus*) and five bush dogs (*Speothos venaticus*). Promastigotes were quantified by quantitative PCR (SYBR Green method) with a calibration curve. The amplification target was the DNA polymerase gene.

wolves and bush dogs are capable of infecting female *L. longipalpis*, parasite loads in engorged female sand flies were low.

3.3. Quantitative PCR

At the first time point (February), the number of parasites/μg of DNA varied from 0 to 150.2. Two animals were negative coinciding with serological results as well as quantification of promastigotes by microscopy (Tables 3 and 4, Fig. 2). At the second time point, the number of parasites/μg of DNA varied from 3.6 to 16.1 (Fig. 3B).

Although averages of parasitic loads were higher at the first time point (February), there were no significant differences for both maned wolves ($P = 0.1071$) and bush dogs ($P = 0.0837$).

4. Discussion

This study demonstrated that the endangered maned wolves (*C. brachyurus*) and bush dogs (*S. venaticus*) are capable of infecting female sand flies (*L. longipalpis*), which is the most important invertebrate vector of *L. infantum* in Brazil. In the past decades, VL has become an urban disease in Brazil (Diniz et al., 2008). Considering the density of domestic dogs, which are efficient reservoirs of the disease (Diniz et al., 2008), and the abundance of *L. longipalpis* (Resende et al., 2006), captive wild animals kept in zoological gardens within endemic urban areas are often exposed to *L. infantum* (Luppi et al., 2008; Lombardi et al., 2014). Therefore, studies about the susceptibility and transmissibility of *L. infantum* are highly desirable for conservational as well as for public health reasons.

Several species of the Canidae family are considered susceptible to *L. infantum*, with reports of wild canids that developed clinical disease (Luppi et al., 2008), although most of the studies are based on serological methods, which does not correlate well with the clinical condition or transmissibility (Curi et al., 2006; Luppi et al., 2008; Jusi et al., 2011). Thus, this study represents an additional step for assessing the potential role of wild canids in transmission of VL. There are several reports of xenodiagnosis in the domestic dog. Some of these studies indicate that dogs with clinical signs of VL are more capable of transmitting the pathogen to the invertebrate vector, while asymptomatic dogs do not transmit *Leishmania* to female sand flies (Verçosa et al., 2008; Travi et al., 2001). However, other studies demonstrated that asymptomatic dogs are also capable of transmitting the pathogen to sand flies, although with lower intensity when compared to symptomatic dogs (Courtenay et al., 2002; Costa-Val et al., 2007; Michalsky et al., 2007; Amorim et al., 2011; Soares et al., 2011; Laurenti et al., 2013). In humans, regardless of the clinical condition or presence of clinical signs, when the patient infected with *L. donovani* reaches a high parasitemia (>1000 parasites per milliliter of blood) the transmission to the invertebrate vector occurs more efficiently (Costa et al., 2000; Seblova et al., 2013), although it is noteworthy that *L. donovanni* and *L. infantum* have different infectiousness potential to their respective invertebrate vectors.

In this study, none of the animals had classical clinical signs of VL, but they were capable of transmitting *Leishmania* to female sand flies. However, the number of positive engorged female sand flies was low as well as the parasite loads in the positive sand flies (<200 parasites/phlebotomine). Based on qualitative xenodiagnosis, 77.5% of the animals were positive at the first time point, whereas 100% were positive at the second time point. A study of
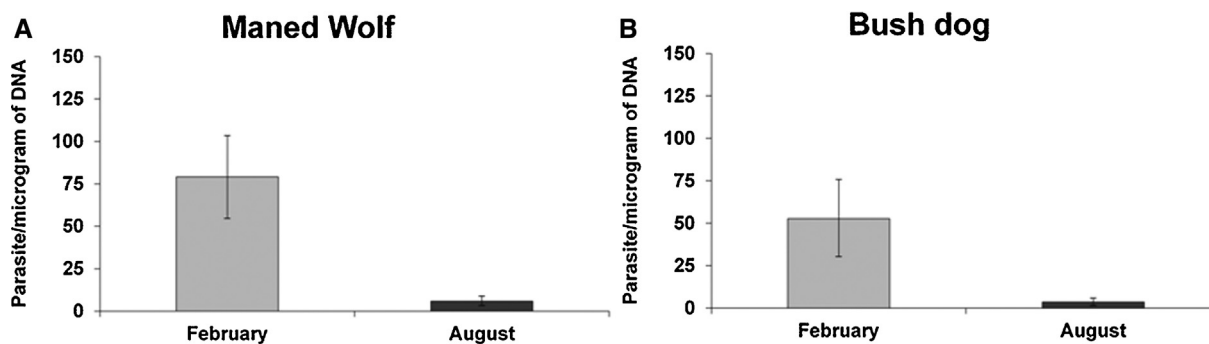
Fig. 3. Seasonal variation of parasite loads in engorged female sand flies (*Lutzomyia longipalpis*) exposed by a xenodiagnosis protocol to (A) four maned wolves (*Chrysocyon brachyurus*), and (B) five bush dogs (*Speothos venaticus*). Xenodiagnosis was performed in February and August. The number of parasites per microgram of DNA was estimated by quantitative PCR. Averages were compared by the nonparametric Mann–Whitney test, and averages were not significantly different ($P > 0.05$).

xenodiagnosis in asymptomatic free ranging crab-eating foxes (*C. thous*) that were PCR and culture positive for *Leishmania*, demonstrated that none of the engorged sand flies became infected (Courtenay et al., 2002). Importantly, a previous study in which xenodiagnosis was performed in one single symptomatic crab-eating fox resulted in infection of all 10 female sand flies evaluated (Deane and Deane, 1954). Therefore, it is reasonable to hypothesize that clinical signs may correlate with efficiency of *Leishmania* transmission from crab-eating foxes to female sand flies (Deane and Deane, 1954; Courtenay et al., 2002). Our results support the notion that maned wolves and bush dogs are capable of transmitting *Leishmania* to female sand flies even in the absence of clinical signs.

Although our results demonstrate unequivocally that *Leishmania* can be transmitted from maned wolves and bush dogs to sand flies, the epidemiological relevance of these findings remain to be established since the number of *Leishmania* that establishes infection in the phlebotomine digestive tract appears to be critical for the ability of the sand fly to transmit the disease to another vertebrate host. Although there has been a research effort to establish the limits for transmission, the actual parasite load in the sand fly that is required for efficient transmission remains poorly known (Maia et al., 2011). High parasite loads are required to generate obstruction of the anterior midgut, which causes regurgitation before the sand fly is capable of bloodfeed (Bates, 2007). The infective dose varies according to the species of *Leishmania* as well as the species of the invertebrate vector. Experimental infection of *L. longipalpis* with *L. infantum* results in 10^5 –10^6 parasites per sand fly (Ranasinghe et al., 2008). *L. infantum*-infected sand flies inoculate 10–10,000 promastigotes, but 75% of the sand flies inoculate less than 300 parasites (Secundino et al., 2012). Therefore, based on our results, it is not clear whether sand flies infected by feeding on maned wolves and bush dogs are capable of transmitting the parasite to another susceptible vertebrate host since less than 200 parasites/phlebotomine indicates low permissivity for transmission of *L. infantum* (Travi et al., 2002) that requires blocking of the sand fly midgut (Bates, 2007).

Regarding the potential for transmission of *L. infantum* by maned wolves and bush dogs at the period when the *L. longipalpis* population is the highest (February) or the lowest (August) in Belo Horizonte (Resende et al., 2006), there were no significant differences between these two time points. In Belo Horizonte (State of Minas Gerais, Brazil), *L. longipalpis* have four generations per year, with a mean interval of three months between generations, which coincides with the incubation period of *L. infantum* in dogs (Resende et al., 2006). Therefore, our results indicate that maned wolves and bush dogs are infective for female sand flies at different seasons of the year.

5. Conclusion

This study demonstrated that maned wolves (*C. brachyurus*) and bush dogs (*S. venaticus*) kept within a metropolitan area endemic for VL, and naturally and asymptomatically infected with *L. infantum* are capable of transmitting *L. infantum* to the invertebrate host *L. longipalpis*. However, the low parasite loads in engorged phlebotomines exposed to these animals possibly indicate that they do not efficiently function as reservoirs of VL.

Acknowledgements

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