Effect of *Leishmania* spp infection on the survival, life expectancy, fecundity and fertility of *Lutzomyia longipalpis s.l.* and *Lutzomyia pseudolongipalpis*

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We evaluated the effects of Leishmania *spp infection on several population parameters of* Lutzomyia longipalpis sensu lato *and* Lutzomyia pseudolongipalpis*, vectors of visceral leishmaniasis in Venezuela, under experimental conditions during the first post-feeding period. Females of both species were allowed to feed and engorge on a suspension of fresh washed human red blood cells in foetal calf serum. These blood cells were either non-infected or infected with one of the four* Leishmania *spp strains and were offered through a chicken skin membrane. The longevity, life expectancy and the fecundity of uninfected flies were similar in both species, but the fertility was significantly lower in uninfected* Lu. longipalpis *females. In all cases, the infection of* Lu. longipalpis *and* Lu. pseudolongipalpis *by the* Leishmania *strains resulted in significant detrimental effects, which exerted a fitness cost expressed by reduced survival and life expectancy, as well as decreased fertility and fecundity compared with the control groups. Nevertheless, differences in these parameters were observed between these vector species depending on whether they were infected with the autochthonous Venezuelan* Leishmania infantum *strain (NESA) or the Brazilian reference strain (PP75). The experimental data obtained agree with field data on the natural infection of these vector species and the significance of this scenario is discussed.*

Key words: survival - life expectancy - fertility - fecundity - *Lutzomyia longipalpis s.l.* - *Lutzomyia pseudolongipalpis* - experimental sandfly infection - *Leishmania* spp

Controversial assumptions have been made about the effects of arthropod-borne parasite infections on their blood-sucking vectors. Previously, it was thought that the infection had no detrimental effects, but several studies have shown that it might, in fact, have an adverse impact on the vector species. In natural parasite-vector associations, the infection has no effect on vector longevity (Freier & Friedman 1987, Chege & Beier 1990, Hamilton & Hurd 2002). However, parasites of the genus *Plasmodium* cause a high mortality in their natural vectors. The death of the infected mosquitoes is associated with an invasion of the intestinal wall by ookinetes and is strongly correlated with the intensity of the infection (Klein et al. 1986, Kittayapong et al. 1992, Lyimo & Koella 1992, Hogg & Hurd 1995) and the age of the mosquitoes (Dawes et al. 2009).

Furthermore, in many blood-sucking insects of various taxa, the process of oogenesis is disrupted by their associated parasites, which results in the loss of reproductive fitness. A natural infection of *Anopheles gambiae* by *Plasmodium falciparum* caused a decrease in the number of eggs laid (Hogg & Hurd 1997). Similar observations have been reported for other parasite-vector combinations (Javadian & MacDonald 1974, Christensen 1981, Renshaw & Hurd 1994).

Williams (1976) showed that *Leishmania* parasites cause disturbances in the physiology of phlebotomine sandflies, for example, disruptions in the ovarian cycle. Killick-Kendrick et al. (1977) reported some damage to nearby cells of the stomodeal valve in two specimens of *Lutzomyia wellcomei* possibly infected with *Leishmania braziliensis*. Subsequently, it was found that *Leishmania major* causes degenerative damage to the cardia of its natural vector, *Phlebotomus papatasi*. This damage is possibly due to the action of a *Leishmania* chitinase that cause the valve to remain open, which, in turn, facilitates the regurgitation and ejection of metacyclic promastigotes from the fly (Schlein et al. 1992).

This scheme is now thought to be the prevailing mechanism of *Leishmania* transmission and it has also been observed in associations between *Phlebotomus duboscqi-L. major* and *Lutzomyia longipalpis-Leishmania chagasi* (Volf et al. 2004). In addition, infections with *L. major* and *Leishmania infantum* promastigotes led to a significant reduction in the longevity and fecundity of *Ph. papatasi* and *Phlebotomus langeroni* (El Sawaf et al. 1994). Rogers and Bates (2007) investigated the behaviour of *Leishmania*-infected sandflies and demonstrated the mechanisms by which the parasites manipulate the vector feeding behaviour, which resulted in an enhanced transmission. This interaction was related to the secretion of a gel-plug associated with the differentiation of mammal-infective transmission stages that

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were achieved in both experimental and natural parasite-sandfly combinations. These authors also reported that the infection with *Leishmania mexicana* or *L. infantum* caused a significant reduction in the longevity of *Lu. longipalpis*. However, it did not affect the reproductive fitness measured as the number of eggs laid (fecundity). The outcome of the association between parasite and vector seems, therefore, to have varying effects on the population parameters of different sandfly species. Thus, this variability differentially influences the specific vectorial competence and the *Leishmania* transmission dynamics. In this study, we investigated the effect of four infecting strains of *Leishmania* on the survival, life expectancy, fecundity and fertility of two closely related species in the longipalpis complex. We hope that this research will lead to a greater understanding of the different epidemiological situations of visceral leishmaniasis in Venezuela.

MATERIALS AND METHODS

Sandflies - Two colonies of phlebotomine sandflies housed at the National Reference Center for Phlebotomine Sandflies in Maracay, Venezuela, were used in the experiments. A *Lutzomyia pseudolongipalpis* colony was established in 1991 with 420 females and 210 males captured in the small village of El Brasilar, La Rinconada, Curarigua (state of Lara; 9º59'N 69º55'W). A *Lu. longipalpis s.l.* colony was established in 2000 with 850 females caught in El Layero, Parapara (state of Guarico; 9º41'N 67º16'W). Both localities are endemic foci for visceral leishmaniasis. The specimens of *Lu. pseudolongipalpis* used in the experiments ranged between the 67th-70th generation, while those of *Lu. longipalpis* ranged between the fourth-seventh generation. After the emergence, groups of 40 females and 10 males were maintained in plastic pots with the bottoms lined with plaster and the tops covered with fine gauze. Each group was provided with a cotton swab moistened with a sucrose solution *at libitum*. In addition, females were fed on healthy hamsters and the larvae received an autoclaved powdered mixture of rabbit faeces, fish food and sand in a 2:1:1 ratio. The room temperature was maintained at 26 ± 1ºC and the relative humidity (RH) in the polystyrene cages where the insects were kept at 80-90% with a moistened towel paper; the photoperiod was set to 12:12 h (L:D).

Parasites - *Lu. pseudolongipalpis* and *Lu. longipalpis* were infected with three strains of New World *L. (L.) infantum* (syn. *L. chagasi*): (i) an autochthonous strain (MHOM/VE/98/NESA) isolated from the bone marrow aspirate of a two-year-old girl from Margarita Island (Zerpa et al. 2001), (ii) an international reference strain from Brazil (MHOM/BR/PP75) and (iii) an autochthonous strain (IEVA/VE/93/UCNA-2) isolated from *Lutzomyia evansi* captured in Guayabita, state of Aragua (Feliciangeli et al. 1999), and an international strain of *L. (Viannia) braziliensis* (MHOM/BR/LBT300). The parasites were maintained in vitro by a passage every three days through a biphasic culture medium that consisted of rabbit blood agar at 10% and liver infusion tryptose (LIT) medium (OMS 1990).

Sandfly infection - Females of each sandfly species, three-five days old, from the same generational batch were randomly assigned to one of five groups. Each group was fed a suspension containing promastigotes of one of the strains cited above: (i) NESA, (ii) PP75, (iii) UCNA-2, (iv) LBT300 or (v) a control group that was given a parasite-free suspension. The density of promastigotes was 3 x 10^6/mL in Schneider medium (Gibco BRL) supplemented with 15% foetal calf serum (HyClone) and fresh washed human red blood cells at a ratio of 1:1, which were offered through an artificial feeder using chicken skin membranes (Maroli 1985). Preliminary tests in which the concentration of parasites applied was increased from 1 x 10^6 to 10 x 10^6 cells/mL at a rate of 1 x 10^6 parasite/mL were performed to establish the minimum infective concentration under our laboratory conditions; the value obtained was 3 x 10^6 promastigotes/mL. Samples of parasites from the cultures were counted in a Neubauer haemocytometer. We used promastigotes in the exponential growth phase because of their greater number and activity. Fed sandfly females were isolated and maintained under the same conditions as the sandfly colony in the polystyrene plastic containers. Each of these containers had a cotton swab dipped in a 30% sucrose solution and a moistened paper in the bottom to maintain an RH of 80-90%.

It is well known that even under optimal laboratory conditions, phlebotomine females will rarely survive to oviposition. This limitation makes the construction of life tables and the calculation of population parameters for this group of insects difficult because data can only be obtained, in general, for the first post-feeding period. To compare control groups and infected groups of the two species, females were observed daily and the following data were recorded for each group: (i) number of dead females, (ii) number of females with retained eggs, (iii) number of eggs laid per female and, later, (iv) number of eggs hatched in each pot. Each dead female was dissected to determine the presence of parasites in their guts and only females in which parasites were observed were included in the analysis. The experiment was repeated three times for each parasite-vector combination and for the control groups.

Statistical analysis - To evaluate the sandfly infection, a database was constructed in Microsoft Excel and exported to EpiInfo 6.4. Comparisons between the two vector species with regards to their infection by the *Leishmania* strains were made by chi-square tests, using the Yates corrected values, estimates of odds ratios and 95% confidence intervals. The longevity and life expectancy of the sandflies in each treatment and for each replicate were calculated according to Rabinovich (1980) after feeding. These parameters were then compared among the experimental groups and their controls using the Friedman non-parametric test [Friedman (1937), cited by Siegel and Castellan (1995)]. The survivorship curves obtained for the different parasite-vector combinations were compared using an application of the Mantel and Haenszel non-parametric test known as the Log Rank test (Mantel 1966, Santos & Luque 1996). With

regards to fecundity and fertility, the Mann-Whitney *U* test [Mann and Whitney (1947) cited by Siegel and Castellan (1995)] was used to compare the average number of eggs laid (fecundity) and the number of eggs hatched (fertility) for each parasite-vector combination. In all cases, the significance level applied was $p < 0.05$. The data were analysed using the statistical program Statistix v.7 (Analytical Software 1998).

RESULTS

Sandfly infection - Table I shows the results of the experimental infection of *Lu. longipalpis s.l.* and *Lu. pseudolongipalpis* with different strains of *Leishmania* spp. Significant differences were observed between the sandflies infected with the autochthonous Venezuelan strain from Margarita Island (NESA) and the Brazilian PP75 reference strain. Conversely, no differences were observed for sandflies infected with the continental Venezuelan strain, *L. infantum* obtained from *Lu. evansi* (UCNA-2) and the non-species specific reference strain, *L. braziliensis* (LBT300). Moreover, a higher infection rate was observed in *Lu. longipalpis* when fed the Venezuelan continental strain (UCNA-2) in comparison with all the other strains. In contrast, the infection rate in *Lu. pseudolongipalpis* was significantly higher when fed with either of the two autochthonous strains (NESA and UCNA-2) in comparison with the Brazilian (PP75) and the non-species specific strain *L. braziliensis* (LBT300).

Longevity and life expectancy - The median age reached after feeding by uninfected *Lu. longipalpis* and *Lu. pseudolongipalpis* females before death (longevity) was 8.5 and eight days, respectively, and this difference was not significant. Individuals infected with promastigotes of any of the *Leishmania* strains, however, showed a significant decrease in longevity ($p = 0.023$ for *Lu. longipalpis* and $p = 0.017$ for *Lu. pseudolongipalpis*) (Table II).

TABLE I

Experimental infection of *Lutzomyia longipalpis* and *Lutzomyia pseudolongipalpis* by different strains of *Leishmania* spp

	Lu. longipalpis		*Lu. pseudolongipalpis*				
Parasite strain	Fed (n)	Infected n (%)	Fed (n)	Infected n (%)	χ^2	p	OR (95% CI)
NESA	225	59 (26.2)	66	55 (83.3)	67.48	0.0000	0.07 (0.03-0.15)
PP75	285	77 (27)	480	60 (12.5)	24.66	0.0000	2.59 (1.75-3.84)
UCNA-2	72	53 (73.6)	120	74 (61.7)	2.36	0.1246[a]	1.73 (0.87-3.46)
LBT300	212	55 (25.9)	178	39 (21.9)	0.86	0.4186[a]	0.80 (0.49-1.31)

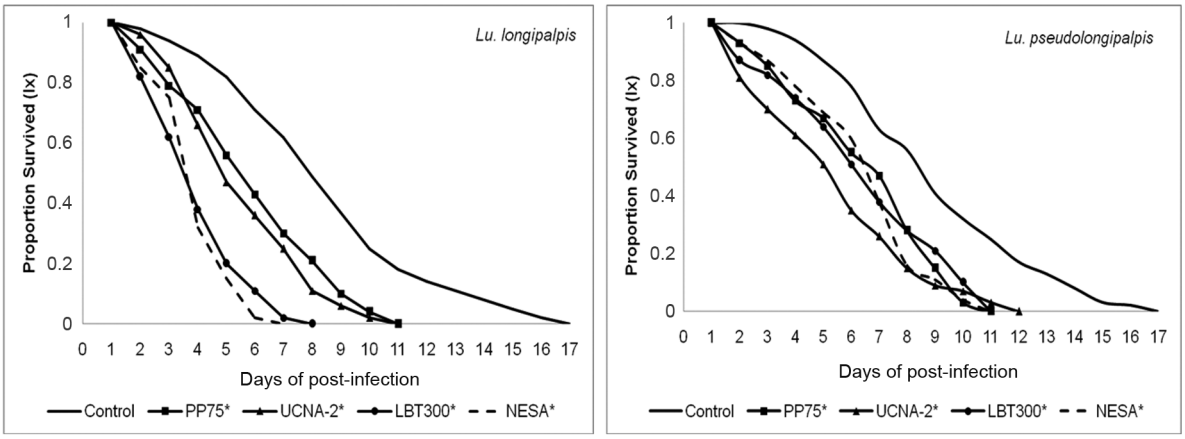
a: not significant; CI: confidence interval; OR: odds ratio.

TABLE II

Longevity and life expectancy of *Lutzomyia longipalpis* and *Lutzomyia pseudolongipalpis* after infection by different *Leishmania* spp species

	Longevity (days) median (range)				
Species	NESA	PP75	UCNA-2	LBT300	Control
Lu. longipalpis	3.5 (1-7) n = 59	6 (1-11) n = 77	5 (1-11) n = 53	4 (1-8) n = 55	8.5 (1-17) n = 65
Lu. pseudolongipalpis	6 (1-11) n = 55	6 (1-11) n = 60	6.5 (1-11) n = 74	6 (1-11) n = 39	8 (1-17) n = 63
	Life expectancy median (range)				
Species	NESA	PP75	UCNA-2	LBT300	Control
Lu. longipalpis	2.7 (2.3-2.9) n = 59	4.2 (4.2-5.3) n = 77	4.3 (3.8-4.8) n = 53	2.6 (2.5-2.8) n = 55	6.8 (6.2-8.2) n = 65
Lu. pseudolongipalpis	4.8 (4.4-5.8) n = 55	5.5 (4.2-5.6) n = 60	4.1 (3.8-4.2) n = 74	5.2 (4.8-5.2) n = 39	7.6 (7.5-7.9) n = 63

n: total sample of fed females from three replicates.



Survivorship curves of *Lutzomyia longipalpis* and *Lutzomyia pseudolongipalpis* post-infection by different species and/or strains of *Leishmania*. Asterisks mean $p < 0.01$ vs. control group.

The life expectancy of uninfected females (calculated from the 1st day after feeding) was 6.8 days for *Lu. longipalpis* and 7.6 days for *Lu. pseudolongipalpis*; these values decreased significantly for females infected with promastigotes of the *Leishmania* strains (*Lu. longipalpis*: $p = 0.024$, *Lu. pseudolongipalpis*: $p = 0.048$) (Table II).

Survival curves - As shown in the Figure, the survival rate of *Lu. longipalpis* dropped to 0.82 and 0.37 at days 5 and 9, respectively, after a parasite-free suspension was offered; in *Lu. pseudolongipalpis,* the survival values were 0.87 and 0.41 at days 5 and 9, respectively, and the maximum survival was 17 days for both species ($p = 0.604$). The infection with *Leishmania* promastigotes significantly decreased the survival ($p < 0.0001$ for all parasite-vector combinations) from 17 days for uninfected females to an average of seven days in *Lu. longipalpis* and 11-12 days in *Lu. pseudolongipalpis* (Figure). However, *Lu. longipalpis* was more affected than *Lu. pseudolongipalpis* by the NESA and LBT300 strains ($p = 0.001$). Thus, on the seventh day, the proportion of survivors was 0.11 and 0.02 for *Lu. longipalpis* females infected with NESA and LBT300 promastigotes, respectively, and 0.38 for *Lu. pseudolongipalpis* females with both strains (Figure). The survival of *Lu. pseudolongipalpis* and *Lu. longipalpis* females was equally affected after the infection with PP75 and UCNA-2.

Fecundity and fertility - Table III summarises the results obtained from the three replicates of each experiment. Although the control females of *Lu. pseudolongipalpis* laid a greater number of eggs than the control females of *Lu. longipalpis*, no statistically significant differences were found between the two species in relation to the number of eggs laid (fecundity). Nevertheless, the proportion of fertile eggs was significantly higher in the control females of *Lu. pseudolongipalpis* compared to the control females of *Lu. longipalpis* ($p = 0.023$). The infection with any of the *Leishmania* strains significantly decreased the fecundity and fertility of both sandfly species. The fecundity of the control females of *Lu. longipalpis* was 14 eggs per female, which was significantly higher than the number of eggs laid per female in the groups infected with *Leishmania* promastigotes (6-8 eggs per female). Similarly, non-infected *Lu. pseudolongipalpis* oviposited 19 eggs per female, whereas infected females laid seven-12 eggs per female. Lastly, the fertility was also reduced to approximately half in both sandfly species after the infection with any of the *Leishmania* spp (Table III).

DISCUSSION

The aim of this study was to investigate the effects of the infection by *Leishmania* spp on several population parameters of the vectors *Lu. longipalpis s.l.* and *Lu. pseudolongipalpis* in natural and experimental vector-parasite combinations. The ultimate goal of this study was to improve our understanding of the *Leishmania* transmission dynamics in some visceral leishmaniasis foci in Venezuela. Under controlled laboratory conditions, we observed that survival, life expectancy and fecundity were similar between control females of *Lu. longipalpis* and *Lu. pseudolongipalpis*, but the fertility was significantly lower in the former. Second, we noted that the infection with different strains of *Leishmania* spp led to different percentages of infection. *Lu. longipalpis*, originally from a continental area (state of Guarico), was more susceptible to the sympatric Venezuelan strain (UCNA-2) than to the allopatric insular strain (NESA), the non-autochthonous *L. infantum* from Brazil (PP75) or the experimental strain *L. braziliensis* (LBT300). This outcome could indicate a relative specificity or the evolution of a close vector-parasite association over a long period of time. Interestingly, *Lu. pseudolongipalpis* was more susceptible than *Lu. longipalpis*; significantly higher infection rates were recorded with both (natural) autochthonous continental (UCNA-2) and insular strains of *L. infantum* (NESA) compared with the (experimental) foreign (PP75) or non-species specific (LBT300) strains. These differences in parasite-vector relationships between the two studied species confirm the consistency of their separation within the longipalpis complex.

TABLE III

Effect of *Leishmania* spp infection on fecundity and fertility of *Lutzomyia longipalpis* and *Lutzomyia pseudolongipalpis*[a]

	Females that laid eggs/ infected females (%)	Females with eggs retained/ infected females (%)	Total eggs laid/ median (range)	Fertile eggs n (%)
		Lu. longipalpis		
PP75	24/77 (31.2)	34/77 (44.2)	183 6 (2-21)[b]	43 (23.5)[b]
UCNA-2	15/53 (28.3)	16/53 (30.2)	118 7 (2-14)[b]	33 (28)[b]
NESA	9/59 (15.3)	26/59 (44.1)	83 8 (2-15)[c]	24 (28.9)[c]
LBT300	21/55 (38.2)	31/55 (56.4)	152 6 (2-14)[b]	56 (36.8)[b]
Control	30/65 (46.1)	6/65 (9.2)	495 14 (2-32)	218 (44)
		Lu. pseudolongipalpis		
PP75	16/60 (26.7)	13/60 (21.7)	123 7.5 (1-17)[b]	39 (31.7)[b]
UCNA-2	32/74 (43.2)	12/74 (16.2)	310 8 (1-19)[b]	98 (31.6)[b]
NESA	22/55 (40)	18/55 (32.7)	218 7 (2-24)[b]	72 (33)[b]
LBT300	15/39 (38.5)	16/39 (41)	179 12 (1-26)[b]	58 (32.4)[b]
Control	45/63 (71.4)	3/63 (4.8)	770 19 (2-31)	504 (65.4)

a: total obtained from three replicates; *b*: $p < 0.01$ vs. control group; *c*: $p < 0.05$.

Rogers and Bates (2007) demonstrated that the two events that directly enhance transmission of *Leishmania*, metacyclogenesis and the secretion of the gel-like plug composed of filamentous proteophosphoglycan accompanied by the differentiated mammal-infective transmission stages (from amastigotes to metacyclic promastigotes), can be produced using both experimental and natural parasite-sandfly combinations and that the exponential growth phase causes both events to appear earlier and in greater amounts.

All the treatments in our experiments, using promastigotes from cultures in the exponential growth phase with natural and experimental strains, gave rise to *Leishmania* infection in *Lu. longipalpis* and *Lu. pseudolongipalpis*, which are both natural vectors in Venezuela. However, the autochthonous (natural) strains resulted in greater infection rates in comparison with the foreign (PP75) and non-specific (experimental) strains (LBT300) (Table I).

After an infection with either *L. infantum* or *L. braziliensis*, the longevity and life expectancy significantly decreased in both vector species in comparison with the controls, even though the latter is not naturally transmitted by species in the longipalpis complex. Similarly, El Sawaf et al. (1994) found that infection with promastigotes of *L. major* and *L. infantum* significantly reduced the longevity and fecundity of not only their natural vector, *Ph. papatasi*, but also of an experimental vector, *Ph. langeroni*.

Rogers and Bates (2007) also reported a reduction in the longevity of *Lu. longipalpis* after an infection with *L. infantum* and *L. mexicana*. However, they did not observe any adverse effects on its fecundity and fertility. As a consequence, they concluded that re-diverting resources destined for egg production to ameliorate a reduced longevity as seen in malaria vectors (Hurd 2003) is not a strategy in *Leishmania* species. In contrast, we observed a consistent and significant reduction in fecundity and fertility in all infected groups vs. the controls.

The *Leishmania* strains had more detrimental effects on the survival, life expectancy, fecundity and fertility of *Lu. longipalpis* than on *Lu. pseudolongipalpis*. To determine whether these differences play a role in the *Leishmania* transmission dynamics, we attempted to relate our experimental results to data obtained in the field. Small populations of *Lu. longipalpis* have been observed in longitudinal studies carried out on the continental Guayabita focus (Feliciangeli et al. 1999) and on Margarita Island (Feliciangeli et al. 2003). It could be argued that this small population size is due not only to the lower fertility of this species, but also to a reduced

longevity and life expectancy as a result of the interaction with *Leishmania* parasites, as observed in this study. The natural infection reported in Guayabita (UCNA-2) was 0.28% (Feliciangeli et al. 1999), while on Margarita Island (NESA) it was more than 1% (Feliciangeli et al. 1998, Rodriguez et al. 2005). In contrast, our experiments show greater infection rates for this species with the sympatric *Leishmania* strain from Guayabita (UCNA-2), but the individuals survived longer than those infected with the allopatric *Leishmania* spp isolated from Margarita Island (NESA). This pattern could indicate a remarkable species-specific vector-parasite relationship and a regulatory mechanism working towards the establishment of equilibrium between the parasite and the vector. On the other hand, *Lu. pseudolongipalpis*, the species in the complex up until now restricted to the state of Lara is an abundant species in its natural habitat, which fits with the high fecundity and fertility observed in our laboratory colony. A high proportion of individuals became infected during the experimental trials; in the field, however, this species exhibits a very low infection rate (0.01%) (Arrivillaga & Feliciangeli 2001, Feliciangeli et al. 2006). In spite of these controversial results, it is reasonable to suppose that, in its quest for successful transmission, when the *Lu. longipalpis s.l.* population density is low, the proportion of *Leishmania*-infected flies increases. Conversely, when the population density of a species is high, such as the case of *Lu. pseudolongipalpis*, the infection rates could be expected to decrease because a high natural infection would severely impact the susceptible vector population. Such an impact could potentially disrupt the host-parasite equilibrium.

In this study, as a preliminary approach based on experimental and field data, we showed that several vector population parameters are influenced by parasite infection. These results led us to postulate that the parasite infection might play an important role in the establishment and maintenance of *Leishmania* transmission in nature. However, further studies are needed to fully understand the dynamics of this parasite-vector relationship.

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