

## Development of *Leishmania (Leishmania) infantum chagasi* in Its Natural Sandfly Vector *Lutzomyia longipalpis*

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**Abstract.** We analyzed the development of *Leishmania (Leishmania) infantum chagasi* in its natural sandfly vector *Lutzomyia longipalpis*. In addition, we compared sandfly infections initiated with axenic amastigotes or promastigotes. Our data showed no important difference between *Lu. longipalpis* infection rates resulting from either type of infections. Furthermore, development of infection was equivalent in both cases. All promastigote forms were found inside the sandfly and, after blood digestion, most of the population consisted of procyclics and nectomonads. A low percentage of metacyclic forms was coincident with a high number of nectomonads during late stages of infection, but which form gives rise to metacyclic forms in *L. infantum chagasi* is unknown. These results also show that the promastigote infection model, at least for this situation, is suitable for obtaining of infected sandflies because it is easier and less laborious.

### INTRODUCTION

Leishmaniasis are neglected diseases endemic to 98 countries or territories. *Leishmania* infections range from mild, self-healing skin lesions to a fatal visceral form, depending on the *Leishmania* species involved. Approximately 500,000 cases of visceral leishmaniasis (VL) are estimated to occur per year, and leishmaniasis is the ninth most common infection disease worldwide. More than 90% of cases are concentrated in Bangladesh, Ethiopia, India, Nepal, Sudan, and Brazil.<sup>1</sup> In developing countries of the Western Hemisphere, the urbanization of VL caused by *Leishmania infantum chagasi* has been increasingly reported in many cities, and its proven main vector, the sandfly *Lutzomyia longipalpis*,<sup>2</sup> is becoming highly adapted to artificial environments.<sup>3–5</sup>

Development of *Leishmania* inside its vector is a complex process. After the sandfly has had an infected blood meal, ingested amastigotes (non-flagellate forms) differentiate into dividing promastigotes (flagellate forms) to establish the parasite life cycle. However, there are numerous adverse conditions to overcome in the midgut of the host, including digestive enzyme activities<sup>6</sup> and the synthesis of a physical barrier (the peritrophic matrix; PM),<sup>7</sup> and the need to bind to the midgut cells<sup>8,9</sup> to avoid excretion. Lipophosphoglycan, the major *Leishmania* surface glycoconjugate, protects the parasites from the enzyme activities of its host and mediates parasite attachment to the midgut of the sandfly.<sup>8–18</sup> After digestion, successful infection in a sandfly vector results in development of several promastigotes forms types named, according to their morphology, as procyclic, haptomonad, nectomonad, paramastigote, and metacyclic forms.<sup>19</sup> Only metacyclic forms transmitted through sandfly bites are able to begin an infection in vertebrate hosts.<sup>20,21</sup>

Studies on *Leishmania*-vector interactions are needed to understand the processes involved in parasite development and transmission. Much information regarding Old World *Leishmania* species and their vectors is available, as reviewed recently by Sacks and others.<sup>22</sup> In contrast, there are few published data relating to New World species; for example,

there are only a few studies on *L. infantum chagasi* and *L. mexicana* in *Lu. longipalpis*<sup>21,23,24</sup> and *L. amazonensis* and *L. braziliensis* in *Lu. migonei* and *Lu. intermedia*.<sup>25–27</sup> However, there is little detailed information in the literature about the developmental biology of the *L. infantum chagasi* in its natural vector *Lu. longipalpis*. This finding is somewhat unexpected, given that *L. infantum chagasi* is the causative agent of the American visceral leishmaniasis, the most severe form of the disease.

Successful experimental infection of sandflies to resemble natural transmission depends on providing amastigotes (the parasitic form found in vertebrate host) during an infective blood meal. Promastigote differentiation into axenic amastigotes is achieved as a result of changes in pH, temperature, and CO<sub>2</sub> concentration.<sup>28–31</sup>

We provide an *in vivo* analysis of the development *L. infantum chagasi* throughout its life cycle in its natural sandfly vector *Lu. longipalpis* from establishment of infection to the metacyclogenesis, a process that enables the parasite to be transmitted to its vertebrate host. In addition, we compared infections initiated with axenic amastigotes and promastigote forms.

### MATERIALS AND METHODS

**Parasite cultures.** In the current study, we used *L. infantum chagasi* World Health Organization reference strain MHOM/BR/1970/BH46. Promastigotes was cultured in medium 199 supplemented with 10% fetal bovine serum and other components<sup>16</sup> at 26°C. Axenic cultures of *in vitro* amastigotes were initiated from stationary-phase promastigotes, which were placed in a concentration of 5 × 10<sup>6</sup> cells/mL in medium 199 containing 20% fetal bovine serum and 25 µg/mL of hemin at 37°C in an atmosphere of 5% CO<sub>2</sub> (Araújo MS and others, unpublished data). Amastigote transformation dynamics were evaluated upon observation of parasite morphology and RNA expression.

**Identification of A2 amastigote-specific protein by reverse transcription polymerase chain reaction.** Total *L. infantum chagasi* RNA was extracted by using Trizol<sup>®</sup> (Invitrogen, Carlsbad, CA) and treated with DNase (Invitrogen) from log-phase promastigotes at 24, 48, 72, and 96 hours and from transformed axenic amastigotes. First-strand cDNAs were generated from 2 µg of RNA by using oligo dT (15) primer (Promega, Madison, WI) and M-MLV reverse transcriptase (Promega). The cDNAs were amplified with gene-specific

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primers A2 (GenBank accession no. S69693), 5'-GACCGAG-CACAATGAAGATC-3' (forward), 5'-GTCACCATGCC-TCATGGCAT-3' (reverse); and  $\alpha$ -tubulin (GenBank accession no. DQ129864.1), 5'-CGTGTGCATGATTGCCAACT-3' (forward), 5'-GAATTGTCCGCTTCGTCTTGAT-3' (reverse). The polymerase chain reaction mixture contained Taq Platinum DNA polymerase (1 unit) (Invitrogen), 200 mM of each dNTP (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, pH 8.5, and 10 pmol of each specific primer set in a 25- $\mu$ L reaction. Thermal conditions were 94°C for 45 seconds, specific annealing temperatures (60°C for A2 and 62°C for alpha tubulin) for 45 seconds and 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. Amplified products were resolved by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

**Sandfly infections.** Wild-caught *Lu. longipalpis* sandflies were collected in the Lapinha Cave, a non-endemic leishmaniasis area located at Lagoa Santa, Brazil (43°57'W, 19°3'S) using CDC light traps. Unfed female sandflies were separated into batches of 150 insects. They were kept in an insectary of the Laboratory of Medical Entomology of the Centro de Pesquisas René Rachou for at least two days before infection experiments. The experimental infections were carried out according to the protocol of Tesh and Modi.<sup>32</sup> Sandflies were allowed to feed through a chick skin membrane in an artificial feeding device containing heparinized mouse blood with heat-inactivated serum and seeded with  $4.8 \times 10^7$  parasites/mL. Blood-engorged females were separated and allowed to feed *ad libitum* on a 50% sucrose solution at 25°C and a humidity of 95% until they were dissected for parasite development analysis.

**Parasite detection and development.** Infected flies were dissected daily from the first to the tenth day after the infective blood meal. Insects were quick immobilized in a freezer and dissected individually in drops of phosphate-buffered saline. The gut of each sandfly was also homogenized in microfuge tube containing 30  $\mu$ L of phosphate-buffered saline at pH 7.2. The number of parasite was counted in a hemocytometer by using phase-contrast microscopy. The same material was used to prepare slide smears, which were stained with quick Romanovsky-type stain (Panótico Rapid; Laborclin, Pinhais, Brazil) for detection of relative proportions of developmental forms of the parasite. These forms were recognized by morphology and classified according to terminology established by Lawyer and others<sup>19</sup> and subsequently used by several authors<sup>25, 27, 33</sup> as shown in Figure 1.

**Statistical analysis.** Results were analyzed by using the Mann Whitney test and the *t*-test. *P* values > 0.05 were considered significant.

## RESULTS

### *In vitro* prepared *L. infantum chagasi* axenic amastigotes.

Amastigotes completed differentiation from promastigotes at 96 hours, as confirmed morphologically by light microscopy (Figure 2A). They were visualized as non-motile, round, or slightly elongated cells lacking a free flagellum. They were viably active cells, as shown by their ability to revert to promastigotes and to infect BALB/c macrophages. Moreover, A2 protein, a well-known amastigote-specific protein,<sup>34</sup> was expressed abundantly in the axenic amastigotes, but was largely absent in promastigotes at 96 hours of cultivation, as demon-



FIGURE 1. Developmental forms of *Leishmania* promastigotes showing from left to right an amastigote ingested from an infective bloodmeal; a procyclic promastigote, a short, ovoid, slightly motile, first promastigote that appears in the sandfly; a nectomonad promastigote, a long slender form; a haptomonad promastigote, a shorter and broader form; a paramastigote promastigote, a rare form with the kinetoplast adjacent to the nucleus; and a metacyclic promastigote, a short, slender, highly active, infective form for the vertebrate host.

strated by reverse transcription polymerase chain reaction (Figure 2B). These parameters ensured that axenic amastigotes would be useful for successful infection of sandflies.

***Lutzomyia longipalpis* infection with *L. infantum chagasi*.** *Lutzomyia longipalpis* was able to sustain *L. infantum chagasi* infection for the experimental period (10 days post-feeding) when the metacyclogenesis is completed and the parasites can be transmitted to vertebrate hosts. Infection rates beginning with either promastigotes or axenic amastigotes ranged from 79% to 94% and from 83% to 100%, respectively. The decreased infection rates was observed at days 3 and 4 (79% and 83%), respectively for promastigotes and amastigotes. After digestion, the parasite number increased again with migration to midgut regions, as observed until the end of the analysis (Figure 3). No significant difference was observed in the infection rates of *Lu. longipalpis* initiated with either promastigote or axenic amastigote (*P* > 0.05), except on day 6. The parasite number at 48 hours, before excretion of the blood meal, was  $2.4 \times 10^4$ /sandfly. This number decreased by day 3 because of digestion of the blood meal, increased again to approximately  $2.4 \times 10^4$ /sandfly until days 6 and 7, respectively, to promastigotes and amastigotes, and was finally maintained at approximately  $1 \times 10^4$ /sandfly by day 10 (Figure 3). The significant difference observed in the infection rates on day 6 can be explained by the transformation time of axenic amastigotes (slower) and promastigotes (faster) needed to achieve the maximum density of parasites.

Colonization of the stomodeal valve and a gelatinous substance similar to the promastigote secretory gel described by Rogers and others<sup>21</sup> in *Lu. longipalpis* infected with *L. amazonensis*, a non-natural vector-parasite pair, were

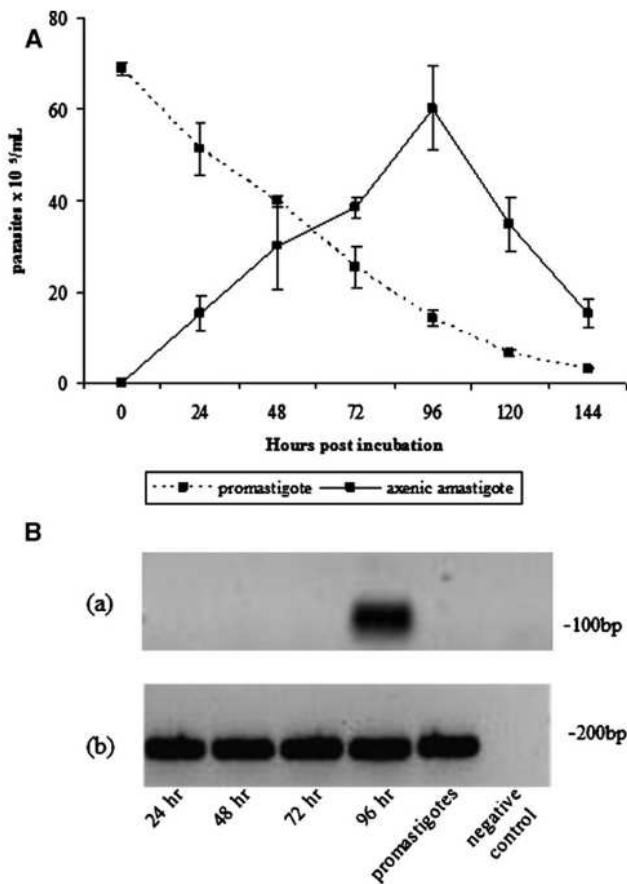


FIGURE 2. **A**, Evaluation of amastigote transformation dynamics of parasite morphology and cell viability in samples stained with trypan blue. **B**, reverse transcription polymerase chain reaction analysis of expression of A2 amastigote-specific protein in axenic *Leishmania infantum chagasi* amastigotes. **a**, A2 primer; **b**,  $\alpha$ -tubulin primer. Lanes 1–4 = axenic amastigotes 24, 48, 72, and 96 hours post *in vitro* incubation, respectively; lane 5 = cultivated promastigotes; lane 6 = negative control. The dataset represent the analysis of two independent experiments. bp = basepairs. Error bars indicate mean  $\pm$  SD.

observed at day 5 in most of the *L. infantum chagasi* females infected with either promastigotes or amastigotes (Figure 4).

#### Developmental forms of *L. infantum chagasi* in *Lu. longipalpis*.

The proportion of *L. infantum chagasi* morphotypes also changed during development of infection within *Lu. longipalpis* (Figure 5 and Table 1). In infections initiated with promastigotes, procyclic forms were observed on day 2, but at low proportions (4.7%). At day 4, the number of procyclic forms was approximately the same (5%) but decreased after day 6. Nectomonads were seen on day 2 and were the predominant form (> 80%) until day 10, which was the last day of the experiments. In contrast, paramastigotes were rarely seen (0.1%). Haptomonads were observed on day 2 (2.8%) and never exceeded 4%. Only a few metacyclic forms were detected until day 4. However, they accounted for 13% of the entire population by day 10.

A similar trend was seen in infection initiated with amastigotes but with some delay in their differentiation to the promastigote forms. On day 2, undifferentiated amastigotes accounted for 5.9% of the parasite population, but they had disappeared completely by day 3, when procyclic forms became

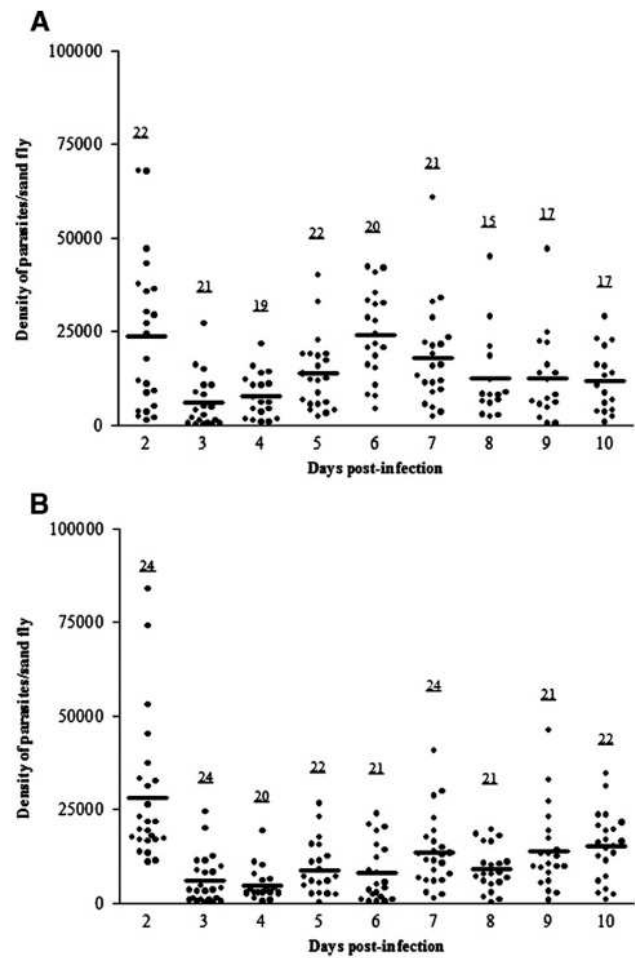


FIGURE 3. *Lutzomyia longipalpis* infected midgut with **A**, promastigote and **B**, axenic amastigote forms of *Leishmania infantum chagasi*. Numbers above the points indicate the number of dissected females. The dataset represent the analysis of four independent experiments. Horizontal lines indicate means.

predominant (81.7%). In contrast, nectomonads accounted for 12.3% of forms by day 2 and became the predominant form by day 3 (96.2%). Procyclic promastigotes decreased to 2.1% of the total forms by day 3 and persisted at low levels throughout the course of infection. Metacyclic promastigotes were only found at day 9 (0.2%), but represented 2.7% of the population by day 10 (Figure 5 and Table 1).

## DISCUSSION

Promastigotes are the easiest parasite form to obtain from *Leishmania* parasites in culture to use in laboratory experiments. Amastigotes of some species were originally cultivated axenically in cell-free media.<sup>30,31,35–37</sup> Acidic pH and a higher temperature induce developmentally regulated changes in shape and gene expression of promastigotes, which generate amastigotes that resemble the animal tissue-derived amastigotes.<sup>28,30,31,38</sup> *In vitro* cultivation of amastigotes provides an excellent source of parasites that are free from host-derived components. They have been used in drug evaluation, molecular cloning, identification of developmentally regulated genes, and vaccine production.<sup>39</sup> In this study, we have

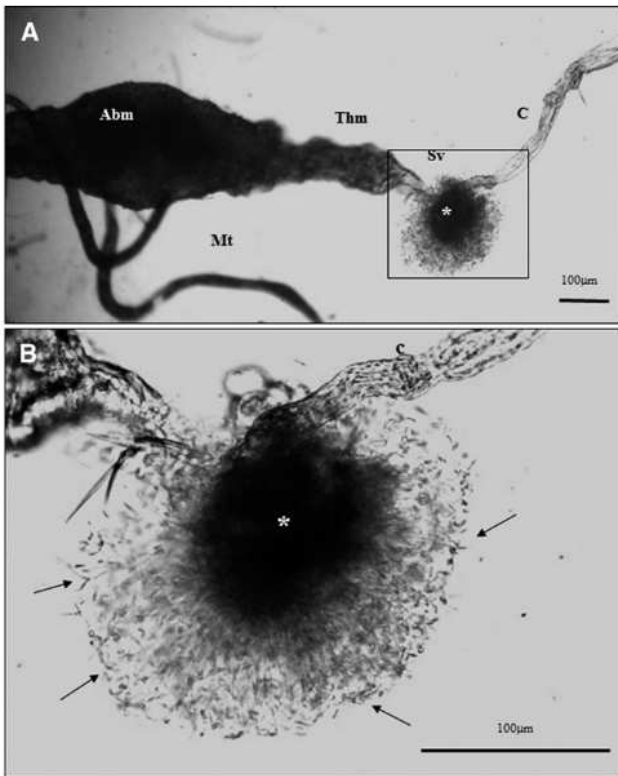


FIGURE 4. **A**, Dissected midgut of *Lutzomyia longipalpis* showing the promastigote secretory gel plug (\*) in a sectioned stomodeal valve (Sv). Abm = abdominal midgut; Thm = thoracic midgut; C = crop; Mt = midgut. **B**, Enlargement showing details of the promastigote secretory gel plug (\*) with a massive concentration of promastigotes (arrows). C = crop.

used axenically cultivated *Leishmania* amastigotes to infect sandfly vectors. To verify *L. infantum chagasi* amastigote viability, we used criteria such as morphology, ability to revert into promastigotes in culture-dependent temperature, ability to infect macrophages, and expression of A2 protein, a well-known amastigote-specific protein.<sup>34</sup> Our data show that axenic *L. infantum chagasi* amastigotes resemble morphologically and physiologically animal tissue-derived amastigotes, including their expressing of the A2 protein and their ability to infect *Lu. longipalpis*, the natural vector of the parasite. In addition, infection with amastigote, the natural mode of infection, was used to compare their development with infection initiated with cultured promastigotes. Interestingly, no significant difference was observed between *Lu. longipalpis* infection rates initiated with either promastigote or amastigote forms. This result shows that the infection model using promastigotes, at least for the *L. infantum chagasi* *Lu. longipalpis* model system, is suitable for obtaining infected sandflies. It is also easier and less laborious than other options.

Experimental infections in sandflies exhibit different developmental patterns of *Leishmania* species inside the host insect gut. These patterns are determined by colonization of different anatomic regions of the gut and the appearance of distinct promastigote forms. Differentiation of infective metacyclic forms is crucial to determine the vectorial capacity of a sandfly.<sup>25</sup> To evaluate the impact of the type of infection (amastigotes versus promastigotes) on *L. infantum chagasi* development, sandfly midguts were examined daily. Parasite

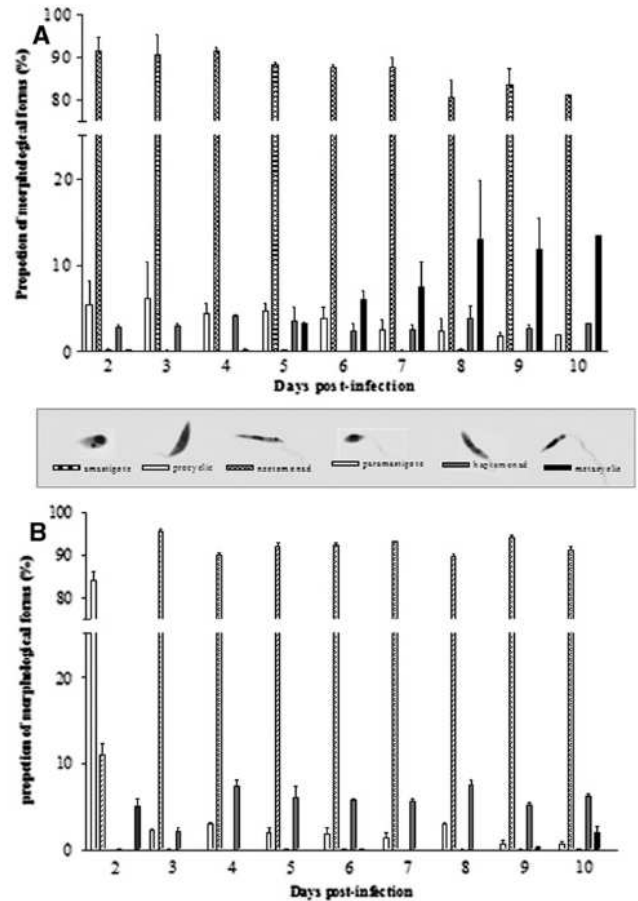


FIGURE 5. *Lutzomyia longipalpis* infected with either **A**, promastigotes or **B**, axenic amastigotes of *Leishmania infantum chagasi*. Samples of 12 sandflies were prepared for morphologic analyses and at least 300 parasites were analyzed in each dissected midgut. Data represent the geometric mean of flies analyzed from two independent experiments. The data set represents the analysis of approximately 30,000 individual parasites. Different forms are all displayed at the same magnification. Error bars indicate mean  $\pm$  SD.

multiplication was higher on the second day of infection. However, in all sandflies, there was a significant decrease in parasite number during the early events of blood meal digestion. Transformed promastigotes inside the sandfly gut have to overcome potentially lethal conditions; for example, approximately 50% of *L. major* ingested by *Phlebotomus papatasi* during initial infection died during this early stage.<sup>7</sup> Borovsky and Schlein<sup>6</sup> suggested that trypsin-like activity in the midgut of the *P. papatasi* prevented survival of *L. donovani*. Pimenta and others<sup>7</sup> observed that the midgut environment, in the first few hours after blood feeding, is harmful even for a strain of *L. major* that is capable of complete development in the sandfly. These studies also showed that the addition of soybean trypsin inhibitor to the blood meal prevent much of the early parasite deaths. In *Lu. longipalpis*, trypsin-like proteins were identified that displaying high sequence similarities to those from *P. papatasi*.<sup>40, 42</sup> Also, mutants lacking lipophoglycan were more susceptible to digestion by enzymes.<sup>10</sup> Our data consistently showed high parasite mortality at the early stages of *L. infantum chagasi* infection, which is probably caused by enzymatic activity.

TABLE I  
*Lutzomyia longipalpis* infected with promastigotes (Pro) or axenic amastigotes (Ama) of *Leishmania infantum chagasi*\*

Days post-infection	Morphologic forms (%)											
	Amastigote		Procyclic		Nectomonad		Paramastigote		Haptomonad		Metacyclic	
	Pro	Ama	Pro	Ama	Pro	Ama	Pro	Ama	Pro	Ama	Pro	Ama
2		5.9	4.7	81.7	92.2	12.3	0.1	0.0	2.8	0.0	0.2	0.0
3		0.0	5.3	2.1	91.5	96.2	0.1	0.1	3.0	1.7	0.1	0.0
4		0.0	4.6	3.1	91.2	90.4	0.0	0.0	4.0	6.4	0.2	0.0
5		0.0	4.7	2.5	88.3	92.7	0.1	0.1	3.6	4.7	3.3	0.0
6		0.0	3.7	2.6	87.4	91.8	0.1	0.1	2.6	5.5	6.2	0.0
7		0.0	2.5	1.9	87.5	92.8	0.0	0.0	2.5	5.3	7.4	0.0
8		0.0	2.5	3.0	80.8	90.1	0.1	0.0	3.9	6.9	12.7	0.0
9		0.0	1.9	1.2	83.5	93.2	0.0	0.0	2.7	5.4	11.9	0.2
10		0.0	2.0	0.9	81.3	90.4	0.0	0.2	3.3	5.9	13.5	2.7

\*Samples were prepared for morphologic analyses and at least 300 parasites were analyzed. Data represent the geometric mean of flies analyzed from two independent experiments. Data set represents the analysis of approximately 30,000 individual parasites.

The PM is a semi-permeable barrier that enables gradually diffusion of sandfly hydrolytic enzymes<sup>43</sup> and, for this reason, protects the parasites from them.<sup>7</sup> Although the PM eventually disintegrates, this process was described to occur more quickly in infected sandflies,<sup>24,44</sup> which suggested a contribution of insect and *Leishmania* chitinases.<sup>45</sup> Parasite escape from PM is an essential step to avoid their expulsion with the non-digested blood meal. At this point, appearance and variation of distinct promastigote forms are considered sequential steps necessary for metacyclogenesis.<sup>25</sup> This process culminates with development of thin, high-motile and infective metacyclic forms<sup>46</sup> that are able to infect the vertebrate host. Morphology of *L. infantum chagasi* parasites within the *Lu. longipalpis* midgut changed during infection process. After blood digestion (day 2), the parasite population consisted almost entirely of procyclic forms and nectomonads in infections started either with amastigotes or promastigotes. Procyclic forms have the capacity to multiply, initiate, and sustain infection during the digestion process.

In the sandflies fed with amastigotes, the new transformed procyclic population decreased on day 3. According to Pimenta and others,<sup>7</sup> the susceptibility to gut proteases is extremely high in transitional-stage parasites during transformation of amastigotes to promastigotes. After expulsion of the blood meal, nectomonads became the main promastigote form present in the gut until the end of experiment. In contrast, in the sandflies infected with promastigotes, the procyclic population decreased early, before expulsion of the blood meal and, on the second day, the predominant forms were nectomonads.

In the current study, all promastigote forms described and classified by Lawyer and others<sup>19</sup> were found. However, we could not find a so-called haptomonad (a morphotype with disc-like expansion of flagellar tip), proposed as a new morphologic category by Rogers and others.<sup>21</sup> This finding could be caused by differences in the *Leishmania* strain and sandfly vector pair used because these Rogers and others used a non-natural model: *Lu. longipalpis* infected with *L. mexicana*. Nevertheless, similarly to the results reported by Rogers and others, we also observed formation of a promastigote secretory gel like substance located in the stomodeal valve, but we were not able to determine the morphotype involved in its formation.

The appearance of *L. infantum chagasi* metacyclic forms in *Lu. longipalpis* was coincident with the presence of a high number of nectomonads during late stages of infection. In other studies using different vector-parasite pairs, appearance

of metacyclic forms overlapped with paramastigotes in the foregut.<sup>25,47</sup> However, we did not observe this phenomenon. Despite careful sequential observation on the appearance of morphologic promastigotes types, the question of which form gives rise to *L. infantum chagasi* metacyclic forms remains unanswered. However, *L. infantum chagasi* and other New World *Leishmania* species<sup>25,27,48</sup> develop low percentages of metacyclic forms, a feature that differs from the Old World species,<sup>33</sup> which appear to be better adapted to generate higher numbers of infective parasite forms.

*Lutzomyia longipalpis* has been the focus of studies because of its importance as a vector of VL in Latin America. Therefore, interaction studies with this sandfly are needed to understand its competence as a vector.

A study comparing sandfly infection with amastigotes from lesion and promastigotes of *L. infantum* did not show any difference in parasite development in *Lu. longipalpis* (Jacobina).<sup>49</sup> Similarly, using amastigotes and promastigotes and another *Lu. longipalpis* population (Lapinha) and *L. infantum chagasi* strain (BH46), we also observed this trend. Comparative studies are of interest because differences in development of parasites might exist if *Lu. longipalpis* is considered a species complex,<sup>50</sup> with currently unknown underlying differences between geographically distinct populations with regard to *Leishmania* interaction and, more importantly, vectorial competence.

Despite minor differences in morphotypes, it is clear that infection of *Lu. longipalpis* with *L. infantum chagasi* promastigotes is an easier way to obtain infected vectors. Importantly, the proportion of metacyclic forms inside the sandfly midgut was higher in promastigote-initiated infection than in amastigote-initiated infection. This finding is probably caused by a two-day delay needed for transformation of amastigotes into promastigotes inside the vector. In conclusion, promastigote infection can be a reliable tool for obtaining infected sandflies and for interaction studies. It is less laborious, quicker, and enables metacyclic differentiation before considerable vector mortality occurs.

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