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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

Leishmaniases 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.[1] 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*,[2] is becoming highly adapted to artificial environments.[3–5]

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[6] and the synthesis of a physical barrier (the peritrophic matrix; PM),[7] and the need to bind to the midgut cells[8,9] 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.[8–18] 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.[19] Only metacyclic forms transmitted through sandfly bites are able to begin an infection in vertebrate hosts.[20,21]

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.[22] 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*[21,23,24] and *L. amazonensis* and *L. braziliensis* in *Lu. migonei* and *Lu. intermedia*.[25–27] 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_2 concentration.[28–31]

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[16] at 26°C. Axenic cultures of *in vitro* amastigotes were initiated from stationary-phase promastigotes, which were placed in a concentration of 5×10^6 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_2 (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® (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′-GACCGAGCACAATGAAGATC-3′ (forward), 5′-GTCACCATGCCTCATGGCAT-3′ (reverse); and α-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 $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.5, and 10 pmol of each specific primer set in a 25-μL 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.[32] 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 μL 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[19] and subsequently used by several authors[25–27,33] 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,[34] was expressed abundantly in the axenic amastigotes, but was largely absent in promastigotes at 96 hours of cultivation, as demonstrated by reverse transcription–polymerase chain reaction (Figure 2B). These parameters ensured that axenic amastigotes would be useful for successful infection of sandflies.

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.

***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 2.4×10^4/sandfly. This number decreased by day 3 because of digestion of the blood meal, increased again to approximately 2.4×10^4/sandfly until days 6 and 7, respectively, to promastigotes and amastigotes, and was finally maintained at approximately 1×10^4/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[21] in *Lu. longipalpis* infected with *L. amazonensis*, a non-natural vector-parasite pair, were

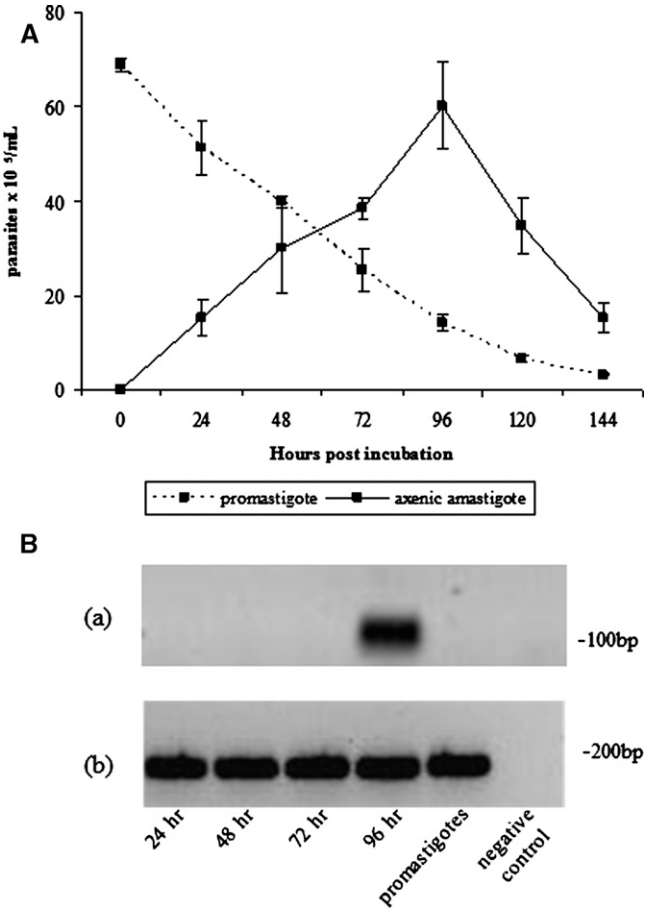

FIGURE 2. **A**, Evaluation of amastigote transformation dynamics of parasite morphology and cell viability in samples stained with trypan blue. **B**, reverse transcription polymerase chain reaction analysis of expression of A2 amastigote-specific protein in axenic *Leishmania infantum chagasi* amastigotes. **a**, A2 primer; **b**, α-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 ± SD.

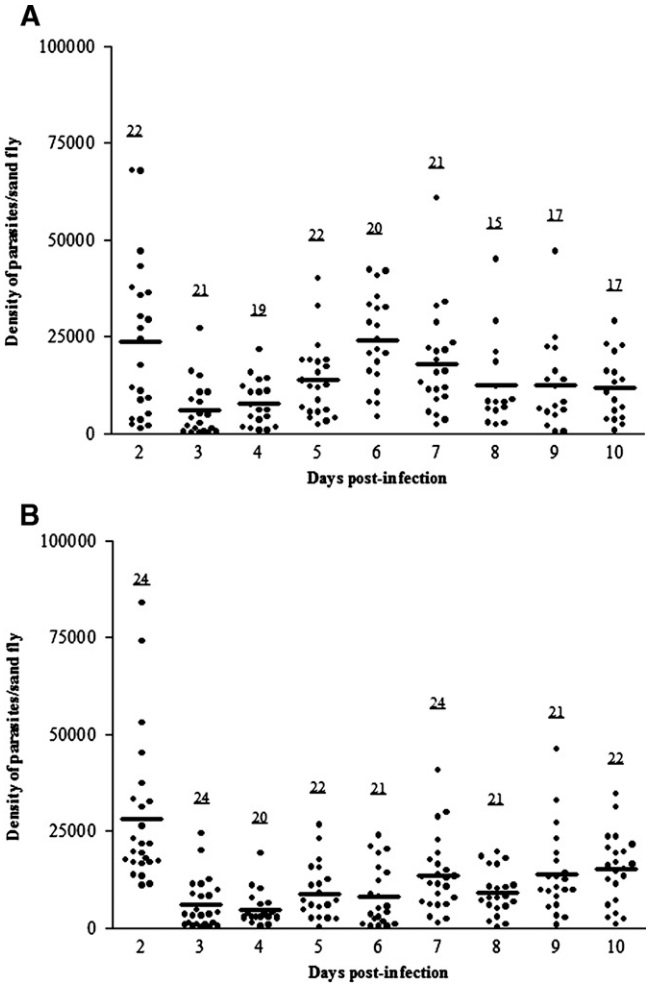

FIGURE 3. *Lutzomyia longipalpis* infected midgut with **A**, promastigote and **B**, axenic amastigote forms of *Leishmania infantum chagasi*. Numbers above the points indicate the number of dissected females. The dataset represent the analysis of four independent experiments. Horizontal lines indicate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

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 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.[30,31,35–37] 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.[28,30,31,38] *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.[39] In this study, we have

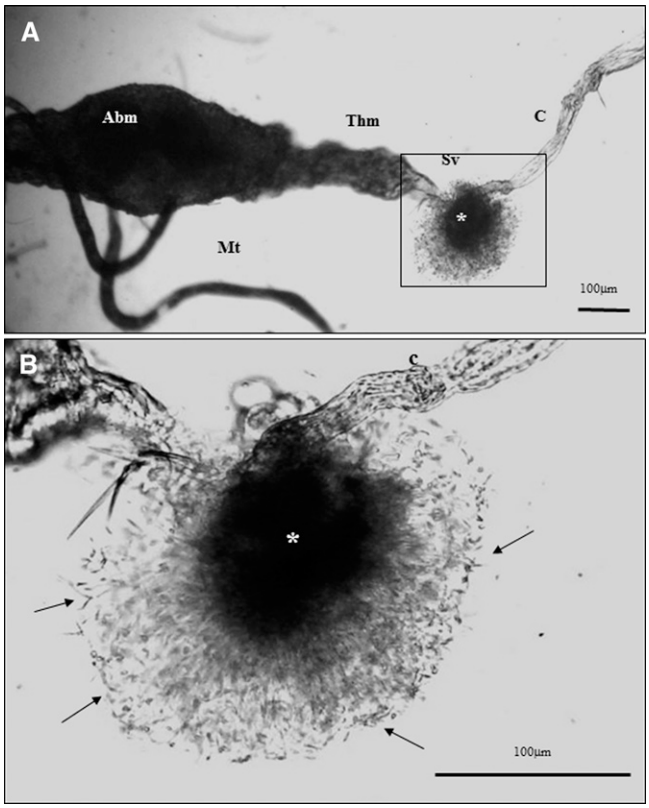

FIGURE 4. **A**, Dissected midgut of *Lutzomyia longipalpis* showing the promastigote secretory gel plug (*) in a sectioned stomodeal valve (Sv). Abm = abdominal midgut; Thm = thoracic midgut; C = crop, Mt = midgut. **B**, Enlargement showing details of the promastigote secretory gel plug (*) with a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

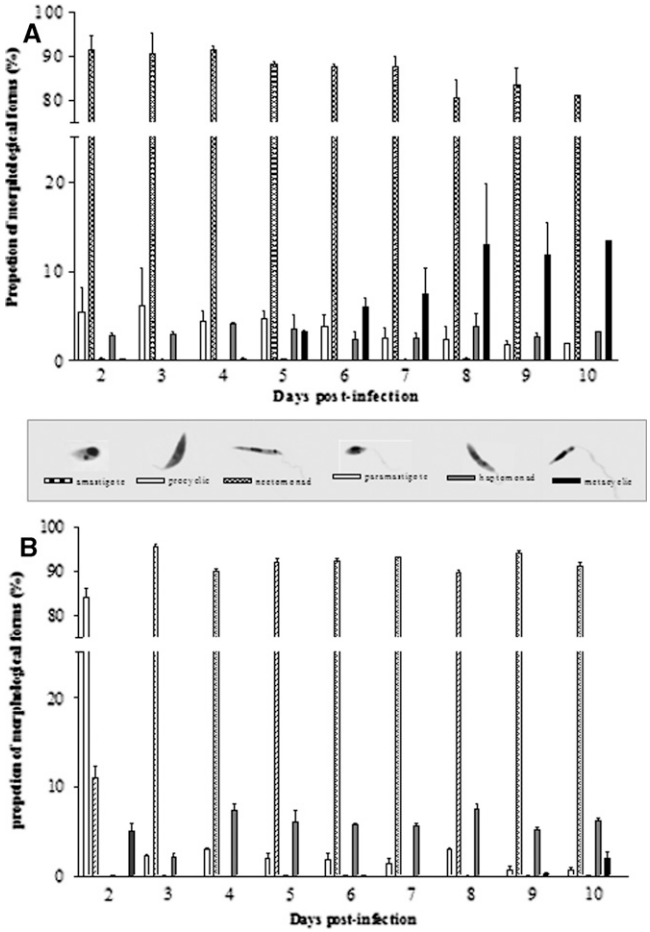

FIGURE 5. *Lutzomyia longipalpis* infected with either **A**, promastigotes or **B**, axenic amastigotes of *Leishmania infantum chagasi*. Samples of 12 sandflies were prepared for morphologic analyses and at least 300 parasites were analyzed in each dissected midgut. Data represent the geometric mean 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 ± SD.

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.[34] 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.[25] To evaluate the impact of the type of infection (amastigotes versus promastigotes) on *L. infantum chagasi* development, sandfly midguts were examined daily. Parasite 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.[7] Borovsky and Schlein[6] suggested that trypsin-like activity in the midgut of the *P. papatasi* prevented survival of *L. donovani*. Pimenta and others[7] 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*.[40–42] Also, mutants lacking lipophosphoglycan were more susceptible to digestion by enzymes.[10] Our data consistently showed high parasite mortality at the early stages of *L. infantum chagasi* infection, which is probably caused by enzymatic activity.

TABLE 1

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

	Morphologic forms (%)											
	Amastigote		Procyclic		Nectomonad		Paramastigote		Haptomonad		Metacyclic	
Days post-infection	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[43] and, for this reason, protects the parasites from them.[7] Although the PM eventually disintegrates, this process was described to occur more quickly in infected sandflies,[24,44] which suggested a contribution of insect and *Leishmania* chitinases.[45] 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.[25] This process culminates with development of thin, high-motile and infective metacyclic forms[46] 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,[7] 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[19] 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.[21] 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 stomedeal 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.[25,47] 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[25,27,48] develop low percentages of metacyclic forms, a feature that differs from the Old World species,[33] 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).[49] 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,[50] 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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REFERENCES

1. World Health Organization, 2010. *Control of the Leishmaniases. Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, March 22–26, 2010*. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf. Accessed March 16, 2011.
2. Lutz A, Neiva A, 1912. Contribuição para o conhecimento das espécies do gênero *Phlebotomus* no Brasil. *Mem Inst Oswaldo Cruz 4:* 84–95.
3. Michalsky EM, Rocha MF, Lima AC, França-Silva JC, Pires MQ, Oliveira FS, Pacheco RS, dos Santos SL, Barata RA, Romanha AJ, Fortes-Dias CL, Dias ES, 2007. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotominae sandflies. *Vet Parasitol 147:* 67–76.
4. Michalsky M, França-Silva JC, Barata RA, Lara-Silva FO, Loureiro AM, Fortes-Dias CL, Dias ES, 2009. *Phlebotominae* distribution in Janaúba, an area transmission for visceral leishmaniasis in Brazil. *Mem Inst Oswaldo Cruz 104:* 56–61.
5. Cerbino Neto J, Werneck GL, Costa CH, 2009. Factors associated to the incidence of urban visceral leishmaniasis: an ecologic study in Teresina, Brazil. *Cad Saude Publica 25:* 1543–1551.
6. Borovsky D, Schlein Y, 1987. Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. *Med Vet Entomol 1:* 235–242.
7. Pimenta PF, Modi GB, Pereira ST, Shahabuddin M, Sacks DL, 1997. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sandfly midgut. *Parasitol 115:* 359–369.
8. Pimenta PF, Turco SJ, McConville M, Lawyer PG, Perkins PV, Sacks DL, 1992. Stage-specific adhesion of *Leishmania* promastigotes to sandfly midgut. *Science 256:* 1812–1815.
9. Kamhawi S, Ramalho-Ortigão M, Pham VM, Kumar S, Lawyer PG, Turco SJ, Barrillas-Mury C, Sacks DL, Valenzuela JG, 2004. A role for insect galectins in parasite. *Cell 119:* 329–341.
10. Pimenta PF, Saraiva EM, Rowton E, Modi GG, Garraway LA, Beverley SM, Turco S, Sacks DL, 1994. Evidence that the vectorial competence of phlebotomine sandflies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. *Proc Natl Acad Sci USA 91:* 9155–9159.
11. Sacks DL, Saraiva EM, Rowton E, Turco SJ, Pimenta PF, 1994. The role of lipophosphoglycan of *Leishmania* in vector competence. *Parasitol 108:* 55–62.
12. Sacks DL, Pimenta PF, McConville MJ, Schneider P, Turco SJ, 1995. Stage-specific binding of *Leishmania donovani* to the sandfly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. *J Exp Med 181:* 685–697.
13. Sacks DL, Modi G, Rowton E, Spath G, Epstein L, Turco SJ, Berveley SM, 2000. The role of phosphoglycans in *Leishmania* sandfly interations. *Proc Natl Acad Sci USA 97:* 406–411.
14. Butcher BA, Turco SJ, Hilty BA, Pimenta PF, Panunzio M, Sacks DL, 1996. Deficiency in β1,3-galactosyltranferase of a *Leishmania major* lipophosphoglycan mutant adversely influences the *Leishmania*-sandfly interaction. *J Biol Chem 271:* 20573–20579.
15. Kamhawi S, 2000. The biological and immunomodulatory properties of sandfly saliva and its role in the establishment of *Leishmania* infections. *Microbes Infect 2:* 1765–1773.
16. Soares RP, Macedo ME, Ropert C, Gontijo NF, Almeida IC, Gazzinelli RT, Pimenta PF, Turco SJ, 2002. *Leishmania chagasi*: lipophosphoglycan characterization and binding to the midgut of the sandfly *Lutzomyia longipalpis*. *Mol Biochem Parasitol 121:* 213–224.
17. Soares RP, Margonari C, Secundino NF, Macedo ME, Costa SM, Rangel EF, Pimenta PF, Turco SJ, 2010. Differential midgut attachment of *Leishmania* (*Viannia*) *braziliensis* in the sandflies *Lutzomyia* (*Nyssomyia*) *whitmani* and *Lutzomyia* (*Nyssomyia*) *intermedia*. *J Biomed Biotechnol 2010:* 439174.
18. Coelho-Finamore JM, Freitas VC, Assis RR, Melo MN, Novozhilova N, Secundino NF, Pimenta PF, Turco SJ, Soares RP, 2011. *Leishmania infantum*: lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts. *Int J Parasitol 41:* 333–342.
19. Lawyer PG, Ngumbi PM, Anjili CO, Odongo SO, Mebrahtu YB, Githure JI, Koech DK, Roberts CR, 1990. Development of *Leishmania major* in *Phlebotomus duboscqi* and *Sergentomyia schwetzi* (Diptera: Psychodidae). *Am J Trop Med Hyg 43:* 31–43.
20. Sacks DL, 1989. Metacyclogenesis in *Leishmania* promastigotes. *Exp Parasitol 69:* 100–103.
21. Rogers ME, Chance ML, Bates PA, 2002. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. *Parasitol 124:* 498–507.
22. Sacks DL, Lawyer P, Kamhawi S, 2008. The biology of *Leishmania*-sandfly interactions. Myler P, Fasel N, eds. *Leishmania: After the Genome*. Norfolk, United Kingdom: Caister Academic Press, 205–238.
23. Lainson R, Shaw JJ, 1988. Observations on the development of *Leishmania* (*L.*) *chagasi* Cunha and Chagas in the midgut of the sandfly vector *Lutzomyia longipalpis* (Lutz and Neiva). *Ann Parasitol Hum Comp 63:* 134–145.
24. Walters LL, Modi GB, Chaplin GL, Tesh RB, 1989. Ultrastructural development of *Leishmania chagasi* in its vector *Lutzomyia longipalpis* (Diptera: Psychodidae). *Am J Trop Med Hyg 41:* 259–317.
25. Nieves E, Pimenta PF, 2000. Development of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* in the sandfly *Lutzomyia migonei* (Diptera: Psycodidae). *J Med Entomol 37:* 134–140.
26. Nieves E, Pimenta PF, 2002. Influence of vertebrate blood meals on the development of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* in the sandfly *Lutzomyia migonei* (Diptera: Psychodidae). *Am J Trop Med Hyg 67:* 640–647.
27. Miranda JC, Secundino NF, Nieves E, Souza AP, Bahia-Nascimento AC, Prates DB, Pimenta RN, Pinto LC, Barral A, Pimenta PF, 2008. Studies of the influence of the presence of domestic animals on increasing the transmission probabilities of leishmaniasis. *Ann Med Entomol 17:* 9–15.
28. Zilberstein D, Shapira M, 1994. The role of pH and temperature in the development of *Leishmania* parasites. *Annu Rev Microbiol 48:* 449–470.
29. Saar Y, Ransford A, Waldman E, Mazareb S, Amim-Spector S, Plumblee J, Turco S, Ziberstein D, 1998. Characterization of developmentally-regulated activities in amastigote of *Leishmania donovani*. *Mol Biochem Parasitol 95:* 9–20.
30. Debrabante A, Joshi MB, Pimenta PF, Dwyer DM, 2004. Generation of *Leishmania donovani* amastigotes: their growth and biological characteristics. *Int J Parasitol 34:* 205–217.
31. Dias Costa J, Soares R, Finkelstein LC, Corte-Real S, Meirelles MN, Porozzi R, 2009. Fast high yield of pure *Leishmania* (*Leishmania*) *infantum* axenic amastigotes and their infectivity to mouse macrophages. *Parasitol Res 105:* 227–236.
32. Tesh RB, Modi GB, 1984. A simple method for experimental infection of phlebotomine sandflies with *Leishmania*. *Am J Trop Med Hyg 33:* 41–46.
33. Saraiva EM, Pimenta PF, Brodin TN, Rowton E, Modi GB, Sacks DL, 1995. Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* on *Phelebotomus papatasi*. *Parasitol 111:* 275–287.
34. Charest H, Matlashewski G, 1994. Developmental gene expression in *Leishmania donovani*: differential cloning and analysis

of an amastigote-stage-specific gene. *Mol Cell Biol 14:* 2975–2984.
35. Pan AA, 1984. *Leishmania mexicana*: serial cultivation of intracellular stages in a cell-free medium. *Exp Parasitol 58:* 72–80.
36. Bates PA, Robertson CD, Tetley L, Coombs GH, 1992. Axenic cultivation and characterization of *Leishmania mexicana* amastigote-like forms. *Parasitol 105:* 193–202.
37. Bates PA, 1994. The development biology of *Leishmania* promatigotes. *Exp Parasitol 79:* 215–218.
38. Doyle PS, Engel JC, Pimenta PFP, Da Silva PP, Dweyer DM, 1991. *Leishmania donovani*: long term culture of axenic amastigotes at 37°C. *Exp Parasitol 73:* 326–334.
39. Gupta N, Goyal N, Rastogi AK, 2001. *In vitro* cultivation and characterization of axenic amastigote of *Leishmania*. *Trends Parasitol 17:* 150–153.
40. Dillon RJ, Ivens AC, Churcher C, Holroyd N, Quail MA, Rogers ME, Soares MB, Bonaldo MF, Casavant TL, Lehane MJ, Bates PA, 2006. Analysis of ESTs from *Lutzomyia longipalpis* sandflies and their contribution toward understanding the insect-parasite relationship. *Genomics 88:* 831–840.
41. Telleria EZ, Pitaluga AN, Ortigão-Farias JR, Araújo APO, Ramalho Ortigão JM, Traub-Cseko YM, 2007. Constitutive and blood meal-induced trypsin genes in *Lutzomyia longipalpis*. *Arch Insect Biochem Physiol 66:* 53–63.
42. Pitaluga AN, Beteille V, Lobo AR, Ortigão-Farias JR, Davila AMR, Souza AA, Ramalho-Ortigão JM, Traub-Cseko YN, 2009. EST sequencing of blood-fed and *Leishmania*-infected midgut of *Lutzomyia longipalpis*, the principal visceral leishmaniasis vector in the Americas. *Mol Genet Genomics 282:* 307–317.
43. Lehane MJ, 1997. Peritrophic matrix structure and function. *Annu Rev Entomol 42:* 525–550.
44. Schlein Y, Jacobson RL, Schlomai J, 1991. Chitinase secreted by *Leishmania* functions in the sandfly vector and implement parasite transmission by bite. *Proc R Soc Lond 245:* 121–126.
45. Ramalho-Ortigão JM, Kamhawi S, Joshi MB, Reynoso D, Lawyer PG, Dwyer DM, Sacks DL, Valenzuela JG, 2005. Characterization of an activated chitinolytic system in the midgut of the sandfly vectors *Lutzomyia longipalpis* and *Phlebotomus papatasi*. *Insect Mol Biol 14:* 703–712.
46. Sacks DL, Perkins PV, 1985. Development of infective stage *Leishmania* promastigotes within phebotomine sandflies. *Am J Trop Med Hyg 34:* 456–459.
47. Warburg A, Schlein Y, 1986. The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *Am J Trop Med Hyg 35:* 926–930.
48. Elnaeim DA, Ward RD, Young PE, 1994. Development of *Leishmania chagasi* (Kinetoplastida: Trypanosomatidae) in the second blood-meal of its vector *Lutzomyia longipalpis* (Diptera: Psychodidae). *Parasitol Res 80:* 414–419.
49. Gossage SM, Rogers ME, Bates PA, 2003. Two separate growth phases during the development of *Leishmania* in sandflies: implications for understanding the life cycle. *Int J Parasitol 33:* 1027–1034.
50. Bauzer LG, Souza NA, Maingon RD, Peixoto AA, 2007. *Lutzomyia longipalpis* in Brazil: a complex or a single species? A mini-review. *Mem Inst Oswaldo Cruz 102:* 1–12.