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Two separate growth phases during the development of *Leishmania* in sand flies: implications for understanding the life cycle

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Abstract

The life cycle of *Leishmania* alternates between two main morphological forms: intracellular amastigotes in the mammalian host and motile promastigotes in the sand fly vector. Several different forms of promastigote have been described in sandfly infections, the best known of these being metacyclic promastigotes, the mammal-infective stages. Here we provide evidence that for *Leishmania* (*Leishmania*) *mexicana* and *Leishmania* (*Leishmania*) *infantum* (syn. *chagasi*) there are two separate, consecutive growth cycles during development in *Lutzomyia longipalpis* sand flies involving four distinct life cycle stages. The first growth cycle is initiated by procyclic promastigotes, which divide in the bloodmeal in the abdominal midgut and subsequently give rise to non-dividing nectomonad promastigotes. Nectomonad forms are responsible for anterior migration of the infection and in turn transform into leptomonad promastigotes that initiate a second growth cycle in the anterior midgut. Subsequently, leptomonad promastigotes differentiate into non-dividing metacyclic promastigotes in preparation for transmission to a mammalian host. Differences in timing, prevalence and persistence of the four promastigote stages were observed between *L. mexicana* and *L. infantum* in vivo, which were reproduced in cultures initiated with lesion amastigotes, indicating that development is to some extent governed by a programmed series of events. A new scheme for the life cycle in the subgenus *Leishmania* (*Leishmania*) is proposed that incorporates these findings.

Keywords

Leishmania; life cycle; sand fly; promastigote; differentiation

1. Introduction

The life cycle of *Leishmania* involves alternation between a mammalian host and a phlebotomine sand fly host. In the mammalian host the developmental biology of the parasite is relatively simple and consistent between species: metacyclic promastigotes (infective forms) are introduced into the skin by the bite of the sand fly (reviewed by Killick-Kendrick, 1990). These are taken up by macrophages and transform into intracellular amastigotes (reviewed by Handman and Bullen, 2002), remaining in this form for the duration of the life cycle in the mammalian host. In contrast the developmental biology of the parasite in the sand fly host is more complex and less well understood (reviewed by Walters, 1993). A different pattern of development is observed in the two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*). Members of the subgenus *Leishmania*

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develop exclusively in the midgut and foregut of their vectors (suprapylarian development), whereas members of the subgenus *Viannia* also include a phase of development in the hindgut (peripylarian development) (Lainson and Shaw, 1987). In both subgenera, there is a convincing body of evidence for the existence of a distinct mammal-infective form of promastigote, the metacyclic promastigote, which is the end product of development in the vector (reviewed by Sacks and Kamhawi, 2001). However, the number of other developmental stages, their functions and relationships are not fully understood. For example, the term procyclic promastigotes has been introduced into the literature, and is sometimes used to refer collectively to such non-metacyclic promastigotes (Pinto-da-Silva et al., 2002; Soares et al. 2002), creating the idea that there are three *Leishmania* life cycle stages: amastigotes, procyclic promastigotes and metacyclic promastigotes. This view has been re-inforced by in vitro culture analyses, where exponentially growing or logarithmic phase promastigotes and stationary phase promastigotes are often regarded as synonymous with procyclic and metacyclic promastigotes, respectively. Unfortunately, this simple view of the life cycle does not incorporate evidence from a significant body of work examining *Leishmania* development in sand flies, and which clearly demonstrates the existence of several other life cycle stages (Walters, 1993; Sacks and Kamhawi, 2001). Such work is not widely appreciated because the relationships between the various forms, and their functional significance, have been uncertain. Recent work on *L.* (*L.*) *mexicana* has gone some way to rectify this situation, and also provided clear evidence for a new life cycle stage, the leptomonad promastigote, with a role in the production of the promastigote secretory gel plug and developmental precursor for the mammal-infective metacyclic promastigote form (Rogers et al., 2002). This study defined four major developmental forms of promastigote: procyclic promastigote, nectomonad promastigote, leptomonad promastigote and metacyclic promastigote. In addition haptomonad promastigotes that possess an expanded flagellar tip and paramastigotes were observed. In the current report we extend this analysis to *L.* (*L.*) *infantum* and describe further experiments on *L.* (*L.*) *mexicana*. We provide evidence for the sequential appearance of these four developmental stages in vivo and in vitro, we analyse their ability to divide and increase the size of the parasite population, and we demonstrate the alternation of dividing and non-dividing phases in the life cycle. Cumulatively these data call for a revision of the life cycle in the subgenus *Leishmania* (*Leishmania*).

2. Materials and Methods

2.1. Parasites

L. mexicana (MNYC/BZ/62/M379) and *L. infantum* (syn. *L. chagasi*) (MHOM/BR/76/M4192) infections were maintained in BALB/c mice. All procedures involving animals were performed in accordance with UK Government (Home Office) and EC regulations. Rump lesions of *L. mexicana* were generated by subcutaneous injection of 10^6 freshly isolated lesion amastigotes; spleen infections of *L. infantum* were generated by tail vein intravenous injection of 10^6 promastigotes enriched for metacyclic forms (Zakai et al., 1998). *L. mexicana* and *L. infantum* amastigotes were isolated by dissection and homogenisation in promastigote culture medium: Medium 199 supplemented with 10% foetal calf serum, BME vitamins (Gibco) and 25 ug per ml gentamicin sulphate (Sigma). Estimation of the yield of amastigotes was by haemocytometer counting and microscopic analysis of Giemsa-stained slides. Lesion amastigotes were immediately used to infect sand flies or initiate cultures.

2.2. Analysis of developmental forms in vivo

Lutzomyia longipalpis females were infected by membrane feeding with rabbit blood seeded with 2×10^6 *L. mexicana* or *L. infantum* amastigotes per ml as described (Rogers et al., 2002). In each experiment ten infected female flies were dissected each day for 10

consecutive days and the number of parasites per fly determined by haemocytometer counting. Amastigote forms in bloodfed midguts immediately post-feeding and on subsequent days, were enumerated by pipetting 1 μl volumes of homogenate onto microscope slides, fixing and Giemsa-staining, and systematically scanning the whole sample for amastigote forms. Giemsa-stained slides were prepared for each fly and the % of different life cycle stages determined by light microscopy as described (Rogers et al. 2002).

2.3. Analysis of developmental forms in vitro

Amastigotes prepared as above were used to seed in vitro cultures at 5×10^5 cells per ml in promastigote culture medium. Tissue culture flasks were maintained at 26°C and small aliquots removed daily for 10 days to prepare Giemsa-stained slides for developmental analysis. Parasites were classified as dividing if they possessed two nuclei and/or two kinetoplasts as revealed in Giemsa-stained slides.

3. Results

3.1. Sequential appearance of developmental forms in vivo

Previously we reported the existence of several different morphological forms of *L. mexicana* during the development of this parasite in *Lu. longipalpis* (Rogers et al. 2002). To investigate the relationships of these forms further sand fly infections were initiated with lesion amastigotes and the progress of infections monitored over a 10 day period. These experiments confirmed that four major promastigote forms of *L. mexicana* are found in vivo and occur in the sequence procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes (Fig. 1A). These forms comprise a series of precursors and products, i.e. procyclic promastigotes are the precursors of nectomonad promastigotes, and so on. *L. mexicana* can undergo development in *Lu. longipalpis* and is transmissible by the bite of this sand fly; nevertheless this is an experimental combination of parasite and vector that does not occur naturally. Therefore, to test whether these conclusions can be extended to other members of the subgenus *Leishmania*, the development of *L. infantum* (*L. chagasi*) in *Lu. longipalpis* was examined following infection with spleen amastigotes (Fig. 1B). This natural combination of parasite and vector is responsible for the transmission of visceral leishmaniasis in South America. Comparison of the results shown in Fig. 1A and Fig. 1B revealed both similarities and differences. One important point to note is that the sand fly host is the same for both parasites and therefore, any differences observed are due to the behaviour of the parasites alone. The timing and sizes of the peaks for each equivalent developmental form of *L. infantum* (Fig. 1B) were not exactly the same as in *L. mexicana* (Fig 1A). Thus the procyclic form peaked earlier in *L. infantum* (day 1 vs. day 2), but these forms persisted as a minor population for longer; nectomonad promastigotes were the major population for a longer period of time in *L. infantum* (days 2-6 vs. day 3); the peak of leptomonad forms was earlier in *L. mexicana* (day 4-5 vs.day 8); and metacyclic promastigotes were slower to appear in *L. infantum* but also reached a high prevalence by day 10. Significantly, however, the overall order in which these forms appeared was the same in both species, indicating that the precursor-product relationships of these different forms are the same in *L. infantum* as in *L. mexicana*. In addition to the forms described above haptomonad promastigotes were noted at low prevalence, less than 10% of the total, from day 4 onwards in both species. Paramastigotes were very rarely seen in either species.

3.2. Sequential appearance of developmental forms in vitro

The pattern of development in vivo described above is presumably the result of various interactions between the parasite and the environment it encounters in the sand fly host. Progression through the developmental sequence and differentiation of the various forms

could be programmed into the parasite and/or be the result of responses to external signals. To test these ideas lesion (*L. mexicana*) or spleen (*L. infantum*) amastigotes were used to initiate in vitro cultures, thus removing the influence of the sand fly host. Conditions used were suitable for transformation to promastigote forms and their subsequent morphological development was examined in this first passage in vitro. Interestingly, the same overall sequence of developmental forms previously seen in vivo was also observed in vitro, for both *L. mexicana* (Fig. 1C) and *L. infantum* (Fig. 1D). Comparison of the development of *L. mexicana* in vitro (Fig. 1C) and in vivo (Fig 1A) reveals a remarkable similarity: the occurrence of procyclic and nectomonad forms is very similar, as is the early onset of metacyclogenesis. One difference was in the extended peak of *L. mexicana* leptomonad forms observed in vitro. Similarly, the development of *L. infantum* in vitro (Fig. 1D) was also very similar to that seen in vivo (Fig 1B): the early peak of procyclic forms persisted, nectomonad promastigotes showed an extended peak, the timing of the leptomonad peak and onset of metacyclogenesis were all similar. This replication of in vivo development in a culture flask indicates that the development of the parasite in the sand fly host must be, at least to some extent, a programmed series of changes. It is important to note that these in vitro data were all obtained with freshly isolated tissue amastigotes allowed to transform and grow as promastigotes for the first time. The pattern of development described above was not reproduced when subpassaging cultured promastigotes, which appear to lose the synchronicity and timing of development after adaptation to in vitro culture (not shown).

3.3. Definition of dividing and non-dividing stages

In order to complete development in the sand fly vector the parasite must generate sufficient metacyclic promastigotes and facilitate transmission of these to a mammalian host. Amongst other things, this requires both an increase in overall numbers and differentiation into metacyclic promastigotes. To assess the potential contribution of the four major promastigote forms described above to expansion of the parasite population, their capacity to undergo cell division was examined. Any population of cells, in this case a particular developmental form, that is capable of division will at a given point in time contain a proportion of cells actually in the process of division, in the mitotic (M) phase of the cell cycle, whilst the remainder will be at various points in interphase (G1, S and G2 phases combined). For the purposes of the current investigation a cell in M phase was defined as one with two nuclei and/or kinetoplasts that had not completed cytokinesis, as revealed by examination of Giemsa-stained slides. Such data was collected for *L. mexicana* and *L. infantum* from both in vivo infections and in vitro cultures (Table 1). This analysis showed that for both species of parasite, procyclic and leptomonad forms were the main life cycle stages capable of division, both in vivo and in vitro, i.e. a significant proportion of these forms were found in the process of division. Dividing forms were found in higher proportions in *L. mexicana* than in *L. infantum*, in particular procyclic promastigotes, with approximately 50% of such forms observed in division in vivo or in vitro. This is also reflected by general experience with in vitro cultures of these parasites, *L. mexicana* typically demonstrating more vigorous growth than *L. infantum*. In contrast to procyclic and leptomonad forms, nectomonad promastigotes were only very rarely found in division, and were essentially a non-dividing stage, certainly in *L. infantum*. The only possible exception to this were a small number of *L. mexicana* nectomonad forms in vitro, although these were still far fewer in proportion than dividing cells in leptomonad or procyclic populations. No metacyclic promastigote was ever found in the process of division, which was a strictly non-dividing life cycle stage.

3.4. Temporal separation of dividing forms in vivo

The data described above indicates that there are two phases of growth in the sand fly vector, resulting from division of procyclic and leptomonad promastigotes, respectively. To

confirm this conclusion the actual numbers of procyclic, nectomonad and leptomonad promastigotes were determined for in vivo infections (Fig. 2). This analysis clearly demonstrates two separate sequential phases of growth in vivo for both species, with the occurrence of an intermediate peak of non-dividing nectomonad forms. There is an interesting difference in the numbers seen in the two species: *L. mexicana* achieved much higher densities than *L. infantum*, principally due to the expansion in leptomonad forms from days 3-6 of infection, even though the former is an experimental combination and the latter a natural combination of parasite and vector. This correlates with the higher proportion of dividing forms seen in *L. mexicana* as described above.

4. Discussion

The results of this investigation indicate that two species of parasites of the subgenus *Leishmania* (*Leishmania*), *L. mexicana* and *L. infantum*, have two independent cycles of multiplication during development in *Lutzomyia longipalpis*: the first as procyclic promastigotes (bloodmeal phase) and the second as leptomonad promastigotes (sugarmeal phase). These two cycles appear to be separated both in time and space. They are linked together by non-dividing nectomonad promastigotes, which also act to establish infection in the sand fly beyond the bloodmeal phase (Sacks and Kamhawi, 2001), and are also the form responsible for anterior migration of the infection (Rogers et al., 2002). Leptomonad promastigotes that cease division differentiate into metacyclic promastigotes, which are a strictly non-dividing stage. Based on this data a revised life cycle for these *Leishmania* (*Leishmania*) species is proposed (Fig. 3).

Several interesting points arise from the current study. The overall sequence of developmental forms was well conserved between *L. mexicana* and *L. infantum* despite important biological differences between the parasites: they cause different types of disease, cutaneous and visceral leishmaniasis, they are naturally transmitted by different vectors, *Lutzomyia olmeca olmeca* and *Lutzomyia longipalpis*, and they have different animal reservoir hosts, forest rodents and domestic dogs being the most important, respectively. This conservation of developmental pattern indicates that it is likely to apply to other species within the subgenus *Leishmania*, although this remains to be verified experimentally, particularly for species developing in *Phlebotomus* sand flies. Overlaid on this common developmental framework, various differences in the prevalence and persistence of the different stages were noted when comparing results from the two *Leishmania* species. The precise reasons for such differences are unknown at present, but presumably they reflect the adaptation of each species to maximise the probability of transmission under natural conditions. This is a complex phenomenon that is dependent on several factors including: the necessity to generate sufficient metacyclic promastigotes in the correct place and at the correct time to coincide with vector feeding behaviour; the need to generate the promastigote secretory gel plug to assist transmission (Rogers et al., 2002); the natural longevity of the sand fly vector in question; and the influence of infection on longevity (Hurd, 2003).

Common barriers to development experienced by all *Leishmania* within sand flies identified to date relate to the influence of the physiology of blood meal digestion. Principally, these are the influence of proteolytic enzymes secreted by the midgut epithelium and the loss of parasites through defecation of undigested blood meal remnants (Sacks and Kamhawi, 2001). Regarding enzymes, transforming amastigotes have been shown to be susceptible to the presence of midgut trypsin (Pimenta et al., 1997). However, there is a body of evidence that shows that *Leishmania* may be able to modulate the digestive capabilities of the sand fly host (Schlein and Romano, 1986; Borovsky and Schlein, 1987; Dillon and Lane, 1993; Schlein and Jacobson, 1998), thus, potentially promoting its survival. Moreover, survival

post-blood meal is facilitated by the attachment of nectomonad promastigotes to the midgut epithelium (Killick-Kendrick et al., 1974a; Sacks and Kamhawi, 2001). Therefore, the developmental program of *Leishmania* may to a certain extent reflect adaptation to these barriers faced within the sand fly, as evidenced by the similarity between in vivo and in vitro development shown in this study. Further, the ability of leptomonad promastigotes to recover through replication may be a necessity for the generation of transmissible infections in the vector.

Another interesting difference between *L. mexicana* and *L. infantum* was the more vigorous growth of the former in vivo. In culture this is typical and usually ascribed to variation in the suitability of in vitro conditions for growth of different species, but in *Lutzomyia longipalpis* the converse might be predicted, since this is a natural host for *L. infantum* and thus presumed to be favourable to development of this parasite. Therefore, these results indicate rather that overall growth rate (in vivo or in vitro) is an intrinsic difference between the species, and one possible explanation is that *L. infantum* is regulating its own growth to a lower level than *L. mexicana*, despite being exposed to similar conditions. If true this may explain the occurrence of mechanisms reminiscent of programmed cell death in *Leishmania* (Zangger et al., 2002). Such mechanisms may be employed by the parasites to regulate their own growth as part of their programmed adaptation to life in a particular sand fly vector. For example, it may be beneficial for the parasite to influence the burden of infection on the sand fly host, thus improving the chance of transmission (Hurd, 2003).

The development of both *L. mexicana* and *L. infantum* (*L. chagasi*) has been examined previously in sand flies and, although the various forms described in these earlier studies were not fully quantitated, these previous findings are consistent with the current study (Lawyer et al., 1987; Walters et al., 1987; 1989). The nomenclature introduced by Lawyer et al. (1990) was an important and useful advance on previous systems, which we now propose to revise based on the data presented in this study and Rogers et al. (2002). Thus, Lawyer et al. (1987) working with *L. mexicana* describe short, ovoid, slightly motile promastigotes (= procyclic forms), long slender nectomonad forms, short broad dividing promastigotes (= leptomonad forms), and metacyclic promastigotes. Similarly, Walters et al. (1987) describe nectomonad forms and short promastigotes (= leptomonad forms) of *L. mexicana*. Likewise, in a study of *L. infantum* (*chagasi*), Walters et al. (1989) describe division stage I and II promastigotes (= procyclic forms), nectomonad forms, and metacyclic promastigotes. It is important to note that there are still unresolved issues and morphological forms exist that require incorporation into the scheme shown in Figure 3. Two of these merit particular mention: haptomonad forms and paramastigotes. Haptomonad promastigotes were originally named by Killick-Kendrick et al. (1974b) to describe two populations: small, broad electron-lucid forms that could be found free in the gut lumen and others of similar appearance that were attached to cuticular surfaces of the gut via hemidesmosome-like expansions of the flagellar tip. Such forms can be found attached to the hindgut, stomodeal valve or foregut of the sand fly, depending on *Leishmania* species. These attached haptomonad forms were a significant but numerically minor population in *L. mexicana* and *L. infantum*, therefore, it has not proved possible to determine their developmental origin yet, although it is likely that they are derived from leptomonad or nectomonad forms (Rogers et al., 2002; current study). Interestingly, those haptomonads that were observed were very rarely in division, in contrast to the leptomonad population. However, it is important to note that other authors have continued to used the term haptomonad to describe small non-attached free swimming promastigotes, (Lawyer et al, 1987, 1990, Saraiva et al. 1995, Nieves and Pimenta 2000), which would be called leptomonad promastigotes according to the current study. However, we prefer and recommend the use of leptomonad promastigote for such forms for reasons of etymology (leptos = small; haptos = attached), and to distinguish these from the attached haptomonad forms that probably have different functions and whose exact origins remain to

be determined. Paramastigotes are even more enigmatic, and have been variously described as infective stages, a form derived from haptomonad promastigotes or degenerate forms. Typically they are found in the foregut in later stages of infection and can be found both free swimming and attached. As with haptomonad forms they are a relatively minor subpopulation and their role in the life cycle, if any, is uncertain.

Two non-dividing forms were identified in the current study: nectomonad promastigotes and metacyclic promastigotes. Since the different life cycle stages do not instantaneously change from one form to another it was not surprising to find a small number of dividing nectomonad forms. However, these were rare, and the general conclusion is that these are essentially a non-dividing stage. Interestingly, it has been suggested that exposure to saliva and/or depletion of hemin from the bloodmeal may trigger the differentiation of procyclic promastigotes to non-dividing nectomonads, such as may occur when these forms escape from the peritrophic matrix and begin their anterior migration (Charlab and Ribeiro 1993, Charlab et al 1995). The trigger for resumption of growth as leptomonad forms in the anterior midgut is unknown, but exposure to sugar meals is a possibility. It is not impossible that a minor population in mature infections, for example procyclic promastigotes, could give rise to metacyclic forms. However, the data presented in the current study and Rogers et al. (2002) strongly support the conclusion that leptomonad promastigotes are the precursors of metacyclic promastigotes. Metacyclic promastigotes were never found in division, in agreement with the consensus supported by many studies (Walters 1993, Sacks and Kamhawi 2001). Further, it is very likely that metacyclic promastigotes are fully committed for amastigote differentiation and cannot resume growth as promastigotes.

In conclusion, these data provide a new interpretation of the life cycle of *Leishmania* (*Leishmania*) species in their sand fly hosts. The next task is to identify molecular or biochemical markers that can help to define these stages in other species and which will give further insights into their roles in development.

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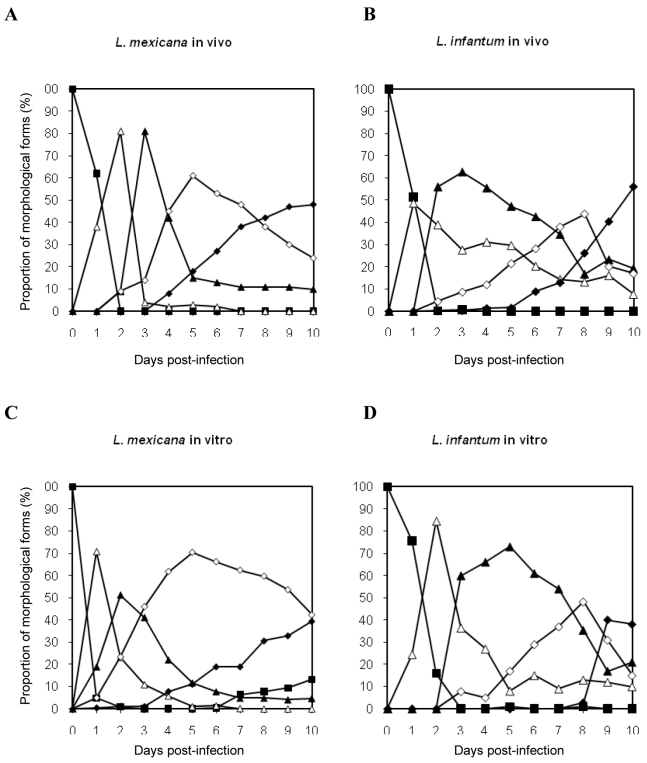

Figure 1.

Developmental profiles of *L. mexicana* and *L. infantum* in vivo and in vitro. Flies were infected with tissue amastigotes of *L. mexicana* (**A**) or *L. infantum* (**B**) and the relative proportions of amastigotes (■-■), procyclic promastigotes (Δ-Δ), nectomonad promastigotes (▲-▲), leptomonad promastigotes (◇-◇), and metacyclic promastigotes (◆-◆) determined. Similarly, in vitro cultures were initiated with tissue amastigotes of *L. mexicana* (**C**) or *L. infantum* (**D**). Each experiment was repeated and the results combined to yield the data shown.

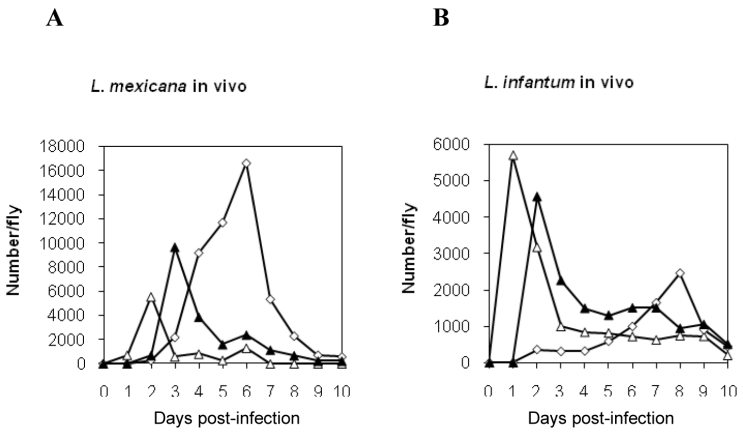

Figure 2.

Occurrence of dividing life cycle stages and nectomonad forms (non-dividing) in vivo. The number of procyclic promastigotes (Δ-Δ), nectomonad promastigotes (▲-▲) and leptomonad promastigotes (◇-◇) per fly for *L. mexicana* infections (**A**) and *L. infantum* infections (**B**) in *Lutzomyia longipalpis*. Combined data from two independent experiments with each species.

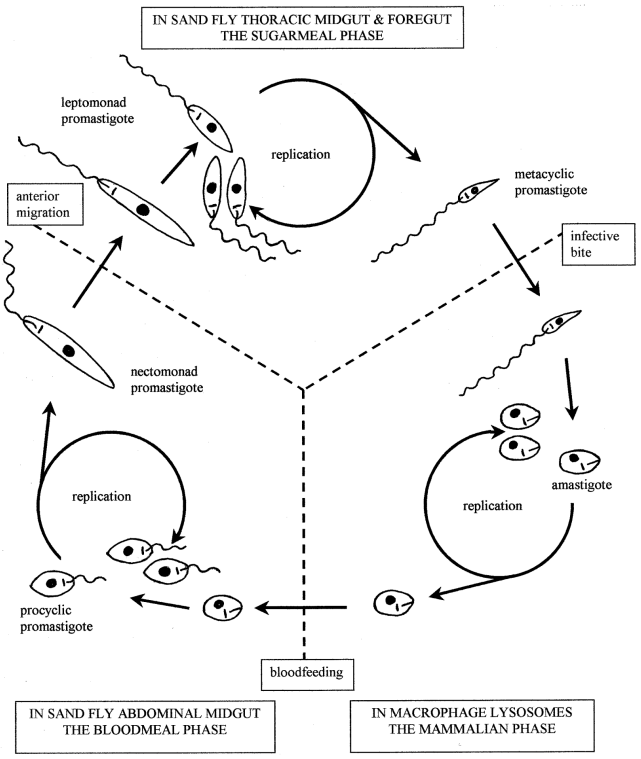

Figure 3.
Revised life cycle of *Leishmania* (*Leishmania*) species in *Lutzomyia longipalpis*. Replication of parasites occurs at three points: amastigotes in macrophage phagolysosomes; procyclic promastigotes in the abdominal midgut; and leptomonad promastigotes in the thoracic midgut. These growth cycles are linked by various non-dividing or transmission stages as shown.

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Table 1

Percentage of dividing forms in different life cycle stages of *Leishmania*. Parasites were examined by light microscopy and Giemsa staining. Dividing forms were defined as cells having two nuclei and/or kinetoplasts. n is the number of cells examined. In vivo data were obtained from infected *Lutzomyia longipalpis*.

Leishmania mexicana

Life cycle stage	In vivo	In vitro
Procyclics	42·4 (n = 118)	56·5 (n = 161)
Nectomonads	1·0 (n = 204)	4·9 (n = 264)
Leptomonads	13·5 (n = 200)	12·0 (n = 217)
Metacyclics	0·0 (n = 300)	0·0 (n = 150)

Leishmania infantum

Life cycle stage	In vivo	In vitro
Procyclics	6·0 (n = 1253)	9·9 (n = 121)
Nectomonads	0·1 (n = 1707)	0·2 (n = 408)
Leptomonads	10·0 (n = 738)	4·8 (n = 186)
Metacyclics	0·0 (n = 1133)	0·0 (n = 78)