Biology of the *Leishmania*–Sand Fly Interaction

Paulo F. P. Pimenta, Vanessa C. de Freitas, Carolina C. Monteiro, Ana Clara M. A. Pires, and Nágila Francinete C. Secundino

Leishmaniasis is a spectrum of diseases transmitted by sand-fly vector caused by a protozoa parasite from the genus *Leishmania* (Trypanosomatida: Trypanosomatidae) This vector-borne disease is transmitted to humans exclusively through sand-fly bites. The *Leishmania* genus was given to honor Sir William Boog Leishman, an assistant professor of pathology in the British Army Medical School, who discovered the parasite for the first time on a slide spleen smear in 1903.

The *Leishmania* genus is divided into three sub-genera according to parasite development in the vector (Lainson and Shaw 1979). (1) Hypopylarian: Parasites develop within the sand-fly hindgut. This group was previously classified as a subgenus of *Leishmania*, but now it is accepted as Sauroleishmania. (2) Perypylarian: These parasites can establish initial infection in the pyloric region and hindgut of the sand fly, where they are attached to the cuticle (Nieves and Pimenta 2000). This group includes the Vianna subgenus species, such as *L.* (V.) *braziliensis*, and it can be found only in the New World. (3) Suprapylarian: The development of these parasites is restricted to the sand-fly midgut. This *Leishmania* species are classified as *Leishmania* subgenus, and they are distributed in the New World and Old World. Only parasites classified with the perypylarian and suprapylarian patterns have medical importance. The majority of *Leishmania* species that affect humans are from the suprapylarian pattern, and the information presented in this chapter includes a detailed description of this group of *Leishmania* considering its life cycle within its natural vectors.

P. F. P. Pimenta (✉) · V. C. de Freitas · C. C. Monteiro · A. C. M. A. Pires
N. F. C. Secundino
Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz,
Belo Horizonte, Minas Gerais, Brasil

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Infective Blood Meal and Promastigote Forms Inside the Sand Fly

The interaction between the parasite and the vector starts at the time when the female sand fly can feed on infective blood to develop its eggs. After choosing an appropriate vertebrate host, the sand-fly mouthparts penetrate the skin, lacerating the epidermis in different spots, forming a blood pool that will be sucked by the insect. During this blood meal, the female occasionally ingests the *Leishmania* parasite present in the infected vertebrate host in a form called "amastigote," an intracellular tissue form found mainly inside macrophages. After the meal is consumed by the sand fly, the recently ingested *Leishmania* amastigotes are stored with the blood in the midgut for digestion. Immediately a differentiation process transforms the amastigote into a flagellated parasite form called "promastigote," the extracellular form that colonizes the vector during its lifetime. Distinct from that of the vertebrate, the life cycle of *Leishmania* in the sand-fly vector is extracellular and occurs inside the digestive tract.

The first promastigotes derived from the differentiation of ingested amastigotes inside the blood meal have high multiplicative capacity, and this form is called "procyclic." The procyclics multiply by binary fission, and subsequently, during the parasite life cycle, they differentiate into morphologically distinct flagellate forms. Different authors have given promastigotes forms distinct designations; however, in this chapter we use the simple nomenclature proposed by Lawyer and colleagues 1990, which has been used in several studies of *Leishmania*–sand fly interaction. Between the second and fifth days after a blood meal, a new promastigote population progressively emerges, the form of which is called "nectomonad." After blood-meal digestion, around the third and fourth days after infection, the nectomonads colonize different parts of the digestive tract according to the sub-genera (as described previously). The nectomonad population decreases with colonization along the midgut, and new promastigote forms emerge, called "haptomonads" and "paramastigotes." Gradually, after total excretion of the blood meal, the "metacyclic," the infective form of vertebrate, appears. The metacyclics migrate to the foregut region of the vector, where they will be ready to be transmitted by the sand-fly bite to a new vertebrate host during the next blood meal.

Natural Barriers Encountered by *Leishmania* Within the Sand-Fly Vector

Development of *Leishmania* within the sand-fly vector is a complex process. Several main events occur during the *Leishmania*–sand fly interaction that constitute barriers to parasite development: (a) *Leishmania* species must be susceptible to the vector when it is present in the vertebrate blood; (b) the action of digestive enzymes on the ingested blood meal; (c) the perithrophic matrix formation, which protects

parasites from digestive-enzyme action; (d) the transformation of amastigotes (vertebrate multiplicative form) into promastigotes (sand-fly multiplicative form); (e) escape of the promastigotes from the perithrophic matrix to the foregut region due to parasite production of chitinase; (f) promastigote differentiation and subsequent adhesion to the midgut epithelium, which is dependent on the expression of species-specific molecules (lipophosphoglycan [LPG]) on the parasite surface; (g) parasite migration to the cardia region around the stomodeal valve after complete blood digestion; (h) promastigote differentiation into metacyclic, the infective form able to be injected and infect the vertebrate host at the time of the bite; (i) the successful feeding of an infected sand fly, which inoculates metacyclics into a new vertebrate host during the bite; (j) actions of the saliva produced by the sand-fly vector and the proteophosphoglycan (PPG) produced by promastigotes in the skin-bite site on the vertebrate host, which are able to intensify and modulate the infection; and, finally, (i) recent data suggesting that native microbiota play a role in vector interaction.

In conclusion, due to all the possible barriers to the development of *Leishmania* inside the sand fly, which determine vectorial competence, this phenomenon should be considered a complex dynamic process that is still open to analysis. Further we will discuss the main natural barriers that exist during *Leishmania*–sand fly interaction.

Amastigote Transformation into Promastigote Inside Sand-Fly Midgut

Sand flies, like others blood-sucking insects, digest the blood meal producing amylases, glycosidases, lipases and proteases (mainly trypsin and chymotrypsin), which are secreted by intestinal epithelium cells. Digestive enzymes can be harmful to parasites. The survival and infectivity ability of all microorganisms transmitted by haematophagous insects, including *Leishmania*, is directly associated with resistance to digestive-enzyme action produced by vectors.

The first barrier to *Leishmania* development inside the vector is the transformation of amastigote into promastigote. Studies have indicated that *Phlebotomus papatasi* infected with *Leishmania major* shows about a 50% reduction in parasite numbers during this transformation (Pimenta et al. 1997). Although amastigote differentiation into promastigote is an important step due to their cellular coating, which confers protection against the sand flies' intestinal protease action, many parasites do not complete this process. Thus, the number of parasites can dramatically decrease or even disappear within the vector during blood-meal digestion. Therefore, the sand-fly digestive process causes a drastic decrease in the vector-infection rate in a population (the percentage of infected sand flies) because many insects heal the infection.

After a blood meal and during the digestive process in sand flies, the concentration of digestive enzymes can increase up to 20 times. Some experimental studies have shown that parasite death can be avoided by adding specific proteolytic inhibi-

tors to the infected blood meal (Borosvsky and Schlein 1987; Pimenta et al. 1997). Interestingly it has also been demonstrated that parasite resistance to digestive-enzyme action is stage-specific: Promastigotes are more resistant than amastigotes (Pimenta et al. 1997). This question was explored by comparing amastigotes until they were completely transformed into promastigotes. The transitional form (amastigotes differentiating to promastigotes) is susceptible to death induced by digestive-enzyme action. It was observed that transitional parasite forms are the most susceptible to digestive-enzyme action because this is a critical stage for parasite survival inside the sand fly (Pimenta et al. 1997). After complete transformation, promastigotes acquire greater resistance to digestive-enzyme action in the gut. This resistance is related to surface expression in the promastigote of a molecule not present in amastigotes, a surface lipophosphoglycan (LPG). LPG forms an organized surface coat that covers and protects the promastigote from proteases lysis (Schlein et al. 1990; Pimenta et al. 1991). This observation is consistent with the fact that *Leishmania* promastigote mutants not expressing LPG on their surfaces are killed during their early hours inside the sand-fly midgut. However, when LPG expression is restored, parasites regain the ability to survive in the vector midgut (Pimenta et al. 1994; Sacks et al. 2000; Myskova et al. 2007; Secundino et al. 2010).

Sand-Fly Competence for *Leishmania* Related to Digestive-Enzyme Action

Susceptibility to digestive-enzyme action is species-specific; therefore, different species of *Leishmania* can modulate proteases action differently within their appropriate sand-fly vector. The ability to resist or modulate these enzyme actions is a determining factor for parasite survival; consequently it is related to vector competence. Telleria and colleagues (2010) showed that trypsin expression decreases by about 20% in *Lutzomyia longipalpis* infected with *L. (L.) infantum chagasi*. The enzymatic activity modulation within the vector has been also studied in detail in the *Phlebotomus* genus. Adler and colleagues (1938) noted that *Phlebotomus papatasi* infected by several unsusceptible *Leishmania* species are killed during blood-meal digestion. This matter was taken up in several studies of Schlein and collaborators in 1986, 1987, and 1990, which demonstrated the ability of compatible *L. major* to partially block digestive-enzyme synthesis in its natural vector, *P. papatasi*. This result was not observed with *L. donovani* parasites, which induced a higher enzyme-production level, causing parasite destruction in this unsusceptible vector. Similar studies developed by Pimenta and colleagues (1997) showed that *P. papatasi/L. major* survival is associated with trypsin-enzyme production. Thus, levels of *Leishmania* species and the enzymes secreted by epithelial cells by a specific sand-fly vector in response to a blood meal are determining factors for infection development.

It is interesting to note that *L. major* and *L. donovani* LPG-deficient mutants surviving in their natural vectors while the blood meal is still present (Sacks et al. 2000;

Boulanger et al. 2004; Myskova et al. 2007; Svárovská et al. 2010; Secundino et al. 2010) does not necessarily decrease LPG's contribution to the resistance to initial parasite death in the midgut because in some cases a modest reduction in LPG-mutant survival during initial infection has been noted (Sacks et al. 2000; Myskova et al. 2007; Secundino et al. 2010). Secundino and colleagues (2010) have observed that the addition of PGs derived from LPGs provided to *L. major* LPG-mutants induce resistance in sand fly *P. duboscqi* against lysis due to midgut proteases.

In conclusion, many *Leishmania* ingested along with a blood meal are killed during digestion in sand-fly midgut because of adverse natural conditions. However, a sufficient number of individuals resistant to enzymatic action survive and multiply inside the digestive tract in order to beat the other barriers and maintain infection in the vector.

The Second Blood Meal and Action of Digestive Enzymes in the *Leishmania*–Sand Fly Interaction

Sand flies in nature ingest successive blood meals; however, the interaction studies cited previously were conducted using only one blood meal. Short (1928) demonstrated that a second blood meal shows no deleterious effects to *Leishmania*, and Adler (1964) confirmed that re-feeding sand fly with a normal blood meal does not decrease the *Leishmania* infection rate. However, Elnaiem et al. (1997) showed an increase in the proportion of *L. infantum chagasi* metacyclic in infected *L. longipalpis* after a second normal blood feeding, suggesting that the deleterious effect from the digestive enzymes was not observed in parasites of the established infection. Vivenes and colleagues (2001) also studied the effects of a second normal blood meal in Lu. *evansi* infected with *L. mexicana*, and they concluded that a second meal favors midgut colonization by promastigotes. Furthermore, Nieves and Pimenta (2002), studying *Lu. migonei* (*Migonemia migonei*) infected with *L. braziliensis*, confirmed that re-feeding increases the number of promastigotes in the gut and therefore plays an important role in transmission because a proliferation of the metacyclic population occurs.

Our group has advanced the studies related to second meals in sand-fly vectors. We used the sand-fly model, *L. intermedia* (*Nissomyia intermedia*), infected with *L. braziliensis*, and re-fed the infected sand flies with blood from several animals (Miranda et al. 2008). The sand flies appeared to ingest the same blood amount independent of the animal source, but infection patterns in the vectors changed according to the blood source. There were increased parasite numbers in sand flies that re-fed on chicken and donkey blood but a moderate increase in vectors that re-fed on human, horse, dog, and cattle blood. In contrast, a decreased parasite number was observed in infected vectors that re-fed on pork and lamb blood. As we know, proteolytic-enzyme action is a crucial factor for infection development; it is likely that the second blood meal serves as a new nutrient source; and blood types stimu-

late *Leishmania* total population growth, thus increasing the number of promastigotes that can modulate enzyme activity. Curiously, the metacyclic proportion did not significantly change in any experimental group of sand flies. The blood-meal influence on *Leishmania* infection is probably related to ingested blood components and hence to the vector digestive process. It is possible that, depending on the specific pair, the re-feeding effect varies depending on each natural pair. Sand flies have a marked ability to opportunistically feed on different vertebrates. It appears that the second blood meal of infected sand-fly vectors from certain domestic animals can increase their ability to transmit *Leishmania* in endemic areas where those animals are found. This may provide a selective advantage to vector competence for some sand-fly species in transmitting the *Leishmania* parasite to vertebrates.

Leishmania Escape from Sand-Fly Perithrophic Matrix

A crucial barrier to *Leishmania* development within sand-fly midgut is the structure called the "perithrophic matrix" (PM). Immediately after blood-meal ingestion, midgut epithelial cells start to synthetize the PM components that will be completely structured in 24 h, and this will persist until the end of the digestive process when the rest of the blood meal is secreted. Similar to other Diptera, sand-fly PM consists mainly of chitin and associated proteins and proteoglycans (Gemetchu 1974; Secundino et al. 2005).

Some classical functions of PM inside insect midguts—such as (a) epithelium protection against damage from food elements (Richards and Richards 1977; Berner et al. 1983); (b) food compartmentalization and digestive-enzyme permeabilization (Terra 1990; Terra and Ferreira 1994); (c) protection of midgut epithelium against ingested pathogens (Peters 1992; Miller and Lehane 1993); (d) flow control of small molecules, such as digestive enzymes and digestion products, toward midgut cells due to PM porosity (Tellam 1996); (e) detoxification by heme sequestering produced by hemoglobin digestion (Pascoa et al. 2002); and, finally, (f) provision of an important physical barrier against pathogen development (Pimenta et al. 1997)—have been considered in the literature.

PM involvement in parasite−vector interaction has been adequately demonstrated in several models (Feng 1951; Billingsley and Rudin 1992; Walters et al. 1992; Shahabuddin et al. 1993; Pimenta et al. 1997). A classic example was developed by Shahabuddin and colleagues (1993) demonstrating that malaria ookinetes secrete a chitinase and partially degrade PM in infected mosquitoes, which allows *Plasmodium* to invade the midgut epithelium to complete its life cycle. Similarly, *Leishmania* species also escape from PM after becoming promastigote, the parasite form that is able to produce chitinase (Schlein et al. 1991; Shakarian and Dwyer 1998; Malta et al. 2016). Schlein and colleagues (1991) found that chitinase and N-acetylglucosaminidase are secreted by cultured *L. major* promastigotes and suggested that these enzymes can catalyze PM degradation, thus allowing parasite escape. Shakarian and Dwyer (2000) identified a chitinase gene in *L. donovani* and

demonstrated that the gene locus and the enzymatic activities are conserved in all different species strains.

PM properties—such as thickness, biochemical composition, synthesis and degradation—are related to parasites' ability to survive in sand-fly vectors. Previously we discussed the role of digestive-enzyme action in *Leishmania* drastically decreasing parasite numbers during blood digestion. However, despite the protection conferred by PM to the insect, the parasite uses the PM to protect itself from these digestive enzymes. This fact was well shown in sand flies infected with *L. major* (Pimenta et al. 1997; Pruzinova et al. 2015). The absence of PM possibly arises from feeding sand flies with blood meal containing exogenous chitinase. This lack of PM formation in sand-fly midgut has a deleterious effect on *Leishmania* infection because almost all parasites are killed within the vector. If PM is not synthesized, *Leishmania* is quickly destroyed by digestive-enzyme action. The few parasite survivors are excreted with final digestion products because they did not complete their differentiation into promastigote; therefore, they cannot adhere to the midgut epithelium because such adhesion depends on LPG. This is discussed later in the text.

During the development of infection, a great many free promastigotes remain in the sand-fly midgut lumen after excretion. To explain this fact, Vaidyanathan (2004, 2005) proposed an alternative mechanism to parasite adhesion to the midgut epithelium. A peptide secreted by *L. major* promastigote cells, when added to phlebotomine meal, was demonstrated to be capable of inhibiting *P. papatasi* midgut contraction. Thus, the parasites would not be excreted. This may also be related to PM degradation. In infected sand fly, degradation occurs in the up-front of the PM portion by promastigote chitinase action in contrast to healthy insects, where degradation occurs in the final portion of the abdomen.

In conclusion, the delicate balance between sand-fly vector and *Leishmania* protozoa is PM dependent. Initially the PM protects parasites from the direct action of digestive enzymes at the time of their differentiation into promastigotes. However, the promastigotes must produce a chitinase at the right time to escape from the PM and then to adhere to the midgut epithelium (Pimenta et al. 1992) in order to not be excreted in final step of the digestive process.

Leishmania Specific Adhesion to Sand-Fly Midgut Epithelium

Another important barrier to *Leishmania* within the sand-fly vector is its ability or not to adhere to midgut epithelial cells. Midgut epithelium adhesion is a vital phenomenon for *Leishmania* infection maintenance (Fig. 1). After escaping the PM, *Leishmania* promastigotes must adhere to the midgut epithelium. This adhesion prevents parasite excretion with the rest of the undigested blood and thus allows their proliferation and subsequent differentiation.

The molecule that enables and controls the adhesion of promastigotes is LPG. Importantly, *Leishmania* LPG surface-molecule expression is promastigote specific and is not present on the amastigotes' surface. This observation was impor-

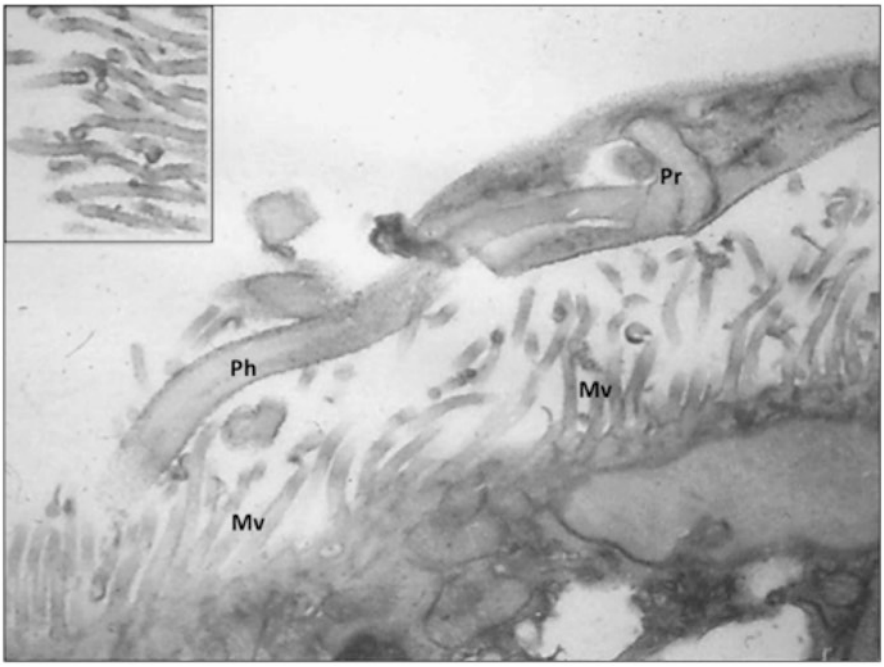

Fig. 1 *L. major* attached to the midgut by the flagellum. The *L. major* promastigote (Pr) is seen attached by the flagellum (Fl) on the microvilli (Mv) of the midgut epithelium of the sand fly *P. papatasi.* Left inset. LPG binding sites over the epithelium microvilli labeled with anti-LPG/gold colloidal particles. Electron micrograph from Paulo Pimenta. (Originally published in Saraiva et al. 1995)

tant and led the first study in 1992 by Pimenta and colleagues about LPG's specific functions in the interaction process with insect vectors. This was the first parasite molecule to be recognized as being active in the interaction process in any pathogen−vector studies. LPG function was first studied in the natural pair *P. papatasi* and *L. major* (Pimenta et al. 1992). LPG was found to expressed specifically in *Leishmania* promastigote, and it covers its entire surface, including the flagellum, being organized as a dense filamentous cellular coat (Pimenta et al. 1989, 1991); since then, it has been found in all *Leishmania* species studied to date (Sacks et al. 2000; Sacks 2001). The characterization of LPG from *L. infantum chagasi* and *L. braziliensis*, two important species circulating in Brazil (Soares et al. 2002, 2005), has also been elucidated.

The LPG molecule consists of a glycan core with a highly conserved lipid anchor and variable oligosaccharide units. LPG's performance in promastigote binding is performed through its saccharide units, which bind specifically to the microvilli of the sand-fly midgut epithelial cells (Pimenta et al. 1992; review Sacks et al. 1995). LPG's role in mediating adhesion was confirmed by studies using promastigote mutants, which are deficient in specific saccharide-unit expression (Butcher et al. 1996)

or deficient in total molecule expression (Pimenta et al. 1995; Svárovská et al. 2010; Secundino et al. 2010). In the experiments with LPG-deficient mutants, it was impossible to retain the sand-fly infection after blood-meal excretion because there was no promastigote adhesion to the midgut epithelium. Other studies showed that the vector competence of the sand flies to become infected by certain *Leishmania* species is controlled by an LPG polymorphism on the promastigote surface (Pimenta et al. 1995). The LPG molecule has a large polymorphism on the parasite surface varying its saccharide units in accordance with the *Leishmania* species observed (McConville and Blackwell 1991). Comparative studies conducted with *L. amozonensis*, *L. donovani*, *L. infantum* and *L. major* revealed that in all these species, the ability of promastigotes to adhere to the gut is directly controlled by the LPG structural polymorphism, which is associated with specific receptors present in the microvilli of specific sand-fly species (Pimenta et al. 1995; Kamhawi et al. 2004). In 1994, it was suggested that *Leishmania* LPG should have specific cell receptors (lectin types) in the midgut and that this receptor would be responsible for promastigote adhesion on the epithelium (Pimenta et al. 1994). Only 10 years later, Kamhawi and colleagues (2004) demonstrated for *P. papatasi* and *L. major* the existence of such specific receptors (*P. papatasi* galectin) connecting LPG with sand-fly midgut epithelial microvilli. The results of this study demonstrated that the adhesion mechanism via LPG, which was performed using the natural vector pair *P. papatasi* and *L. major*, plays an important role in the transmission of cutaneous leishmaniasis (Fig. 2). However, it is worth noting thus far that the literature does not clearly demonstrate adherence to the epithelium in other sand-fly species and the possible role of other molecules in this interaction.

Leishmania Metacyclogenesis Inside Sand-Fly Midgut: Promastigote Differentiation into Vertebrate Infective Forms

The parasites' differentiation into metacyclic and subsequent transmission to vertebrate hosts can be considered one of the latest barriers to the process of *Leishmania*–sand fly interaction. After promastigote adhesion to midgut epithelium, which ensures life-cycle continuity within the sand fly, a series of multiplication, changes and differentiation in parasite forms, called "metacyclogenesis," occurs, and this imperative phenomenon warranties competence. Metacyclogenesis is a differentiation process that produces infective metacyclic promastigotes that are able to be transmitted by sand-fly bites and initiate infection in a vertebrate. Metacyclic is considered the only *Leishmania* promastigote form able to initiate the parasite life cycle in a vertebrate. Since the initial studies demonstrating that leishmaniasis are transmitted by sand-fly vectors, there has been suspicion of a specific promastigote form within the vector that adapted to live in vertebrates. Studies by Sacks and colleagues (1984, 1987), comparing promastigotes obtained at subsequent days after sand-fly infective blood feeding, demonstrated conclusively that *Leishmania* parasites are not uniform in transmitting

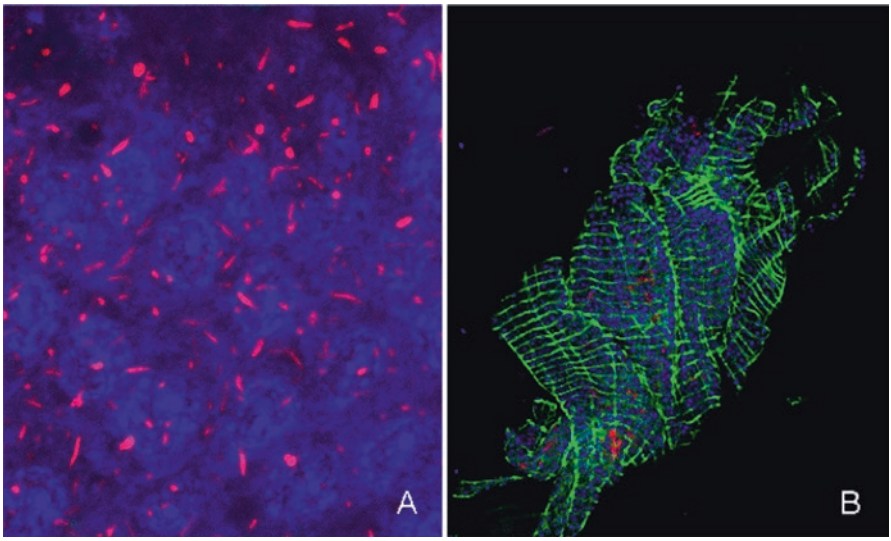

Fig. 2 Scanning confocal microscopy of *P. duboscqi* midguts infected with *L. major*. (**a**) Midgut dissected 9 days after infection. Note in blue (DAPI) the epithelial cells and in red the *Leishmania* promastigote adhered to the midgut epithelium. (**b**) *P. duboscqi* infected midgut dissected 14 days after infection. Note muscle fibers in green (FTC-phalloidin), midgut epithelial cells in blue (DAPI); and red parasites (RFP-expressing strain of *L. major*). (Unpublished photos from Nágila Secundino)

infection to vertebrates. These investigators noted that promastigote populations with the ability to infect vertebrates were gradually obtained only after the fourth day of vector infection. Thus, this experiment demonstrated the existence of a process within the sand flies, i.e., metacyclogenesis, that allowed the development of infective promastigotes being able to initiate infection in a vertebrate host.

After this initial study, many other studies have contributed to our improved knowledge of the metacyclogenesis process because it was possible to obtain metacyclic promastigotes in old axenic cultures and to purify them by agglutination methods with lectins (Sacks et al. 1985). These studies demonstrated that metacyclics are highly differentiated with distinct morphological characteristics, particularly the presence of a very dense cellular coat constituted essentially by LPG (Pimenta et al. 1989, 1991). Subsequently, it was demonstrated that during metacyclogenesis, considerable structural changes occur in the promastigote surface with LPG changing the types and number of saccharide units. The metacyclic LPG molecule becomes about three to five times longer than that in other promastigotes, thus increasing the thickness of the *Leishmania* cell coat (Pimenta et al. 1989). The use of different techniques associated with electron microscopy, immune-staining and cryofracture by Pimenta and collaborators (1989) showed that LPG was present on the *Leishmania* surface as densely filamentous structures distributed exclusively on the metacyclic surface. Other additional studies with different LPG fractions demonstrated that these modifications are crucial for procyclic adhesion and metacyclic

subsequent release from midgut epithelium microvilli of sand-fly vectors (Pimenta et al. 1992). The occurrence of these associated phenomena allows *Leishmania* movement to the anterior portion of the sand-fly digestive tract so they can be inoculated into a new vertebrate host. In addition, studies from the same research group with infected sand fly, using a technique that forced infected sand-fly feeding through microcapillaries, demonstrated that only metacyclic inoculated by sand flies and these promastigotes are identical, including their LPG expression, to those obtained *in vitro* (Saraiva et al. 1995).

So, after metacyclogenesis, the metacyclic migrate to the foregut region and can be accumulated in the cardia (a ring coated with cylindrical epithelial cells whose function is control and direct the blood flow by preventing the blood to be regurgitated). Promastigote adhered to this organ can make cuticle damages causing a dysfunction on the blood flow control (Schelein et al. 1992; Volf et al. 2004), than the infected vector regurgitate the metacyclics on the vertebrate skin in the moment of the bite. Moreover, because the insect is not able to ingest enough blood, it repeatedly stings the same or even other vertebrates providing a more efficient parasite transmission.

Finally, studies of metacyclic interaction with mammals (da Silva et al. 1989; Puentes et al. 1990) showed that the metacyclic cell coverage is what permits *Leishmania* survival within the vertebrate. Metacyclics have resistance to complement mediated lysis, which probably promotes their adherence and ingestion by macrophages by way of appropriate receptors. However, it is interesting to note that, after several attempts, the experimental *Leishmania* transmission to vertebrates only occurred in the year 2003 (Kamhawi et al. 2003) with *P. papatasi* infecting mice with *L. major*. This was an important study model to study the roles of sand-fly bite site and saliva in vertebrate *Leishmania* transmission.

In conclusion, metacyclogenesis is a phenomenon occurring inside the sand-fly midgut that makes the parasite pre-adapted to survive within the vertebrate, thus ensuring the continuity of their life cycle.

The Role of the Sand Fly Saliva in *Leishmania* Transmission to the Vertebrate Host

Studies of the role of the vector's saliva *Leishmania* transmission to the vertebrate host were important and affirmed that the insect is not only leading parasites to their target. The initial study in this field was performed by Titus and Ribeiro (1988) and opened up a whole new study field that came to the top with the production of DNA vaccines based on saliva components to combat leishmaniasis. This will presented and discussed next.

The chemical components present in saliva not only modulate the vertebrate's host response, they also assist in locating the blood vessel and determining the parasite's transformation. During the sand-fly bite to obtain the blood meal, the sand fly introduces the mouthparts into the host skin along with its saliva, which assists in the rapid localization of blood vessels. After these vessels are lacerated, the saliva is

responsible for blood-flow maintenance, thus promoting rapid blood feeding by the sand fly. Next follows a series of events, i.e. adaptive strategies for the more effective acquisition of blood. The main saliva functions include platelet-aggregation inhibition, anticoagulation, vasodilation and anesthetic function (Kamhawi review in 2000). After blood-vessel laceration, vertebrate host response occurs through hemostasis (platelet activation and coagulation factors, fibrinogen conversion to fibrin) and inflammatory reactions such as erythema, swelling and pain (Ribeiro et al. 1995; Kamhawi 2000).

More than a decade ago, Ribeiro and Titus reported that a small amount of sand-fly saliva could increase infection when inoculated with *Leishmania* promastigotes, thus demonstrating for the first time the role of sand-fly saliva role in disease development (1988). The lesion size and the parasite numbers were increased dramatically when saliva was added to the inoculum during experimental laboratory animal infection. Furthermore, the capacity of the vector's saliva to increase the infectivity of *Leishmania* is restricted to sand-fly saliva because saliva of other arthropods did not produce the same effect (Howard 1986; Ribeiro and Titus 1988). In addition, the increased infectivity due to the saliva was demonstrated with susceptible and resistant mice co-inoculated with *L. major* along with the saliva of *P. papatasi*, both vectors and parasites existing in the Old World (Mbow et al. 1988; Belkaid et al.1998). Until recently, the *L.* salivas of *L. longipalpis* and *P. papatasi* are the most studied species because they are more easily colonized and are recognized as having immunomodulatory activity (see review in Kamawahi 2000).

A pathogenic effect of saliva components was suggested by Warburg et al. (1994). They proposed a possible role in the clinical manifestation related to the variability of *Leishmania* infection inducing or not the *Leishmania* visceralization. They started from the initial observation that *L. infantum chagasi* transmitted by *L. longipalpis* normally causes visceral leishmaniasis in Brazil and Colombia but not in Central America where the infections usually result in skin lesions. The authors found that the molecule maxidilan, a vessel dilator and erythema inducer, found exclusively in sand-fly saliva, has more potent activity in sand fly *L. longipalpis* found in Brazil and Colombia than in Costa Rica. The authors concluded that the maxidilan amount inoculated by the *L. longipalpis* infected *L. infantum chagasi* determines whether the parasites migrate or not to the vertebrate viscera.

In 1998, Belkaid and colleagues developed a natural cutaneous leishmaniasis model in mice to study the effect of saliva in detail. The authors infected mice ears and observed the subsequent lesion development and the acute and chronic inflammation. The authors confirmed that *P. papatasi* saliva enhances infection by *L. major*. The substances present in the saliva causing the increased skin lesion probably are similar to the components found in the saliva of *L. whitmani*, a New World vector species, because the same phenomenon was also shown in mice infected with *L. braziliensis* by this vector (Bezerra and Teixeira 2001). It was also shown that sand-fly saliva antigenic substances are capable of inducing delay-type hypersensibility (DTH) as observed in experimental and human hosts (Belkaid et al. 2000). In Brazil, a significant correlation between the anti-gland immunoglobulin G levels and DTH response in children was found in an endemic area of visceral leishmaniasis (Barral et al. 2000).

Furthermore, protection against *L. major* infection has been shown in mice models when they are pre-exposed or pre-immunized with the saliva of uninfected vectors saliva (Volf et al. 2000; Belkaid et al. 1998). Further studies have shown that saliva components may be candidates for a leishmaniasis vaccine because mice pre-exposed to saliva and inoculated with parasites were protected and associated with a strong DTH reaction (Valenzuela et al. 2002; Kamhawi et al. 2000a, Elnaiem et al. 2005). Exacerbation of the infection was abolished in pre-exposed mice to *P. papatasi* saliva and challenged with *L. major* (Belkaid et al. 1998). Recent data suggest that the response to vector saliva can also be used to monitor human exposure and that of other vertebrate hosts bitten by the sand flies; it can be used as a risk marker for *Leishmania* transmission in endemic areas. In addition to findings from relevant saliva studies, the 15-kDa *P. papatasi* saliva protein was able to protect mice against infection with *L. major*. A vaccine containing the cDNA for this 15-kDa protein led to the same protection in B-cell knockout mice. These results indicated that the DTH response against saliva confers the vaccine protective effect, and salivary components or their cDNAs can be used in vaccine production against leishmaniasis (Valenzuela et al. 2001). Updated literature on this subject can be found in the papers from Valenzuela's group (Anderson et al. 2006; Oliveira et al. 2008; Teixeira et al. 2010; Hosseini-Vasoukolaei et al. 2016; Ferreira et al. 2016).

All studies on the effect of vector saliva related with exacerbation or protection against *Leishmania* infection were performed using laboratory-colonized vectors. However, using the murine model, recent studies have shown significant differences in the exacerbation effect caused by colonized, or wild, *L. longipalpis* salivary gland lysates (Laurenti et al. 2009a, b). The saliva of colonized sand flies co-inoculated with *Leishmania* parasites showed a stronger exacerbation effect, causing injuries twice larger than those caused by the saliva of wild sand-flies inoculated with parasites. Furthermore, it was suggested that the wild-insect saliva does not have the same chemotactic factors encountered in the saliva of colonized sand flies. The protein amount and composition found in the saliva of both groups were also significantly different (Laurenti et al. 2009a). In another study, the same authors suggested that this difference could explain the lesser modulation effect on the *Leishmania* infection observed in mice co-inoculated with parasites and saliva from wild sand fly compared with those co-inoculated with parasites and saliva from colonized sand fly (Laurenti et al. 2009b). Similar studies using the natural pair *P. papatasi* and *L. major* have shown similar results (Ahmed et al. 2010).

Leishmania–Transmission Mechanisms by Infected Sand-Fly Bite

After metacyclogenesis, it is possible to detect metacyclic forms in the foregut region, and the insect is able to transmit *Leishmania*. However, it is believed that simply being infected may influence the vector behavior as we will discuss below.

Different researchers believe in a physical or biological blockage of the *Leishmania*, which is predominant for transmission efficient because fast blood

intake is lacking, meaning that the sand fly must bite the host's skin multiple times. Killick-Kendrick and Molyneaux (1981) suggested a transmission mechanism in which metacyclic interferes directly in the mouthpart sensilla, which controls probing and feeding, thus influencing the blood rate and direction in the alimentary canal and promoting the parasite's release on the skin. The stomodeal-valve physical damage was proposed attributed to the *Leishmania* chitinase action (Schlein et al. 1992; Volf 2004). The authors suggested that valve physical damage promotes or facilitates infective metacylic regurgitation into the vertebrate's skin.

Other investigators, however, have described the transmission block to biological parasite masses being embedded in a gel-like matrix in the stomodeal valve (Warburg et al. 1986; Lawyer et al. 1987, 1990; Walters et al. 1987, 1989 Stierhof et al. 1999; Rogers et al. 2002). This gel-like substance secreted by the parasite in the vector midgut is called promastigote secretory gel (PMG), and it is also responsible for mechanical dysfunction on the stomodeal valve formed by plug pressure. This gel was found in *L. longipalpis* infected with *L. mexicana* and *P. papatasi* infected with *L. major*. This obstruction is common in all *Leishmania*−vector combinations studied so far (Stierhof et al. 1999; Rogers et al. 2002, 2004), and it is caused by PPG, a mucin-like substance secreted by parasites inside the midgut. High concentration and limited phlebotomine gut space induce PPGs in the form of PSG (Stierhof et al. 1999). Thereby, it is believed that PSG blocking alters sand fly−probing behavior, which increases the number of attempts to bite and the feeding time (Killick-Kendrick et al. 1977; Beach et al. 1985; Rogers et al. 2002; Rogers and Bates 2007; Bates 2008).

In conclusion, after vessel laceration, saliva is responsible for blood-flow maintenance, thus promoting rapid feeding. These events, both physical and biological, could cause changes in the insect's behavior to facilitate parasite deposition. Actions such as (a) vertebrate tissue injury caused by the bite, (b) PSG, and (c) saliva would help the sand fly retain its meal and facilitate successful parasite entry, thus exacerbating the infection (Schlein et al. 1992; Volf et al. 2004; Rogers et al. 2004; Peters et al. 2008; Oliveira et al. 2008).

After several attempts, *Leishmania* experimental transmission occurred only in the year 2003 (Kamhawi et al. 2003) with the model of *P. papatasi* and *L. major* infecting mice. This model allowed several groups to understand disease transmission from the vector−host point of view, i.e. by bite-like natural transmission. In summary, two aspects were relevant: the bite and the *Leishmania* dose transmitted to the host as follows:

(a) Sand-fly bite: The infected sand-fly female inoculates exclusively infective metacyclic promastigote forms into the host's skin. These are phagocytosed by macrophages, directly or after neutrophil infection, and are rapidly recruited to the bite site (van Zandbergen et al. 2004; Peters et al. 2008). Images of the parasite-transmission process showed a significant role of neutrophils, which are rapidly attracted to the bite site, thus ensuring parasite survival in the early period of infection (Peters et al. 2008).
(b) Sand-fly transmitted dose: In the literature, all knowledge generated about the infection process and the establishment of *Leishmania* in vertebrate hosts has come from studies on subcutaneous or intradermal parasite inoculation.

The traditional, routine experiments use different amounts of parasites (10^2–10^7 parasites/mL) (Belkaid et al. 1998, 2000). The parasite number in the inoculum was appropriate due to the need to establish infection in the natural host without, however, considering the *Leishmania* number inoculated by the sand-fly vector. It has been suggested that the amount of *L. mexicana* regurgitated by *L. longipalpis* varies (10–10,000 parasites). However, this study was conducted using an experimental model, and the parasites were collected through a membrane apparatus (Rogers et al. 2004). Finally, a quantitative polymerase chain reaction study using *P. papatasi* infected with *L. major* demonstrated that the sand fly could inoculate 100−100,000 parasites into the host's tissue. Dose distribution showed a binomial profile: About 75% of the sand flies released ≤600 promastigotes, whereas the other 25% injected >1000 parasites. High doses of infection were strongly associated with sand-fly infection that presented at least 30,000 parasites/midgut (Kimblin et al. 2008). A similar study established a transmission model of *L. infantum chagasi* by the bite of *L. longipalpis*, the vector of American visceral leishmaniasis. The parasites were successfully transmitted by infected sand-fly bites to mice and hamsters (Fig. 3), thus indicating that both animals are good experimental models. The *L. infantum chagasi* dose that was transmitted in each single bite ranged from 10−10,000 parasites, but 75% of the sand flies transmitted <300 parasites (Secundino et al. 2012). These two studies elucidated the *Leishmania*-transmission mechanism by vector bites of both New World and Old World sand flies.

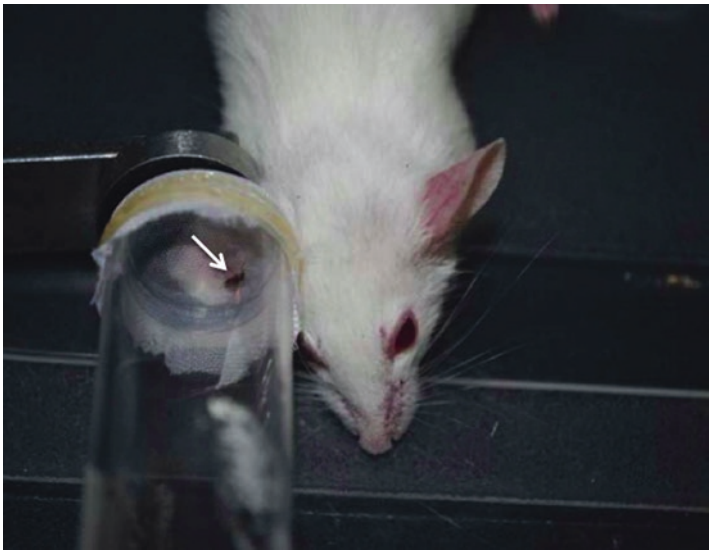

Fig. 3 Experimental transmission of *L. infantum chagasi* by the bite of infected sand fly *L. longipalpis* in an ear of Balb/C mouse. A single sand fly was confined within a vial and allowed to bite the animal's entire ear. Note the blood-meal engorgement of the sand fly (white arrow). (Originally published in Secundino et al. 2012)

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