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Research Article

Differential Midgut Attachment of *Leishmania (Viannia) braziliensis* in the Sand Flies *Lutzomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia*

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The interaction between *Leishmania* and sand flies has been demonstrated in many Old and New World species. Besides the morphological differentiation from procyclic to infective metacyclic promastigotes, the parasite undergoes biochemical transformations in its major surface lipophosphoglycan (LPG). An upregulation of β-glucose residues was previously shown in the LPG repeat units from procyclic to metacyclic phase in *Leishmania (Viannia) braziliensis*, which has not been reported in any *Leishmania* species. LPG has been implicated as an adhesion molecule that mediates the interaction with the midgut epithelium of the sand fly in the Subgenus *Leishmania*. These adaptations were explored for the first time in a species from the Subgenus *Viannia, L. (V.) braziliensis* with its natural vectors *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (Nyssomyia) whitmani*. Using two in vitro binding techniques, phosphoglycans (PGs) derived from procyclic and metacyclic parasites were able to bind to the insect midgut and inhibit *L. braziliensis* attachment. Interestingly, *L. braziliensis* procyclic parasite attachment was ~11-fold greater in the midgut of *L. whitmani* than in *L. intermedia*. The epidemiological relevance of *L. whitmani* as a vector of American Cutaneous Leishmaniasis (ACL) in Brazil is discussed.

1. Introduction

Leishmania (Viannia) braziliensis, the etiological agent of American Cutaneous Leishmaniasis (ACL), has the widest geographic distribution in the Americas and can be transmitted by the sand flies *Lutzomyia (Psychodopygus) wellcomei*, *Lutzomyia (P.) complexa*, *Lutzomyia migonei*, *Lutzomyia (Nyssomyia) whitmani*, *Lutzomyia (N.) intermedia* [1, 2], and *Lutzomyia (N.) neivai* [3]. From those, *L. (N.) whitmani* is considered of the highest epidemiological importance [4].

Leishmania parasites in their sand fly vectors spend their life cycle as flagellated promastigotes within the gut, where they must confront several challenges to survive including the activity of digestive enzymes, the need to escape from the peritrophic matrix, elimination of unattached parasites from the sand fly gut with the digested blood products, and the need to develop infective forms which can be transmitted to the vertebrate host [5–8].

Many studies have demonstrated that the promastigotes' dominant surface lipophosphoglycan (LPG) protects the parasites against those adverse conditions preventing loss of the parasite in the gut for many species of the subgenus *Leishmania*. LPG has been biochemically characterized and implicated in the *Leishmania* specificity to

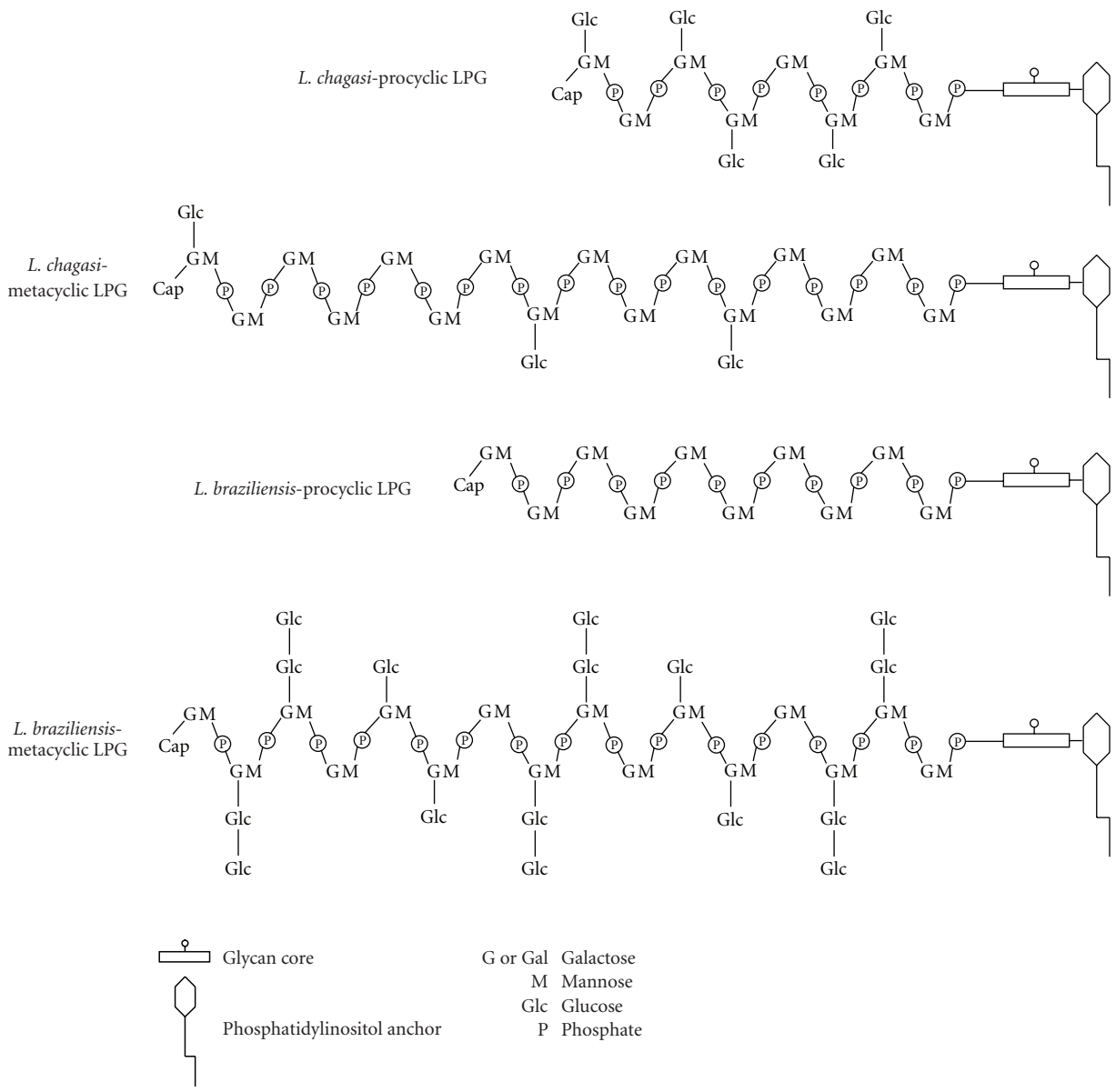

Figure 1: Opposite mechanisms in the glucose regulation in the LPGs from procyclic and metacyclic *L. chagasi* (syn. *L. infantum*) and *L. braziliensis* [9, 10]. The structure of the glycan core is Gal(α1,6)Gal(α1,3) Gal$_f$(α1,3)[Glc(α1-PO$_4$)-6]-Man(α1,3)Man(α1,4)GlcN(α1,6) linked to 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The repeat units are 6-Gal(β1,4)Man(α1)-PO$_4$.

different vectors [9, 11–17]. All LPGs have a conserved glycan core region of Gal(α1,6)Gal(α1,3)Gal$_f$(β1,3)[Glc(α1)-PO$_4$]Man(α1,3)Man(α1,4)GlcN(α1) linked to a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. LPG intra- and interespecific polymorphisms are in the size and variability of side-chains attached to the repeat unit Gal(β1,4)Man(α1)-PO$_4$ backbone and in the cap [18]. In *L. braziliensis* LPG, a novel mechanism in the carbohydrate regulation in the LPG side-chains was observed. The LPG from the procyclic form is devoid of side chains, while in the metacyclic phase it contains one or two β(1,3)glucose residues as side chains [10]. In other species such as the Indian strain of *L. donovani* and *L. infantum*, both belonging to the subgenus *Leishmania*, the opposite occurs, resulting in downregulation of β(1,3)glucose residues during metacyclogenesis (Figure 1). The consequence is a loss of attachment by metacyclic LPG and midgut detachment of these *Leishmania* species [9, 15].

The developmental patterns of *Leishmania* within the sand fly gut do not depend on the vector but rather on the inherent behavior of the parasite species involved. Those behavior patterns were used to determine the currently accepted classification of *Leishmania* into the subgenera *Viannia* and *Leishmania* for species that show peripylarian and suprapylarian patterns, respectively [19]. Development of *Leishmania* (*Leishmania*) spp is restricted to anterior regions of the pylorus, beginning in the thoracic and abdominal midgut [20]. However, *Leishmania* (*Viannia*) spp involves an obligatory phase in the posterior gut (mainly in

the pylorus) prior to anterior migration and establishment of the parasites in the abdominal and thoracic midgut with following colonization of the foregut and mouth parts [21]. In this manuscript, we report for the first time the interaction of LPG with *L. whitmani* and *L. intermedia* midguts, vectors of ACL in Brazil.

2. Materials and Methods

2.1. Parasites. *Leishmania braziliensis* World Health Organization reference strain (MHOM/BR/75/M2903) was used. Starter cultures of promastigotes were grown in Medium 199 supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), streptomycin (50 μg/mL), 12.5 mM glutamine, 0.1 M adenine, 0.0005% hemin, and 40 mM Hepes, pH 7.4 at 25°C. Cells were grown at 26°C to a density of 1–1.2 $\times$ 10^7 cells/mL [10].

2.2. Purification of Metacyclic Cells. Parasites from stationary phase were harvested and resuspended in Medium 199 containing peanut agglutinin (PNA) from *Arachis hypogaea* (35 μg/mL). After 30 minutes incubation at room temperature, procyclic parasites that were agglutinated by the lectin (PNA+) were removed by low-speed centrifugation (150 *g*, 5 minutes, 4°C). Non-agglutinated metacyclic cells remaining in the supernatant (PNA−) were washed 2 times by centrifugation with phosphate-buffered saline (PBS) (2100 *g*, 15 minutes, 4°C) [10]. The yield of PNA+ and PNA− parasites was approximately 1.0 $\times$ 10^{11} and 5.0 $\times$ 10^9 cells, respectively.

2.3. Extraction and Purification of LPG from L. braziliensis. LPGs from procyclics (PNA+) and metacyclics (PNA−) parasites were extracted in solvent E (H_2O/ethanol/diethyl ether/pyridine/NH_4OH; 15:15:5:1:0.017), dried by N_2 evaporation, and resuspended in 0.1 N acetic acid/0.1 M NaCl. Then, they were applied to a column of phenyl-Sepharose (2 mL) equilibrated in the same buffer and LPG was eluted using solvent E. For binding studies, purified LPG was treated with PI-specific phospholipase C from *Bacillus cereus* (16 hours, 37°C). The dilapidated PG was separated from the cleaved lipid anchor by passage through a column of phenyl-Sepharose (2 mL) [22].

2.4. Midgut Binding Studies. *Lutzomyia whitmani* and *L. intermedia* sand flies were captured in Corte de Pedra, Bahia state, Brazil (13°26′23′′S, 39°39′3′′W). Taxonomical identification was performed prior to dissecting the midguts using the taxonomic key of Young and Duncan [23]. Binding of promastigotes was quantified by an in vitro technique [12]. Blood-unfed females maintained on 30% sucrose were dissected in PBS. Midguts (6–13 per group) were opened along the length of the abdominal segment with a fine needle, placed in concave wells of a microscope chamber slide, and incubated for 30 minutes with procyclic and metacyclic promastigotes (2 $\times$ 10^7 cells/mL, 50 μl). Then, guts were washed in successive drops of PBS and the number of attached parasites is determined with a Neubauer-counting *chamber*. In a second experiment, the midguts were incubated for 20 minutes with PGs (10 μg/mL) derived from procyclic and metacyclic promastigotes, washed with PBS, and then incubated with procyclic promastigotes (2.0 $\times$ 10^7 cells/mL) for 20 minutes at room temperature. Controls were dissected guts incubated only with procyclic promastigotes. The guts were then individually washed and counted as described above.

For binding of purified PG to midguts in vitro, opened, dissected midguts were fixed with 2% formaldehyde in PBS (4°C, 20 minutes). After several washes in PBS, the guts were incubated for 20 minutes with PG (10 μg/mL) from procyclic or metacyclic parasites. After several washes, the guts were incubated in a 1:400 dilution of ascites containing the anti-LPG antibody CA7AE followed by incubation with fluorescein antimouse IgG (FITC) (1:1000). Stained guts were examined with a fluorescence microscope [9].

2.5. Data Analysis. The D'Agostino-Pearson omnibus test was made to test the null hypothesis—that the data are sampled from a Gaussian distribution—*P* value ($P < .01$) shows that the data deviate from Gaussian distribution. For this reason, nonparametric Kruskal-Wallis was performed to test equality of population medians among groups and independent samples. Data were analyzed by GraphPad Prism 4.0 software and $P < .05$ was considered significant.

3. Results and Discussion

Leishmania parasites have to face adverse conditions to accomplish their life cycle either in the invertebrate or in the vertebrate hosts [24]. For that purpose, the parasites developed a range of molecules represented by the secreted acid phosphatases (*s*APs), glycoinositolphospholipids (GIPLs), filamentous proteophosphoglycans (*f*PPGs), lipophosphoglycans (LPGs), and lectins [25–27]. From these molecules, LPG is the most studied molecule and considered a multivirulent factor in *Leishmania* [28]. During the life cycle of *L. braziliensis* and other species of the subgenus *Viannia*, a crucial step for parasite survival is attachment to different parts of midgut. In this article, we focused on the interaction of this species with the midgut epithelium, where the parasites have to attach prior to arrival in the foregut and mouth parts. In the vector midgut, the ingested amastigotes need to transform into promastigotes. After metacyclogenesis, procyclic promastigotes differentiate in metacyclics, the infective forms to be passed to a new vertebrate host [20]. The relationship between stage differentiation and midgut adhesion has been previously reported for *L. major*, *L.infantum,* and *L. donovani* (India and Sudan). In those species, procyclic promastigotes attach to the midgut using the LPG, while metacyclic forms detach. In the case of *L. major*, the side chains comprising the LPG repeat units from procyclic parasites are often terminated with β(1,3)galactose, while metacyclic LPG side chains are capped with α(1,2)arabinose. In *L. donovani* from Sudan, procyclic LPG is devoid of side-chains, but metacyclic LPG increases in size, resulting in masking of the cap. The procyclic LPG of

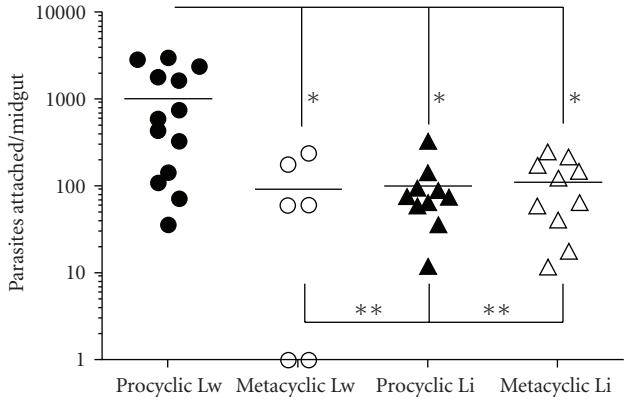

Figure 2: Differential attachment of *L. braziliensis* (procyclic and metacyclic) to *L. whitmani* (Lw) and *L. intermedia* (Li) midguts in vitro. $^{*}P < .01$; $^{**}P > .05$. Data are the representation of two experiments.

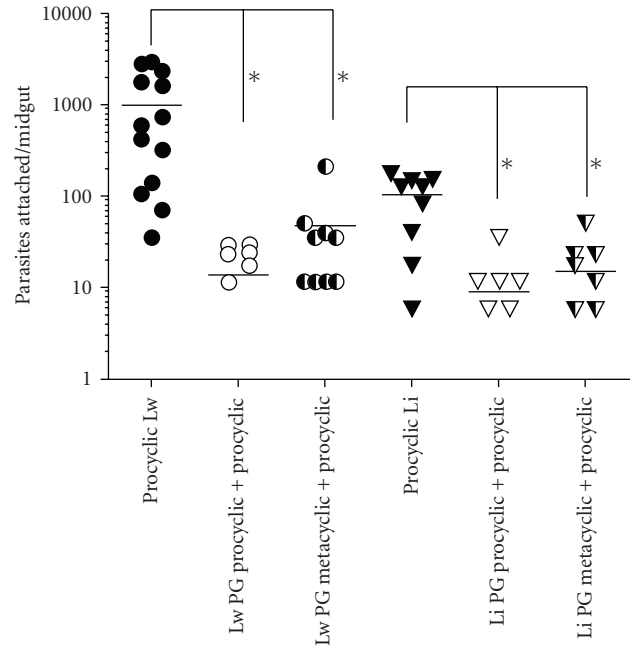

Figure 3: Inhibition of attachment of procyclic *L. braziliensis* to the midgut of *L. whitmani* (Lw) and *L. intermedia* (Li) in the presence of phosphoglycans (PGs) (10 μg/mL). Midguts were incubated with PGs from procyclic and metacyclic *L. braziliensis* and further incubated with procyclic promastigotes. Control midguts were incubated only with procyclic promastigotes. PG procyclic: PG derived from procyclics; PG metacyclic: PG derived from metacyclics. $^{*}P < .0001$. Data are the representation of two experiments.

L. infantum and *L. donovani* (India) has β-glucose residues that are downregulated in the metacyclic phase [9, 12, 14, 15].

Using a similar quantitative in vitro assay for the attachment of living *L. braziliensis* promastigotes, dissected midguts from *L. intermedia* and *L. whitmani* were analyzed. A differential pattern of attachment was observed in procyclic and metacyclic *L. braziliensis*. In *L. whitmani*, the average number of attached procyclics was ~11-fold compared to the metacyclics (1027.71 ± 299.30 versus 90.00 ± 40.25, $P = .0014$). For *L. intermedia*, the attachment of procyclics (101.40 ± 16.10) and metacyclics (112.80 ± 27.10) was very low and not statistically different ($P = .70$) (Figure 2). While comparing procyclic attachment between the two sand fly species, in *L. whitmani* the number of parasites that attached was ~10-fold higher than in *L. intermedia* (1027.71 ± 299.30 versus 101.40 ± 16.10, $P = .001$). Those data suggest that procyclic *L. braziliensis* were able to attach to the midgut with a mechanism different from hemidesmosomes seen in the pyloric and anterior regions [21], since they were able to easily detach and being counted in our in vitro system.

The number of metacyclic promastigotes that attach was very low in both *L. whitmani* and in *L. intermedia* ($P = .71$) (Figure 2) and therefore, only procyclics were used for the subsequent inhibition experiments. To test if the parasite attachment could be intermediate by LPG molecules, a competitive binding experiment was developed, where midguts were previously incubated with PGs derived from procyclic and metacyclic forms. Both PGs strongly blocked (~10-fold) the attachment of procyclic *L. braziliensis* in *L. whitmani* and *L. intermedia* midguts (Figure 3) ($P < .0001$). Interesting observation arose regarding the unexpected binding of metacyclic PG (Figure 4) to the midguts and its inhibition of parasite attachment (Figure 3). The possible explanation is that the attachment of *L. braziliensis* metacyclics to the midguts of *L. whitmani* and *L. intermedia* was very low in numbers. Furthermore, *L. braziliensis* has 10–20 less LPG molecules expressed on its cell surface than species from the subgenus *Leishmania* [10] and thus much less ligands for midgut attachment. These data also suggest the existence of an LPG-ligand in the sand fly midguts, which is necessary for *L. braziliensis* to establish and maintain the infection with further passage of the parasites towards the mouth parts. A galectin receptor for *L. major* LPG (subgenus *Leishmania*) in *P. papatasi* midgut was recently reported [29].

To confirm if PGs from procyclic and metacyclic promastigotes were recognizing any ligand in the midguts, the molecules were incubated with the dissected organs and revealed by immunocytochemical fluorescent staining with a specific antibody CA7AE, which recognizes PGs from procyclic and metacyclic *L. braziliensis* [10] (Figure 4). Consistent with our observations using live parasites, a similar pattern of stage-specific bindings was observed, where procyclic and metacyclic PGs were able to attach to opened *L. whitmani* and *L. intermedia* midguts (Figure 4(a) and 4(b)). The attachment of both types of PGs in this model may be attributed to the presence of β(1,3)glucose residues in the procyclic promastigote cap and in the repeat units of metacyclics [10]. The role of β(1,3)glucose residues in attachment was also demonstrated in *L. donovani* (India) [15] and *L. infantum* (Brazil) [9]. Those data suggest that upregulation of glucoses is necessary for *L. braziliensis* development. By adding glucoses in its LPG, perypilariam metacyclics might have to traverse midgut following their

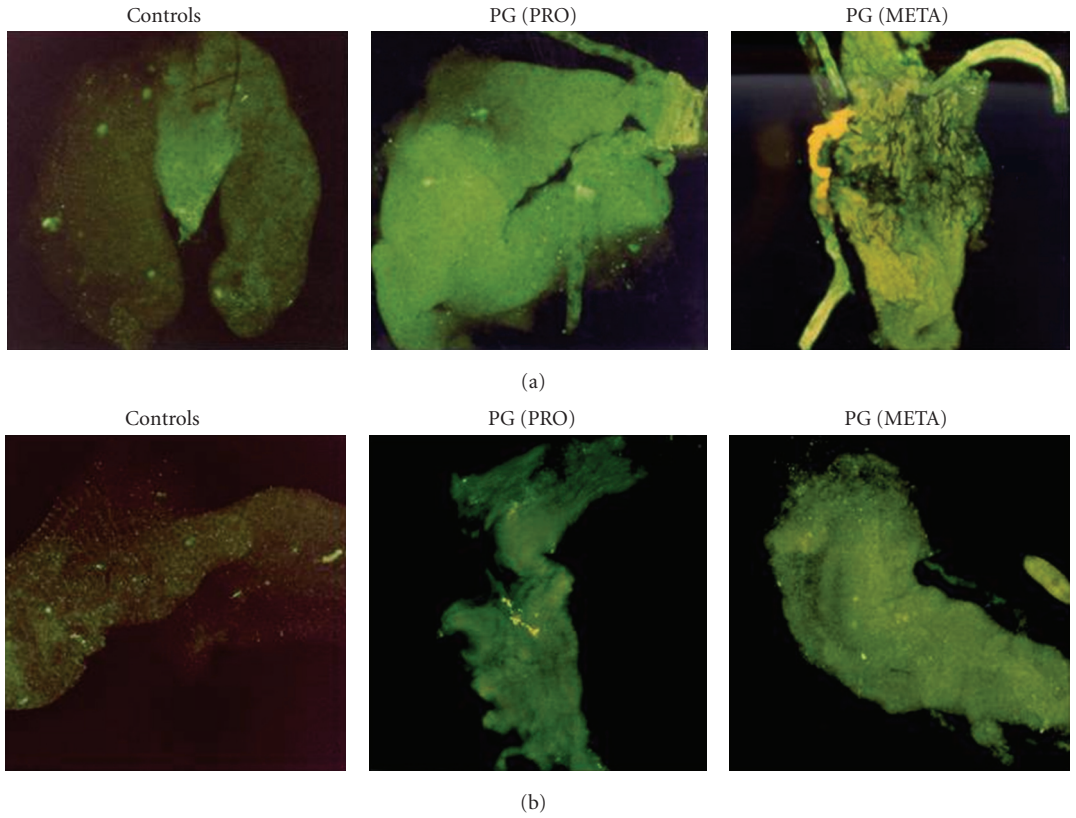

Figure 4: Fluorescent staining of *L. intermedia* (a) and *L. whitmani* (b) midguts incubated with PGs from procyclic (PRO) and metacyclic (META) *L. braziliensis*, probed with CA7AE antibody (1:400) and developed with FITC (1:1000). Control midguts were incubated with primary and secondary antibodies only.

early development in the hindgut. Given that the term metacyclic normally refers to the detached infective stage available for transmission in the subgenus *Leishmania*, in the subgenus *Viannia*, this term should perhaps be renamed as suggested elsewhere [26]. However, how those parasites would detach from the midgut is still a missing step, which cannot be demonstrated in our in vitro system. A possibility is that a "second metacyclic-like" stage, if any, could detach from midgut prior to migration to foregut.

Although both studied sand flies are known to be successful vectors, the adhesion of the parasites in *L. whitmani* was more pronounced (~11-fold) than in *L. intermedia*. Another interesting result is that procyclics were able to attach more than the metacyclics in *L. whitmani*, while in *L. intermedia* this attachment was low for both forms (Figure 2). This finding has a major epidemiological relevance, once *L. whitmani* is known to be the most important and widely distributed ACL vector [2, 4, 30–32] mainly in the Northeast of Brazil. On the other hand, *L. intermedia* is more concentrated in Southeast, especially in the State of Rio de Janeiro where is the main vector of *L. braziliensis* [2, 33–36]. However, in Corte de Pedra, State of Bahia, the border state between Northeast and Southeast, where the insects were captured for this study, both species occur sympatrically being able to transmit ACL all over the year [37].

Recently, it was shown that *L. longipalpis* was more efficient vector of *L. infantum* than *Lutzomyia evansi* [38]. Infection success was dependent on the establishment of the parasite in the midgut, which was very irregular in *L. evansi*. Consequently, those results explain the irregularity in the Visceral Leishmaniasis transmission where *L. evansi* occurs. To be considered a good vector, many conditions have to be followed: the distribution of the sand fly vector has to be coincident with the human disease; the insect must be found infected in the peridomestic or domestic areas and it has to feed avidly on man and many hosts [39]. However, if differences in *Leishmania* attachment have an impact on the efficacy of disease transmission by *L. whitmani* and *L. intermedia* still awaits further investigation.

4. Conclusions

In the Subgenus *Leishmania*, there are strong biochemical and genetic evidences that LPG is a critical molecule for the attachment process to sand fly midguts. However, for *L. braziliensis,* which makes less LPG [10], the role of this molecule seems to be necessary at least during the transient parasite passage through the midgut. The term metacyclics refers to the vertebrate infective parasite stage able to detach from the midgut as it was observed in the species from the subgenus *Leishmania*. Nonetheless, in

the subgenus *Viannia,* we found two patterns of metacyclic attachment: (1) similar to the Subgenus *Leishmania* as observed for *L. whitmani*, a very competent vector and (2) very low as observed in *L. intermedia*, where LPG seems to have a less important role. We thus conclude that the unusual pattern of attachment of *L. braziliensis* in the midgut may be a result of its perypilarian behavior related with the vector susceptibility/specificity. Yet to be elucidated is how other glycoconjugates may be critical on the anterior migration of the parasite to the mouth part in the sand fly digestive tract. In the species of the Subgenus *Viannia*, the pattern of membrane glycoconjugates is different from those of the Subgenus *Leishmania,* which results in higher expression of GIPLs in *L. braziliensis* and *L. panamensis* [40]. However, the current study reinforces our understanding that LPG may still play a key role in the interaction with those vectors.

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