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Microbes Infect. Author manuscript; available in PMC 2010 March 16.

Published in final edited form as:
Microbes Infect. 2007 March ; 9(3): 317–324. doi:10.1016/j.micinf.2006.12.010.

A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies

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Abstract

Leishmaniases are serious parasitic diseases the etiological organisms of which are transmitted by insect vectors, phlebotominae sand flies. Two sand fly species, *Phlebotomus papatasi* and *P. sergenti*, display remarkable specificity for *Leishmania* parasites they transmit in nature, but many others are broadly permissive to the development of different *Leishmania* species. Previous studies have suggested that in 'specific' vectors the successful parasite development is mediated by parasite surface glycoconjugates and sand fly lectins, however we show here that interactions involving 'permissive' sand flies utilize another molecules. We did find that the abundant surface glycoconjugate lipophosphoglycan, essential for attachment of *Leishmania major* in the specific vector *P. papatasi,* was not required for parasite adherence or survival in the permissive vectors *P. arabicus* and *Lutzomyia longipalpis*. Attachment in several permissive sand fly species instead correlated with the presence of midgut glycoproteins bearing terminal N-Acetyl-galactosamine and with the occurrence of a lectin-like activity on *Leishmania* surface. This new binding modality has important implications to parasite transmission and evolution. It may contribute to the successful spreading of *Leishmania* due to their adaptation into new vectors, namely transmission of *L. infantum* by *Lutzomyia longipalpis*; this event led to the establishment of *L.infantum/chagasi* in Latin America.

Keywords

Parasite transmission; Emerging infectious disease; Trypanosomatid protozoan

1. Introduction

Parasitic protozoa of the genus *Leishmania* are transmitted by the bite of bloodsucking female phlebotomine sand flies (Diptera: Phlebotominae). Parasites taken up with the blood meal undergo a period of replication and development in the midgut, after which they differentiate to an infective metacyclic stage adapted for transmission to mammals [1]. Initially, procyclic promastigotes, the flagellated parasite developmental stages, are enclosed by the sand fly peritrophic matrix, which decays within a few days. Promastigotes of suprapylarian species of *Leishmania* (subgenus *Leishmania*) then attach to the midgut epithelium via insertion of flagella between microvilli [2, 3]. This attachment is an essential part of *Leishmania* life cycle as it enables the parasite to avoid expulsion from the midgut when the remnants of the digested blood meal are defecated by the sand fly. It has been

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proposed that only *bona fide* sand fly vectors can support the establishment of parasites via this attachment mechanism [1, 2].

A series of studies performed with *L. major* in *P. papatasi* showed that the attachment is controlled by species-specific modifications of the major surface glycoconjugate of *Leishmania* promastigotes, lipophosphoglycan (LPG) [1, 4-6]. In this parasite – sand fly duo, the LPG phosphoglycan repeating units are modified by galactosyl residues, which selectively bind to the midgut galectin receptor PpGalec [1, 7]. Midgut detachment during metacyclogenesis arises following a further modification of the LPG, into an arabinosyl-capped form unable to bind to the midgut galectin [7]. In other *Leishmania* – sand fly pairs, the role of LPG in attachment has not been investigated in such detail as in the *L. major* - *P. papatasi* model. Nonetheless, the available data were consistent with the model invoking LPG as the major adhesin responsible for midgut binding in all *Leishmania* species [4, 8-11].

Laboratory studies examining the development of different *Leishmania* in a range of sand fly species suggest that sand flies fall into two groups. Certain species are specific vectors as they are refractory to the development of most *Leishmania* species; i.e. *P. papatasi* supports development of *Leishmania major* but not of any other parasite species tested [4]. Another example of a specific vector is *P. sergenti*, the vector of *L. tropica* [8]. In contrast, most sand fly species examined to date support the development of a broad range of *Leishmania* species and fall into a second group, which we propose to call “permissive” vectors. These include *Lutzomyia longipalpis* [12], *P. argentipes* [4], *P. halepensis* [13] and *P. arabicus* [14]. Evidently, the parasites are able to develop in any permissive sand fly species, if given the opportunity. Mechanisms underlying this broad permissivity have not been fully elucidated, and a variety of candidate molecules have been proposed to mediate this process, such as relatively conserved promastigote surface proteins like flagellar proteins [3]. Based upon the LPG-dependent model briefly introduced above, it was hypothesized that midguts of broadly permissive vectors possess a receptor for a conserved oligosaccharide on LPG, which *P. papatasi* and *P. sergenti* lack [2]. In this study we show that another LPG-independent lectin-like mediated process exists in the permissive sand flies, which plays a dominant role in parasite attachment to the sand fly midgut.

To investigate the role of LPG in permissive sand flies, we made use of the *L. major* mutant *lpg1*$^{-}$ which specifically lacks LPG through deletion of the *LPG1* galactofuranosyl transferase required for synthesis of the LPG core [15]. The data obtained argued that LPG is unlikely to play a major role in the adherence of *Leishmania* in permissive sand fly species. Thus analyses of sand fly midguts was undertaken to detect insect molecules mediating *Leishmania* attachment. Special attention was paid to glycosylation of polypeptides, as this post-transcriptional modification is known to play a key role in parasite-host interactions [16]. Our study demonstrated the importance of sand fly midgut glycoproteins bearing N-Acetyl-galactosamine (GalNAc) and revealed the presence of the new binding modality in sand flies otherwise shown to be permissive for a broad range of *Leishmania* species.

2. Materials and methods

2.1 Reagents

All reagents, if not otherwise stated, were purchased from Sigma.

2.2. Parasites and sand fly colonies

Laboratory colonies of seven sand fly species were used: *Lutzomyia longipalpis* (origin from Jacobina, Brazil), *Phlebotomus papatasi* (Turkey), *P. sergenti* (Turkey), *P. halepensis*

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(Jordan), *P. arabicus* (Israel), *P. perniciosus* (Italy) and *P. argentipes* (India). Experiments were done with females 5-10 day old. *Leishmania infantum* MHOM/TR/2000/OG-VL and two lines of *L. major* LV39 (MRHO/SU/1959/Neal P), the wild type (*wt*) and LPG null mutant (*lpg1⁻*), were maintained on medium 199 supplemented with 20% foetal calf serum (Gibco BRL) and gentamicin (50 μg/ml). For the *lpg1⁻* mutant, hygromycin 15 μg/ml and puromycin 11 μg/ml was added to the culture medium. Promastigotes from the low number (3-4) of *in-vitro* passage were used for the experiments.

2.2. Leishmania development in sand flies

Female sand flies were fed through a chick skin membrane with 5-day-old promastigotes at cell density 5×10^6 promastigotes/ml in heat-inactivated rabbit blood. Blood-engorged females were maintained at 28 °C and sacrificed for microscopical examination and enumeration of parasites in the midguts before defecation (day 2) or four days after defecation (day 7 for *Lutzomyia longipalpis* and day 8 for *P. papatasi* and *P. arabicus*). Parasite density was graded according to criteria reported previously [13]. The experiments were repeated twice.

2.3. Detection of glycoproteins in sand fly midgut lysates

Midgut glycoproteins were analysed by SDS PAGE (10 % gel, reducing conditions, 10 μg protein per lane) followed by western blotting. The nitrocellulose membrane was incubated with Tris-NaCl-Tween (20 mM Tris, 150 mM NaCl, pH 7.6) with 5 % bovine serum albumin and then with biotinylated *Helix pomatia* lectin (HPA, 1 μg/ml) or concanavalin A (0.5 μg/ml). After repeated washing the blots were incubated with streptavidin peroxidase (2.5 μg/ml) in Tris-NaCl-Tween and developed in 4-chloro-1-naphtol solution. The specificity of lectin reactions was controlled by addition of 250mM GalNAc for HPA and methyl-mannopyranoside for ConA. To study sensitivity of glycoconjugates to trypsin exposure, lysates of ten midguts of *P. arabicus* (in 15 μl of Tris-NaCl) were incubated with 4 or 20 units of bovine trypsin at 37 °C for 1 hour and than analysed by SDS PAGE and western blotting with HPA.

2.4. Binding of sand fly midgut lysates to Leishmania promastigotes

The four-day culture of *L. major* was washed and diluted to final density of 10^7 promastigotes/ml, spotted onto a slide, air-dried and fixed using methanol. Slides were incubated for 30 min with supernatant (100000 g / 30 min / 4 °C) of *P. halepensis* or *P. papatasi* midgut lysates diluted in Tris-NaCl and for another 30 min with FITC-HPA (125 μg/ml) in Tris-NaCl. To check the specificity of binding of midgut molecules the *Leishmania* were incubated first with 250 mM GalNAc. Then the unbound GalNAc was washed out and parasites were incubated with supernatant of midgut lysate followed by FITC-HPA. In negative controls either the parasites were preincubated with 250 mM GalNAc or Tris-NaCl buffer only. Sections were mounted in Vectashield mounting medium with propidium iodide (Vector Laboratories) and checked under the fluorescent microscope Olympus BX51.

2.5. Localization of HPA-binding epitopes on sand fly midguts

Abdomens of *Lutzomyia longipalpis* females were fixed using 2% paraformaldehyde and embedded into LR-White resin. Sections 2 μm thick were incubated for 2 hours with 2% bovine serum albumin and then for 1 hour with FITC-HPA diluted in Tris-NaCl (final concentration 100 μg/ml). Finally, the sections were post-stained with Evans blue, mounted and checked as described above. In negative controls the reaction was blocked by addition of 250 mM GalNAc into FITC-HPA-containing solution.

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3. Results

3.1. Leishmania major lpg1- mutants develop in different permissive sand flies whereas they do not develop in *P. papatasi*

Experiments with *Leishmania* mutants deficient in LPG revealed a striking difference between various sand fly species with respect to the parasite development. In the specific vector *P. papatasi, lpg1*⁻ mutants did not adhere nor survive well following bloodmeal digestion (Fig.1). In contrast, the *L. major lpg1*⁻ survived normally in *P. arabicus* and *Lutzomyia longipalpis*, two permissive species not implicated as natural *L. major* vectors. Within both species *lpg1*⁻ mutans were able to attach within the midgut, and yield a high percentage of sand flies with high parasite loads (Fig.1).

3.2. GalNAc-displaying glycoproteins are present in the midgut lysates of the permissive sand flies

Initially, midgut proteins of two specific vectors, *P. papatasi* and *P. sergenti*, and the midguts of two permissive sand fly species, *Lutzomyia longipalpis* and *P. halepensis,* were compared by lectin blotting. All midgut lysates displayed peptides that bind concanavalin A, a lectin detecting terminal mannose residues present on high mannose-, hybrid- and complex-type of N-linked glycans (Fig. 2A). In contrast, *Helix pomatia* agglutinin (HPA) showed reactivity only with the two permissive sand flies, showing strong reactivity with a small number of molecules ranging from 30 to 70 kDa (Fig. 2A). This lectin is specific for GalNAc, a carbohydrate typically associated with O-linked glycan-displaying glycoproteins such as mucins.

We extended these studies to other sand fly colonies representing three other species, *P. argentipes, P. perniciosus* and *P. arabicus*. The first two represent proven vectors of parasites of the *L. donovani* complex [17]. Since the susceptibility of *P. arabicus* to *Leishmania*, other than *L. tropica* [14], had not been previously reported, we fed *P. arabicus* on blood containing either *L. infantum* or *L. major* (Fig. 3). On day 8 post bloodmeal, many *P. arabicus* females harboured heavy *L. infantum* loads (≥ 1000 promastigotes per midgut), this parameter being fully comparable to those previously obtained with proven vectors. Similarly, *P. arabicus* supported the late-stage development of *L. major* confirming that it belongs to permissive sand fly species (Fig. 3).

The extended lectin studies corroborate the previous experiment. As before, midguts of all permissive sand fly species strongly bind HPA, indicating the presence of terminally exposed GalNAc residues (Fig. 2B). These bands react also with soybean agglutinin, another GalNAc-binding lectin (data not shown). The main HPA-reactive molecules within permissive sand fly species varied in apparent molecular mass (35 to 55 kDa), and did not yield ‘sharp’ SDS PAGE bands which is a common characteristic of glycoconjugates (Fig. 2B). If the midgut lysates were incubated with trypsin prior to SDS PAGE these bands were no more detectable suggesting that they were glycoproteins (Fig. 4).

3.3. GalNAc-displaying glycoproteins from midguts of permissive sand flies bind to Leishmania promastigotes

To further investigate whether the HPA-binding glycoproteins could be the ligands of the sand fly to which the promastigotes attach, parasites were incubated with midgut lysates followed by fluoresceinated HPA (FITC-HPA) to observe binding. With permissive *P. halepensis* lysates strong reactivity was seen (Fig. 5A), whereas only control background binding was seen with midgut lysates from the ‘selective’ species *P. papatasi* (Fig. 5E). HPA reactivity was abolished by incubation with GalNAc (Fig. 5C) as expected. Notably this also occurred if GalNAc was preincubated with *Leishmania* and washed out prior to incubation

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of parasites with *P. halepensis* lysate followed by HPA labelling (Fig. 5B). Thus, permissive sand fly HPA-reactive glycoproteins are similarly capable of binding to *Leishmania* in a GalNAc-dependent manner. Since *Leishmania* themselves did not react with HPA (Fig. 3D), this argues that a parasite lectin-like activity recognizes sand fly GalNAc-containing conjugates.

3.4. GalNAc epitopes are present on the luminal surface of the sand fly midgut

We visualized the location of the HPA-binding molecules by incubating sections of the abdomen of the permissive vector *L. longipalpis* with FITC-HPA. Notably, FITC-HPA showed specific localisation to the microvillar border of the midgut (Fig. 5F), the location where the parasites are expected to attach. Binding was prevented to occur by preincubation with GalNAc (Fig. 5G) and no specific reactivity was seen outside of the midgut region.

4. Discussion

In previous studies of 'specific' *Leishmania*-sand fly interactions exemplified by *P. papatasi* and *L. major*, LPG was found to be a major determinant for midgut binding, a key step allowing the *Leishmania* development to fully proceed to the metacyclic stage [1, 2, 4-7]. Critical to this conclusion was the fact that LPG-deficient mutants, such as the *lpg1*- ones studied in this context, failed to bind to the *P. papatasi* midgut and did not survive in this specific sand fly vector. In contrast, we found that the *lpg1*- parasites resembled wild type *Leishmania* in two other sand flies qualified as permissive vectors, *L. longipalpis* and *P. arabicus*, as they attach to the midgut and produced high proportion of sand fly females with heavy parasite loads. As a control, we confirmed previous results showing that the same *lpg1*⁻ mutant did not survive following bloodmeal digestion in specific vector *P. papatasi* [2]. The results are consistent with those obtained with another *Leishmania* species, as Rogers et al. [18] found that *lpg1*⁻ mutants of the New World species *L. mexicana* could survive and complete their development in *L. longipalpis*. This showed that LPG was not essential for attachment of *L. major* and *L. mexicana* in this permissive vector.

The binding of *L. major* to *P. papatasi* midguts is mediated by galactose-modified phosphoglycan repeating units which comprise the bulk of LPG, as shown by the inability of the LPG-galactosylation mutant Spock to survive in the sand flies [6]. Galactose-modified phosphoglycan repeating units also occur on other abundant parasite surface glycoconjugates such as proteophosphoglycans, which are unaffected in the *lpg1*- mutants [15]. The *lpg2*- mutant, which lacks phosphoglycans through deletion of the golgi GDP-mannose transporter required for phosphoglycan repeating unit synthesis [19], failed to develop in sand flies due to their sensitivity to midgut conditions [2, 20], and thus parasite survival cannot be used to assess the adherence step of their development in the sand fly midgut. We explored also the use of the parasite binding assay to sand fly midguts described by Pimenta et al. [4, 5] to confirm the role of GalNAc – displaying glyconjugates more directly. However, this method did not yield consistent parasite binding (data not shown), and further technical advances will be required to develop improved methods to quantify parasite binding in permissive sand flies.

Two further observations support the hypothesis that LPG is not essential for attachment of *Leishmania* in permissive sand fly species and that GalNAc-containing glycoproteins are involved in the novel mechanism of attachment. Firstly, these glycoproteins specifically bound to the surface of *Leishmania* promastigotes (Fig. 5 A-E). Secondly, HPA lectin showed specific localization of GaLNAc-containing epitopes on the microvillar border of the midgut (Fig. 5 F-G), the location required for the attachment of parasites inside the sand fly midgut. A similar location of HPA-binding epitopes was previously demonstrated in *L. longipalpis* by electron microscopy [21].

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Our data suggest that in several sand flies that are permissive to development of different *Leishmania*, the dominant factor mediating parasite-midgut interaction does not arise from interactions of LPG with sand fly lectins. Instead, the "adherence paradigm" may be inverted, in that there appears to be a lectin-carbohydrate interaction involving sand fly GalNAc-containing glycoproteins with a parasite lectin-like receptor. Potential candidates for this receptor are heparin-binding proteins and lectin-like molecules, reported previously in various *Leishmania* species, some of which occur on the cell surface and bound GalNAc [22-25]. Heparin-binding and lectin-like activities are known to participate in adhesion of *Leishmania* to host phagocytes [22, 26, 27] and our studies now extend their function into permissive sand fly vectors.

Identification of a conserved sand fly GalNAc ligand – parasite interaction in several permissive sand fly vectors provides a useful perspective on the successful adaptation of *Leishmania* to other sand flies than the specific ones, often as a result of humans activities. An important example is the introduction of *L. infantum* (syn. *L. chagasi*) from the Mediterranean to Latin America [28]. In Southern Europe, this parasite is transmitted to dogs and to humans by *P. perniciosus* [17] (a species with GalNAc-containing glycans – Fig. 2B) and related species of subgenus *Larroussius* [17]. When European colonists arrived with their dogs in Latin America, the parasite was able to switch to a new permissive host/vector, *Lutzomyia longipalpis*. Similar explanations may underly other parasite-host/vector interactions or vectorial shifts that are due to modification of surface molecules of the parasite, such as transmission of atypical *L. tropica* strains in northern Israel [29]. Due to modifications of LPG [30] this *L. tropica* is not able to develop in the specific vector *P. sergenti* [14], but can develop in a permissive vector *P. arabicus* [14], species the midgut of which was shown to contain GalNAc-displaying glycoproteins (Fig. 2B).

Our findings are not in conflict with the ones that establish the role of LPG receptors in specific vectors that lack midgut glycoproteins with terminal GalNAc. In these species, alternative parasite recognition sites expressed by *P. papatasi* and *P. sergenti* has in turn led to development of highly branched and species-specific LPG structures in *L. major* and *L. tropica*. This has enabled these parasites to take advantage of two widely distributed sand fly species that are inherently refractory to other *Leishmania* species. On the other hand, *Leishmania* species, like *L. donovani* complex, that express unsubstituted or poorly substituted LPG rely on broadly permissive sand fly vectors with GalNAc epitopes in the midgut. Thus, while the *L. major LPG* / *P. papatasi* PpGal interactions assessed an elegant example of parasite-vector co-evolution [1, 7], the present data and other recent analysis indicate that another couple of interacting molecules may dominate in certain permissive sand flies. We suppose that this new mechanism of attachment is relevant also for midgut phase of development of the peripylarian *Leishmania* of the subgenus *Viannia*.

We wish to emphasize that the present distinction within the sand fly species into two categories, e.g., 'specific' or 'permissive' to *Leishmania* species, should be considered provisional. Indeed, many parasite-sand fly combinations have not been tested, due to the difficulty of carrying out such experiments, and experimental techniques differ amongst investigators. As knowledge grows these concepts will be further refined, however they provide a useful working frame presently. Similarly, whether the LPG-independent development seen within the permissive species *L. longipalpis* or *P. arabicus* hosting *lpg1-L. major* or *L. longipalpis* hosting *lpg1- L. mexicana* [18], will be generalized to all permissive sand flies remains to be determined. In fact, previous studies showed that LPG-deficient *L. donovani* mutants were unable to survive within *P. argentipes* [2], a broadly permissive vector with GalNAc epitopes expressed on midgut glycoproteins (Fig. 2B). Potentially this 'LPG-dependent permissive' fly interaction reflects differences in experimental methods, specific parasite and/or sand fly colonies studied. Our studies

establishing the presence of a dominant, LPG-independent interaction of parasites within certain permissive sand fly vectors is clearly a step in advancing our knowledge of this complex biological system. The next step would be a more detailed characterization of molecules involved in this new attachment mechanism.

Acknowledgments

We thank D.L. Sacks, S. Kamhawi and E. Rowton for providing *P. perniciosus* and *P. argentipes* larvae. We would like to acknowledge D.E. Dobson and R.L. Jacobson for discussion and critical comments. This work was supported by projects MSM21620828, LC06009, 195/2005/B-Bio, Czech Science Foundation (206/06/0967), NIH grant AI31078 (SMB) and the Wellcome Trust (PV, JP and MS).

Abbreviations

LPG	lipophosphoglycan
HPA	*Helix pomatia* lectin
GalNAc	N-Acetyl galactosamine
FITC	fluorescein isothiocyanate

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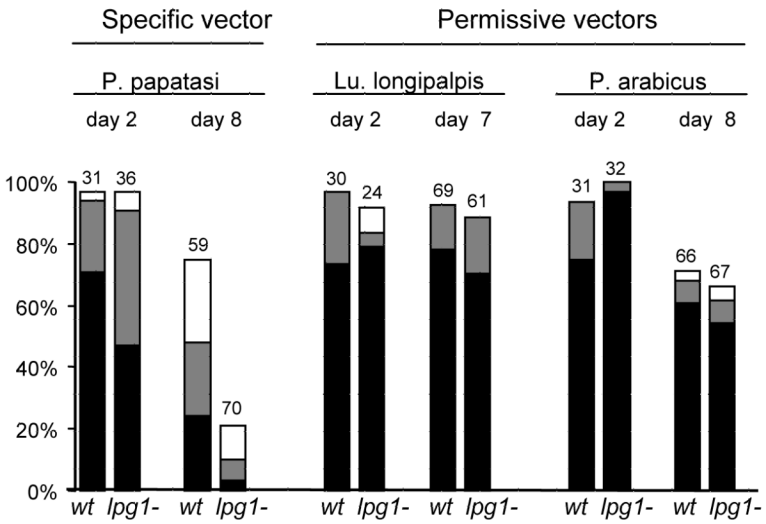

Figure 1. LPG is not required for *Leishmania major* development in permissive sand flies

Phlebotomus papatasi, L. longipalpis and *P. arabicus* fed on blood containing *Leishmania major* lacking LPG (*lpg1⁻)* and the wild type (wt). Parasite loads were classified into three cathegories: heavy (more than 1000 promastigotes per gut) - black bars, moderate (100-1000) - grey bars, light (1-100) - white bars. Numbers above the bars indicate the number of dissected females. χ^2 test was used to compare the parasite load and the rate of successful parasite development between and within sand fly groups. In *P. papatasi*, the percentage of positive females and the parasite loads were significantly decreased in *lpg1* group on day 8 post-infection ($p<0,05$). In *L. longipalpis* and *P. arabicus* both parameters did not differ between groups.

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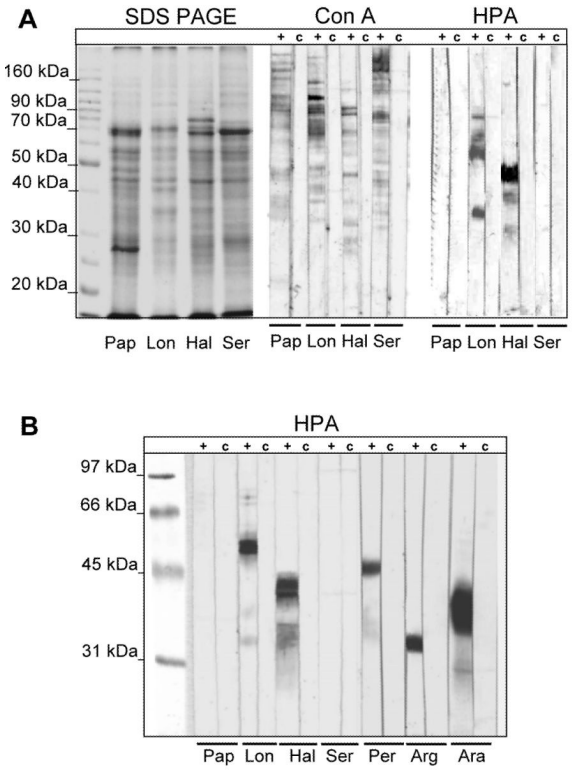

Figure 2. GalNAc-containing glycoconjugates are present only in the midgut lysates of permissive sand flies

A, SDS PAGE and blotting of midgut lysates with lectins concanavalin A and HPA, comparison of four different sand fly species: pap - *Phlebotomus papatasi*, lon - *Lutzomyia longipalpis*, hal - *P. halepensis*, ser - *P. sergenti*.

B, Western Blot of midgut lysates incubated with lectin HPA; comparison of seven sand fly species. pap - *P. papatasi*, lon - *Lu. longipalpis*, hal - *P. halepensis*, ser - *P. sergenti*, per - *P. perniciosus*, arg - *P. argentipes*, ara - *P. arabicus*.

+, reaction of the lectin; **c**, control inhibition with specific sugar (GalNAc for HPA, mannose for ConA). HPA reacted specifically with lysates of all five permissive species studied.

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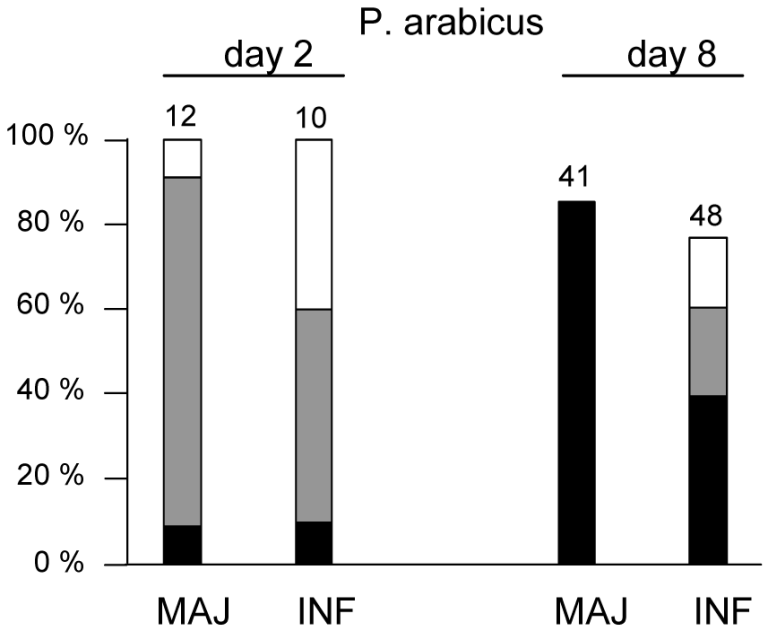

Figure 3. *Phlebotomus arabicus* allows the development of *L. major* and *L. infantum*

Females fed on blood containing *L. major* **(MAJ)** or *L. infantum* **(INF)**. Parasite development was evaluated as described in Fig.1. Day 2 - dissection before defecation (48hours post-infection), day 8 - dissection after defecation. Numbers above the bars indicate the number of dissected *P. arabicus* females.

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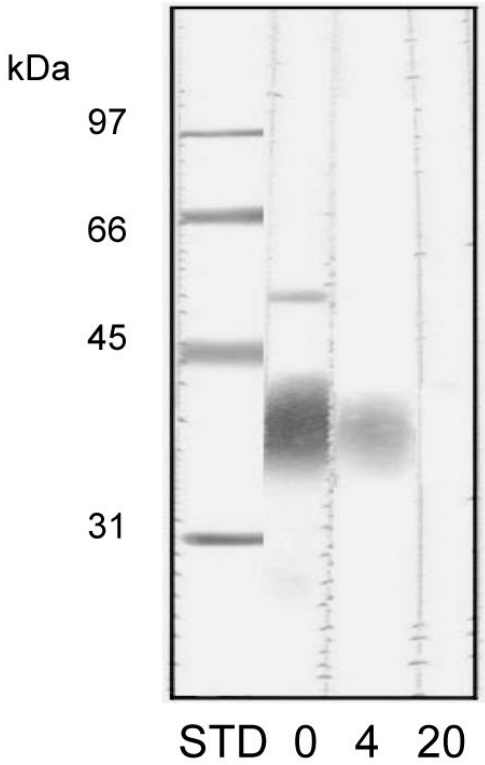

Fig. 4. GalNAc-containing glycoconjugates are sensitive to trypsin exposure

Lysates of *P. arabicus* midguts were incubated with bovine trypsin and analysed by SDS PAGE and Western Blot with HPA. Two concentrations of trypsin were used: **4** and **20** units. In control (**0**), the extract was mixed with Tris-NaCl buffer only.

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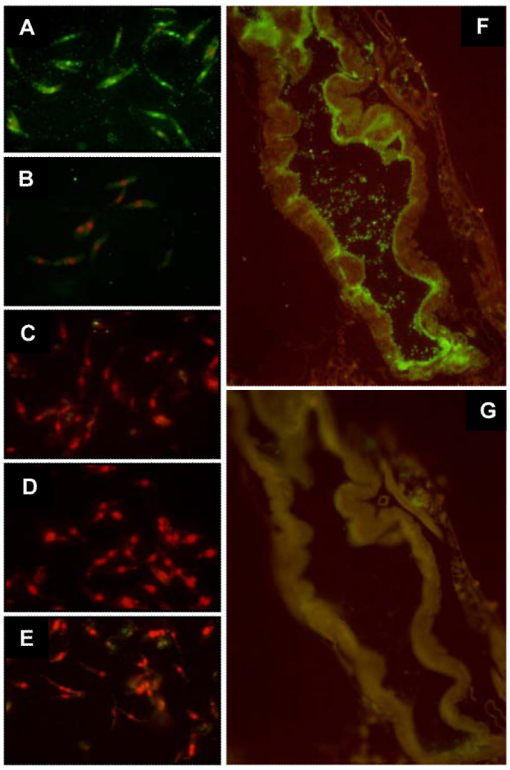

Figure 5. A-E. GalNAc-containing glycoproteins bind to *Leishmania* surface and are present on microvillar surface of the midgut

A, reaction of midgut lysate of permissive species *P. halepensis* visualized by fluorescein-labelled lectin HPA. **B,** the binding of HPA-reactive midgut proteins inhibited by preincubation of *Leishmania* with GalNAc (parasites incubated with 250 mM GalNAc, washed and incubated with midgut lysate followed by FITC-HPA as in panel A. **C,** reaction inhibited by pre-incubation of FITC-HPA with GalNAc. **D,** control without midgut lysate. **E,** as in panel **A** but midgut lysate of specific vector *P. papatasi* was used instead of *P. halepensis*. **F,** section of *Lutzomyia longipalpis* midgut, positive reaction of FITC-HPA, **G,** another section, negative control (lectin preincubated with GalNAc).