Leishmaniasis transmission biology: Role of Promastigote Secretory Gel as a transmission determinant

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2013

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Thesis submitted for the degree of Doctor of Philosophy of the University of London

Funded by:

Abstract

Leishmaniasis is a complex vector-borne disease caused by *Leishmania* sp. (Kinetoplastida: Trypanosomatidae) and is transmitted by female sand flies (Diptera: Psychodidae). It is endemic in 88 countries where the prevalence is 12 million and 350 million people are at risk. The study of vector biology is essential to understand leishmaniasis epidemiology and to implement cost-effective vector control measures.

This thesis aims to describe the role of Promastigote Secretory Gel (PSG) in disease transmission. Transmission is a crucial event in the *Leishmania* life *c*ycle, requiring sufficient infective metacyclic promastigotes to be injected in the host by sand fly bite in a favourable ecotope. PSG is produced by immature *Leishmania* forms within the female sand fly gut and it is comprised of filamentous proteophosphoglycan (fPPG). It is known to modify sand fly behaviour. Here, we investigate its biological role in development and enhancement of transmission by parasite selection using a *Lutzomyia longipalpis-Leishmania mexicana* experimental model.

Briefly, a *Lutzomyia longipalpis* colony was established and maintained at LSHTM and all parasite developmental forms were generated. PSG was obtained from experimentally infected flies. PSG role in development, specifically in attachment and detachment of promastigotes to the midgut, was studied by *ex vivo* competitive midgut binding. The role of PSG in parasite selection was studied by *in vitro* PSG slide attachment, capillary migration and parasite video-tracking. In order to study the underlying binding mechanisms to both PSG and midgut, mutant parasites were used. Ligand analysis was completed by Western Blotting.

PSG was found to favour promastigote detachment from the midgut and thus, parasite migration and maturation. It binds to immature parasitic forms and not to infective metacyclic promastigotes, creating a biological sieve that retains immature forms and enriches the bite for metacyclics. Collectively, the results show that PSG is an essential vector competence determinant allowing successful transmission.

Acknowledgements

First, I would like to thank Matthew Rogers for taking me as his first PhD student and for his guidance during these years. I would also like to thank Mary Cameron for her supervision and Paul Ready for introducing me to sand fly taxonomy. I would also like to acknowledge all the people that help me in one way or another in the completion of this thesis. I thank Rod Dillon and Paul Bates from Lancaster University for kindly donating the eggs to initiate the LSHTM sand fly colony, base of the current thesis. Thanks to Álvaro Acosta-Serrano, from The Liverpool School of Tropical Medicine, for welcoming me in his laboratory. My gratitude to Ross Paveley for his invaluable help with parasite video-tracking and to Emily Giraud, Rachel Gregory and Liz McCarthy, for their help with the CCD microscope. Thanks to Marie Ridoff for her collaboration in the preliminary optimisation assays. Thanks also to Andrew Davies and John Raynes for their assistance with the ultracentrifugation. Thanks to Pat Aiyenuro for kindly donating sand fly cages for the colony and to Carolynne Stanley for helping with the admin. Thank you to the people in the *Leishmania* laboratory, Vanessa Yardley, Katrien Van Bocxlaer, Hollie Burrell-Saward, Karina Corware and Lisa Sanderson. Thanks to my colleagues in 440, Will, Vanessa, Alex, Shahida, Sarah and Ian. And of course, a big thank you to Cheryl Whitehorn and Seth Irish for their friendship and advice.

I am incredibly grateful to my family and friends in Bilbo, Madrid, London and elsewhere. Special thanks to my mum, Esther, Mikel, Lidia, Alain, Valérie, Iñaki, María Jesús and José Alberto for their encouragement. Also to François, Ederne, Mary, Alessandra, Laura C, Belén, Monica, Ainara, Nerea, Izaro, Miren, Alfonso, Aida, Nuria, Garazi, Celia, Laura A, Reyes, Merche, Maruxi, Juanma, Rosa, Inés, Marta, Chari, Angela, Melissa, Shani, Lena, Ghislaine, Halima, Rachel, Marieke, Sophie, Manuela, Karina, Natali, Emma, Felipe and Duncan. Thank you all for being there, for your love and support. Thank you for your visits, phone calls, emails and long pub evenings. You are the best!!!!

Finally, I am greatly indebted to the Basque Government and taxpayers for the financial support that made this PhD possible (fellowship no. BFI09.183).

Thank you! Mila esker bihotz-bihotzez!

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LIST OF ABBREVIATIONS

ACL	Anthroponotic Cutaneous Leishmaniasis
AVL	Anthroponotic Visceral Leishmaniasis
DAT	Direct Agglutination Test
fPPG	Filamentous Proteophosphoglycan
GIM	Grace´s Insect Medium
HIFCS	Heat Inactivated Foetal Calf Serum
GPI	Glycosylphosphatidylinositol
GPILS	Glycoinositol phospholipids
ICT	Immunochromatographic test
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Nets
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
NNN	Novy-MacNeal-Nicolle
OWCL	Old World Cutaneous Leishmaniasis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween™ 20
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment length polymorphism
PCR-RT	Real Time Polymerase Chain Reaction Polymerase Chain Reaction
PGs	Phosphoglycans
PM	Peritrophic membrane
PKDL	Post Kala –Azar Dermal Leishmaniasis
PPGs	Proteophosphoglycans
PSG	Promastigote Secretory Gel
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
sAP	Secretory Acid Phosphatase
SE	Scanning Electron
SEM	Standard Error of the Mean
WHO	World Health Organization
WT	Wild Type
ZCL	Zoonotic Cutaneous Leishmaniasis
ZVL	Zoonotic Visceral Leishmaniasis

1 General introduction:

Leishmaniasis is a complex vector-borne disease caused by a protozoan parasite (Kinetoplastida: Trypanosomatidae) from the genus *Leishmania* (Subgenus *Leishmania* and *Viannia*) and naturally transmitted by female phlebotomine sand flies (Diptera, Psychodidae: Phlebotominae) (**Fig. 1**). Occasionally, non-vector transmission occurs by blood transfusion, needle sharing or congenitally[1]. Leishmaniasis is endemic in 88 countries where the overall prevalence is 12 million and 350 million people are at risk, particularly in developing countries[2]. Published burden estimates place leishmaniasis second in mortality and fourth in morbidity among all tropical diseases and it is associated with 2.4 million disability-adjusted life years[3]. The course of the disease is variable, ranging from skin ulcers (Cutaneous Leishmaniasis, CL) to a lethal systemic disease[4] (Visceral Leishmaniasis, VL) and it is usually fatal in the absence of treatment[5].

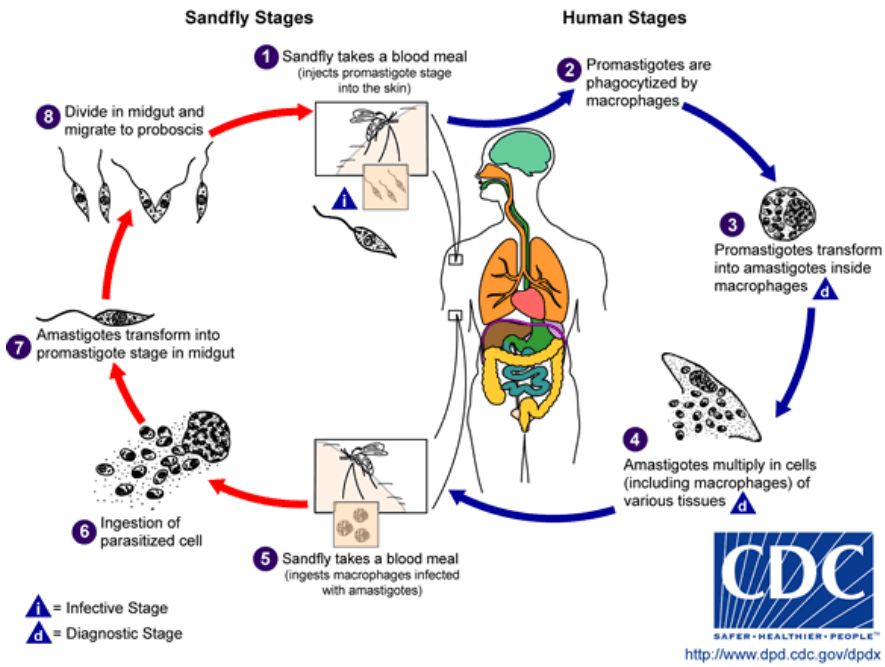

Figure 1 *Leishmania* life cycle (CDC).

The burden of Leishmaniasis is increasing due to HIV, conflicts and disruption of health systems in endemic zones, migration and environmental changes, such as unsustainable urbanisation and deforestation that affect ecological relationships between humans, vectors and reservoirs [2,6,7]. According to the World Health Organization (2007-2011), 0.2-0.4 million cases of VL and 0.7-1.2 million cases of CL occur annually with up to 20000-40000 deaths per year[8]. It is epidemiologically diverse and distributed worldwide with marked regional differences in the vector and parasite species, transmission route, environment, reservoirs and clinical profile[3]. It can be anthroponotic (human reservoir) or zoonotic (animal reservoir), resulting in four major eco-epidemiological profiles: zoonotic and anthroponotic visceral leishmaniasis (ZVL and AVL) and zoonotic and anthroponotic cutaneous leishmaniasis (ZCL and ACL).

1.1 Epidemiology and clinical features:

Visceral Leishmaniasis:

Visceral leishmaniasis occurs in Central and South America, the Mediterranean basin, Central Asia, Indian subcontinent, Middle East and Africa (**Fig. 2**). In Asia and Africa, VL is caused mainly by *Leishmania (Leishmania) donovani* (Kala-Azar) and is transmitted by *Phlebotomus (Euphlebotomus) argentipes* in Asia and *P. (Larroussius) orientalis* and *P. (Synphlebotomus) martini* in Africa[8-11]. Humans act as reservoirs (Anthroponotic Visceral leishmaniasis). In the Mediterranean basin, *Leishmania (L.) infantum* is responsible for VL and it is transmitted mostly by *P. (La.) perniciosus* and *P. (La.) ariasi.* Dogs are the main reservoirs (Zoonotic Visceral Leishmaniasis). In the New World, *L. (L.) infantum*(syn *L. chagasi*) is the causative parasite and *Lutzomyia (Lutzomyia) longipalpis* is the main vector[9-11] with dogs, foxes and jackals as reservoirs[6].

Cells of the reticuloendothelial system are the target of the parasite, causing fever, weight loss, hepatosplenomegaly and pancytopaenia with anaemia, thrombopaenia and immunosuppression. Lymphadenopathy is common in Sudan and hyperpigmentation is described in Indian patients with prolonged disease (kala Azar, "black fever" in Hindi)[12]. Occasionally, a cutaneous form of the disease appears, usually post-treatment, with multiple nodular lesions, especially on the face, called Post Kala Azar Dermal Leishmaniasis (PKDL)[13]. PKDL is unpredictable and variable, occurring in 50% of treated patients in East Africa and 5-15% of treated patients in India [8].

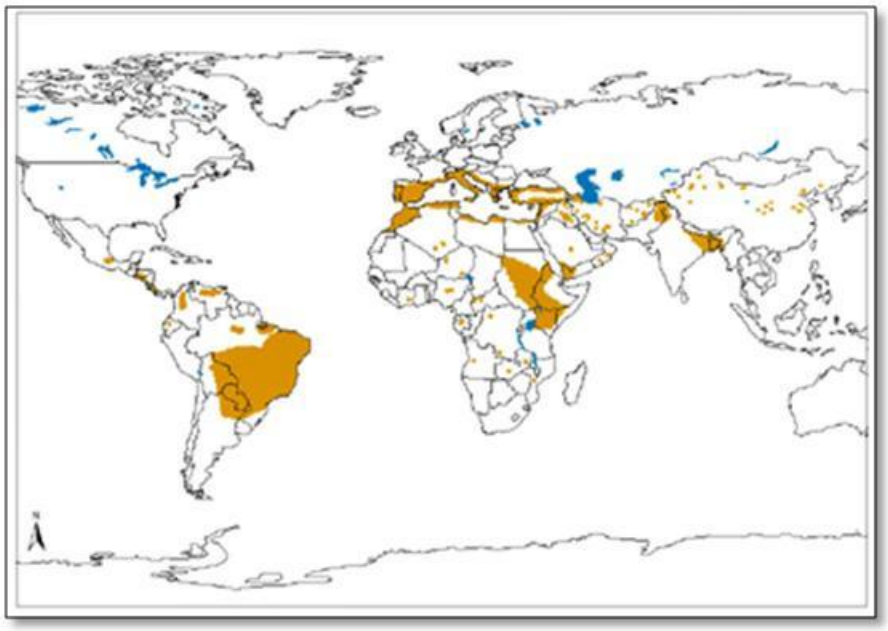

Figure 2 Geographic distribution of visceral leishmaniasis (WHO, October 2010).

Cutaneous and mucocutaneous leishmaniasis:

Old World Cutaneous Leishmaniasis (OWCL) occurs mainly in North Africa, the Mediterranean basin, the Middle East, the Indian Subcontinent and Central Asia (**Fig. 3**). It can be anthroponotic (ACL) or zoonotic (ZCL), where rodents are reservoirs. ACL is mostly caused by *L. (L.) tropica* and transmitted among others by *Phlebotomus (Paraphlebotomus) sergenti*. In Sub-Saharan Africa, it is transmitted by

P. (La.) guggisbergi. ZCL is mainly caused by *L. (L.) major,* and *P. (Phlebotomus) papatasi* and *P. (P.) duboscqi* are the primary vectors[9-11]. It can be localised to skin, causing a painless papule that evolves to an ulcer that heals spontaneously often leaving a scar. The lesion can be nodular instead of ulcerative. Secondary bacterial infections are common.

Occasionally, lesions can be diffuse or disseminated and be accompanied by lymphangitis. Diffuse OWCL is mainly seen in Africa and is caused by *L. (L.) aethiopica* and transmitted by *P. (La.) longipes* and *P. (La.) pedifer*[9-11]. Rarely, OWCL can affect mucosal tissue[11,14].

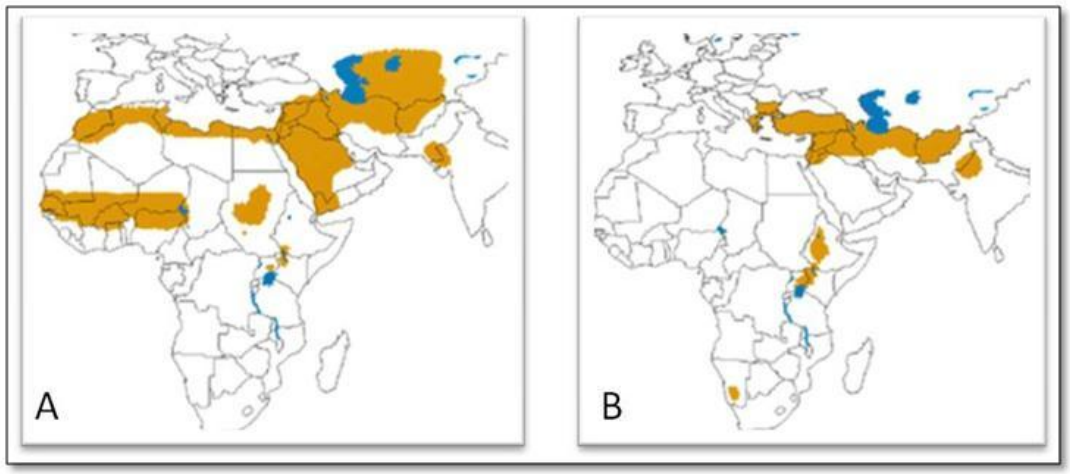

Figure 3 Geographic distribution of cutaneous leishmaniasis in the Old World due to **A/** *Leishmania tropica*, *L. aethiopica* and related species; **B/***Leishmania major*. (WHO, October 2010).

New World or American Cutaneous Leishmaniasis occurs in Central and South America, mainly in Bolivia, Brazil and Peru (**Fig. 4**). It is caused mostly by *L. (Viannia) braziliensis* followed by *L. (V.) guyanensis*, *L. (V.) panamensis, L. (L.) amazonensis and L. (L.) mexicana*. *Lutzomyia (Psychodopygus) wellcomei*, *Lu. (Nyssomyia) whitmani, Lu. (Ny.) trapidoi*, *Lu. (Ny.) flaviscutelata*, *Lu. (Ny.) olmeca olmeca* are vector sand flies among others[9-11,15]. A wide variety of rodents and big mammals such as marsupials, monkeys and edentates, act as reservoirs [6,16]. The clinical profile is the same as described for OWCL.

Some strains of these parasites, especially *L. (V.) braziliensis,* can affect oral and nasal mucosae, causing disfiguring mucocutaneous[10,11] lesions or Espundia [17].

Figure 4 Geographic distribution of cutaneous and mucocutaneous leishmaniasis in the New World (WHO, October 2010).

1.2 Diagnosis and treatment:

Apart from the clinical features of disease, both parasitological and immunological techniques are used for diagnosis. Clinical differential diagnosis includes tuberculosis, carcinoma and dermatomycoses in cutaneous leishmaniasis and malaria, syphilis, tuberculosis, typhoid fever, brucellosis, histoplasmosis and schistosomiasis in visceral leishmaniasis[1].

Parasitological techniques for *Leishmania* detection comprise direct microscopic examination of Giemsa stained skin biopsies, scrapings and impression smears (cutaneous and mucocutaneous) and aspirates from lymph nodes, bone

marrow, liver and spleen (visceral). Highest sensitivity is obtained with spleen aspirates (95%) but this technique is invasive and carries a risk of spleen rupture. Microscopy is still the gold standard due to its high specificity but sensitivity is low and variable[1,17]. To increase sensitivity, parasites can be grown in culture media (NNN medium) and immunochemistry techniques can be performed.

For both *Leishmania* detection and identification, amplification of *Leishmania* DNA by Polymerase Chain Reaction (PCR,PCR-RT,PCR-RFLP) has been proved to be a good diagnostic method but it is expensive for developing countries and technically demanding[17]. Molecular techniques are also used to quantify parasite load, treatment monitoring, determination of virulence or drug resistance and parasite tracking in epidemiology. The main problem with these techniques is their wide range of targets and thus, the lack of standardization and quality control[1]. The most common targets include kinetoplast DNA (kDNA) and small subunit ribosomal RNA (SSU rRNA) gene[17].

Immunological techniques include Montenegro-Leishmanin Skin test (Delayed Type Hypersensitivity), antigen detection in urine (by latex agglutination), serodiagnosis by indirect immunofluorescense and ELISA. Rapid methods such as rK39 ICT (Immunochromatography) and DAT (Direct Agglutination Test)[18] are also used.

First-line treatment for visceral leishmaniasis are pentavalent antimonials (Sodium Stibogluconate-Pentostam® and Meglumine antimoniate-Glucantim®), Amphotericin B (Fungizone® and its liposomal formulation AmBisome®) and pentamidine[19]. Despite their toxicity, antimonials are widely used, but treatment failure has been reported especially in Bihar (>60%) and Sudan[20,21]. For CL, pentavalent antimonials are the first choice and miltefosine, topical paramomycin, imiquimod or antifungal azoles are also used[19].

1.3 Disease control:

Vector borne parasitic diseases are epidemiologically complex with intricate and heterogeneous parasite-vector-host interactions. Accurate epidemiological data is essential for the implementation of control measures, often difficult to obtain in Neglected Tropical Diseases[8]. It is crucial to comprehend sand fly-*Leishmania*-host interactions to understand disease transmission[22] and therefore be able to prevent and control the disease. Control measures need to be cost-effective and environmentally sustainable[9].

Unfortunately, there is no mass drug or vaccination available for human leishmaniasis control[4]. Current control measures include early diagnosis and treatment of cases, integrated vector management, disease surveillance, control of reservoirs and education[2,12].

Leishmaniasis control is complicated due to the geographic diversity of vectors, parasites and reservoirs and the limitation to identify breeding and resting sites[11]. Most of the species are nocturnal andcrepuscular and they feed and rest outdoors in humid and cool places (i.e. they are exophagic and exophilic). Larvae are terrestrial and they need humidity and organic matter[11]. This poor characterisation of breeding sites makes the control of larval sources unfeasible, unlike in other vector borne diseases, such as malaria. Exceptionally, in some species, destruction of rodent burrows reduces rodent population and sand fly populations associated with them[11]. Insecticide impregnated dog collars protect dogs and have an added community benefit[23]. There are two canine vaccines available in BraziI, and a new canine vaccine has been recently commercialised in Europe[24,25]. Indoor Residual Spraying (IRS) of houses and animal shelters and Insecticide Treated Nets (ITNs) are used with fairly different success rates due to variations in vector behaviour or cultural habits[26]. Currently, insecticide resistance is not reported as a serious problem for sand flies with just a few exceptions with DDT in India[9].

Alternative tools such as use of sugar baits containing insecticides, cultivation of plants noxious to sand flies and pheromone driven insecticide targets are presently under investigation[9]. There is also some evidence of the reduction of sand fly populations using entomopathogenic fungi[27]. For sylvatic and occupational transmission, personal protection with repellents is the best option.

Sand fly control is key for Leishmanias control and it also entails the reduction of biting nuisance, control of Carrion´s disease (*Borrelia bacilliformis*) and arboviruses transmitted by sand flies in endemic areas [9].

2 Sand fly vectors

2.1 Vector identification and incrimination

As with other vector borne diseases*, Leishmania* fitness is intimately linked to the fate of the sand fly[28]. Biological, environmental and behavioural factors determine vector-parasite relationship dynamics. Leishmaniasis transmission requires both specificity and co-evolution /co-adaptation of these elements[9].

Female sand flies are the natural vectors of *Leishmania,* although interestingly, *Forcipomyia* (*Lasiohelea)* spp. day bitting midges (Diptera: Ceratopogonidae) have been implicated recently in the transmission of Australian *Leishmania* CL in North Australia among red kangaroos[29]. So far, 20 species of *Leishmania* are responsible for disease in humans[17,30,31]. Approximately 1000 species of sand flies have been described worldwide[30] (except New Zealand and Pacific Islands) and divided into six genera*: Chinius, Phlebotomus* and *Sergentomyia* in the Old World; and *Lutzomyia*, *Warileya*, *Brumptomyia* in the New World [9]. Among these, only 32 species are proven vectors of leishmaniasis[10,11] and a further 70 are suspected vectors[9,31]. All proven vectors belong to either *Phlebotomus* or *Lutzomyia* genus. Non-vector species do not support parasite development and/or lack natural contact with humans and/or reservoirs[30].

Identification and incrimination of sand flies is a difficult task due the complexity of their biology and taxonomical features. Since Killick-Kendrick[10,11] , few reviews about sand fly vectors have been made with the exception of Galati et al [32] and a recent comprehensive review by Ready[9] (2013) . Morphology is still the method most widely used for sand fly identification, despite the lack of standardised literature[9] and the impossibility of accurately identifying female specimens of some species (especially in the Neotropics). Alternatively, isoenzymatic and molecular techniques can be used but they do not allow species identification by themselves, requiring the combination of different techniques and expertise for species identification[16]. Cytological, morphometric, alloenzymatic and molecular techniques have had different impacts on sand fly taxonomy compared to mosquitoes and blackflies[9,33]. Morphological identification focuses mainly on sand fly genitalia, pharyngeal armature, antennae and wing venation (for neotropical species) (**Fig. 5**). Recently, new taxonomic features have been included such as the forms of the glands at the base of the spermathecal ducts and the genital atrium armature[9].

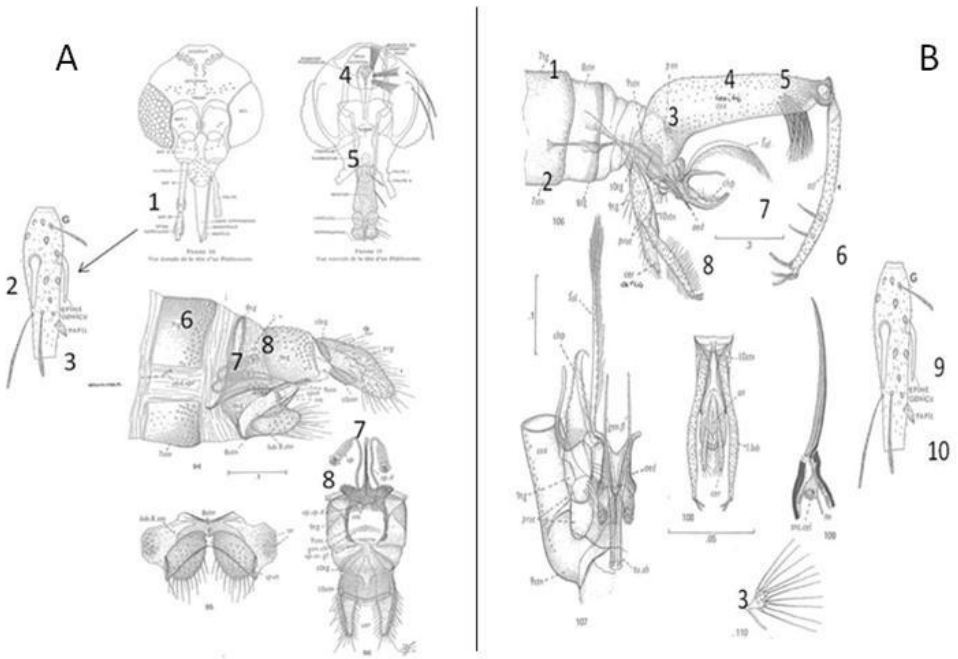

Figure 5 A. Summary of morphological structures examined in female sand flies: **1.** antennal formula, **2.** ascoids, **3.** papilla, **4.** pharyngeal armature, **5.** cibarium, **6.** hairs and socket of abdominal segments 2-6, **7.** spermathecal body segments and **8.** spermathecal

neck and head. **B.** Summary of morphological structures examined in male sand flies: **1.** hairs and sockets of abdominal segment 2-6, **2.** genital filament and pump, **3.** basal process, **4.** coxite, **5.** coxite hairs, **6.** style, **7.** parameres, **8.** aedeagus, **9.** ascoid and **10.** papilla. Adapted from Abonnenc, 1972 and Jobling, 1997 [34,35]

In his latest review , Ready[9] revisited incrimination criteria by Killick-Kendrick[10,11], incorporating molecular and modelling criteria. Updated criteria for medically important vectors are the following:

I.	Promastigotes are isolated and/or typed frequently from several females of the same sand fly species when bloodmeal digested.
II.	Infective parasite forms are found in the anterior midgut and stomodeal valve of naturally or experimentally infected flies.
III.	A fly species is attracted to and bites (both) humans and reservoir hosts.
IV.	Ecological association between fly, humans and any reservoir hosts.
V.	Experimental transmission from a reservoir host.
VI.	Mathematical modelling retrospectively demonstrates that the vector (alone or in combinantion with others) is key for maintaining transmission*.
VII.	Mathematical modelling demonstrates that, based on a control programme, disease incidence significantly decreases following a decrease in the biting intensity of the vector*.

Table 1 Incrimination criteria
**None of the vectors decribed so far fulfills the last two requirements[9].*

For vector incrimination, demonstrating sand fly infection with *Leishmania* (by direct observation and PCR) is essential but the percentage of infected flies in the wild is low[36], requiring a large number of flies for epidemiological studies[37]. Moreover, sand flies can be carriers of the parasite and not vectors, due to the inability of the parasite to carry out metacyclogenesis[36]. In that regard, development of stage specific parasite markers could help overcoming this barrier. Unlike malaria, there is no prophylaxis available, limiting human-landing studies and thus host determination and biting behaviour[9]. Alternatively, blood meal identification (ELISA, PCR) is used for host preference analysis[38,39].

According to these incrimination criteria, sand fly vectors are divided into two categories: proven and suspected vectors. Proven vectors are by definition, anthropophilic sand fly species infected with the same parasite found in both humans and reservoirs, i.e sand fly species that fullfill incrimination criteria (1-4[9]). Suspected vectors on the other hand, are those with compatible geography and inconclusive epidemiological evidence such as proven vector elsewhere or infection with unconfirmed parasite species[16] and they fulfill some of the classical incrimination criteria but not all[9]. Vectors vary greatly between continents, countries or even foci. Killick-Kendrick[10,11] and later Ready[9] reviewed proven and suspected sand fly vectors from both Old and New world. Proven and suspected vectors are summarised below (tables adapted from Ready[9]):

Table 2 *Leishmania* species and their vectors in the Old World[9-11]

Parasite	Proven vectors species	Suspected vectors
Leishmania (L.) donovani	*Phlebotomus (Euphlebotomus) argentipes*	*P. (Synphlebotomus) celiae*
	P. (Larroussius)orientalis	*P. (Paraphlebotomus) alexandri*
	P. (Sy.) martini	*P. (Adlerius) chinensis s.l.*
		P. (Sy.) vansomereneae
L. (L.) infantum	*P. (La.) ariasi*	*P. (La.) langeroni*
	P. (La.) perniciosus	*P. (La.) kandelakii*
		P. (La.) perfiliewi
		P.(La.) longicuspis
		P. (La.) major
		P. (La.) smirnovi
		P. (La.) tobbi
		P. (Ad.) species
L. (L.) tropica	*P. (Pa.) sergenti*	*P. (Pa.) similis*
	P. (La.) guggisbergi	*P. (Pa.) species*
	P. (Ad.) arabicus	
L.(L.) major	*P. (Phlebotomus) papatasi*	*P. (P.) salehi*
	P. (P.) dubosqi	*Sergentomyia garnbami*
		S.(Spelacomyia) darlingi
L. (L.) aethiopica	*P. (La.) longipes*	*P. (La,) aculeatus*
	P. (La.) pedifer	*P. (Pa.) species*

Table 3. *Leishmania* species and their vectors in the New World [9-11,15]

Parasite	Proven vector species	Suspected vectors
Leishmania (L.) chagasi/infantum	*Lutzomyia (Lutzomyia) longipalpis s.l.*	*Lu. (Lu) almerioi*
		Lu. (Lu.) cruzi
		Lu. (Pifanomyia) evansi
L. (Viannia) lainsoni	None	*Lu. (Trichophoromyia) ubiquitalis*
		Lu. (Pf.) nuneztovari
L. (V.) shawi	None	*Lu. (Nyssomyia) whitmani*
L. (V.) colombiensis	None	*Lu. (Helcocyrtomyia) hartmanni*
L. (V.) guyanensis	*Lu. (Ny.) umbratilis*	*Lu. (Ny.) anduzei*
		Lu. (Ny.) whitmani
		Lu. (Ny.) shawi
L. (V.) braziliensis	*Lu. (Psychodopygus) wellcomei*	*Lu. (Ps.) carrerai*
	Lu. (Ny.) neivai	*Lu. (Ny.) intermedia*
	Lu. (Ny.) whitmani	*Lu. (Migonei) migonei* s.l.
	Lu. (Pf.) ovallesi	*Lu. (Pf.) townsendi* s.l.
		Lu.(He.) pescei s.l.
		Lu. (Ps.) panamensis
L. (V.) panamensis	None	*Lu. (Ny.) trapidoi*
		Lu. (Ny.) ylephiletor
		Lu. (Ny.) edentula
		Lu. (Trycholateralis) gomezi
L. (L.) mexicana	*Lu. (Ny.) olmeca olmeca*	*Lu. (Ny.) olmeca bicolor*
		Lu. (Dampfomyia) anthophora
		Lu. (Trycholateralis) diabolica
L. (L.) amazonensis	*Lu. (Ny.) flaviscutellata*	*Lu. (Ny.) olmeca nociva*
		Lu. (Ny.) olmeca reducta
		Lu. (Lu.) longipalpis
L. (V.) peruviana	None	*Lu. (He.) peruviensis*
		Lu. (Pf.) verrucarum
L. (L.) venezuelensis	None	*Lu. (Ny.) olmeca bicolor*
L. (V.) naiffi	None	*Lu. (Ps.) ayrozai*
		Lu. (Ny.) trapidoi

2.2 *Leishmania* development in the fly (**Fig. 6**)

Infection starts when a female sand fly bites and takes **amastigotes** along with the bloodmeal from an infected host. Females of most sand fly species require blood for the production of eggs (anautogenous); species differ in the number of blood meals taken per gonotrophic cycle[9,11]. Sand flies are batch-feeding insects that create a haemorraghic pool with their saw-like mouthparts. Previous studies by Killick-Kendrick et al[40,41] described the development of *Leishmania* promastigotes from these initial amastigotes within the sand fly midgut, pointing out morphogical changes as parasites migrate forward to the foregut of the sand fly[42]. Migration resulted in highly motile slender forms, called metacyclics, pre-adapted for survival in the mammalian host[31,43,44] and therefore the infectious form[40,42]. Metacyclics were further characterised *in vitro*[45] and *in vivo*[42] using monoclonal antibodies. Extensive research in the following years, culminated in a concise description of promastigote developmental forms. Five distinct promastigote stages have been characterised within the sand fly midgut: procyclic, nectomonad, leptomonad, haptomonad and metacyclic promastigotes[46].

Most parasites follow a suprapylarian development within the sand fly, confining their development to the midgut with the exception of species belonging to *Viannia* subgenus, that are peripylarian, entering the hindgut region before migrating forward[44]. A third group of *Leishmania* parasites belonging to the subgenus *Sauroleishmania,* which are non-pathogenic for humans *("*lizard *Leishmania")* are hypopylarian, confining their development to the hindgut[47]. The overall time for full development is 6-9 days depending on the species[44] and environmental conditions[48]. It is essential for *Leishmania* parasites to overcome sand fly defensive barriers to complete their development[49]. The first 48 hours of development are essential for the establishment of transmission, when up to 50%-80% of the parasites are lost[22,46]. In the first 12-18 h, in the posterior midgut, ovoid amastigotes transform into flagellated and poorly motile **procyclic promastigotes;** motility is crucial in parasite development[31]. Procyclic promastigotes multiply within the peritrophic membrane (PM). The PM consists on a meshwork of proteins and chitin secreted by the midgut epithelium which encloses and partially protects the

bloodmeal from digestive proteases, but at the same time, prevents promastigotes migrating anteriorly[44,50,51]. Coping with the digestive enzymes of the fly is the first challenge that parasites encounter to continue with their growth. Midgut proteases peak in the first 18-48 hrs and jointly with lectins, impair promastigote survival. Parasites seem to prevent parasite agglutination by sand fly lectins by an unknown mechanism[22,52,53] and also inhibit and retard the peak activity of midgut proteases[22,44,54]. After 2-3 days, procyclic promastigotes develop into numerous elongated forms called **nectomonad promastigotes.** Their objective is to escape from the PM, migrate forward and attach to the midgut, to prevent being eliminated by defecation. Parasites and sand flies secrete chitinases to break through the PM[50] and a myoinhibitory peptide is secreted by the parasites to reduce sand fly gut peristalsis and delay defecation. Interestingly, PM degradation begins from the anterior and posterior ends[55] and nectomonds often accumulate in the anterior pole of the PM[46,50], which will favour their migration and as a result, midgut attachment and survival. Attachment is a key event in leishmaniasis biology to resist being defecated from the fly[44,56]. Following detachment from the sand fly epithelium, nectomonads migrate forwards to the thoracic midgut and stomodeal valve. Here they transform into the next cell stage, the **leptomonad promastigotes.** Leptomonad promastigotes (4-7 days) multiply vigorously within the thoracic midgut and produce a viscous gel called Promastigote Secretory Gel (PSG), made from a secreted form of proteophosphoglycan[57]. Coincident with anterior midgut colonisation is the appearance of **haptomonad promastigotes** which attach *via* hemidesmosomes to the stomodeal valve damaging its epithelium and initiating the blocking for transmission. It is unclear whether nectomonads or leptomonads are the precursors of haptomonad promastigotes[46,58]. Finally, leptomonad promastigotes terminally differentiate within the PSG into **metacyclic promastigotes**, the infective highly motile and non-dividing forms that accumulate heavily in the cardia region behind the stomodeal valve. Metacyclics are regurgitated and injected by bite along with the PSG and sand fly saliva[46].

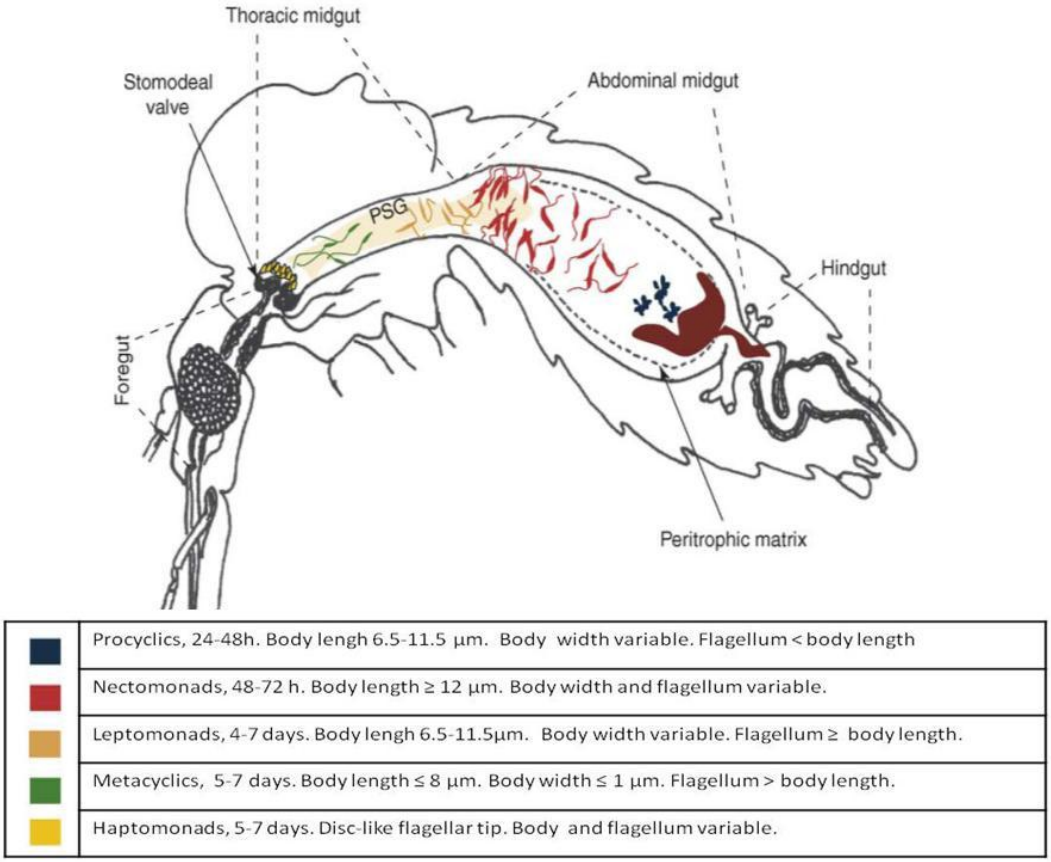

Figure 6 Suprapylarian parasite development within the sand fly, depicting different promastigote developmental forms (in colours), blood meal, peritrophic matrix, PSG and sand fly gut regions (Modified from Rogers et al. and Kamhawi et al[44,46]).

2.3 Vectorial competence

Some sand fly vectors support the full development and successfully transmit several *Leishmania* species ("**Permissive vectors"**) while others are just vectors of one particular species and refractory to the rest ("**Specific/Restrictive vectors"**), even if other species coexist in the same environment[59,60]. A third category, "**Intermediate vectors"** was recently introduced by Volf[61], for those sand flies that support the development of many but not all of the species tested. This classification is mainly based on experimental combinations and might be a simplification of parasite-vector relationships but it is a useful tool for further studies on vectorial competence[61].

The mechanisms responsible for controlling vectorial competence and threfore sand fly susceptibility to *Leishmania* infection and transmission are not well understood[62]. Anatomical, biological and behavioural factors are believed to determine vectorial competence which seems to be under genetic control[60]. In that regard, much research is being carried out to identify the molecules that allow parasites to overcome and survive in sand fly midgut hostile environment. It is essential for *Leishmania* development within the sand fly to manipulate defensive barriers. In this struggle for survival, midgut binding seems to be crucial and therefore key for vectorial competence[44,56].

Early competence experiments were performed by Pimenta et al[63], comparing survival of several *Leishmania* species (*L. donovani*, *L. major*, *L. amazonensis* and *L. tropica*) in both *P. papatasi* and *P. argentipes* after full blood meal digestion. *P. papatasi*, was found to be a restrictive/specific vector, supporting only the full development of *L. major*, whereas in *P. argentipes*, a permissive vector, all the species survived. These findings were later confirmed by Kamhawi et al[59]. *P. sergenti* was also described as a restrictive vector, allowing just the development just of *L. tropica* and not *L. donovani* nor *L. major*[59]. Interestingly , *P. duboscqi*, a sister species of *P. papatasi* and also a natural vector of *L. major*, supports the development of *L. tropica* experimentally[64] but not *L. infantum*[65].

Besides *P. argentipes*, *Lu. longipalpis, P. arabicus, P. halepensis* and *P. perniciosus* are also described as permissive vectors[65,66]. *Lu. longipalpis* is a widely permissive vector, supporting not only *L. chagasi*/*infantum* but also *L. major*[66], *L. mexicana*[46] and *L. amazonensis*[67]. *P. arabicus,* recently incriminated as a *L. tropica* vector[68], allows full development of *L. major* experimentally[66]. *P. halepensis*, fully supports *L. major* and *L. tropica* experimentally but its vector role remains unclear[69]. And finally, *P. perniciosus* vector of *L. infantum,* also allows full development of *L. major*[65].

Table 4 Specific and permissive sand fly species and *Leishmania* species they support :

.

SPECIFIC VECTORS	PERMISSIVE VECTORS
Phlebotomus sergenti[59] - *L. tropica*	*P .argentipes*[63] - *L. donovani* - *L. major* - *L. amazonensis* - *L. tropica*
Phlebotomus papatasi[59,63] - *L. major*	*P. halepensis*[69] - *L. major* -*L. tropica*
Phlebotomus duboscqi[22,64,65*] - *L. major*	*P. arabicus*[66,68] - *L. major* - *L. tropica*
	Lu. longipalpis[46,66,67] - *L. chagasi/infantum* - *L. mexicana* - *L. major* - *L. amazonensis*
	P. perniciosus[65] - *L. infantum* - *L. major*

* *P. duboscqi* does not support *L. infantum* but supports *L. tropica*. It has been described in the literature as both a permissive[22,64] and specific vector[65].

A competent vector will become epidemiologically relevant when it has a critical vectorial capacity[9]. Vectorial capacity is defined quantitatively and it is influenced by vectorial density and longevity in nature[70]. It depends on vectorial competence –which refers to intrinsic (genetic) ability of a vector to transmit a pathogen- but also environmental and behavioural factors that influence pathogen-vector-reservoir-host interactions[9,70]. Vectorial competence must be accompanied by frequent biting of reservoirs and human hosts within a favourable ecotope[24]. Existence of permissive vectors is *per se* epidemiologically significant due to their potential role in the emergence of new foci of infection[31]. The best known example of establishment of new foci is perhaps the introduction of *L. infantum* in the New World, successfully transmitted by *Lu. longipalpis*[31,66].

3 *Leishmania* promastigote binding to the sand fly midgut

3.1 *Leishmania* glycoconjugates and midgut binding

In order to survive and successfully develop within the sand fly midgut, parasites are believed to express and secrete glyconconjugates[71,72]. The main glycoconjugate of *Leishmania* is lipophosphoglycan (LPG), which covers the whole parasite surface including the flagellum[22]. LPG is formed by a linear phosphoglycan chain with repetitive [Gal-Man-PO(4)] phosphosaccarides and terminating cap structures linked to parasite surface *via* a glycosylphosphatidylinositol (GPI) anchor[22,63,73-75]. GPI anchored molecules also include GIPLs (glycoinositol phospholipids) ,GPI-anchored proteins such as gp63 metalloproteinase or Leishmanolysin (**Fig. 7**) and membrane bound proteophosphoglycan (mPPG). Other parasite glycoconjugates are secreted PPG, phosphoglycans (PGs) and secretory acid phosphatase (sAP)[71,72,76] (**Fig. 8**). These glycoconjugates have been described in all *Leishmania* species studied so far[77], however, sAP is secreted to a much lesser extent in *L. major*[65,77] compared to other species.

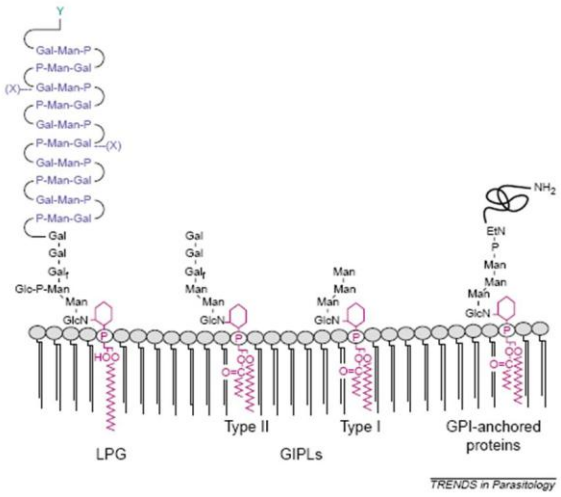

Figure 7 LPG (lipophosphoglycan), GIPLs glycoinositolphospholipids and GPI-anchored proteins. The lipid anchors are shown in purple and LPG structure in blue. (X) denotes additional sugars subject to intra and interspecies variation. (Y) in green, represents terminal cap structure, which is also variable. Figure adapted from Turco et al[76].

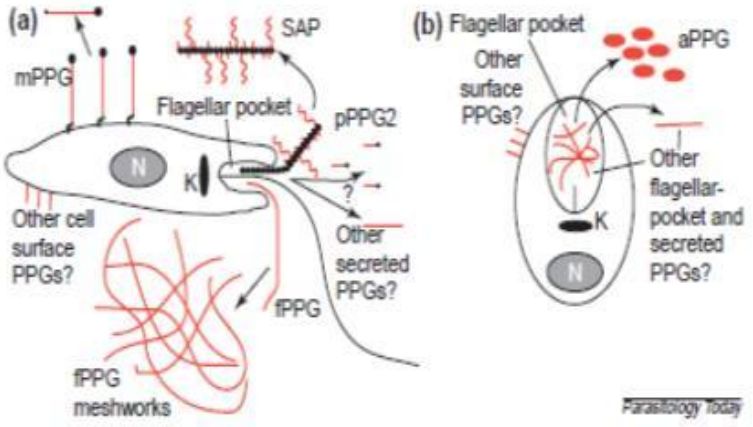

Figure 8 *Leishmania* proteophosphoglycans in promastigotes (a) and amastigotes. SAP fPPG, aPPG and probably pPPG2 are secreted via the flagellar pocket. mPPG isattached to the surface and released slowly. Other surface and secreted PPGs are still uncharacterised. Abbreviations: aPPG, amastigote PPG; fPPG, filamentous PPG; mPPG, membrane bound PPG, promastigote PPG2; K, kinetoplast; N, nucleus; and SAP secreted acid phosphatise. Figure by Ilg, 2000[71].

The specific roles of these glyconcojugates in promastigote early survival and midgut binding have been extensively studied[42,52,59,63,66,71,77-81].

Promastigote midgut binding, in contrast to the binding to cuticle lined gut parts (hindgut, foregut and stomodeal valve), does not occur via flagellar hemidesmosomes[82]. Electron microscopy has shown that parasites insert their anteriorly located flagellum between the midgut epithelium microvilli[31]. However, the lack of ultrastructural changes suggests that this is a receptor-ligand mediated binding[31] assisted by the insertion of the flagellum[22].

Extensive research has concluded that parasite LPG is the best candidate for ligand[22,31,44,77], since its first implication in *L. major* promastigote binding to *P. papatasi* midgut epithelium *in vivo* by Davies et al[42]. Morever, the addition of LPG in midgut binding experiments, completely inhibited promastigote attachment[17,59]. It is believed to allow binding and release of parasites to and from the sand fly midgut[78,83]. Non-LPG glycoconjugates are shown to be involved in survival of parasites within the sand fly conferring protection against midgut proteases and lectins[22,52,53].

In many respects, LPG is functionally and structurally similar to -gram negative- bacteria LPS (LipoPolySaccharide) and crucial for parasite virulence[44,71,84]. Among other functions, it confers resistance to complement-mediated lysis, binds to host macrophages, modulates macrophage signal transduction, confers resistance to the oxidative attack and it is key for the establishment of successful infections[74,78,83,85].

Midgut receptors are still subject of extensive research. For the best interest of the parasite, this binding molecule should be abundant in the midgut as well as essential for fly survival[80,86]. So far lectins, sugar binding proteins, are the best candidates. Lectins or lectin like molecules have been found to date in *Lu. longipalpis* [87,88], *P. duboscqi*[80,87,88], *P. papatasi* [47-50], *P. perniciosus* [87] and *P. halepensis* [87] sand fly species.

The way in which LPG determines binding for different sand fly parasite combinations has been further characterised.

3.2 LPG dependent midgut binding and restrictive vectors

LPG has already been described for several *Leishmania* species: *L. major*[89]; *L. donovani*[81], *L. tropica*, *L. aethiopica*[90], *L. mexicana*[91], *L. chagasi/infantum*[77] and *L. braziliensis*[92]. The lipid anchor and hexasaccharide core are highly conserved while repeating units and cap structures are highly polymorphic[22,63,73] (**Fig. 9)**. It shows both inter and intra-species polymorphism.

LPG is highly substituted in parasites such as *L. major* or *L. tropica*, transmitted by restrictive *P. papatasi* and *P. sergenti,* respectively; and simple in parasites such as *L. donovan*i transmitted by permissive *P. argentipes*[44].

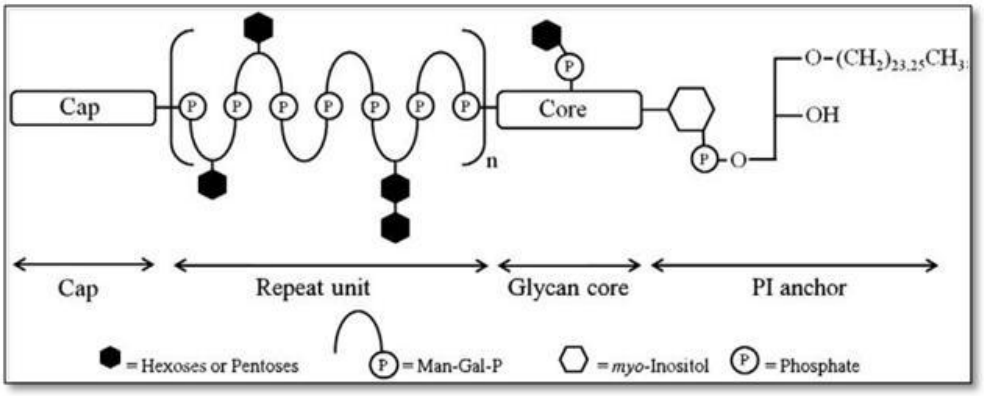

Figure 9 Schematic structure of *Leishmania* LPG with the four domains: conserved phosphatidylinositol-linked lyso-alkylglycerol lipid anchor and glycan core (GPI); and variable phosphorylated dissacharide repeats and cap structure. Figure by de Assis et al[72]. Hexoses or pentoses present in the repeats determine LPG specificity.

Most side chain modifications occur in the C3 hydroxyl group of the galactose residue[77,85] that can be either unsubstituted as in Sudanese *L. donovani* and *L. braziliensis* or partially substituted with glucose residues as in *L. mexicana* and Indian *L. donovani*[81]. In contrast, in *L. tropica* and *L. major*, side chains are almost completely modified with glucose, galactose and arabinose[89]. *L. tropica* has the most complex side chain modifications with over 19 different types of glycans[77,85]. *L. aethiopica*, is the exception with substitution occurring in C2 hydroxyl group of the mannose residue[90] (**Fig. 10**).

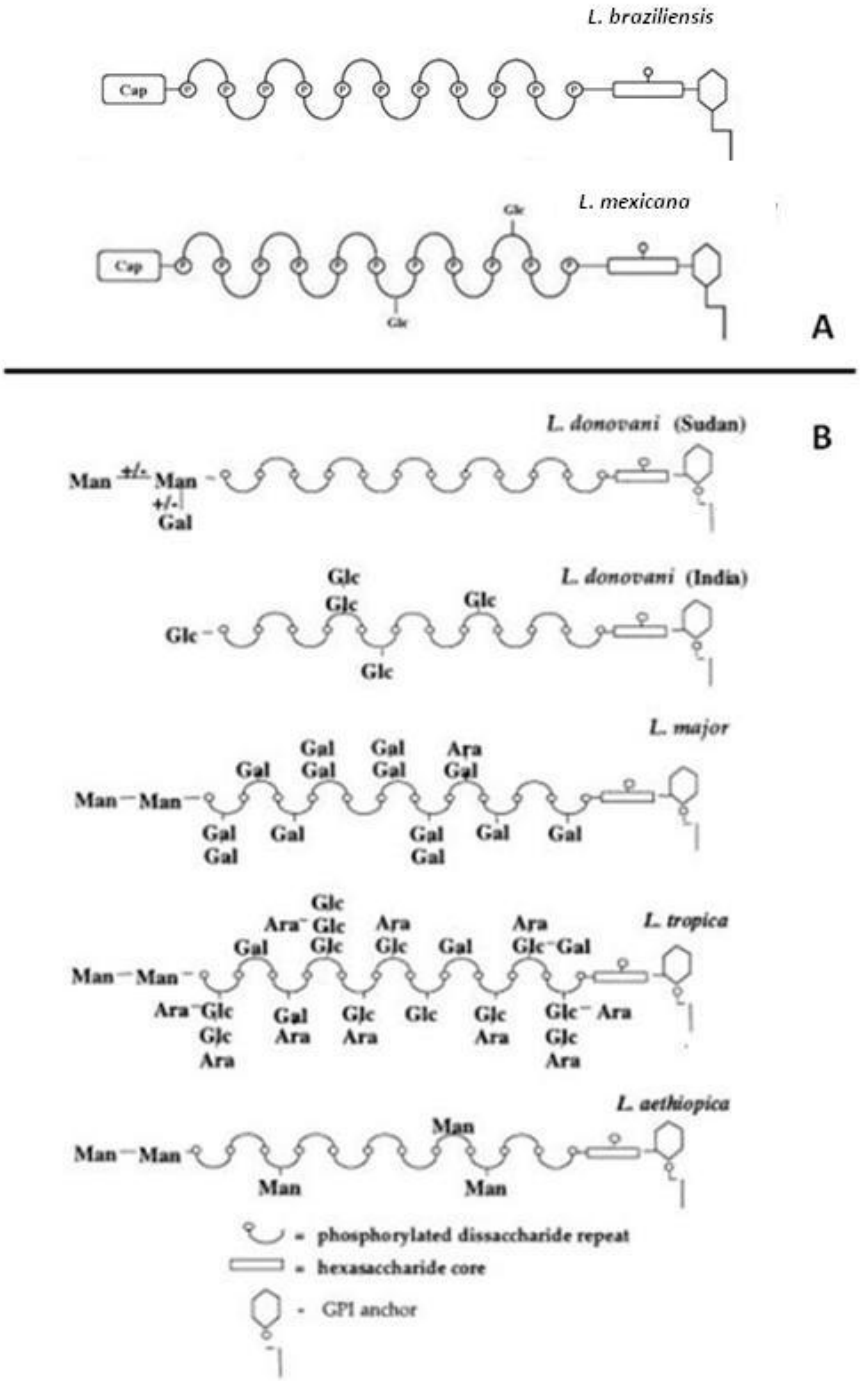

Figure 10 LPG intra and interspecies polymorphism. (**A**) LPG in New World species, *L. braziliensis* and *L. mexicana*[91,92]. Figure adapted from Assis et al[72]. (**B**) LPG in Old World species *L. donovani* Sudan, *L. donovani* India, *L. aethiopica, L. major* and *L. tropica.* Figure from Sacks et al[60]. Note that GPI anchor and hexasaccharide core are conserved in the different species while sugars in phosphorylated disscharide repeats and cap structure differ.

Intraspecies polymporphism includes variations among strains as found in *L. donovani, L. major*, *L. mexicana* and *L. tropica* [60,79,90,91,93] but also among the different developmental forms. LPG is developmentally regulated; metacyclogenesis is accompanied by modifications to LPG structure[22,73,85]. Sacks et al[45,94], described differences between *L. major* metacyclics and non-metacyclic promastigotes *in vitro* using PNA lectin agglutination and monoclonal antibodies later related to a change of composition and expression of repeating phosphorylated saccharide units, almost doubling the expression of repeating units[73]. Metacyclic LPG size was 65-130 KDa, while in the non-metacyclic promastigotes LPG size was 30-65 KDa (**Fig. 11)**. Galactose residues were also found to be substituted with α-arabinose and ß-glucose residues, explaining the lack of binding to PNA displayed by metacyclics[85]. Similar elongation has been found in *L. donovani*. In this case, repeated units remain the same but there are changes in the cap structure, losing the terminal galactose responsible for PNA binding[73]. *L. chagasi* metacyclic LPG was also found to be elongated and to partially lose its side glucose substitutions[77]. In *L. braziliensis* metacyclogenesis resulted in an LPG elongation accompanied by substitution of galactose residues with glucose[92] (**Fig. 12)**. On the other hand, *L. major* amastigotes were found to minimally express LPG molecule, biochemically and antigenically distinct from promastigote LPG, with less repeating saccharide units [64, 85]. LPG was found to be absent in *L. donovani* amastigotes [85]**.**

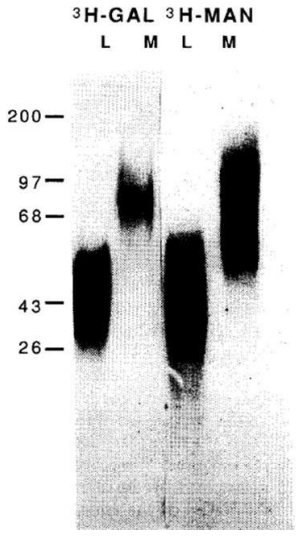

Figure 11. SDS-PAGE analysis of H^3 galactose and H^3 mannose labelled logarithmic phase /non-metacyclic promastigotes (L) and stationary phase/metacyclic (M) promastigotes LPG. (Sacks et al[73] 1990).

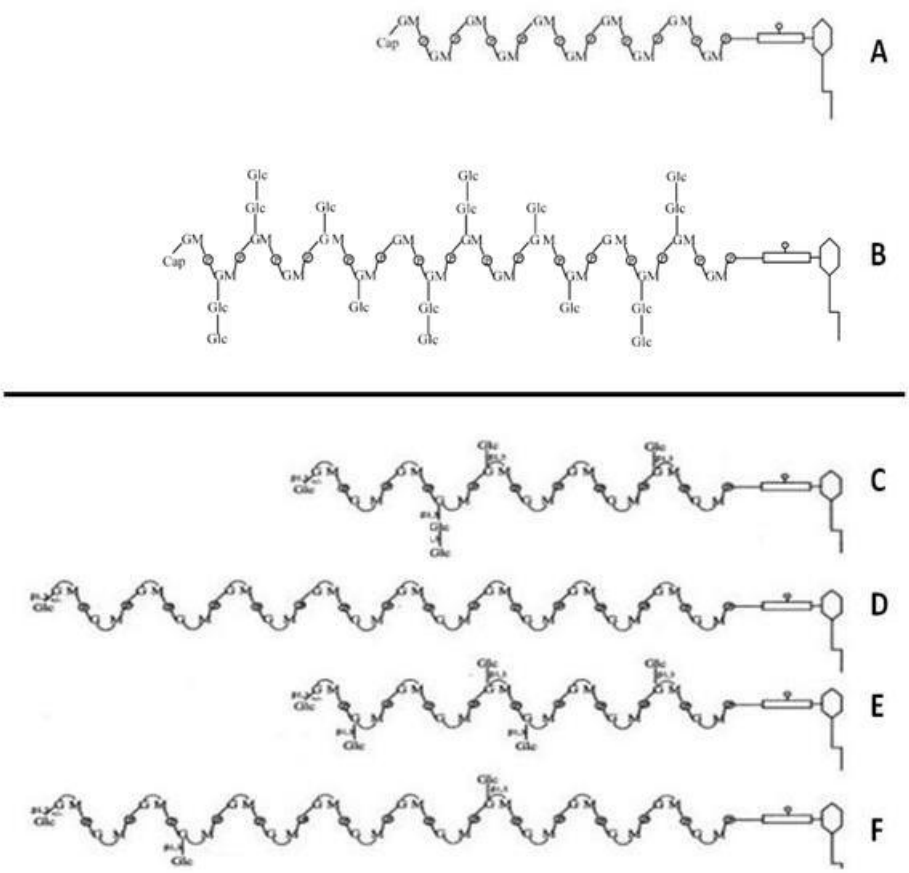

Figure 12. Developmental changes for LPG. LPG in *L. braziliensis* procyclic (**A**) and metacyclic promastigotes (**B**); LPG in Indian *L. donovani* procyclic (**C**) and metacyclic promastigotes (**D**) and in *L. chagasi/infantum* procyclic (**E**) and metacyclic promastigotes (**F**).Abbreviations: GM: Galactose-Mannose. Glc: glucose. Figures from Soares et al, 2005,(A &B)[92] and 2002 (C-F)[77].

This LPG polymorphism was corroborated using SE (scanning electron) microscopy, by Pimenta et al[75], observing in metacyclics a thickening of the cell coat accompanied by densely pack filamentous structure, that was absent in immature non-metacyclic promastigotes (**Fig. 13**). SE imaging was accompanied by stage-specific immunolabelling.

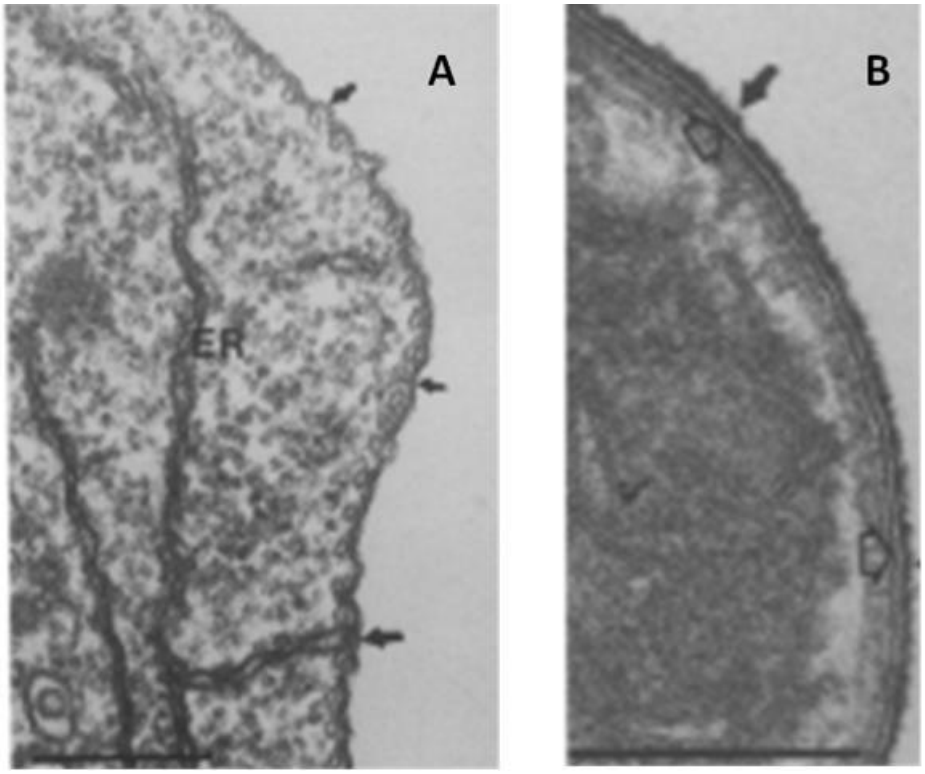

Figure 13 .Promastigote structure by SE microscopy (**A**) SE image of a non-metacyclic promastigote showing a fine cell coat (arrows). ER: endoplasmic reticulum. (**B**) SE image of a metacyclic promastigote showing a thicker cell coat (black arrows) covering the plasma membrane (white arrows). Scale bars: 0.25 µm. Image by Pimenta et al[75].

Competence studies showed that both intra and interspecies LPG polymorphisms determine midgut binding[79]. The repeating units and cap domains of LPG have been shown to be the sites for parasite interactions with its vector[22,77,79-81]. LPG dependent midgut binding has been described to be species-, strain- and stage-specific.

Developmental changes translate into a stage-specific midgut binding [22,42,56,80] that allows the detachment and free movement of mature infective metacyclics to ensure transmission[22,56,86]. Experimentally, metacyclic promastigotes have never been observed attached to sand fly midguts, in contrast to immature promastigotes[44,92]. Immunostaining revealed only non-metacyclic LPG from *L. major* able to bind to *P. papatasi* midgut epithelial cells, with no reaction from amastigote and metacyclic specific markers[42]. In the *L. chagasi-Lu. longipalpis*

combination, Soares et al[77] found by immunofluorescence that phosphoglycans (PGs) from metacyclics did not bind to midgut epithelium while PGs from immature forms did bind. The lack of binding displayed by metacyclics in both *L. major* and the Sudanese strain of *L. donovani* is believed to be related to the loss of galactose residues during metacyclogenesis; on the other hand, the loss of binding in *L. chagasi/infantum* and Indian *L. donovani* is believed to be due to the loss of ß(1,3)glucose residues as a result of metacyclogenesis[77,93,95]. *L. braziliensis* constitutes an exception; peripylarian infective metacyclics attach to the midgut in their long journey to the foregut. Soares et al[95], observed that PGs from both procyclics and metacyclics of *L. braziliensis* attached to open midguts of its natural vectors *L. whitmani* and *L. intermedia*. In this case, elongation and upregulation of the glucose residues of the LPG resulted in sustained binding of metacyclic promastigotes. Binding of promastigotes is believed to be via ß(1,3)glucose residues of the cap structure.

Leishmania species-specific midgut binding seems also to be linked to their LPG structure[79]. Purified LPG from different species was also found to bind differently to both *P. papatasi* and *P. argentipes*, mimicking the results obtained using whole parasites. Only *L. major* was able to bind to *P. papatasi* midguts *in vitro*, while all the species tested bound to *P. argentipes in vitro* [63], reflecting their restrictive and permissive nature. Kamhawi et al [59] continued these LPG-midgut *in vitro* experiments, confirming the specificity of *P. papatasi* and permissivity of *P. argentipes* but also specificity of *P. sergenti*. Interestingly, mutant strains with deficient or atypical LPG did not bind to *P. papatasi* or *P. argentipes*[63]. This suggests that midgut binding is both species and strain-specific, which was later confirmed by Soares et al[79], who found variations in LPG repeat units among *L. tropica* isolates from different sand fly species. *L. tropica* is shown to be very heterogeneous up to the point that some strains are carried by alternative vectors to *P. sergenti*, such as *P. arabicus* in Israel[61,68,79,96,97]. The selection of midgut binding is a dynamic process; Volf et al[98] showed that survival of *L. infantum- L. major* hybrids in *P. papatasi* sand flies was enabled by the expression of *L. major*-like LPG.

In this context, LPG gene deficient lines have proved very useful to further characterise the role of LPG in binding. Important evidence for the role of LPG in *Leishmania*-sand fly interactions has been obtained through the use of specific LPG defective mutants that were unable to attach and sustain infections in the midgut[22,77]. Attachment was re-established when LPG deficiencies were restored by the addition of extrachromosomal copies of the affected genes[22,53], allowing increased survival and development of the restored parasites within the sand fly. In *lpg1*$^{-}$ mutants, LPG expression is selectively affected through the deletion of the LPG1 galactofuranosyl transferase required for the synthesis of the LPG glycan core; while *lpg2*$^{-}$ mutants are defective for all phosphoglycans through deletion of the Golgi GDP-mannose transporter required for the synthesis of the PGs repeated disaccharide units[22,66, 78](**Fig. 14**).

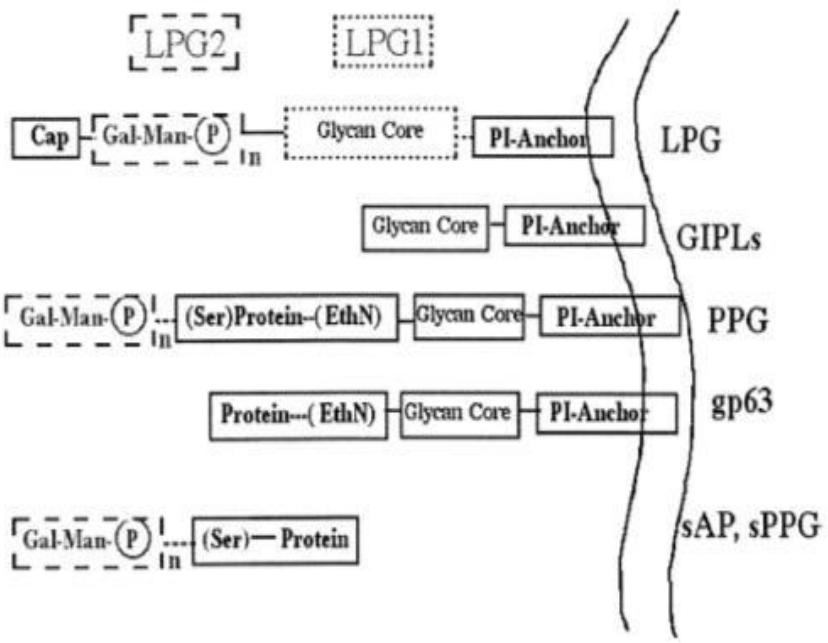

Figure 14 Parasite surface glycoconjugates, showing phosphlycans affected in *lpg1*$^{-}$ (dotted) and *lpg2*$^{-}$ mutants (dashed lines) from Sacks et al [22].

Despite the proven implication of LPG in midgut binding in specific vectors such as *P. papatasi*[42,86] and *P. sergenti*[59], research is still ongoing to identify the

corresponding midgut receptor. In 1999, Dillon & Lane [86] showed that *L. major* purified LPG bound specifically to midgut microvillar proteins by Western Blotting. Five years later, Kamhawi et al[80] identified a galactose binding lectin, named PpGalec, whose expression was upregulated in *P. papatasi* adult females and was abundantly present in the midgut luminal surface. This lectin showed specificity for poly gal (ß1-3) side chains of *L. major* and specific anti-Ppgalec antibodies inhibited midgut binding. Ppgalec was also present in *P. duboscqi*, a proven *L. major* vector, and absent in the restrictive and non-*L. major* vector *P. sergenti*. It was also absent in permissive vectors such as *P. argentipes* and *Lu. longipalpis*.

3.3 LPG independent midgut binding and permissive vectors.

Recently, it has been shown that LPG is not essential for parasite development in all vector-parasite combinations studied[56], nor for establishment of the infection in the mammalian host[84,99]. Therefore, alternative or supplementary binding mechanisms are sought (**Table 4**).

Permissive vectors not only support the development of several *Leishmania* species and strains[72,77] but also allow the development of mutant species. In recent studies, *lpg1*$^{-}$ mutant *L. major* was found to develop in *P. argentipes*, *P. perniciosus*[65], *P. arabicus* and *Lu. longipalpis*[66] and failed to develop in *P. duboscqi*[65]; *L. mexicana lpg1*$^{-}$ successfully develop in *Lu. longipalpis*[57]. *lpg2*$^{-}$ mutants however, were unable to survive. Mutant survival suggests that in these permissive combinations unlike in restrictive combinations, other phosphoglycans will not be important just for early survival but also for attachment. PPGs appear to carry out functions previously reserved for LPG. Interestingly, there are also differences in PPGs between different species and promastigote stages[83].

GalNac (N-acetyl-galactosamine) containing midgut glycoproteins have been suggested as *Leishmania* binding ligands on permissive sand fly guts. These

glycoproteins were found in permissive *Lu. longipalpis*[100], *P. halepensis*, *P. perniciosus*, and *P. arabicus* and were absent in restrictive *P. papatasi* and *P. sergenti*[66]. They were also absent in *P. duboscqi* that does not support mutants or *L. infantum*, but allows development of both *L. major* and *L. tropica* [66].

Table 5 LPG and phosphoglycan requirements for development according to Svarovska et al[65].

Vector (colony origin)	*Leishmania* species	LPG and PGs requirement
P. papatasi (Israel)	*L. major* (natural)	LPG required in late phase
P. papatasi (Turkey)	*L. major* (natural)	LPG required in late phase
P. duboscqi (Mali)	*L. major* (natural)	LPG required in late phase
		PGs required for early phase
P. duboscqi (Senegal)	*L.. major* (natural)	LPG required in late phase
		LPG2 related molecules required from early phase
Lu. longipalpis (Brazil)	*L. major* (unnatural)	LPG independent
		LPG2 related molecules required from early phase
	L. mexicana (unnatural)	LPG independent
P. arabicus (Israel)	*L. major* (unnatural)	LPG independent
P. perniciosus (Spain)	*L. major* (unnatural)	LPG independent
		LPG2 related molecules required from early phase
P. argentipes (India)	*L .major* (unnatural)	LPG independent
		LPG2 related molecules required from early phase
	L. donovani (natural)	LPG possibly required
		PGs required from early phase

The existence of alternative binding mechanisms and the striking diversity of *Leishmania*-sand fly interactions highlight the need to perform more studies about the glycobiology of midgut binding[31] to understand disease transmission.

4 Promastigote Secretory Gel (PSG) and *Leishmania* transmission

4.1 *Leishmania* PSG

In sand flies with mature infections the anterior midgut is blocked by a 3D matrix parasite derived gel[48,49]. It is first visible in the cardia region at day 4 for *L. mexicana* in *Lu. longipalpis* and then expands bidirectionally[46] reaching its peak at day 7 post infection[46,48,49]. This gel, named Promastigote Secretory gel (PSG), is comprised of PPGs secreted by parasites, especially by leptomond promastigotes[46]. Its main constituent is a filamentous phosphoglycan (fPPG), a mucin-like molecule unique to *Leishmania* parasites[48,57]. It is a large and highly glycosylated serine rich LPG-like molecule, composed by [Gal-Man-PO(4)] repeats with a terminal cap-structure linked to a serine rich protein backbone[49,71]. As in LPG phosphoglycans, side sugars can be variably substituted. It is biochemically and morphologically indistinguishable from the fPPG secreted by promastigotes *in vitro*[49,71](**Fig. 15**). In the PSG plug, fPPG is arranged as a mesh-work that surrounds and traps promastigotes[101] (**Fig. 16**). Glycans are responsible for both the matrix like structure and the pathogenicity of fPPG[49].

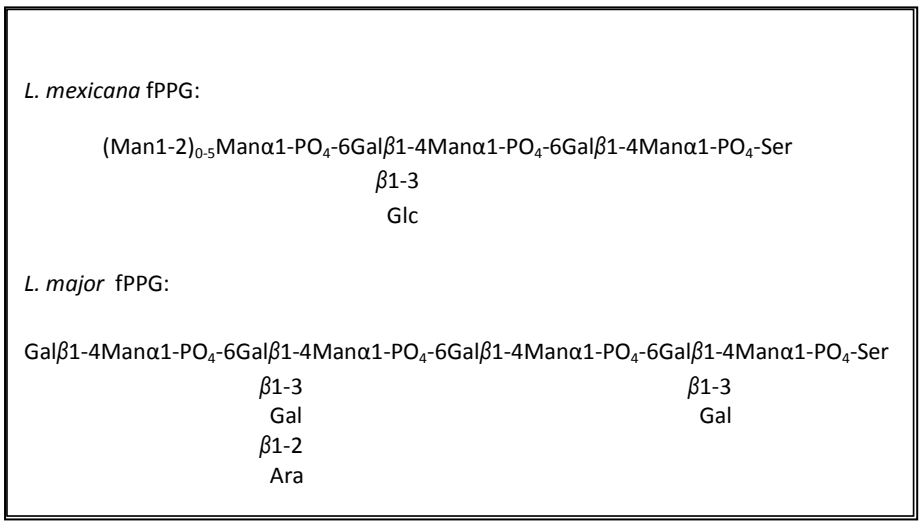

Figure 15 Structure of leishmania filamentous proteophosphoglycan (fPPG). Note the differences in the number of repeating units, sugar substitutions and cap structure among different species. fPPG size: 3-6 nm of diameter and up to 6 µm length. Figure adapted from Rogers[49].

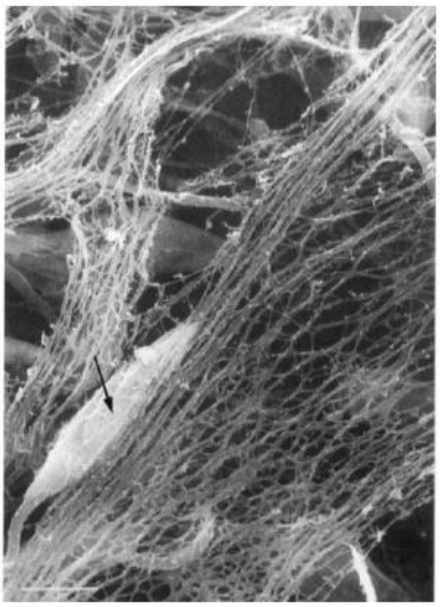

Figure 16 Promastigote (arrow) trapped in fPPG meshwork (SE image; Scale bar=2µm). Image by Stierhof et al[101].

fPPG seems to be made by all species of *Leishmania*, as a large variety of New and Old World *Leishmania* species secrete fPPG in culture. Moreover, this PSG-parasite plug appears to be a common feature in flies with mature infections, being observed in many sand fly–parasite combinations (**Table 6**).

Table 6 List of sand fly-parasite combinations in which PSG plug has been observed and its role in transmission**.** Table adapted from Rogers[49].

***Leishmania* species**	**Sand fly species**	**Combination**	**Role in transmission**
L. donovani	*P. argentipes*	Natural	Unknown
L. infantum	*P. ariasi*	Natural	Unknown
L. major	*P. papatasi*	Natural	Proven
L. major	*P. duboscqi*	Natural	Proven
L. tropica	*P. arabicus*	Natural	Proven
L. tropica	*P. sergenti*	Natural	Proven
L. mexicana	*Lu. longipalpis*	Experimental	Proven
L. mexicana	*Lu. diabolica*	Experimental	Unknown
L. mexicana	*Lu. abboneci*	Experimental	Unknown
L. amazonensis	*Lu. longipalpis*	Experimental	Unknown
L. chagasi/infantum	*Lu. longipalpis*	Natural	Proven
L. panamensis	*Lu. gomezi*	Natural	Unknown
L. braziliensis	*Lu. longipalpis*	Experimental	Proven

Recent work in Leishmaniasis vector biology has been focused on investigating the role of PSG in *Leishmania* transmission[46,48,57]. Traditionally, the actual transmission by bite was rarely included in the *Leishmania*-sand fly interactions studies[22]. In 2004, Rogers et al[57] demonstrated that metacyclic promastigotes of *Leishmania mexicana* are regurgitated from the midgut of the sand fly vector accompanied by PSG and co-delivered with saliva by bite. In the *Leishmania* life-cycle, transmission is a crucial event and it is extremely variable and influenced by the parasite[49]. So far, there are two known parasite mediated events that directly enhance transmission: metacyclogenesis and secretion of fPPG[48,57]. Interestingly, these two events are shown to be linked as there is a positive correlation between metacyclogenesis and the secretion of fPPG [46,48]. Like in other haematophagus vectors, saliva is also a major component of transmission. It is shown to facilitate blood-feeding, inhibit blood coagulation and modulate the immune response[49].

4.2 Manipulation of blood-feeding behaviour and enhancement of transmission

During blood feeding, the PSG plug exerts direct pressure over the stomodeal valve and as a result it tends to remain permanently open[28,48,49] allowing regurgitation of PSG, parasites and saliva with the blood meal. Parasite chitinases are believed to contribute to the damage of the stomodeal valve[31,50], contributing futher to its dysfunction and impairing the intake of blood (**Fig. 17**). The PSG plug alters sand fly feeding behaviour by blocking the sand fly midgut, so it is difficult to blood feed (The blocked fly hypothesis, Shortt & Swaminath, 1928[102]).

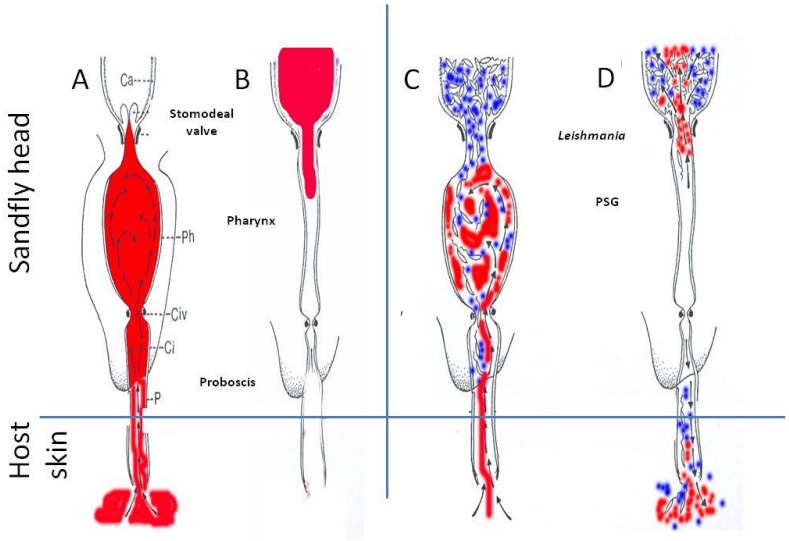

Figure 17 Sand fly and parasite regurgitation (Schlein et al)[103]. Pool feeding in uninfected females (A&B) and infected females (C&D). Blood (red) and PSG (blue).Adapted by Rogers, 2012[49].

In the late 70s, Killick-kendrick et al[104], observed that these blocked flies, probed more and spent more time feeding as a response to the blockage, a behaviour that would enhance *Leishmania* transmission[48].

Sand fly manipulation was further investigated by Rogers & Bates[48] whodemonstrated for the first time that *Leishmania* infection and PSG accumulation manipulates sand fly behaviour. Manipulation of host feeding behaviour to increase parasite fitness is a common strategy in many parasite-vector associations[28], but unlike for leishmaniasis, in the majority of the cases the molecules responsible are still unknown[48]. Sand fly manipulation by *Leishmania* parasites was shown to be an adaptative behavioural change resulting in enhaced *Leishmania* transmission but with detrimental effect on sand fly fitness, reducing their lifespan[48].

PSG blockage increased feeding time per bite, and the persistence of the infected fly when interrupted and encouraged host-seeking, resulting in an enhanced transmission[48,49,59]. Later, Rogers et al[105] demostrated that fPPG

determines not only feeding behaviour but also the dose. Increased feeding persistence was positively correlated with the number of metacyclics and the amount of PSG in the midgut.

Therefore, maximum fly blockage and feeding manipulation coincides with a peak of metacyclogenesis within the fly[48], which is essential for successful transmission. Transmission is associated with the risk of being killed by the defensive reactions of the host to the bite. Therefore, from the perspective of the parasite, encouraging a vector to bite when sufficient numbers of infective forms have been generated is an optimal transmission strategy. In contrast, early delivery of parasites before metacyclogenesis and late delivery could result in death of the vector and parasites[31]. In this regard, PSG appears to manipulate the sand fly for efficient transmission[48].

4.3 Determination of the infective dose

Detailed analysis of the plug revealed a mixture of mature and immature forms[49] which clashes with the metacyclic enriched dose delivered by the sand fly[57]. Relatively little is known about the molecules that induce metacyclogenesis *in vivo*[31]. From *in vitro* studies, it is believed to be triggered by low pH[31,44] and nutrient depletion[31]. PSG also seems to stimulate metacyclogenesis by acidification and anaerobiosis, generated by the high number of trapped cells[49]. *Leishmania* dose delivered by bite was determined experimentally in a few vector-parasite combinations: *P. duboscqi-L. major*[106]; *P. perniciosus-L. infantum*[107], *Lu. longipalpis-L. infantum*[108] and *Lu. longipalpis-L. mexicana*[57]. Delivered dose was heterogenous, especially for the *P. duboscqi* combination that ranged from 10-10000 parasites, but in the majority of the flies, it was <600 parasites, matching data from other combinations (100-600 parasites per bite/fly). In addition, detailed analysis of bite inoculum in voluntary conditions, revealed a striking high percentage of infective metacyclic promastigotes, with 86-98% metacyclics for *L. mexicana-Lu. longipalpis*[57] and up to 95% for the natural *L. infantum-Lu. longipalpis* combination[108]. Interestingly, serial dissections of infected flies revealed that most of the

promastigotes were located behind the pharynx, thus regurgitation is the primary mode of transmission.

Analysis of the promastigote populations embedded in the plug and in the midgut lumen revealed that metacyclics were mainly located in the plug and not free in the midgut. Rogers et al[46] found that metacyclics were mainly located in the anterior pole of the PSG plug, confirming previous observations by Lawyer et al[109] and Saraiva et al[110] suggesting that metacyclic movement is unhindered in PSG. Therefore, the role of PSG in determining both parasite dose and composition should be further examined as it may act as a predictor for successful transmission and infection[49].

4.4 Establishment of infection

Leishmania transmission must be followed by a successful establishment of the infection in the mammalian host. PSG actively participates in the establishment of the infection together with saliva[48,49]. Rogers et al [57] found that egested PSG was correlated with the size of the lesion and final amastigote burden. Jointly, saliva and PSG exacerbate both the skin and visceral phases of infection[108]. PSG and saliva increase *Leishmania* host cells available in the site of infection by influencing recruitment of both neutrophils and macrophages. Moreover, PSG is known to promote *Leishmania* survival within the macrophages by alternative activation of the macrophages (Th2 response). Alternative activation results in an enhacement of macrophage arginase activity and promotes arginine metabolism beneficial for parasite proliferation[105]. Diversion of the substrate L-arginine into polyamine biosynthesis will reduce its availability for inducible nitric oxide synthase and limit its metabolism into nitric oxide which is lethal for the parasite. Saliva also promotes a Th2 response by IL-4 and IL-10 secretion and consequently, arginase activation[111]. Interestingly, PSG and saliva effect do not act synergistically[57] and seem to enhance arginase activity via different pathways[49]. The net result is enhancement of *Leishmania* survival and growth in mice[57]. Since alternatively activated

macrophages are instrumental in wound healing, it has been proposed that PSG manipulates this process in favour of the establishment of the parasite in its mammalian host[49,105].

Due to their immunomodulatory properties, both saliva and PSG are being investigated as vaccine candidates. Vaccine development for *Leishmania* is particularly difficult due to complexities in the immune response[43]. Encouraging results have been obtained with both saliva[112-114]and PSG[43].

AIM: To describe the role of Promastigote Secretory Gel (PSG) in *Leishmania* transmission using a *Lutzomyia longipalpis-Leishmania mexicana* experimental model.

Specific objectives:

1. To investigate the role of PSG in enhancement of *Leishmania* transmission: promastigote selection by stage-specific binding.
2. To study the participation of PSG in parasite development within the sand fly: effect of PSG in promastigote attachment and detachment to sand fly midgut.
3. To investigate promastigote binding mechanism to both sand fly midgut epithelium and PSG.
4. To compare *Leishmania mexicana* and *Leishmania major* binding to PSG and sand fly midgut.

1 | Materials for *in vitro* experiments:

A / Parasites, fPPG and LPG for in vitro experiments

1.1 Cultivation of parasites:

Leishmania mexicana (MNYC/BZ/62/M379) Wild Type (WT) and *lpg1¯*mutant parasites were obtained from lesions on BALB/c mice (Imperial College, London). *L. mexicana lpg1¯* mutant parasites lack the main surface phosphoglycan, lipophosphoglycan (LPG), but preserve all other surface and secreted phosphoglycans. Lesion amastigotes were cultured at a concentration of 5 x 10^5 parasites/ml in Medium 199 supplemented with 10% (v/v) heat inactivated foetal calf serum (HIFCS), 1X BME vitamins (all from Gibco, UK) and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, UK) and a pH of 7.2 at 26°C[36]. Daily follow-up by density measuring (Neubauer hemocytometer) and morphometric analysis by microscopy (with 10% (w/v) Giemsa stain) was performed to obtain highly enriched populations of **procyclics** (24-48h), **nectomonads** (48-72 h) and **leptomonad promastigotes** (4-7 days). To obtain **metacyclic promastigotes** , nectomonad stages were passaged at a concentration of 1x 10^6 parasites/ml into Grace´s Insect Medium supplemented with 10% HIFCS, 1XBME vitamins, 1% L-glutamine (all from GIBCO), 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, UK) at a pH of 5.5 and incubated in 26°C for a further 5-6 days.

For a small number of experiments, *lpg2¯¯* mutant parasites were obtained from liquid nitrogen cryobank stock (LSHTM). *L. mexicana lpg2¯*LPG2 $^{-/-}$ mutant parasites lack membrane bound and secreted PPGs. *lpg1¯* and *lpg2¯* mutants were kindly donated by Dr Thomas Ilg.

L. mexicana mutants were completed with *lpg1¯/*+LPG1 and *lpg2¯*/+LPG2 add-back parasites and were kindly provided by Dr Martin Wiese (University of Strahclyde, Glasgow). *L. major* WT (V39), *lpg1¯, lpg2¯, lpg1¯/*+LPG1 and *lpg2¯*/+LPG2 parasites were kindly donated by Dr Steve Beverley (Washington University). Parasites were grown as described above to obtained nectomonad promastigotes for the experiments.

A cryobank of the different promastigote forms of *L. mexicana* WT and *lpg1*$^{-}$ was created for the experiments by preserving parasites in 10% (v/v) dimethyl sulfoxide (DMSO) in HIFCS at a concentration of 5 x 10^7/ml and stored at 80^{o}C until use (**Fig. 1**).

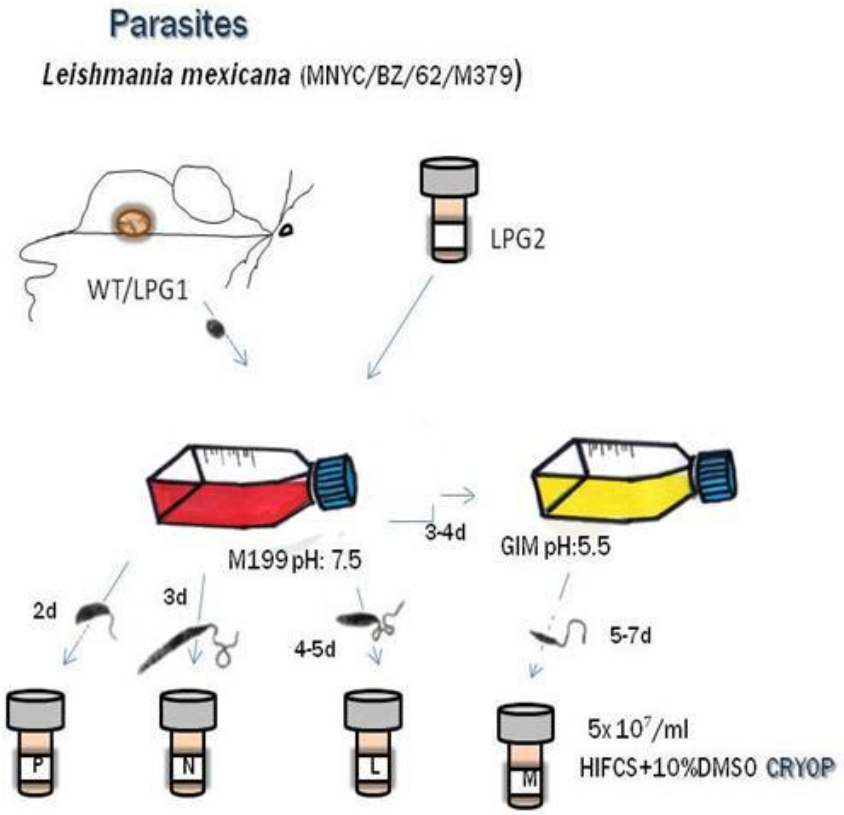

Figure 1 Stage-specific cryobank. (Parasite pictures from Dr Matthew Rogers[46]).

1.2 Purification of fPPG (filamentous proteophosphoglycan)

fPPG and other minor PPGs (proteophosphoglycans) from culture were purified by ultracentrifugation, following the protocol by Sadlova et al, 2010[115]. 250 ml of *L. mexicana* WT late log phase promastigote cultures (≥2.5 x 10^7) were divided into 50 ml BD Falcon tubes and centrifugated at 3000 rpm/ 1650 g (4 ˚C) for 10 minutes to remove parasites and parasite debris. The supernatant was carefully removed and transferred to clean Falcon tubes (BD Biosciences, USA). Samples were centrifuged again as before and the new supernatant transferred to an Ultra Clear™ 38.5 ml, 25 x 89mm thin wall ultracentrifugation tube (Beckman Coulter®, USA). Tubes were placed in a sw28 rotor and ultracentrifuged at 23600 rpm for an hour (equivalent to 100,000 g) using Optima L-90K Ultracentrifuge (Beckman Coulter®, USA) with the help of Dr Andrew Davies and Dr John Raynes (LSHTM). Supernatants were carefully removed and the overall pellet re-suspended in 200 µl of PBS (Phosphate Buffered Saline) and stored at -20˚ C.

1.3 Purification of promastigote LPG (Lipophosphoglycan):

Sample preparation: *L. mexicana* WT nectomonad and metacyclic promastigotes were pelleted by centrifugation at 2-4 x 10^8cells/ml and washed 3 times in PBS for 10 min. Pellets were kept at -80˚C. Overall, 4x10^9 nectomonads and 2x10^9 metacyclics were available. Purification was carried out at the Liverpool School of Tropical Medicine (LSTM) under Dr Álvaro Acosta Serrano´s supervision. Samples were lyophilised by freeze-vacuum drying for 13 hours using a Mini Lyotrap (LTE Scientific, UK) lyophiliser.

Extraction: Pellets were carefully fragmented with a spatula. Chloroform-Methanol 1:1 (Sigma-Aldrich, UK) extraction of the samples was performed twice by bath sonication for 10 minutes followed by centrifugation at 3200rpm/2190 g for 10 minutes. Supernatants containing major and regular phospholipids were removed and 5 ml of Chloroform-Methanol-Water (1:2:0.8) were added to the pellets, followed by sonication and centrifugation as described before. Supernatants

(glycoinositolphospholipids and other lipids) were discarded and pellets were then lyophilised to dry out the excess of C/M/W. Finally, butanol 9% (Sigma-Aldrich, UK) extraction was performed 3 times and supernatants kept for LPG.

Visualisation: Extraction products were visualised in a SDS-PAGE gel stained with Schiff´s stain (Sigma-Aldrich, UK) for carbohydrates and Western Blotting with LPG specific LT-15 monoclonal antibody (provided by Dr Thomas Ilg). Prior to loading, samples concentration was maximised by speed vacuum centrifugation (Hera-Tekno DNA mini).

B/ Sand fly colony & Promastigote Secretory Gel (PSG)

1.4 Colony rearing:

For the study of parasite-vector interactions sand flies from LSHTM colonies were used. *Lutzomyia longipalpis* (Jacobina strain) sand flies have been maintained at school insectaries since February 2011. *Lu. longipalpis* is widely used in experimental transmission models due to its permissive nature and relatively good survival in captivity.

— Immature forms:

Larvae were kept in a 500ml jar (Thermo Scientific Nalgene, UK), perforated and lined with plaster (Fine Casting Plaster, UK) in the bottom, to allow the humidity to come through. The pot was sealed with filter paper (Fisher Scientific, UK), left on a moistened paper towel and placed into a sealed box. Larvae were kept at 24-27 ^{0}C. Keeping the right humidity is extremely important. Larvae were fed daily according to size, with a 50:50 mixture of autoclaved rabbit food and faeces. Mite infestation was avoided by daily wiping pots and boxes with 70% ethanol. Once the adults emerged, the filter was replaced by a piece of netting **(Fig. 2a).**

— Adults:

When adults emerged they were transferred to a fine-mesh cage, supplied with a moistened paper towel and cotton wool on top (soaked with 70% sucrose), placed into a sealed bag and kept at 25 ^{0}C in 12h : 12h light/dark photoperiod in a LMS cooled incubator (Wolf Labs). 5 day old females were artificially blood-fed weekly, using a Hemotek®(Hemotek Ltd. UK) membrane feeding system filled with fresh rabbit blood (Harlan Laboratories, UK) and thinned chick skin as a biological membrane (**Fig. 2b**). For optimal egg laying, blood fed females were carefully transferred to 500 ml pots. Humidity is once again provided by a plaster base placed onto a humid tissue. In order to provide a suitable environment for egg-laying, the oviposition pot wasfilled with corrugated strips of filter paper pieces (**Fig. 2c).**

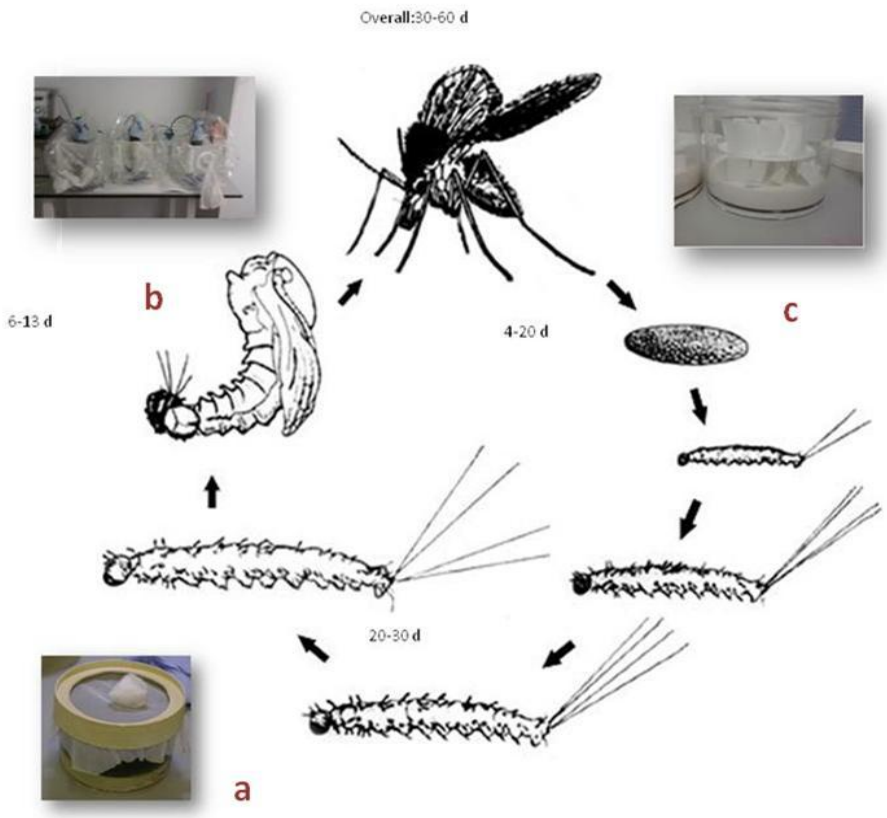

Figure 2 *Lutzomyia longipalpis* life cycle in pictures (illustrations by **Rod Dillon,** http://pcwww.liv.ac.uk/leishmania/life_cycle__habitats.htm)**. a** larvae pot with emerging adults**. b** Hemotek® membrane feeding of adult females **c** oviposition pot.

1.5 Sand fly experimental infection and isolation of PSG.

PSG was obtained from laboratory reared *Lu. longipalpis* flies (from both LSHTM and Liverpool School of Tropical Medicine colonies). Flies were infected through a chick skin membrane with fresh rabbit blood (Harlan, UK) containing *L. mexicana* WT amastigotes (MNYC/BZ/62/M379) at 2x 10^6/ml using a Hemotek™ feeding system. *Lu. longipalpis* is known to successfully support and transmit *L. mexicana* under experimental conditions[36]. On day 8-10, flies were dissected by longitudinal scission of the gut and the PSG plug was obtained from the anterior midgut. PSG plug was transferred in 5 µl of PBS and solubilised with a pipette. PSG was centrifuged 4 times, 1 x 2000 rpm (270 g)/10 minutes and 3 x 7000 rpm (3290 g)/ 5 minutes, to remove the parasites and any cell debris[35]. The supernatant (PSG) was stored at -20˚C for experiments (**Fig. 3**).

PSG from *Phlebotomus duboscqi* female sand flies experimentally infected with *L. major* was obtained from a stock previously obtained by my supervisor using the experimental colonies of Professor Petr Volf (Charles University, Prague, Czech Republic).

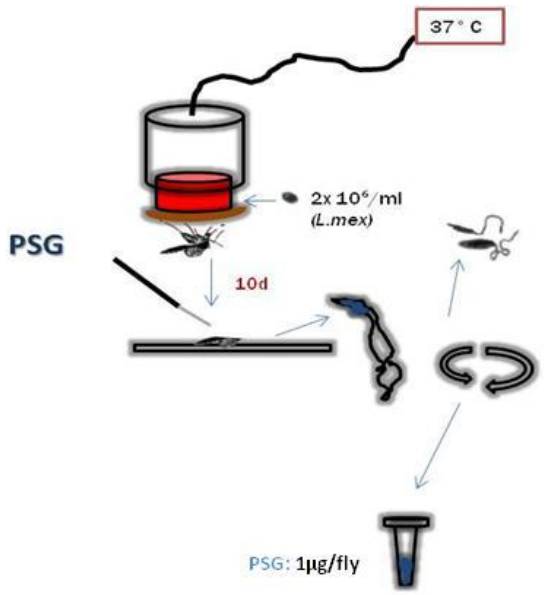

Figure 3 Sand fly infection and PSG isolation *in vitro*. (Sand fly illustration by Rod Dillon (http://pcwww.liv.ac.uk/leishmania/life_cycle__habitats.htm); parasites by Matthew Rogers[46]).

2 | Parasite specific binding*: in vitro* slide attachment:

2.1 Optimisation.

In order to study promastigote stage specific binding to PSG *in vitro*, 16 well Lab Tek™ Chamber Slides (Thermo Scientific, UK) were coated with PSG diluted in M199 and incubated with parasites. Briefly, several experiments were performed to choose the optimal concentration of parasites and PSG per well and optimal number of washes required to wash off the unbound parasites (from the negative PBS control). Using nectomonads, the optimal parasite concentration was found to be $2.5x10^6$ per well (100 µl) and 3 washes were found to be sufficient to remove unbound parasites from the PBS control wells. Due to the difficulty to obtain PSG, the minimum concentration required for parasite binding was carefully chosen by PSG titration assay.

In 16 well chamber slides, 4 different PSG concentrations (0.05 µg, 0.1 µg, 0.5 µg and 1 µg PSG per well) were incubated with 100 µl of M199 culture containing $2.5x10^6$ nectomonad and metacyclic WT promastigotes, in duplicate, as follows:

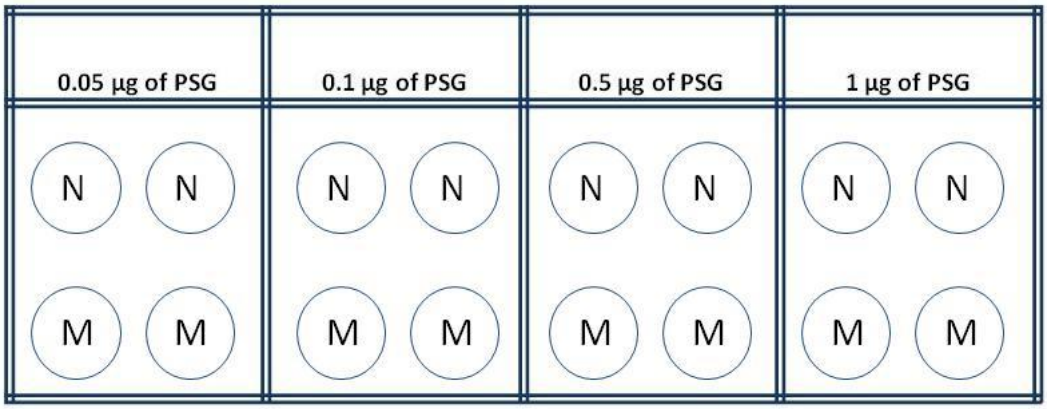

Figure 4 PSG titration assay. 16 well chamber slide coated with increasing concentration of PSG, where N= nectomonads (high binding expected) and M= metacyclics (low binding expected).

Briefly, 50µl of PSG diluted in M199 was pipetted in each well ensuring that the entire bottom of the well was covered. It was incubated for 10 minutes at room temperature followed by 60 minutes at 56˚ C. The slides were left to cool down at

room temperature for 10 minutes and the remaining PSG solution was pipetted out. Just prior to this step, cryopreserved nectomonad and metacyclic parasites were thawed out and washed in M199 + 10% HIFCS culture medium. Their viability was checked microscopically by placing 3 µl of sample under a coverslip and rating flagellar motility. Vials with <75% viability were rejected. Per well, 100 µl of parasites in M199 culture medium ($2.5x10^7$/ml) were added and incubated for 1 hour at 26˚ C. Afterwards, they were pipetted out and washed 3 times with 200 µl of PBS. The well chamber was removed and the slide air dried, fixed with methanol and stained with 10% Giemsa in distilled water for 15 minutes. Parasites in two vertical and two horizontal axes were counted, avoiding the edges. Parasite counts indicated that 0.5 µg of PSG concentration was adequate for the assay (see **figure 1.1** in results).

2.2 PSG binding to slide

PSG binding to slide was detected by immunofluorescence. 2.5 µl of PSG were diluted in 47.5 µl of M199 in a well (Lab Tek™ Chamber Slides Thermo Scientific, UK) and placed in 56 °C for an hour. 50 µl of M199 were used as negative control. Content was pipetted out. 50 µl of 1:300 LT15 monoclonal antibody in PBST were added and left for 1 hour at room temperature (RT) with gentle rocking. LT15 antibody is specific for galactose-mannose disaccharide units present in fPPG[57]. Wells were washed twice with PBST to remove unbound LT15. FITC labeled anti-mouse IgG (1:500) (Sigma Aldrich, UK) in PBST was added and left onto a shaker for 1 hour at RT. Wells were washed again in PBST and ProLong™ Gold Antifade Reagent (Invitrogen, Life Technologies, USA) added to preserve fluorescence. A coverslip was added and sealed with a clear nail varnish.

Images were captured using an Axioplan 2 Upright fluorescence microscope (Carl Zeiss Ltd, UK) linked to a Retiga 2000R Fast CCD camera (QImaging, UK) and analysed using Volocity® software (PerkinElmer, USA).

2.3 Stage-specific binding:

Once the technique was optimized, *L. mexicana* (MNYC/BZ/62/M379) WT stage specific parasite binding was assessed. Quadruplicates were made for procyclic, nectomonad, leptomonad and metacyclic promastigotes for WT (**Fig. 5**).

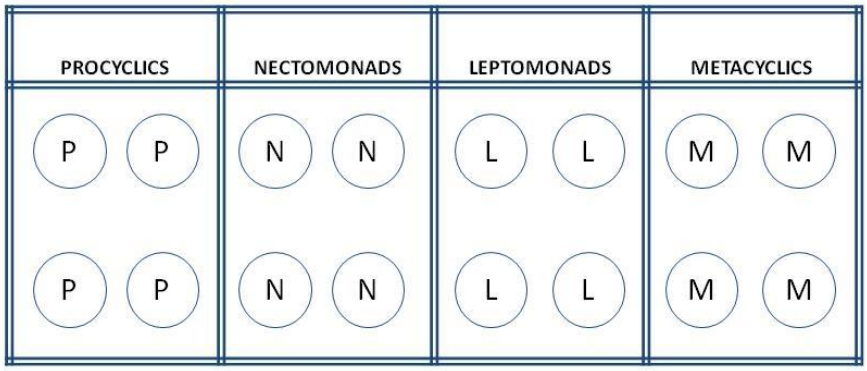

Figure 5 *L. mexicana* WT (MNYC/BZ/62/M379) slide binding assay with 2.5 X10^6 parasites (in 100µl) and 0.5 µg PSG per well (in 50µl).

2.4 *L. major* and *L. mexicana* mutant binding:

L. mexicana PSG *in vitro* binding was completed with the study of mutants and their respective add-backs as described above. Stage-specific binding was studied in *lpg1*¯ mutants, comparing nectomonad, leptomonad and metacyclic promastigote attachment to PSG; procyclics were not available for this study (**Fig. 6**).

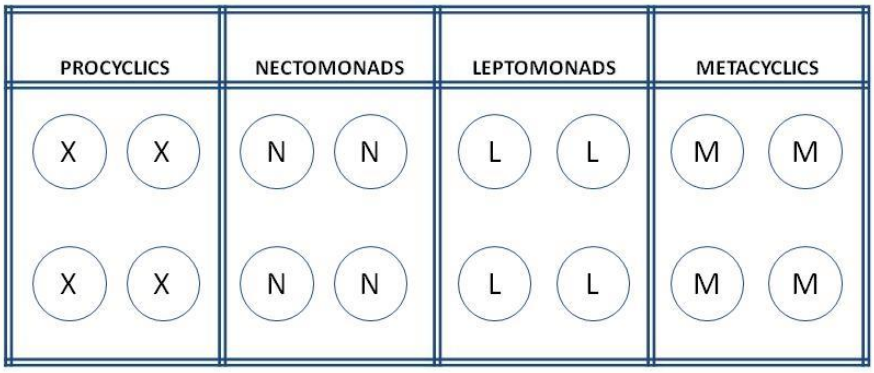

Figure 6 *L. mexicana lpg1*¯ slide binding assay with 2.5 X10^6 parasites (in 100µl) and 0.5 µg PSG per well (in 50µl). X: not available.

To assess the specific effect of LPG in PSG binding, nectomonad slide binding was analysed for *lpg1⁻*, *lpg2⁻*, *lpg1⁻*/+LPG1 and *lpg2⁻*/+LPG2 parasites with *L. mexicana* WT as control group. This last experiment was also performed for *L. major* and *L. major* PSG from *P. duboscqi* under the same conditions.

3 | Parasite dynamics: Migration Assay & Velocity Recording

3.1 Migration assay:

In order to more closely mimic the PSG and parasite interaction within the sand fly gut, parasite migration assays were performed in capillary tubes (Hirschmann ® Labörgerate GmbH & CO.KG, Germany).

Briefly, capillaries were cut into lengths to accommodate 6 µl and filled with either 5 µl of PSG (at a concentration of 1µg/5 µl) or 5 µl of PBS (negative control). In 4 capillaries (2 PBS and 2 PSG) 0.5 µl of $1x10^6$ nectomonad promastigotes were inoculated in one end and another 4 capillaries, (2 PBS and 2 PSG), were loaded with the same density of metacyclic promastigotes. Migration to the other end of the capillary was assessed at 15, 30, 60 and 120 minutes by taking 0.5 µl for microscopy. During the assay, capillaries were kept in a humidified chamber at room temperature to minimise evaporation. After staining with 10% Giemsa the amount of parasites per 10 consecutive fields was recorded, avoiding the edges (**Fig. 7**).

At the same time, a migration assay was carried out with both nectomonads and metacyclics combined (mixed in equal proportion) in 4 capillaries (2 PSG and 2 PBS). As above, 0.5 µl were removed at timed intervals. Immediately after the last time sample (120 min), 0.75 µl were taken sequentially from each capillary to sample the parasites along the length of the tube; from the end (1^{st} sample) to the origin (4^{th} sample) (**Fig. 8).** The same procedure was repeated using *lpg1⁻*mutant parasites.

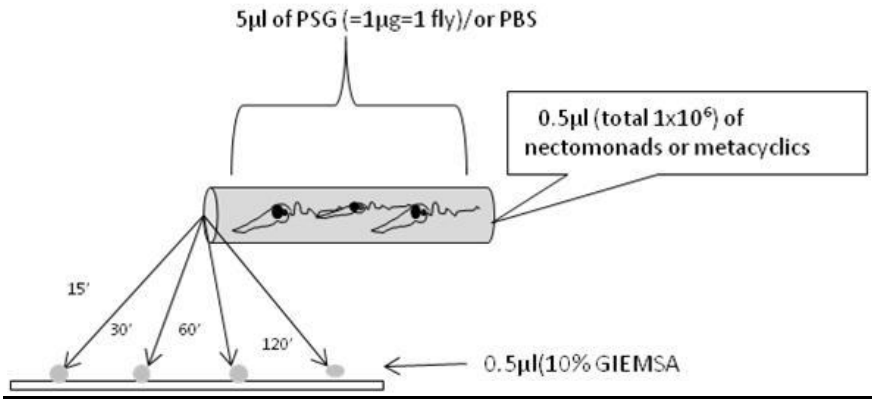

Figure 7 Migration by sampling of parasites at the end of the tube (at 15´, 30´, 60´, 120´).

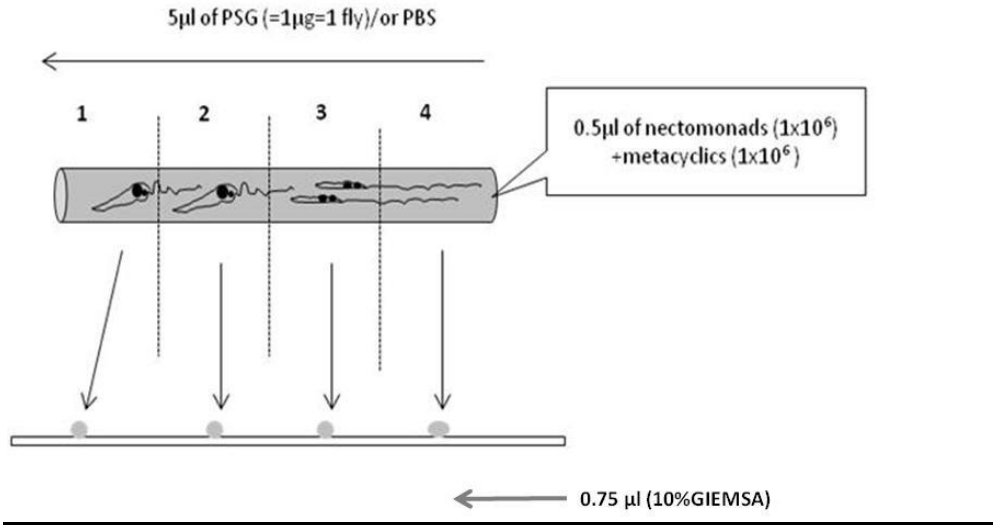

Figure 8 Comparative migration assay in PSG and PBS.

3.2 Velocity recording of parasites:

In order to measure parasites movement patterns and, therefore, real-time binding, video-tracking of cultured parasites was performed.

For that purpose, fresh nectomonad and metacyclic promastigotes were inoculated in duplicate in 384 flat bottom well microplate (Corning Life Science, US) containing PSG and PBS as substrates. Parasites did not undergo any process that could affect their viability such as cryopreservation, centrifugation or fluorescent staining.

Parasite motility was analysed by placing 1 µl of nectomonad or metacyclic promastigotes (at $2X10^7$/ml) in 9 µl of PSG/PBS per well. 50 pictures at a 0.5s interval were captured per well using ImageXpress^{MICRO} HCS microscope (Molecular Devices, UK). Parasites in the 50 time frames were artificially coloured (**Fig. 9**) using Pipeline Pilot software (Accelrys ®, US) by Dr Ross Paveley (LSHTM)[116] for later analysis in Volocity®(PerkinElmer, US) software. Parasites were tracked obtaining numerical data showing movement patterns and velocity. Data obtained in Volocity was exported to Prism 5.0 for statistical analyisis.

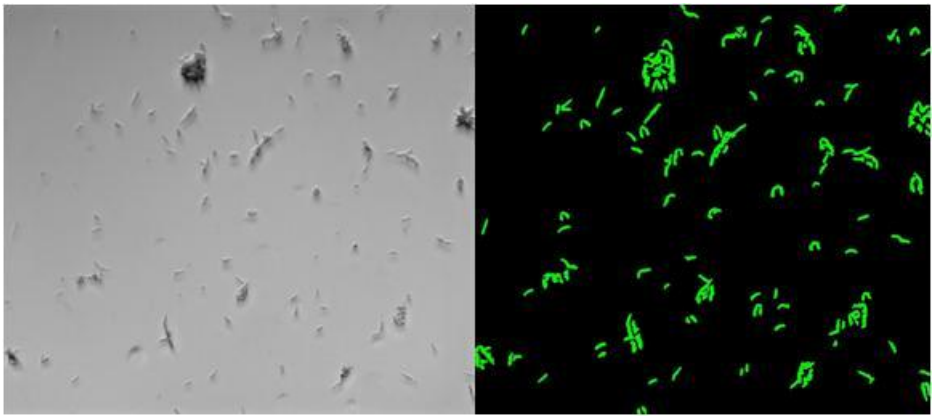

Figure 9 Bright field (**left**) and digitally fluorescent nectomonad parasites (**right**). x400. Images by Dr Ross Paveley.

4 | *Ex vivo* midgut binding:

4.1 Stage-specific binding:

5 to 7 day post blood feed flies were selected for experiments, to match the physiological status of infected flies at the time of midgut attachment[56]. Of those, just females with fully digested bloodmeals and eggs were finally included. Nevertheless, prior to the midgut binding assay, few experiments were performed to study the effect of sand fly age in binding. Briefly, mean midgut binding was found to be superior in 5 days post blood meal flies (666.2), compared to 4 days post blood meal flies (357), 4 day old sugar fed flies (37) and 2 days old unfed flies (28.5).

Fly guts were carefully dissected in small subgroups and placed quickly in PBS prior to longitudinal scission. *L. mexicana* procyclic, nectomonad, leptomonad (in M199) and metacyclic promastigotes (in GIM) were inoculated in quadruplicate at a concentration of $1X10^7$ parasites per well (50 µl) in a 96 well ELISA plate (SLS). Guts were carefully opened by longitudinal scission using a fine BD insulin Ultra-Fine™ needle (BD Biosciences, UK) under a Olympus SZH10 dissecting microscope and transferred into individual wells containing parasites (**Figs.10 & 11, Table 1**)

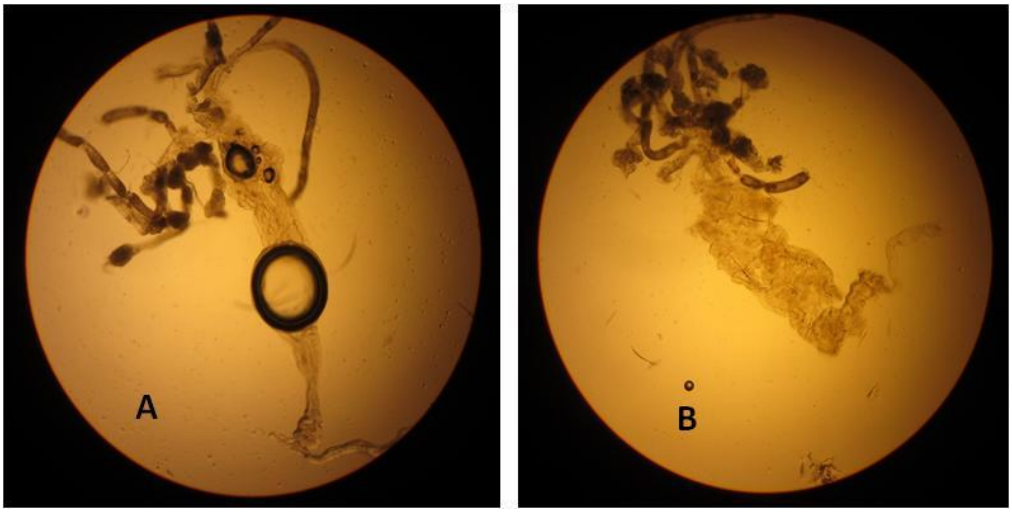

Figure 10. *Lu. longipalpis* female digestive tractbefore (**A**) and after longitudinal scission (**B**)(x40). Leitz Laborlux K microscope Canon IXUS 220 HS.12.1 camera.

Figure 11. *Lu. longipalpis* female midgut (arrow) before (**A**) and after longitudinal scission (**B**) (x100)Leitz Laborlux K microscope Canon IXUS 220 HS.12.1 camera. Malpighian tubes, hindguts and crops were removed for the experiments.

The plate was incubated for 45 minutes at 26 °C. After this time, guts were recovered and washed 3 times in PBS and homogenised in a 1.5 ml Eppendorf tube containing 30 µl of M199 using a conical tissue grinder. 10 µl of the homogenate was stained with Giemsa (10% v/v) and total number of parasites per slide (i.e 10 µl) was counted.

Table 1 Stage-specific midgut binding groups

Parasites $1X10^7$ in 50 µl	Flies	Incubation 45 minutes at 26˚C	Washes in PBS	Attachment # of parasites in 10µl
Procyclics	5-7 days post blood -meal	Procyclics + Midgut	3	Homogenised gut (M199) Giemsa stain
Nectomonads	5-7 days post blood -meal	Nectomonads + Midgut	3	Homogenised gut (M199) Giemsa stain
Leptomonads	5-7 days post blood -meal	Leptomonads + Midgut	3	Homogenised gut (M199) Giemsa stain
Metacyclics	5-7 days post blood -meal	Metacyclic + Midgut	3	Homogenised gut (M199) Giemsa stain

4.2 Competitive binding:

Guts were placed individually into wells containing 1x10^7/50 µl *L. mexicana* WT nectomonads, 0.5µg of PSG (in 50 µl of M199) or both, and incubated for 45 minutes at 26 °C. Different combinations of the above were designed to study nectomonad specific binding (Control), PSG-Nectomonad binding competition, PSG binding blocking and PSG mediated detachment:

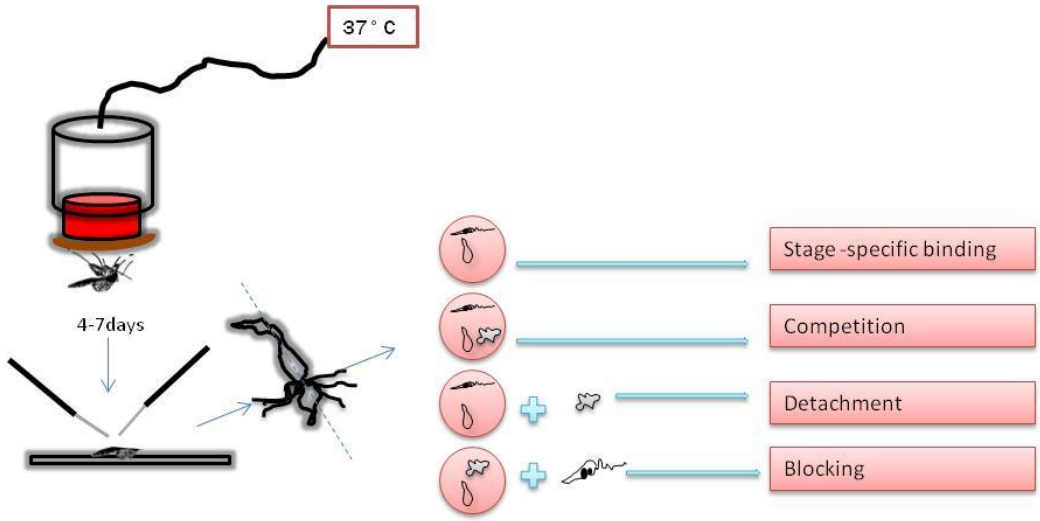

Figure 12 Subgroups in midgut competition assay. Sand fly image by Rod Dillon.

Briefly, guts were divided into 4 groups with 10 replicates per group: in control group, guts were incubated just with nectomonads; in the competition group, guts were incubated simultaneously with PSG and nectomonads; in the blocking group, guts were incubated with PSG and transferred to wells with parasites; and in the detachment group, guts were incubated with parasites, washed and placed into PSG containing wells. In all groups, guts were washed in PBS and homogenised in a tube containing 30 µl of M199. 10 µl of the homogenate was Giemsa-stained for parasite counting (**Table 3**).

Per assay, only 3 replicates of each subgroup were performed, to avoid incubation discordances and minimise the time for processing the guts to avoid gut damage.

Table 2 Display of competition assay.

Line1	Control	Competition	Competition	Competition
Line2	Control	Blocking	Blocking	Blocking
Line3	Control	Detachment	Detachment	Detachment

Table 3 Summary of competition assay (*BM: blood meal).

Group	Flies	Parasites $1X10^7$ in 50 µl	Incubation 1 45 minutes at 26˚C	Washes in PBS	Incubation 2 45 minutes at 26˚C	Washes in PBS	Attachment # of parasites in 10µl
Control	5-7 days post BM*	Nectomonads	Nectomonads + Midgut	3	N/A	0	Homogenised gut (M199) Giemsa stain
Competition	5-7 days post BM	Nectomonads	Nectomonads + Midgut+ PSG	3	N/A	0	Homogenised gut (M199) Giemsa stain
Blocking	5-7 days post BM	Nectomonads	Midgut + PSG	1	Midgut + Nectomonads	3	Homogenised gut (M199) Giemsa stain
Detachment	5 -7days Post-BM	Nectomonads	Nectomonads + Midgut	1	Midgut + PSG	2	Homogenised gut (M199) Giemsa stain

4.3 Detachment & PSG titration:

PSG mediated detachment was studied in detail by PSG titration. 5 different PSG concentrations were tested: 0 µg per well, 0.005 µg per well, 0.05 µg per well, 0.1 µg per well and 0.5 µg per well.

Guts were obtained as before and incubated in $1x10^7$ nectomonad parasites per well for 45 minutes at 26 °C, washed once in PBS and transferred to wells containing different concentrations of PSG for a second incubation. They were washed and homogenised as before. The control group was adjusted by adding a second incubation with just M199 culture medium (Sigma-Aldrich, UK) in the wells. A minimum of 5 replicates were studied per group (**Table 4**).

Table 4 PSG titration (*BM: blood meal).

Group	Flies	Parasites $1X10^7$ in 50 µl	Incubation 1 45 minutes at 26˚C	Washes in PBS	Incubation 2 45 minutes at 26˚C	Washes in PBS	Attachment # of parasites in 10µl
0 µg PSG	5-7 days post BM*	Nectomonads	Nectomonads + Midgut	1	Midgut + M199	2	Homogenised gut (M199) Giemsa stain
0.5 µg PSG	5-7 days post BM	Nectomonads	Nectomonads + Midgut	1	Midgut + PSG	2	Homogenised gut (M199) Giemsa stain
0.1 µg PSG	5-7 days post BM	Nectomonads	Nectomonads + Midgut	1	Midgut + PSG	2	Homogenised gut (M199) Giemsa stain
0.05 µg PSG	5 -7days post BM	Nectomonads	Nectomonads + Midgut	1	Midgut + PSG	2	Homogenised gut (M199) Giemsa stain
0.005 µg PSG	5 -7days post BM	Nectomonads	Nectomonads + Midgut	1	Midgut + PSG	2	Homogenised gut (M199) Giemsa stain

4.4 *L. major* and *L. mexicana* mutant binding to midguts:

The effect of LPG on midgut binding was analysed by adding mutant promastigotes to the experiments described above. *L. mexicana lpg1⁻* and *lpg2⁻*/+LPG2 nectomonad midgut binding was studied with *L. mexicana* WT nectomonads as control group. Competition assays were completed studying the effect of PSG in *lpg2⁻*/+LPG2 nectomonads midgut binding.

Following the *L. mexicana* midgut binding protocol, *L. major* midgut binding to *Lu. longipalpis* was analysed for WT *lpg1⁻, lpg2⁻* and *lpg2⁻*/+LPG2 nectomonad promastigotes. The effect of *L. major* PSG from *P. duboscqi* on *L. major* WT-*Lu. longipalpis* midgut binding was also assessed.

4.5 Midgut binding by immunofluorescence

L. mexicana WT promastigotes expressing DsRed fluorescent protein, kindly provided by Dr Toni Aebischer (Robert Koch Institute, Germany), were used to study promastigote attachment to guts in absence and presence of PSG.

Two guts from 6 days post blood fed flies were dissected and placed into wells containing $1x10^6$ nectomonad-leptomonad DsRed promastigotes alone and with 0.5 µg of PSG, respectively. They were incubated in the dark for 45 minutes at 26 °C and washed 3 times in PBS and placed onto a slide. ProLong™ Gold Antifade Reagent (Invitrogen, Life Technologies, USA) added to preserve fluorescence before covering. Images were captured using an Axioplan 2 Upright fluorescence microscope (Carl Zeiss Ltd, UK) linked to a Retiga 2000R Fast CCD camera (Qimaging, UK) and analysed using Volocity® software (PerkinElmer, USA). Dr Emilie Giraud (LSHTM) helped with the CCD microscope.

5 | Binding detection by Western Blotting

5.1 Biotinylation of fPPG:

fPPG was biotinylated as described by Dillon and Lane, 1999[86].. Prior to biotinylation, fPPG was oxidaised by incubation for 20 minutes with 10 mM Sodium Periodate in acetate buffer (pH=5.5) (Sigma, UK) in ice (1:1) in a light protecting vessel. The solution was filtered at 4°C and 3500 rpm/2250 g for 15 minutes using a 10KDa ultrafilter tube (Centricon 10 Amicon, Millipore, US). The retentate was resuspended in 10mM phosphate buffer (pH=7.20) (Sigma, UK) as washing buffer and refiltered 3 times as above.

Biotin hydrazide (EZ-Link® Hydrazide Biotin, ThermoScientific, UK) was added to the solution at a final concentration of 5 mM and left for 1h and 20 minutes at room temperature (RT). To eliminate the excess of biotin, this mix was ultrafiltered 3 times (as described above) and kept at 4° C for next day use.

5.2 Promastigote LPG attachment to Biotin-fPPG:

Specific attachment of nectomonad and metacyclic promastigotes to fPPG was studied by immunoblotting (**Fig. 13**).

2 vials of nectomonad LPG and 2 vials of metacyclic LPG were recovered from the LPG stock and concentrated by vacuum-freeze-dry for 1-2 hours. Each vial contained LPG from 1.6 x 10^8cells. 20 µl of SDS loading buffer were added per vial and heated at 100° C for 5 minutes. Loading buffer composition: SDS (0.4 g), glycerol 100% (2 ml), 0.5 M Tris-HCl pH 6.8 (2.5 ml), bromophenol blue (0.02 g), ß-mercaptoethanol (0.4 ml) and up to 20 ml dH_2O (Courtesy of Dr Sam Alsford, LSHTM). Sample heating with SDS at 100 ° C will eliminate any differences in charge between the samples that could influence their binding to biotin-fPPG. Unlike the native gel, SDS-PAGE will separate molecules according to their molecular weight only and not their structure or charge and allow us to identify more accurately the molecule involved in attachment.

Briefly,1 vial was loaded per lane and separated on a Pre-Cast NuPAGE® 4-12% Bis Tris Gel (Invitrogen, UK) at a current of 150V for 1h and 10 minutes in a Xcell Sure Lock ®Invitrogen Novex Mini Cell running block (Invitrogen UK) with NuPAGE® MES SDS running buffer (20x)(Invitrogen, UK). ColorBurst Electrophoresis Marker (Sigma, UK) was used as a marker.

Proteins were transferred onto a nitrocellulose membrane (Amersham ®Hybond®-ECL. GE Healthcare) at 25V for 20 min using a semi-dry transfer cell (Bio-Rad Trans-Blot ®SD). The blot was left overnight at 4° C in blocking buffer (PBST +10 % w/v dry milk) with gentle agitation.

After washing the membrane repeatedly in PBST (4x5 minutes), one of the blots was used as positive control and incubated for 1 hour at RT with 1:300 LT15 monoclonal antibody (kindly provided by Dr Ilg). It was washed again and probed with 1:500 biotinylated anti-mouse IgG (Vector Laboratories USA) for another hour at RT.

In parallel, the second blot was incubated with biotinylated fPPG (1:500) for 1 hour at RT.

Once the blots were washed, proteins were detected by the biotin/avidin system using (Vectastain ABC kit Elite, Vector ®VIP) and visualised by chemiluminescense using the Peroxidase Substrate Kit (both from Vector laboratories, Inc, USA) following instructions from the manufacturer.

Marker		Necto-LPG		Meta-LPG		Necto-LPG		Meta-LPG
		LT15 1:300 (in Blocking Buffer)		LT15 1:300 (in Blocking Buffer)		—		—
	B	Wash : PBST (4x5´)	B	Wash : PBST (4x5´)	B	—	B	—
	L	Biotin + Anti-Mouse IgG 1:500 (in PBST)	L	Biotin + Anti-Mouse IgG 1:500 (in PBST)	L	Biotin + fPPG 1:500 (in BB)	L	Biotin + fPPG 1:500 (in BB)
	A	Wash : PBST (4x5´)	A	Wash : PBST (4x5´)	A	Wash : PBST (4x5´)	A	Wash : PBST (4x5´)
	N	ABC kit (in PBST)	N	ABC kit (in PBST)	N	ABC kit (in PBST)	N	ABC kit (in PBST
	K	Wash : PBST (3x5´) PBS (1x5´)	K	Wash : PBST (3x5´) PBS (1x5´)	K	Wash : PBST (3x5´) PBS (1x5´)	K	Wash : PBST (3x5´) PBS (1x5´)
		Peroxidase Substrate Kit		Peroxidase Substrate Kit		Peroxidase Substrate Kit		Peroxidase Substrate Kit
		Stop: dH2O (2x5´)		Stop: dH2O (2x5´)		Stop: dH2O (2x5´)		Stop: dH2O (2x5´)

Figure 13 Blot distribution and probing sequence.

5.3 Immunoblotting of Biotin-fPPG, fPPG, and PSG:

Biotin-fPPG, fPPG and PSG were also studied by immunoblotting. Briefly, samples were pre-heated in SDS loading buffer for 5 minutes at 100°C and loaded onto a SDS /4-12% Acrylamide-BIS gel with an enlarged stacking gel (4%) and separated by electrophoresis at a constant current of 150V for 1h and 10 minutes. Mini-PROTEAN®Tetra Cell system (Bio-Rad) was used for casting and electrophoresis. Samples were electroblotted, blocked and washed as before. The blot was incubated with 1:300 LT15 monoclonal antibody and posteriorly probed with 1:500 Biotinylated anti-mouse IgG (Vector Laboratories USA). Proteins were detected and visualised using Vector kits as above.

6 | Statistical Analysis.

Statistical analysis was performed using GraphPad Prism 5.00 (GraphPad Software Inc., USA) statistical package. Data distribution was examined using D´Agostino–Pearson, Shapiro Wilk and Kolmogorov-Smirnov normality tests and by frequency distribution analysis provided by the software. According to their distribution, non-parametric unpaired Mann-Whitney test (or its extension Kruskal-Wallis test) and parametric unpaired t-test were used for data comparison. Null hypothesis (populations are the same) was rejected when $p<0.05$. Significance levels: p = 0.05-0.01, significant; p = 0.01-0.001, very significant; and $p<0.001$, extremely significant.

RESULTS: CHAPTER 1

Parasites develop and migrate within the sand fly undergoing structural and functional changes until infective metacyclic promastigotes are delivered by bite. Simultaneously, PSG is heavily secreted in the sand fly midgut by immature promastigotes. PSG is proven to block the fly and thus, modify sand fly behaviour[48], but are there other roles in the sand fly that can influence transmission? Rogers et al (unpublished data) observed that following gut puncture, only metacyclics exited the gut from a mixed population of nectomonad, leptomonad and metacyclic promastigotes. This suggests an underlying process of promastigote selection within the gut. Does PSG take part in this selection? Can *Leishmania* bind to PSG and if so, are there any differences between promastigote developmental stages?
Here, we investigate *in vitro Leishmania* attachment to PSG.

1.1 | Optimisation

Up to approximately 1 µg of PSG can be obtained from an experimentally infected female sand fly[57,105]. For our assay, we compared nectomonad and metacyclic attachment to PSG coated slides, expecting opposite results in binding based on previous works.[46,109,110]. We tested four different PSG concentrations: 1 µg, 0.5 µg, 0.1 µg and 0.05 µg. Mean nectomonad attachment for lower concentrations was just 23 ± 18 (0.05 µg) and 25 ± 11 (0.1 µg) making them unsuitable for experiments. Highest nectomonad attachment was obtained with 0.5 µg concentration (80 ± 10) followed by 1 µg, with a mean attachment of 66 ± 18 nectomonads.

In contrast, mean metacyclic attachment was below 10 parasites with all the concentrations studied: 2 ± 2 for 0.05 µg, 7.5 ±0.5 for 0.1 µg, 9 ± 4 for 0.5 µg and 0 ± 0 for 1 µg (**Fig. 1.1**). Differences in attachment related to concentration were non-significant for both nectomonad (p=0.1386) and metacyclic (p=0.1231)

promastigotes, but 0.5 µg of PSG was chosen due to highest binding. P values were obtained by Kruskal-Wallis test.

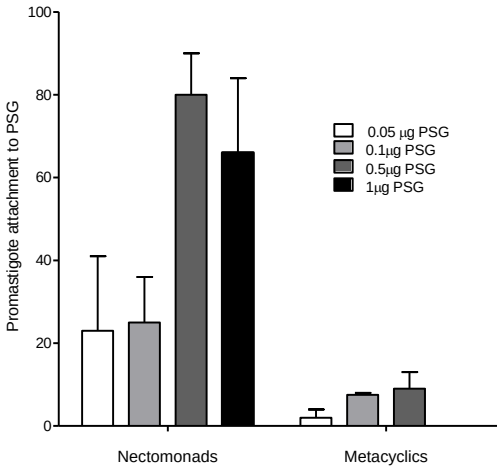

Figure 1.1 Promastigote mean attachment + SEM using different concentrations of PSG.

1.2 | PSG binding to slide

Optimisation was completed by confirmation of slide coating by immunofluorescence. PSG was visualised stuck to the slide after incubation at 56 °C for 1 hour and subsequent probing with LT15 monoclonal antibody (**Fig. 1.2**).

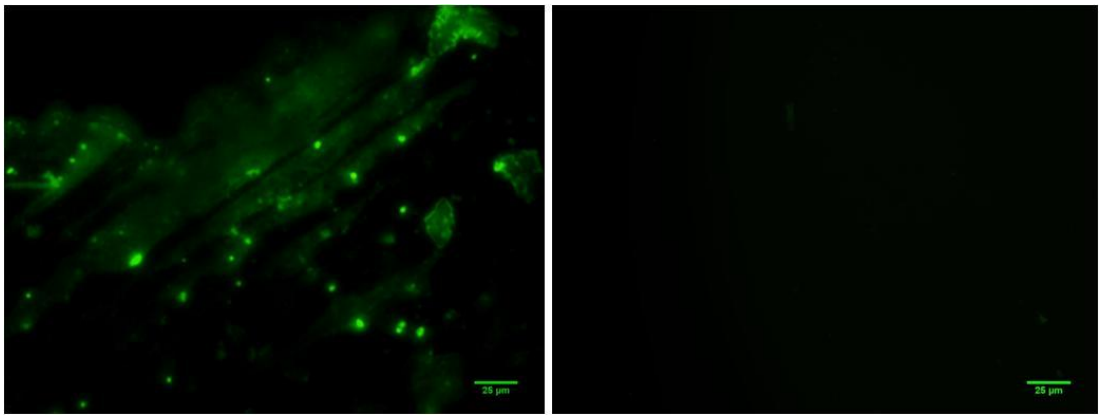

Figure 1.2 On the left, PSG onto a slide probed with LT15; right, negative control (M199 alone). X200. Scale bar 25µm. Axioplan 2 Upright fluorescence microscope (Carl Zeiss Ltd, UK). Retiga 2000R Fast CCD camera (QImaging, UK)

1.3 | Stage-specific promastigote binding

Promastigote attachment to PSG differed notably according to their developmental stage. Nectomonad promastigotes, highly mobile slender forms, were found to bind in greater numbers (471.3 ± 45.98) when compared to the other groups, as observed previously in the PSG titration experiment. The next group to bind to the PSG were the closely related leptomonads promastigotes with a mean attachment of 71.00 ± 11.97. In contrast, first and last developmental stages, procyclics and metacyclics, showed very low binding, with just 9 ± 3.028 and 0.75 ± 0.4787 parasites, respectively. Differences in attachment were significant for all groups when compared to the control nectomonad group ($p<0.05$) (**Fig. 1.3, Table 1.1)**. In this first experiment, binding to PBS was used as negative control, revealing default attachment to wells, significantly lower than the PSG group ($p<0.05$) (**Fig. 1.4**).

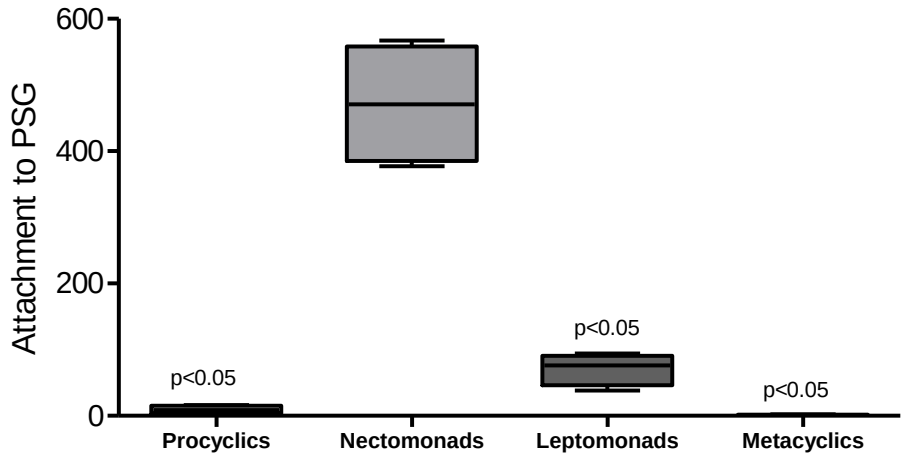

Figure 1.3 Box plots showing data distribution and P values for *L. mexicana* WT stage specific binding to PSG. P values obtained by Mann-Whitney analysis.

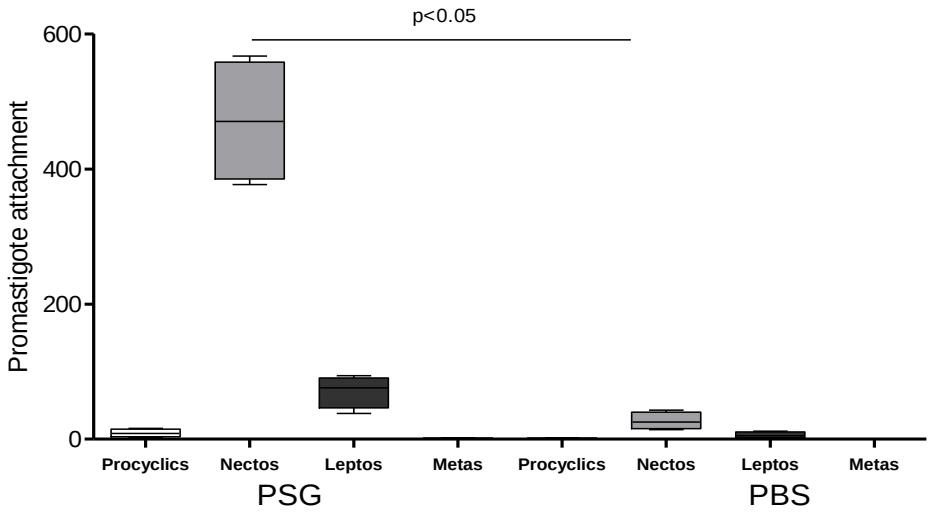

Figure 1.4 Promastigote attachment to PSG *versus* PBS. P values obtained by Mann-Whitney analysis

Figure 1.5 Nectomonad promastigotes in PSG coated slide. Parasites are aligned along the interface of PSG and medium with their flagella projecting into the PSG. Giemsa stain, (x1000). Leitz Laborlux K microscope. Canon IXUS 220 HS.12.1 Camera.

Table 1.1 Summary of statistics for stage-specific PSG binding.

	Mean	SEM	Median	N[a]	p value	Significance[b]
Procyclics	9	3.028	8.5	4	0.0286	*
Nectomonads	471.3	45.98	470.5	4	--------	--------
Leptomonads	71.00	11.97	76	4	0.0286	*
Metacyclics	0.75	0.4787	0.5	4	0.0286	*

[a]N=number of observations. [b]Significance values: p=0.05-0.01, significant (*)

1.4 | Capillary migration

Promastigote movement was analysed within a capillary. In PBS, *L. mexicana* nectomonad and metacyclic promastigotes move steadily through the capillary over time, showing similar dispersal. In PSG, metacyclic migration was unhindered, with a non-significant reduction (p=0.6857) in migration (231 parasites in PBS *vs* 203.5 in PSG), whereas, nectomonad migration was severely affected (p=0.0286) with only 13.86% (52) of the parasites being able to migrate to the end of the capillary after 120 minutes compared to PBS control (375) (**Figs. 1.6 & 1.7**).

This behaviour was also true when nectomonad and metacyclic promastigotes were combined. Comparative migration was observed along the length of the capillary after 120 minutes (**Fig. 1.8**). At the inoculation end of the capillary (segment 4), nectomonads remained the dominant population in PSG (91.5%) compared to metacyclics (8.5%) (p=0.0035). In PBS, the proportions of nectomonads (60%) and metacyclics (40%) were more similar (p=0.0050).

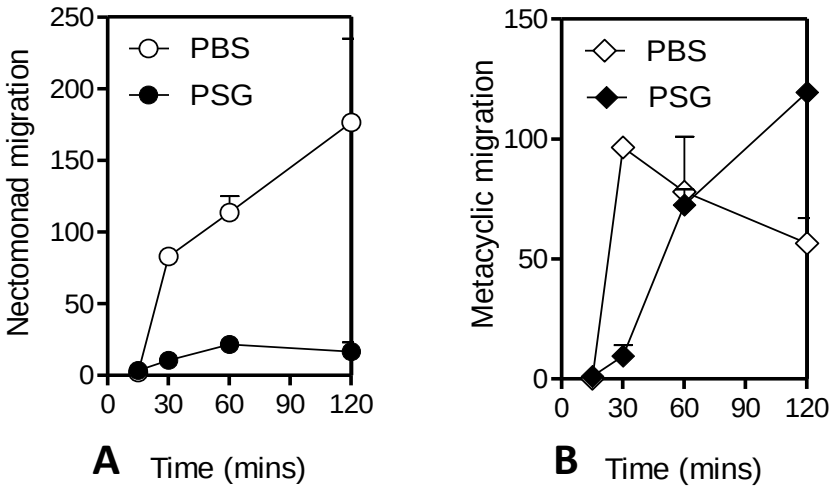

Figure 1.6 Nectomonad (**A**) and metacyclic (**B**) migration pattern (mean +SEM) through the capillary tube over time in PSG and PBS, showing number of parasites per drop and time point.

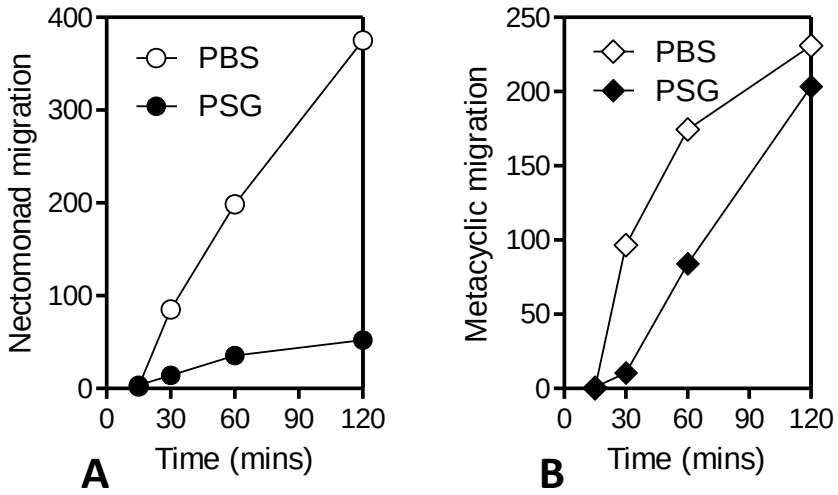

Figure 1.7 Nectomonad (**A**) and metacyclic (**B**) migration (mean) through the capillary tube over time in PSG and PBS, showing cumulative number of migrated parasites per time point.

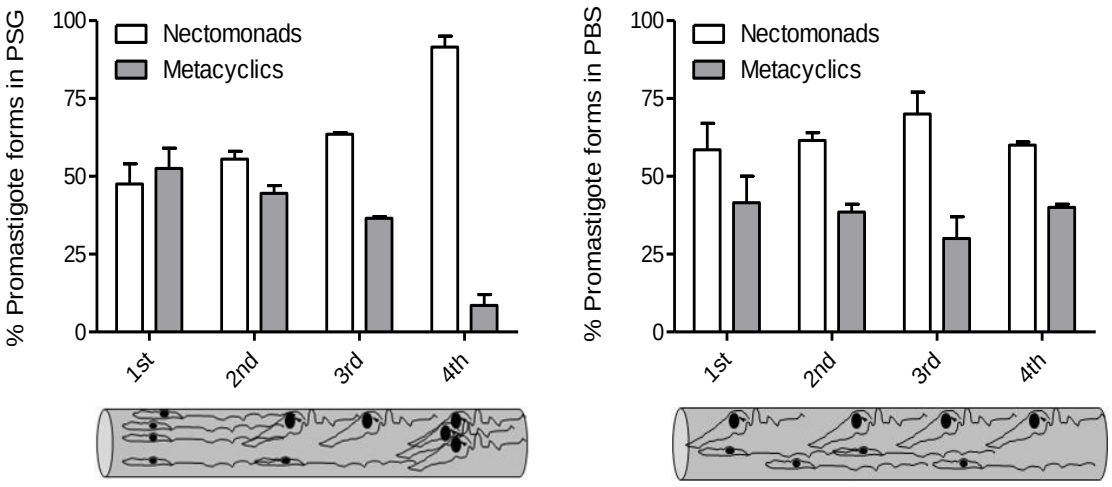

Figure 1.8 Comparative migration assay in PSG(left) and PBS (right).

1.5 | Promastigote video-tracking:

In order to complete the parasite migration study as an indicator of promastigote attachment, nectomonad and metacyclic promastigote movement was also assessed in wells by microscopic video tracking under the supervision of Dr Ross Paveley. Nectomonad and metacyclic promastigote dynamics in PSG and PBS, such as velocity (average speed over track) and movement pattern (total object path) were analysed using Volocity® tracking system in 50 time-points (i.e 25 seconds) (**Fig. 1.9**).

Nectomonad movement was found to be compromised in PSG compared to PBS; their mean track velocity was 70.71 ± 1.820 µm/sec in PSG compared to 144.1 ± 6.277 µm/sec in PBS, with a significantly different distribution ($p<0.05$) (**Fig. 1.10, Table 1.2**).

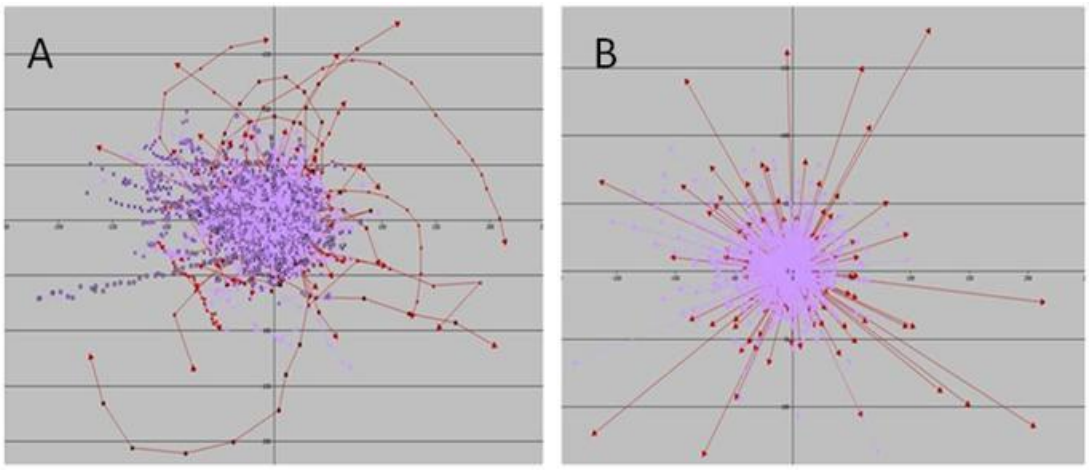

Figure 1.9 (**A**)Nectomonad movement pattern in PSG (in pink) and in PBS (in red) showing centroid measurement of objects for all timepoints linked by tracks (in µm). (**B**)Nectomonad final displacement (in µm) in PSG (in pink) and PBS (in red).

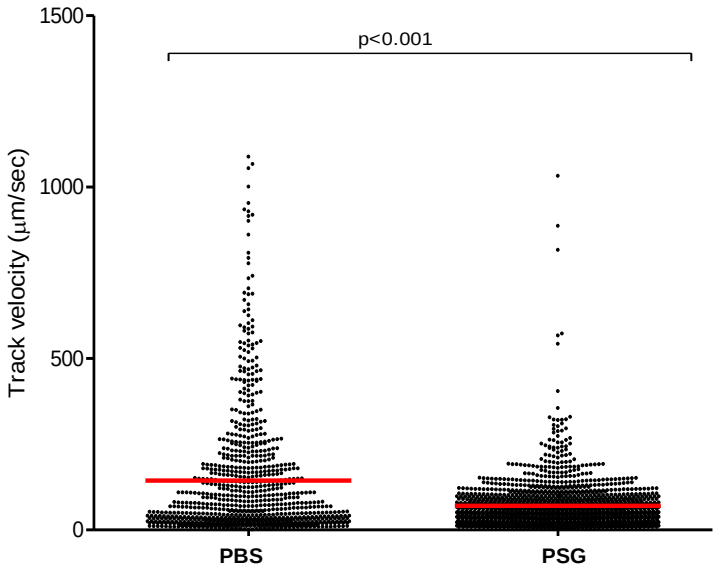

Figure 1.10 Nectomonad velocity per individual track in PBS and PSG. Mean track velocity in red. P value obtained by Mann-Whitney analysis.

Table 1.2 Summary statistics for nectomonad video-tracking.

	Mean	SEM	Median	N[a]	p value	Significance[b]
PSG	70.71	1.820	53.97	1504	p<0.0001	***
PBS	144.1	6.277	72.27	818		

[a]N=number of observations. [b]Significance values: p<0.001 extremely significant (***)

In contrast, metacyclics were not affected by PSG (p>0.05), revealing similar movement pattern, displacement and velocity, with a mean velocity of 78.80 ± 2.091 in PSG and 75.53 ± 2.593 in PBS (**Figs 1.11,1.12, Table 1.3**).

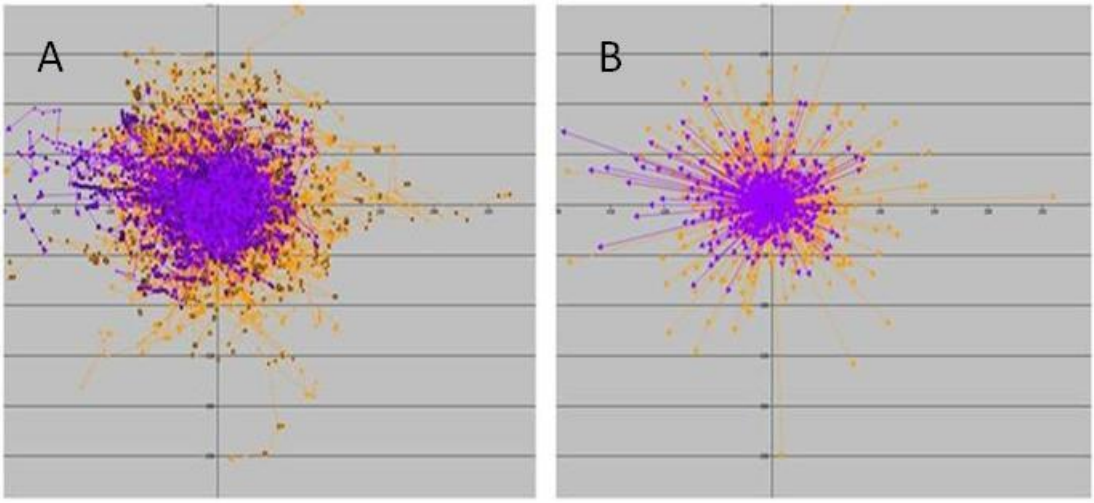

Figure 1.11 (**A**) Metacyclic movement pattern in PSG (in purple) and in PBS (in yellow) showing centroid measurement of objects for all timepoints linked by tracks (in µm).(**B**) Metacyclics final displacement (in µm) in PSG (in purple) and PBS (in yellow).

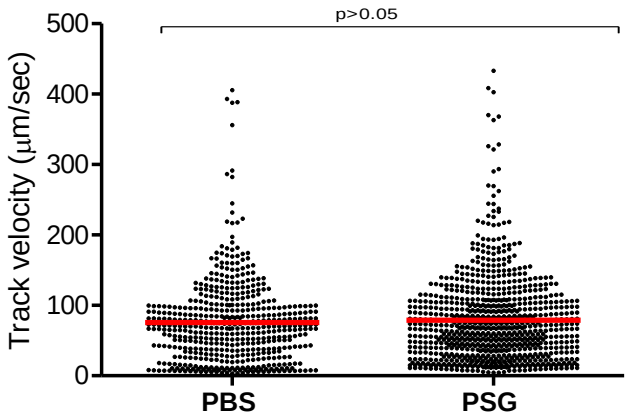

Figure 1.12 Metacyclic velocity per individual track in PBS and PSG. Mean track velocity in red. P value obtained by Mann-Whitney analysis.

Table 1.3 Summary statistics for metacyclic video-tracking.

	Mean	SEM	Median	N[a]	p value	Significance[b]
PSG	78.80	2.091	68.50	824	0.3150	ns
PBS	75.53	2.593	69.57	511		

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns).

Joint analysis showed that overall, nectomonads were faster than metacyclics in PBS ($p<0.05$). In PSG, nectomonad mean track velocity was significantly lower compared to metacyclics ($p<0.05$)(**Fig 1.13, Table 1.4**).

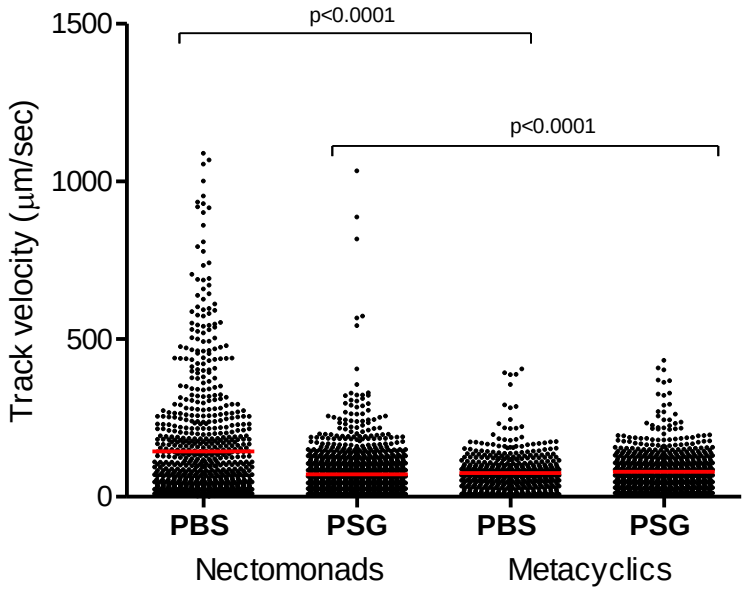

Figure 1.13 Overall track velocity for nectomonad and metacyclic promastigotes in PSG and PBS. Mean track velocity in red.

Table 1.4 Summary statistics for nectomonad and metacyclic promastigotes in PSG and PBS.

	Promastigote	Mean	SEM	Median	N[a]	p value	significance[b]
PSG	Nectomonad	70.71	1.820	53.97	1504	p<0.0001	***
	Metacyclic	78.80	2.091	68.50	824		
PBS	Nectomonad	144.1	6.277	72.27	818	p<0.0001	***
	Metacyclic	75.53	2.593	69.57	511		

[a]N=number of observations. [b]Significance values: p<0.001 extremely significant (***)

RESULTS: CHAPTER 2

Metacyclic promastigotes do not bind to sand fly gut epithelium whereas immature nectomonad promastigotes do[44,92]. This stage-regulated binding is thought to be driven by changes to the LPG[22,81]. In comparison, the process of detachment from the gut is unknown. Here we investigate stage-specific midgut binding but also the role of PSG in attachment and detachment from the gut, as PSG secreted by nectomonads and leptomonads may compete for the binding of these forms and promote their detachment from the gut. Midgut detachment is key for promastigote development and therefore *Leishmania* transmission.

2.1 | Wild type *L. mexicana* stage-specific binding to *Lu. longipalpis* midgut

Slide-counts revealed differences in sand fly midgut attachment according to promastigote stage.

Nectomonad promastigotes were found to attach in greater numbers to guts, with a mean attachment of 355.3 ± 172.8 parasites per 10 µl of gut homogenate, followed by leptomonad promastigotes with 144.0 ± 2.24 parasites. On the other hand, procyclic and metacyclic promastigotes showed very low attachment to midgut with a mean attachment of just 0.7500 ± 0.4787 and 0.2500 ± 0.2500 respectively.

Statistical analysis showed that when compared to the nectomonad control group, difference in attachment was significant in the procyclic ($p<0.05$) and metacyclic groups ($p=0.0286$), but not in the leptomonad group ($p>0.05$) (**Fig. 2.1, Table 2.1**).

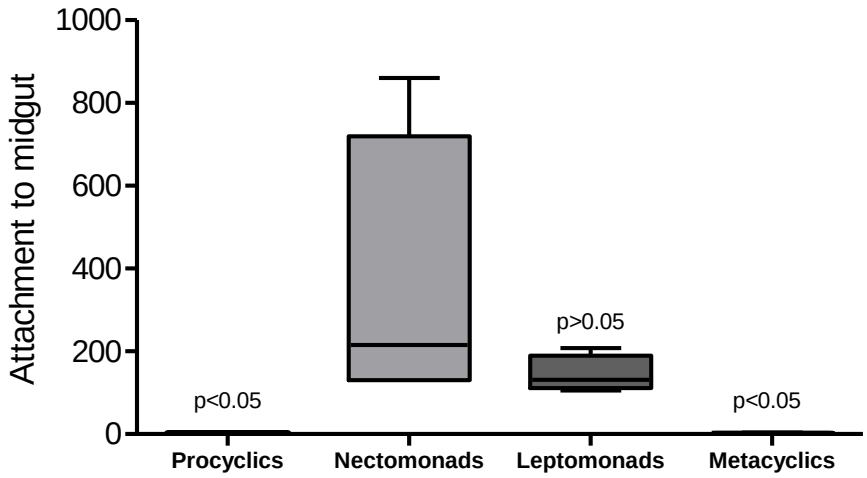

Figure 2.1 Box plots showing data distribution and P values for stage-specific midgut attachment, including median and 25%-75% percentiles. P values obtained using Mann-Whitney analysis, where p<0.05, significant and p>0.05, non-significant.

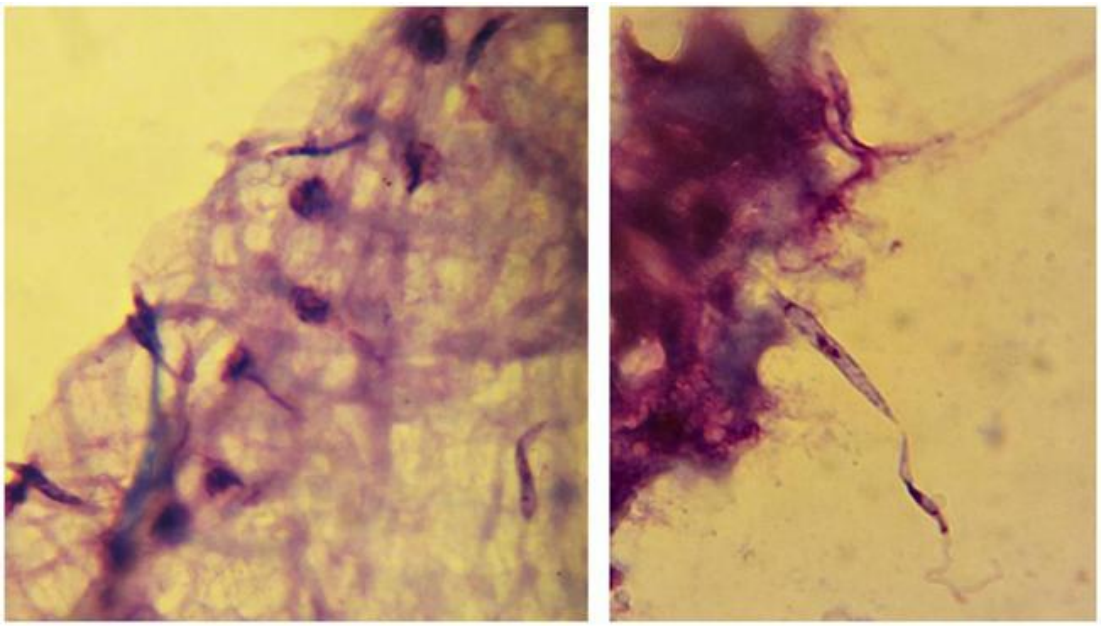

Figure 2.2 Promastigote binding to midgut. Giemsa stain (x1000). Leitz Laborlux K microscope. Canon IXUS 220 HS.12.1 Camera.

Table 2.1 Summary statistics for stage-specific midgut binding

	Mean	SEM	Median	N[a]	p value	Significance[b]
Procyclics	0.7500	0.4787	0.500	4	0.0294	*
Nectomonads	355.3	172.8	215.5	4	--------	--------
Leptomonads	144.0	22.24	131.5	4	0.3836	ns
Metacyclics	0.2500	0.2500	0.0	4	0.0286	*

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.05-0.01, significant (*).

2.2 | Wild type *L. mexicana* binding to *Lu. longipalpis* midgut in the presence of PSG:

Our results showed that PSG does affect nectomonad promastigote binding to midgut epithelium *ex vivo.*

When PSG is simultaneously added to wells containing both parasites and guts (**competition**), attachment dramatically drops to 93.18 ± 33.76 nectomonads, when compared to the **control group** lacking PSG (432.2 ± 80.14). Attachment still occurs but with a 75% reduction in binding.

If PSG is added prior to nectomonad-midgut interaction (**blocking**), attachment is also lower with 71.9 ± 15.74 parasites and a reduction of 83.36% compared to the control group. Finally, if PSG is added once the binding has occurred (**detachment**), binding decreases by 95.56% to 19.17 ± 7.644. These differences were confirmed by statistical analysis. Attachment was found to be significantly lower in all the study groups when compared to the control group ($p<0.05$): competition, $p=0.0002$; blocking, $p<0.0001$; and detachment group $p<0.0001$ (**Fig. 2.3, Table 2.2)**.

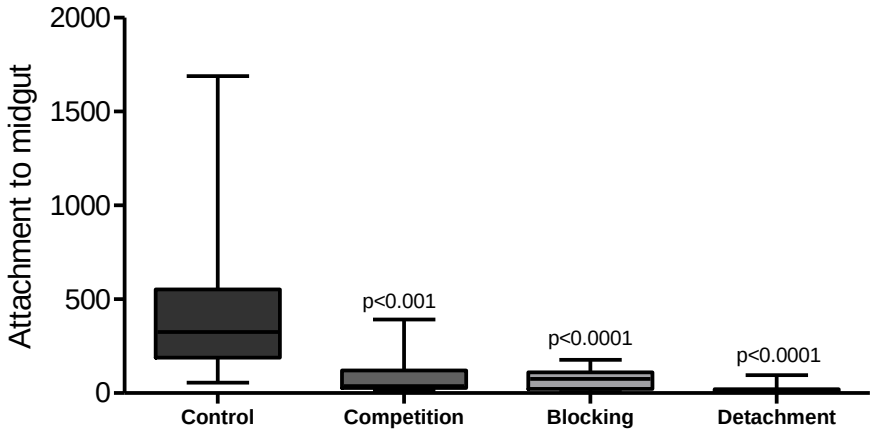

Figure 2.3 Box plots showing data distribution and P values for midgut binding groups including median and 25%-75% percentiles. P values obtained by Mann-Whitney analysis.

Table 2.2 Summary statistics for midgut binding in absence/presence of PSG

	Mean	SEM	Median	N[a]	p value	significance[b]
Control	432.3	80.14	326	21	--------	--------
Competition	93.18	33.76	37	11	0.0002	***
Blocking	71.9	15.74	76	11	<0.0001	***
Detachment	19.17	7.644	15	12	<0.0001	***

[a]N=number of observations. [b]Significance values: p<0.001, extremely significant (***).

2.3 | Wild type *L. mexicana* detachment from midgut in the presence of PSG:

Inclusion of intermediate PSG concentrations (**0.005 µg**, **0.05 µg**, **0.1 µg**) as study groups between the absence (**0 µg**) and average PSG concentration per infected fly (**0.5 µg**), revealed a PSG dose dependant detachment. The number of nectomonad promastigotes attached to guts decreased according to PSG.

concentration increase: mean nectomonad attachment was 286.429 ± 36.32 per 10 µl in the control group (0 µg), 188.2 ± 36.45 in the 0.005 µg group, 116.8 ± 21.85 in the 0.05 µg group, 76 ± 21.23 in the 0.1 µg group and 24 ± 8.533 in the 0.5 µg of PSG group (**Table 2.3**).

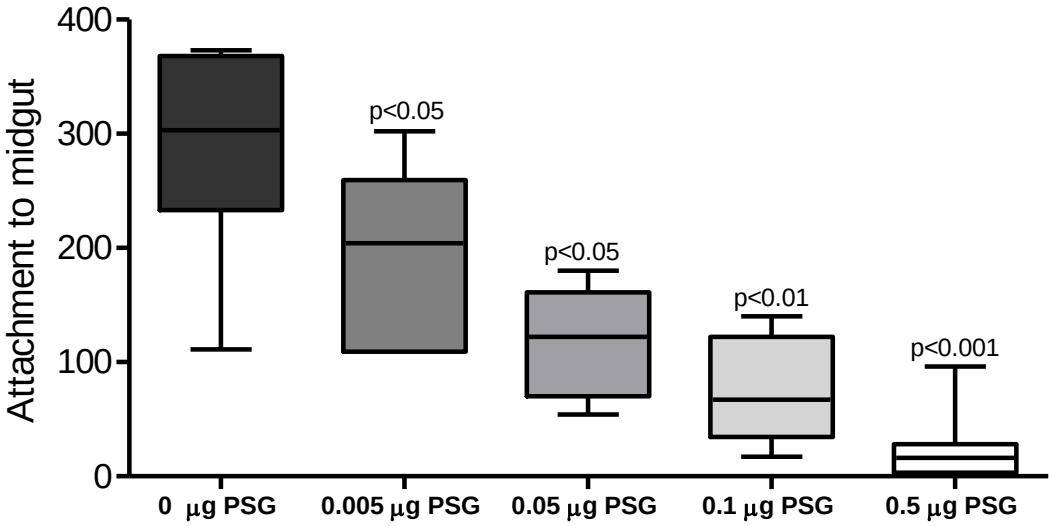

Figure 2.4 Box plots showing data distribution and P values for different PSG groups, including median and 25% -75% percentiles. P values obtained by Mann-Whitney analysis when compared to the control group (µg 0).

Statistical analysis of the groups showed that there was a significant difference in bound nectomonads in absence and presence of PSG (all $p < 0.05$) and that p values increased at higher PSG concentrations (**Fig. 2.4, Table 2.3**). Detailed analysis revealed that this significance prevailed between the different titration groups except when 0.05 µg group was included in the analysis (**Table 2.4**).

Table 2.3 Summary statistics for midgut binding in absence (0 µg of PSG/control group) and titrated presence of PSG.

	Mean	SEM	Median	N[a]	p value	Significance[b]
0 PSG	286.429	36.32	303.0	7	--------	--------
0.005 PSG	188.2	36.45	204.0	5	0.0480	*
0.05 PSG	116.8	21.85	122.0	5	0.0177	*
0.1 PSG	76	21.23	67.00	5	0.0051	**
0.5 PSG	24	8.533	16.00	13	0.0004	***

[a]N=number of observations. [b]Significance values: p=0.05-0.01, significant (*); p=0.01-0.001, very significant (**); and p<0.001 extremely significant (***).

Table 2.4 P values and significance level for each of the combinations studied.

p values	0 PSG	0.005 PSG	0.05 PSG	0.1 PSG	0.5 PSG
0 PSG	--------	0.0480(*)	0.0177(*)	0.0051(**)	0.0004 (***)
0.005 PSG	0.0480 (*)	--------	0.2087(ns)	0.0362(*)	0.0016(**)
0.05 PSG	0.0177(*)	0.2087 (ns)	--------	0.2222 (ns)	0.0043(**)
0.1 PSG	0.0051 (**)	0.0362(*)	0.2222 (ns)	--------	0.0301(*)
0.5 PSG	0.0004(***)	0.0016(**)	0.0043(**)	0.0301(*)	--------

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.05-0.01, significant (*); p=0.01-0.001, very significant (**); and p<0.001 extremely significant (***).

2.4 | Fluorescent promastigote midgut binding with/without PSG

The study of the PSG effect on promastigote midgut binding was completed with the visualisation of DsRed fluorescent nectomonad-leptomonad promastigote binding to *Lu. longipalpis* midguts, obtaining similar results. PSG did affect

binding(**Fig. 2.5**), reducing the attachment in 56 % from 222 parasites / 50 fields (control) to 97 parasites / 50 fields (**Fig. 2.6**).

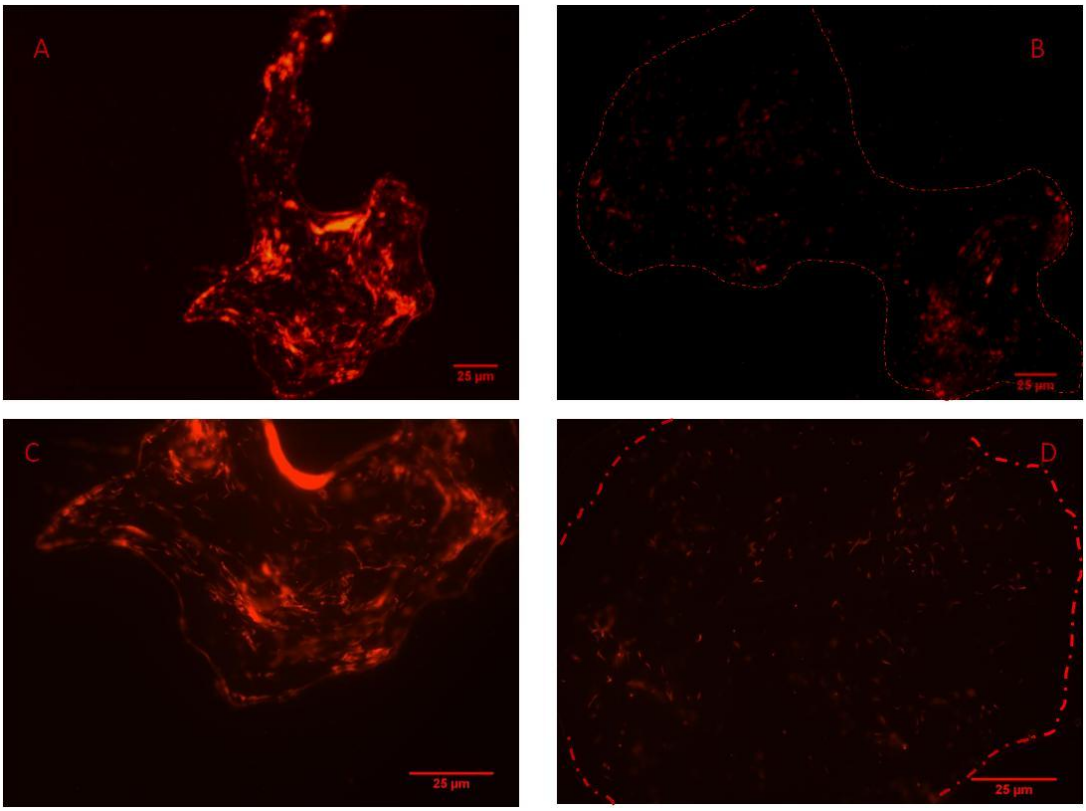

Figure 2.5 DsRed promastigote binding in the absence of PSG x200 (**A**) and x400 (**C**); and in presence of PSG x200 (**B**) and x400 (**D**) Scale bar: 25µm.

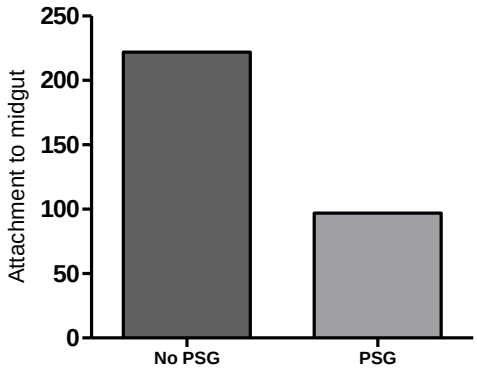

Figure 2.6 DsRed promastigote total attachment to midgut in 50 fields.

RESULTS: CHAPTER 3

The LPG role in midgut binding has been extensively studied [22,31]. In this work, we studied in parallel the role of LPG in midgut binding and PSG/fPPG binding. For that purpose, we assessed *in vitro* binding of *L. mexicana* and *L. major* promastigotes (WT and mutants) to permissive *Lu. longipalpis* midguts and to their PSG, produced respectively in *Lu .longipalpis* (permissive) and *P. duboscqi* (restrictive). For competition studies, PSG was also added to midgut combinations.

Using *the L. mexicana-Lu. Longipalpis* model, the analysis of the role of LPG in PSG binding was completed with capillary migration assays, video-tracking experiments and Western Blot.

3.1 | Promastigote LPG in PSG-slide binding

3.1.1 *L. mexicana-Lu. longipalpis* combination:

L. mexicana-PSG slide-binding was completed with mutants and add-back parasites. First, stage-specific attachment was assessed, revealing that certain stage-specificity is also present in *L. mexicana lpg1¯* promastigotes. Nectomonads were also found to attach in greater numbers, with 41.50 ± 24.51 mean attachment, followed by leptomonads with 15.50 ± 5.331 parasites. Metacyclic attachment was very low (1.750 ± 1.500) as observed previously with WT parasites (**Fig. 3.1**). Unfortunately, we were not able to obtain *lpg1¯* procyclics for this study. Nevertheless, for *lpg1¯* mutant parasites, promastigote stage -dependent reduction in binding was non-significant ($p>0.05$) compared to nectomonad control (**Fig 3.2**). Drop in attachment was slightly less dramatic for *lpg1¯* parasites compared to WT: 62.50% vs 84.93% for leptomonads and 95.78 % vs 99.84% for metacyclics.

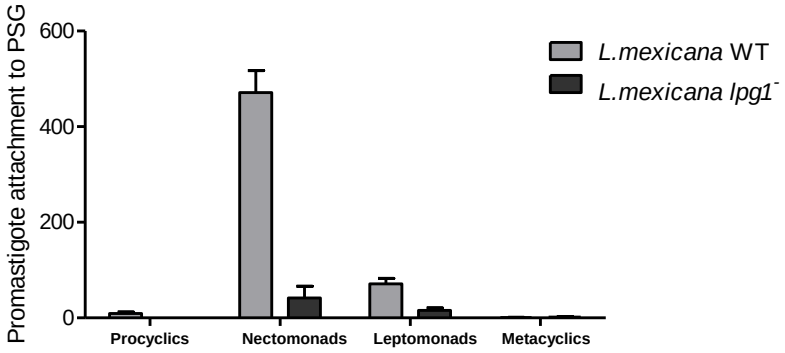

Figure 3.1 Stage-specific PSG binding for *L. mexicana lpg1¯*promastigotes (mean +SEM). Note: *lpg2¯* promastigotes were not available for this experiment.

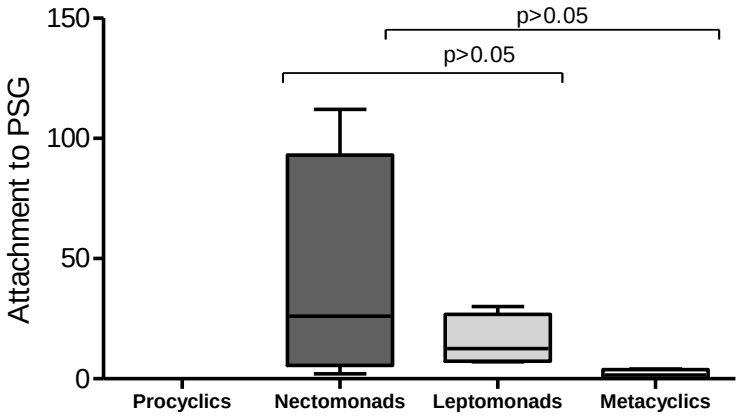

Figure 3.2 Box plots showing data distribution (including median) and P values for *L. mexicana lpg1¯*promastigote stage-specific binding to PSG. P values obtained by Mann-Whitney analysis when compared to nectomonads control; nectomonads *vs* leptomonads: p=0.6857 and nectomonads *vs* metacyclics. p=0.1143.

L. mexicana nectomonad binding was studied in detail, including *lpg2¯* parasites in both PSG and in PBS (negative control) (**Fig.3.3**) and add-back *lpg1/*+LPG1 and *lpg2¯*/+LPG2 parasites (**Fig. 3.4**), revealing LPG-related differences. Compared to *L. mexicana* WT, mean attachment in *lpg1¯*promastigotes was

significantly reduced (p<0.05). There was a 91.1% (41.5 ± 24.51) reduction in binding in *lpg1*¯promastigotes and 92.2% 36.25 ± 16.29) in *lpg2*¯ promastigotes.

Restored *lpg2*¯/+LPG2 parasites attachment was 88% superior to their deficient *lpg2*¯ mutant (p<0.05) and slightly superior to the WT control used in the experiment (p>0.05), with a mean attachment of 308 ± 44.51 vs 288.3 ± 73.76. (**Figs.3.4, Table I**)

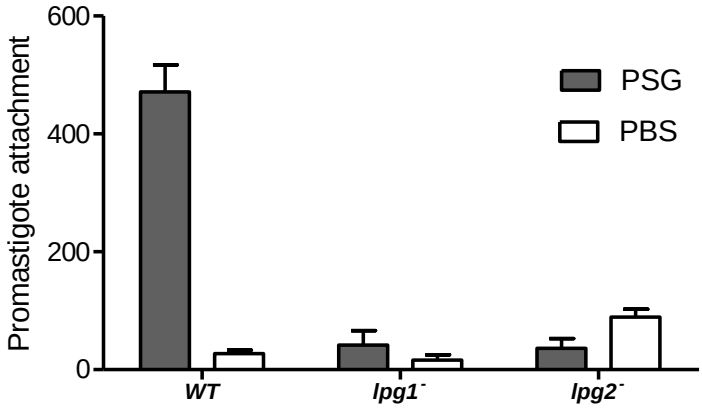

Figure 3.3 *L. mexicana* WT**,** *lpg1*¯ and l*pg2*¯ nectomonad binding to PSG and PBS.

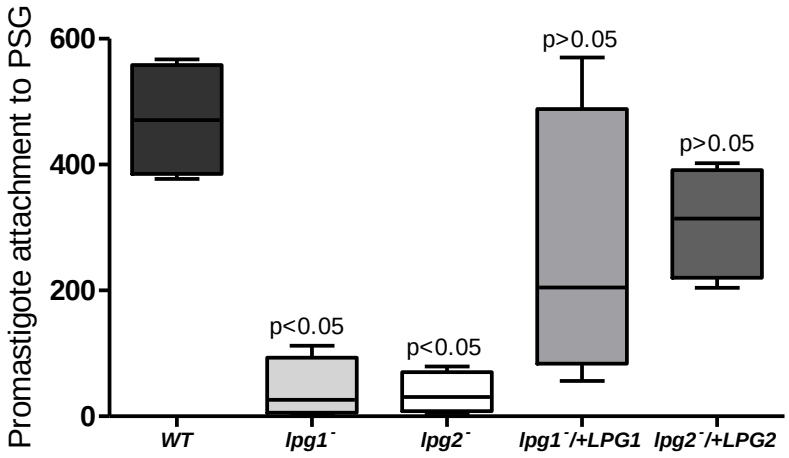

Figure 3.4 Box plots showing data distribution and P values for WT, mutant and restored parasites. P values obtained by Mann Whitney when compared to WT controls.

Table 3.1 Summary statistics for *L. mexicana* LPG mutants and add-back parasites.

	Mean	SEM	Median	N[a]	p value	Significance[b]
L .mexicana WT	471.3	45.98	470.5	4	--------	--------
L. mexicana lpg1⁻	41.5	24.51	26	4	p=0.0286	*
L .mexicana lpg2⁻	36.25	16.29	30.5	4	p=0.0286	*
L. mexicana lpg1/+LPG1	258.8	110.6	204.5	4	P=0.3429	ns
L. mexicana lpg2⁻/+LPG2	308.5	44.51	314	4	p=0.0571	ns

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.05-0.01, significant (*).

3.1.2 *L. major-P. duboscqi* combination

Analysis of *L. major* nectomonad attachment to PSG from experimentally infected *P. dubosqi* revealed that *in vitro* attachment to PSG does also occur and that it actually happens in high numbers. WT nectomonad mean attachment was 870.5 ± 177.7, decreasing when mutants were analysed, with 364.5 ± 43.97 mean attachment for *lpg1⁻* (58.12% reduction) and 100 ± 21.24 for *lpg2⁻* (88.51% reduction). Restoration lead to a marked increase in binding, with 1360 ±246.8 mean attachment for *lpg1⁻*/+LPG1 and 1003± 300.5 for *lpg2⁻*/+LPG2 parasites. Statistical analysis showed that only decrease in binding for *lpg2⁻* was significant ($p<0.05$) compared to WT nectomonads (**Fig.3.5, Table 3.2**).

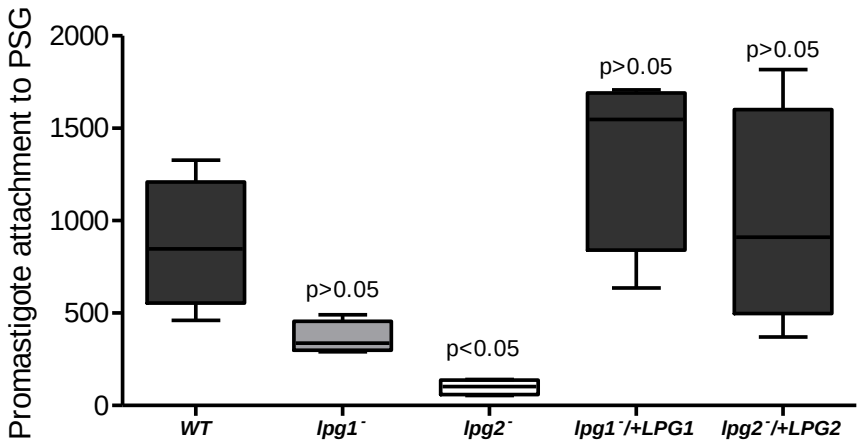

Figure 3.5 Box plots showing data distribution and P values for *L.major* WT, mutant and restored promastigote binding to PSG. P values obtained by Mann Whitney analysis compared to WT controls.

Table 3.2 Summary statistics for *L. major* LPG mutants and add-back parasites.

	Mean	SEM	Median	N[a]	p value	Significance[b]
L. major WT	870.5	177.7	847	4	--------	--------
L .major lpg1⁻	364.5	43.97	338	4	p=0.0571	ns
L. major lpg2⁻	100	21.24	103	4	P=0.0286	*
L. major lpg1⁻/+LPG1	1360	246.8	1547	4	p=0.2	ns
L. major lpg2⁻/+LPG2	1003	300.5	910.5	4	P=0.8571	ns

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.05-0.01, significant (*)

3.1.3 *L. mexicana-Lu. longipalpis vs L. major-P. duboscqi*

Overall, *L. major* promastigotes, WT, mutants and add-backs (AB), were found to attach in greater numbers to PSG compared to their *L. mexicana* counterparts. Mean nectomonad attachment to PSG was 3 times higher in *L. major* (WT + add-back promastigotes) compared to *L. mexicana* (WT+AB), 1078 ± 143.2 vs 352 ± 38; 8.78 times for *lpg1¯*promastigotes; and 2.75 times for *lpg2¯* parasites. This difference was statistically significant for both WT and *lpg1¯* promastigotes ($p<0.05$) but not for *lpg2¯* ($p>0.05$) (**Fig.3.6**). Interestingly, reduction in binding due to LPG and PG deficiency was superior in *L. mexicana*: 91.1% *vs* 58.12% in *lpg1¯* and 92 .2% *vs* 88.5% for *lpg2¯.*

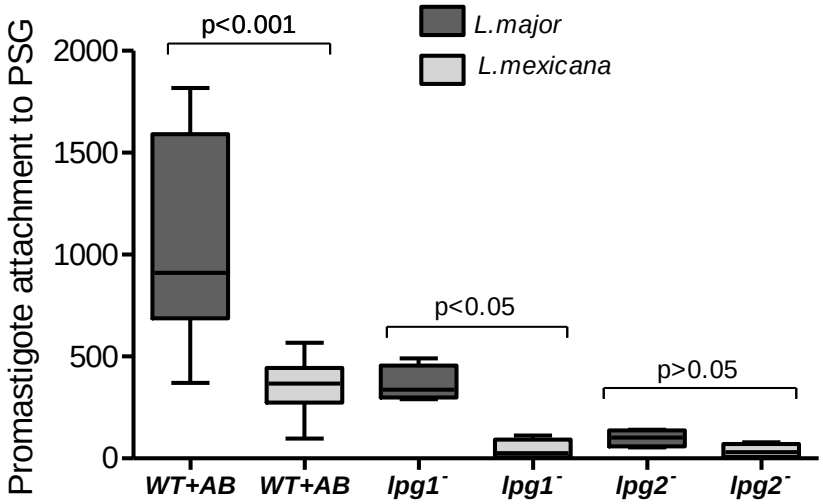

Figure 3.6 *L. major-P. duboscqi* PSG and *L. mexicana-Lu. longipalpis* PSG: data distribution and P values by Mann-Whitney analysis.

Table 3.3 Summary statistics for *L. major-P.duboscqi* PSG binding *vs L.mexicana-Lu. Longipalpis* PSG binding.

	Mean	SEM	Median	N[a]	p value	Significance[b]
L. major WT + AB	1078	143.2	910.5	12	p=0.0002	***
L. mexicana WT + AB	356	38.43	368	12		
L .major lpg1⁻	364.5	43.97	338	4	p=0.0286	*
L. mexicana lpg1⁻	41.5	24.51	26	4		
L. major lpg2⁻	100	21.24	103	4	p=0.1143	ns
L .mexicana lpg2⁻	36.25	16.29	30.5	4		

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.05-0.01, significant (*)and p<0.001 extremely significant (***).

3.2 | Promastigote LPG in capillary migration assay

Capillary migration experiments were performed as described for WT parasites (results: chapter 1). Unlike WT promastigotes, *lpg1⁻* mutant parasite movement was not affected by PSG. Interestingly, nectomonad migration was favoured by PSG compared to PBS, with almost 40% parasites more crossing the capillary at the end of the experiment (p=0.0087). *lpg1⁻* metacyclic migration was not significantly different in PSG and PBS (p=0.5587) **(Figs 3.7 & 3.8)**.

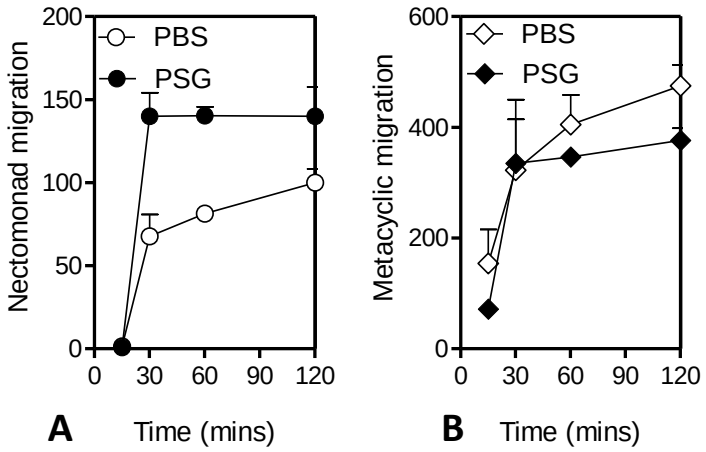

Figure 3.7 *L. mexicana lpg1¯* nectomonad (**A**) and metacyclic (**B**) migration pattern (mean +SEM) through the capillary tube over time in PSG and PBS, showing number of parasites per drop and time point.

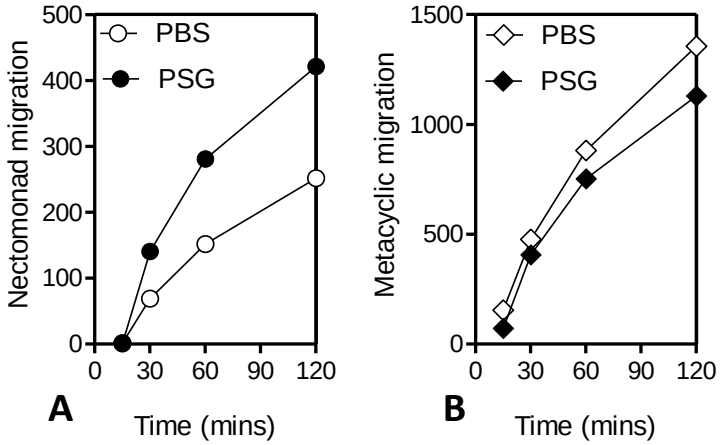

Figure 3.8 *L. mexicana lpg1¯* nectomonad (**A**) and metacyclic (**B**) migration (mean) through the capillary tube overtime in PSG and PBS, showing cumulative number of migrated parasites per time point.

Analysis of WT and *lpg1⁻* nectomonads migration in PSG confirmed LPG-dependent migration differences. *lpg1⁻* nectomonad migration was significantly better (p=0.0095), with an increase of 87.64% compared to WT (421 *vs* 52 parasites)(**Fig 3.9**).

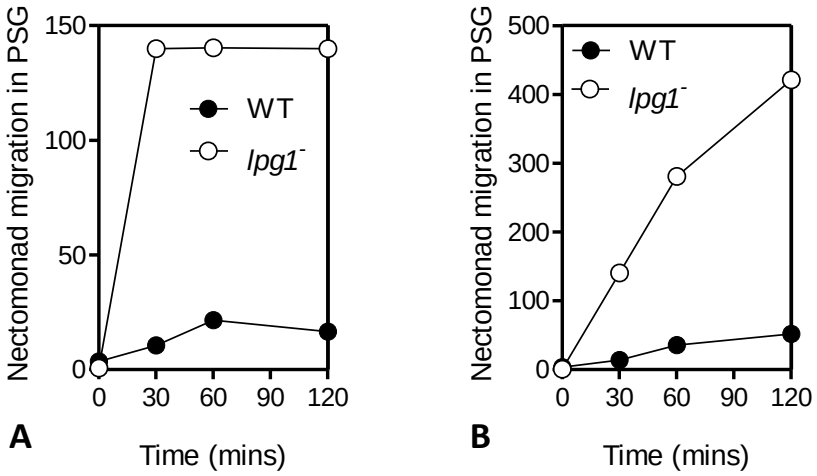

Figure 3.9 A/ *lpg1⁻* and WT nectomonad migration pattern (mean) through the capillary tube over time in PSG showing number of parasites per drop and time point. **B/** migration (mean) through the capillary tube overtime in PSG, showing cumulative number of migrated parasites per time point.

Combined migration in PSG was also studied for mutant parasites. In WT combinations, nectomonad parasites were more abundant in the capillary plug (around 60% of the total in both PSG and PBS capillaries). In mutant parasites however, numbers turned and metacyclics were found to be the most abundant form (63.06% *vs* 36.9%). At the inoculation end, after 120 minutes, nectomonads were proportionally more abundant than metacyclics (63.27% *vs* 36.72%) but this difference was not significant (p=0.0765) (**Fig. 3.10**). Compared to WT experiment, *lpg1⁻* nectomonad were able to move freely and migrate from the point of inoculation (p=0.0027) (**Fig.3.11)**.

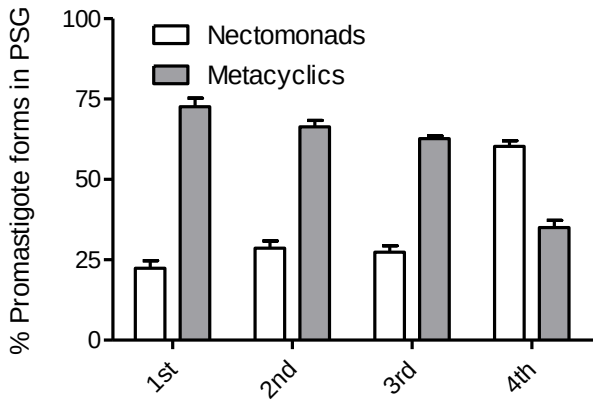

Figure 3.10 Comparative migration assay for *lpg1*¯mutant nectomonad and metacyclic promastigotes in PSG.

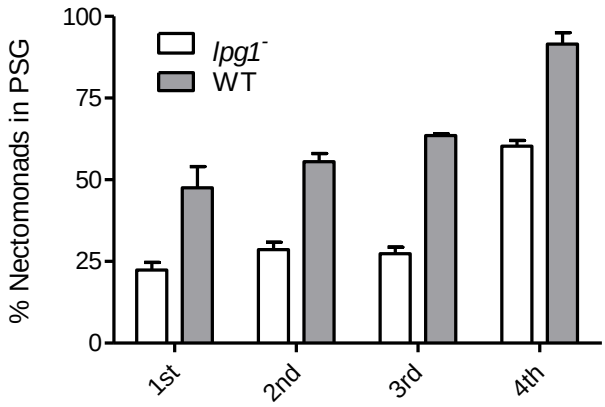

Figure 3.11 Percentage of WT and *lpg1*¯nectomonads in PSG at the inoculation end after 120 minutes.

3.3 Promastigote LPG in parasite video-tracking

L. mexicana WT nectomonad movement *in vitro* was found to be compromised in PSG compared to PBS ($p<0.05$) while metacyclics showed similar velocity in both PSG and PBS ($p>0.05$). Following the same conditions, *lpg1*¯promastigote movement was also video-tracked and analysed using Volocity® tracking system (**Fig. 3.12**).

lpg1¯ nectomonad velocity was lower in PSG than in PBS, with a mean track velocity of 68.01 ± 3.636 µm/sec *vs* 81.50 ± 6.234 µm/sec). In contrast to WT, this difference in velocity was not significant (p>0.05)(**Fig.3.13, Table 3.4**)

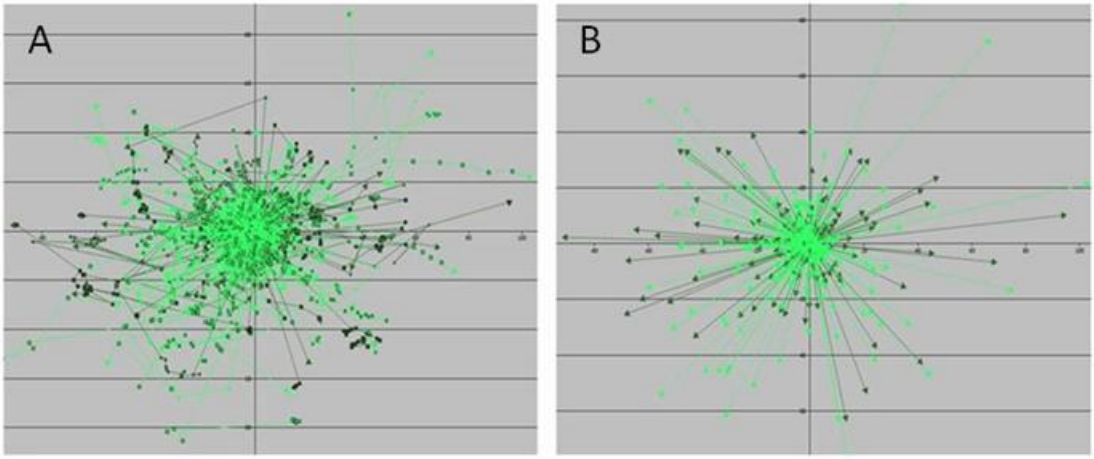

Figure 3.12 (**A**) *lpg1*¯ Nectomonad movement pattern in PSG (light green) and in PBS (dark green) showing centroid measurement of objects for all timepoints linked by tracks (in µm). (**B**) Nectomonads final displacement (in µm) in PSG (light green) and PBS (dark green).

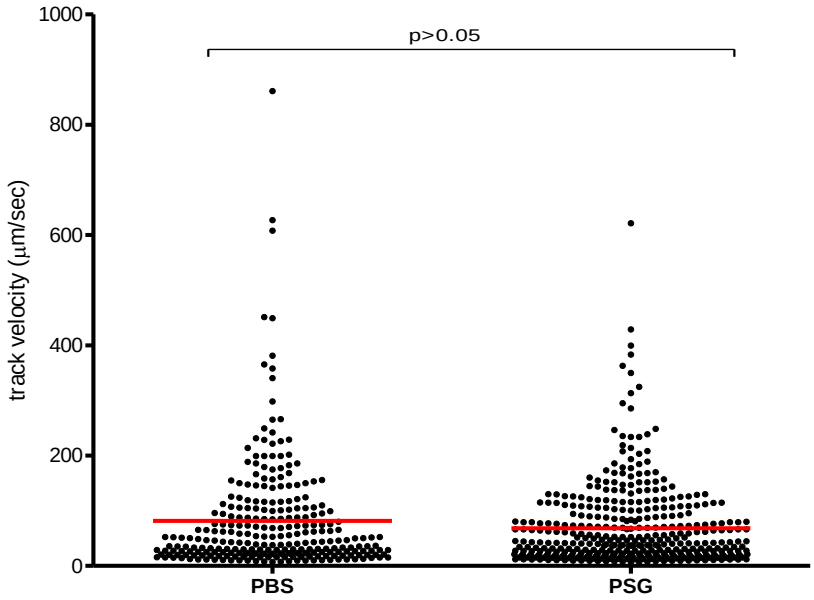

Figure 3.13 *lpg1*¯ nectomonad promastigote velocity per individual track in PBS and PSG. Mean track velocity in red. P value obtained by Mann-Whitney analysis.

Table 3.4 Summary statistics for *lpg1⁻* nectomonad video-tracking:

	Mean	SEM	Median	N[a]	p value	Significance[b]
PSG	68.01	3.636	40.91	406	p=0.1649	ns
PBS	81.50	6.234	44.42	269		

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns).

In addition, *lpg1⁻* and WT nectomonad velocity was analysed jointly by video-tracking (**Fig.3.14**), confirming the role of LPG in parasite movement observed in the capillary and previous video-tracking experiments. *lpg1⁻* nectomonad promastigotes were significantly faster than WT nectomonad in PSG ($p<0.05$) *lpg1⁻* mean track velocity was 142.5 ± 6.137 µm/sec compared to 67.63 ± 1.454 µm/sec for WT nectomonads (**Fig.3.15, Table 3.5**).

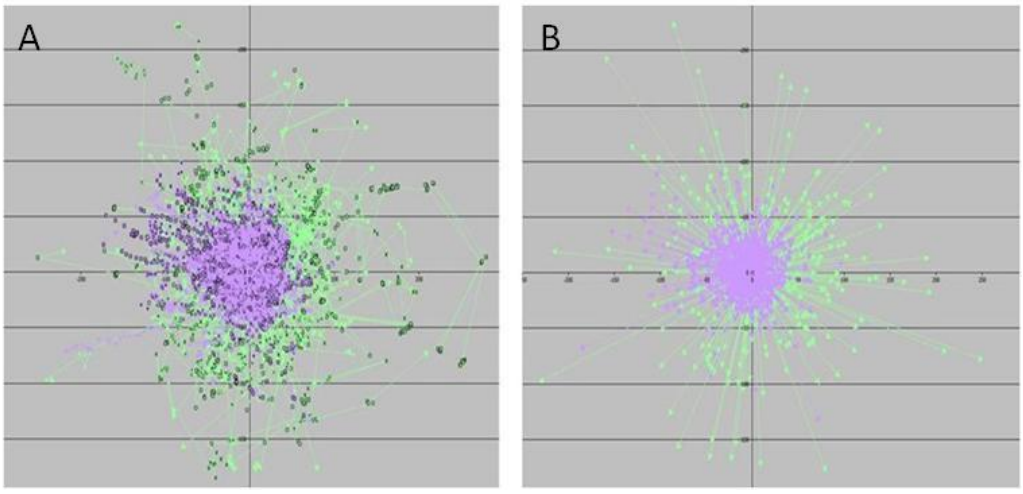

Figure 3.14 (**A**) Movement pattern for *lpg1⁻* (light green) and WT (purple) nectomonad promastigotes in PSG, showing centroid measurement of objects for all timepoints linked by tracks (in µm). (**B**) Final displacement (in µm) for *lpg1⁻* (light green) and WT (purple) nectomonad promastigotes.

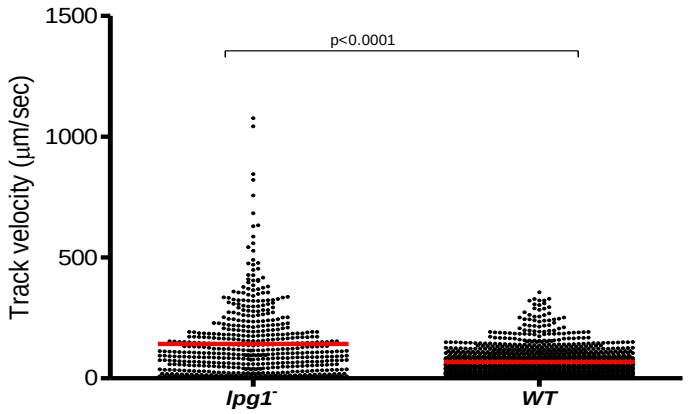

Figure 3.15 Velocity per individual track for *lpg1¯*and WT nectomonads in PSG. Mean track velocity in red. P value obtained by Mann-Whitney analysis.

Table 3.5 Summary statistics for *lpg1¯* and WT nectomonad video-tracking:

	Mean	SEM	Median	N[a]	p value	Significance[b]
lpg1¯	142.5	6.137	106	529	p<0.0001	***
WT	67.63	1.454	54.07	1385		

[a]N=number of observations. [b]Significance values: p<0.001 extremely significant (***)

3.4 | Promastigote LPG in parasite attachment to midgut in absence and presence of PSG

3.4.1 *L. mexicana-Lu. longipalpis* combination:

*L. mexicana lpg1¯*mutant nectomonads were found to bind in significantly lower numbers (p<0.05) to *Lu. longipalpis* midgut, with a mean attachment of 6.50 ± 2.95 *vs* 432.3 ± 80.14 parasites. When LPG deficiency was restored (*lpg2¯*/+LPG2 parasites), nectomonad binding increased again (392.5 ± 202.4) close to WT levels (p>0.05) (**Figs. 3.16)**. *lpg2¯* and *lpg1¯*/+LPG1 parasites were not available for this experiment.

The addition of PSG to restored mutants-midgut combinations had similar effects to WT-midgut combinations. There was a 68.5% reduction in binding in the restored group (compared to 78.4% in WT group), but this difference was found not to be statistically significant (p>0.05) (**Figs. 3.17, Table 3.6**).

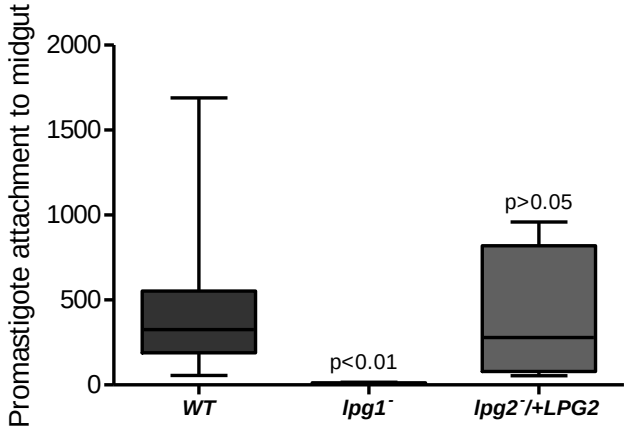

Figure 3.16 Box plots showing data distribution and Pvalues for *L. mexicana* WT, mutant and restored nectomonad midgut binding. P values obtained by Mann-Whitney analysis using WT nectomonad as control group.

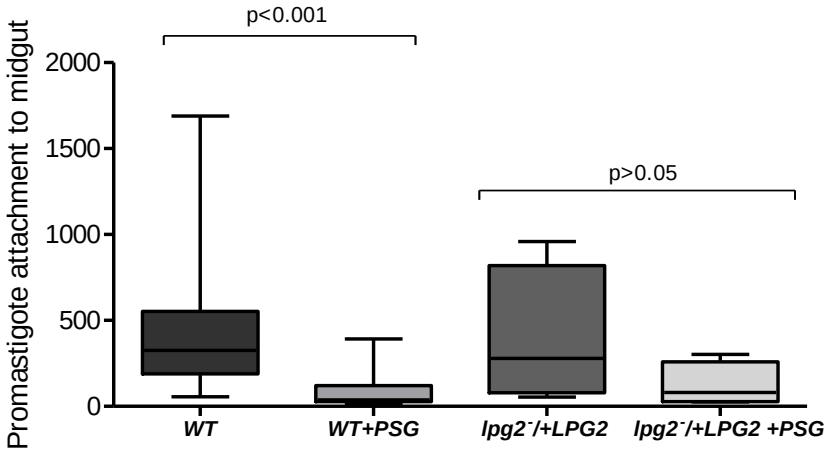

Figure 3.17 Box plots showing data distribution for *L. mexicana* WT and restored nectomonad binding in presence and absence of PSG. P values for WT and restored nectomonad binding ± PSG. P values obtained by Mann-Whitney analysis.

Table 3.6 Summary statistics *for L. mexicana* midgut binding.

	Mean	SEM	Median	N[a]	p value	Significance[b]
L. mexicana WT	432.3	80.14	326	21	--------	--------
L. mexicana lpg1⁻	6.50	2.95	4.5	4	p= 0.0021	**
L. mexicana lpg2⁻/+LPG2	392.5	202.4	278.5	4	p= 0.6299	ns
L. mexicana WT+ PSG	91.18	33.76	37	11	p=0.0002	***
L. mexicana lpg2⁻/+LPG2 +PSG	122.3	64.52	80.5	4	p=0.2	ns

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns); p=0.01-0.001, very significant (**); and p<0.001 extremely significant (***).

3.4.2 *L. major-Lu. longipalpis* combination:

L. major WT nectomonad promastigotes were also found to bind to permissive *Lu. longipalpis* midgut *in vitro* (809.5 ± 380.5). This attachment seems to be mediated by LPG as well, with 199 ± 97.41 *L. major lpg1⁻* parasites bound, but also by other PGs as shown by binding reduction in *lpg2⁻* parasites, with 42 ± 20.81 nectomonads attached. When add-back *lpg2⁻*/+LPG2 parasites were studied, binding increased again up to 1058 ± 389.2 parasites. However, mutant and add-back parasite binding was found not to be significantly different compared to *L. major* WT control (all p>0.05) (**Fig. 3.18, Table 3.7**).

PSG from experimentally infected *P. dubosqi* female sand flies was added to the midgut-parasite combinations to asses its role in midgut binding/ detachment. There was a reduction 54.29 % in binding; attachment dropped from 809.5 ± 380.5 to 370 ± 88.08 nectomonads per midgut. This fall in attachment was also found not to be statistically significant (p>0.05) (**Fig. 3.18, Table 3.7**).

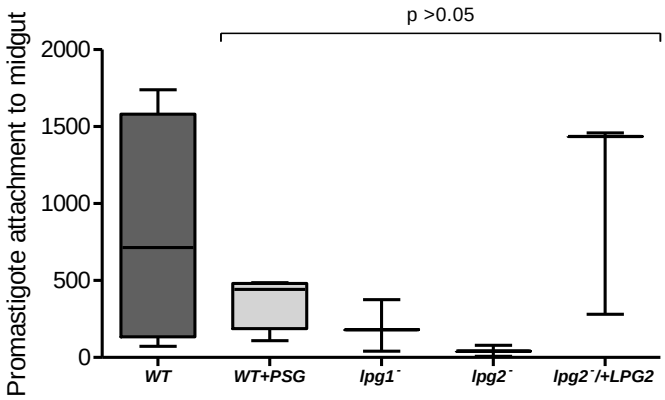

Figure 3.18 Box plots showing data distribution and P values for WT (±PSG) , mutant and add-back promastigote binding. P values obtained by Mann-Whitney analysis when compared to nectomonads WT control group.

Table 3.7 Summary statistics for *L.major-Lu.longipalpis* midgut binding

	Mean	SEM	Median	N[a]	p value	Significance[b]
L. major WT	809.5	380.5	713.5	4	--------	--------
L. major lpg1⁻	199	97.41	181	3	p=0.8857	ns
L. major lpg2⁻	42.00	20.81	40	3	p=0.4000	ns
L. major lpg2⁻/+LPG2	1058	389.2	1436	3	p=0.1143	ns
L .major WT+PSG	370	88.08	442.5	4	p=0.8571	ns

[a]N=number of observations. [b]Significance values: p ≥0.05, non-significant (ns).

3.4.3 *L. mexicana* vs *L. major* in *Lu. longipalpis* midgut:

In midgut binding, attachment was 2.15 times higher for WT+AB and 30.6 times for *lpg1⁻* in *L. major* compared to *L. mexicana*. In both cases, this difference was not statistically significant (p>0.05). As with binding to PSG, the fall in attachment to the gut resulting from a deficiency in LPG was higher for *L. mexicana* compared to *L.major*, 98.49% *vs* 43.37%(**Fig. 3.19, Table 3.8**).

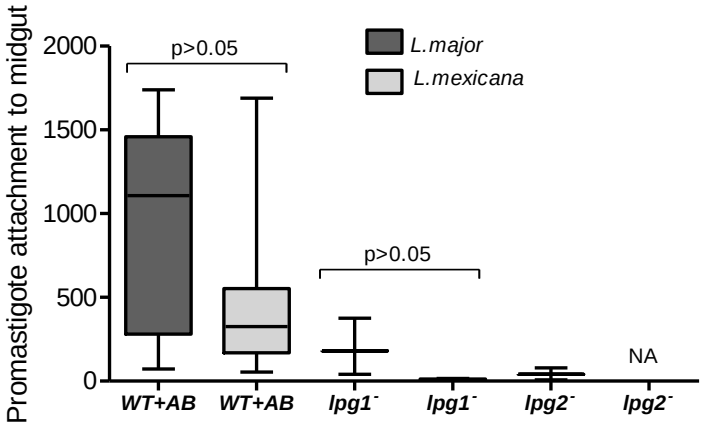

Figure 3.19 Box plots showing data distribution and P values for *L. major* and *L. mexicana* WT and mutant binding to *Lu. longipalpis.* P values by Mann-Whitney analysis. .NA: not applicable.

Finally, the effect of PSG in binding was found to be higher in *L. mexicana* WT, with a 78.9% significant reduction in attachment ($p<0.05$) than in *L. major* WT, with 54.29% reduction($p>0.05$) (**Figs. 3.20**).

Table 3.8 Summary statistics for *L. major vs L. mexicana* promastigote binding to *Lu. longipalpis* midgut.

	Mean	SEM	Median	N[a]	p value	Significance[b]
L. major WT + AB	916.1	256	1108	7	p=0.1212	ns
L. mexicana WT +AB	426	72.97	362	25		
L .major lpg1⁻	199	97.41	181	3	p=0.0571	ns
L. mexicana lpg1⁻	6.50	2.95	4.5	4		
L. major lpg2⁻	42.00	20.81	40	3	----	----
L .mexicana lpg2⁻	----	----	----	----		

[a]N=number of observations. [b]Significance values: $p \geq 0.05$, non-significant (ns).

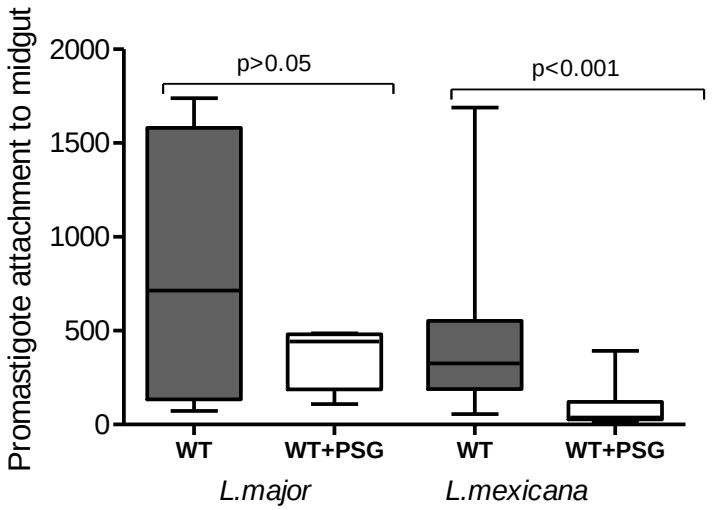

Figure 3.20 PSG effect in midgut binding for *L. major* WT and *L. mexicana* WT: data distribution and p values obtained by Mann-Whitney analysis.

3.5 fPPG binding to LPG by Western Blotting

So far, *in vitro* assays have revealed a selective binding of promastigotes to PSG and/or fPPG. Here we aim to confirm this binding at protein level by Western Blotting. Previously, procyclic promastigote LPG binding to midgut microvillar proteins was studied by Western Blot with biotinylated LPG[86].

Immunoblotting confirmed the stage-specific binding to PSG/fPPG observed in our biological experiments. Selective binding was detected when purified *L. mexicana* nectomonad and metacyclic LPG were immunoblotted using biotinylated-fPPG.

Prior to immunoblotting, quality of fPPG after biotinylation process was assessed by LT15 probing and comparison to both fPPG and PSG; its suitability for experiments was confirmed (**Fig 3.21**). LT15 probing was also positive for nectomonad and metacyclic LPG used in our experiments (**Fig 3.22**).

fPPG was found to bind to nectomonad LPG and not to metacyclic LPG under the same conditions (LPG from 1.6 x 10^8 cells in both). A band of ~17-19 kDa was observed repeatedly in nectomonad LPG line and was always absent in metacyclics (**Fig 3.23**).

Fig 3.21 From left to right: PSG, biotinylated fPPG and fPPG probed with LT15

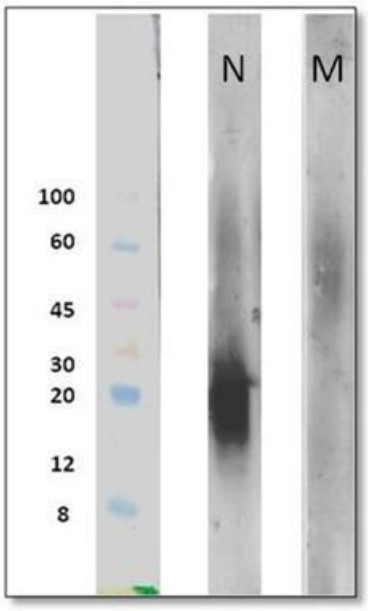

Fig 3.22 Purified LPG from nectomonad (N) and metacyclic (M) promastigotes visualised by Peroxidase Substrate Kit (Vector Laboratories).

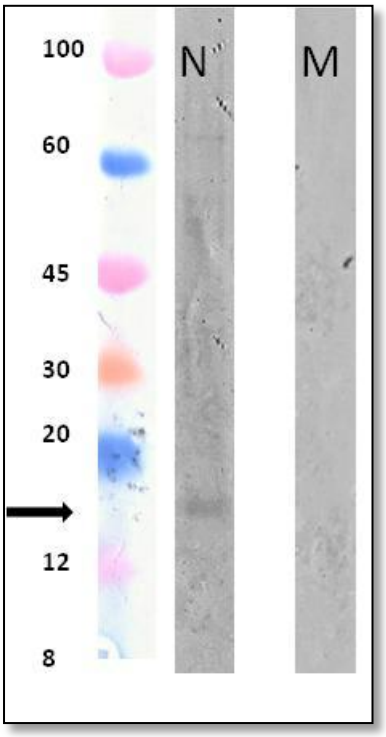

Fig 3.23 Nectomonad (N) and Metacyclic (M) LPG immunoblotting with biotinylated fPPG. A 17-19 kDA band was observed in the nectomonad promastigote line(arrow).

Transmission is a crucial event in the *Leishmania* life-cycle. The chances of successful *Leishmania* transmission benefits from a large inoculum of infective metacyclic promastigotes to be transmitted by sand fly bite[117]. Transmission occurs as a result of complete promastigote development and an appropriate blood feeding behaviour, shown to be regulated by parasites[31,48]. At the end of *Leishmania* development within the sand fly gut, however, there is a mixed promastigote population with both mature and immature parasites present in the anterior midgut[46,109,110] (and **Rogers, unpublished data**). This points to the existence of an underlying promastigote selection process. Here we provide initial evidence of the role of promastigote secretory gel (PSG) in this selection. Additionally, we show preliminary data that suggest that PSG could also play a part in another key event in leishmaniasis development and transmission, the detachment of promastigotes from the midgut epithelium.

1 | PSG in transmission:

Previously, PSG has been described as a behavioural transmission determinant modifying sand fly feeding behaviour[48] (blocked-fly hypothesis) and as a key element in the establishment of infection in the host[49]. From our work, we extend and propose PSG as a biological transmission determinant, acting as a sieve that retains immature parasites to produce a final metacyclic enriched inoculum. Previous works by Lawyer et al[109], Saraiva et al[110] and Rogers et al[46] observed differences in movement among immature and mature promastigote forms within the PSG plug. Rogers and Bates[48] demonstrated that PSG/fPPG determines dose delivered by the sand fly and that presence of PSG was positively correlated with numbers of metacyclics in the midgut, but the actual mechanism that determines inoculum composition remained unaddressed[49].

In this study, we tested the hypothesis that *Leishmania* may bind to PSG in a stage-specific manner allowing it to “sieve” infectious from non-infectious

promastigote forms inside the sand fly midgut and influence the composition of the infective sand fly bite. To do this, attachment to PSG from both experimental (*L. mexicana-Lu. longipalpis*) and natural (*L. major-P. duboscqi*) parasite vector combinations was observed.

Experiments were optimised and performed mainly with *Lu. longipalpis* and *L. mexicana*. Despite being an experimental combination, it has been proven to be a reliable model widely used in competence studies[46,56,57,66]. *Lutzomyia longipalpis* is classified as a permissive vector that supports development and successfully transmits all *Leishmania* species tested so far, wild type and in some species, also mutant lines. Like other permissive sand fly species, it expresses *O*-glycosylated glycoproteins with terminal N-acetylgalactosamine (GalNAc) in the midgut epithelium, shown to be a potential permissive ligand[61,62,66] and lacks PpGalec found in restrictive combinations[80]. *Leishmania mexicana* development *in vitro* has been characterised in detail[49] and its LPG is known to be minimally substituted with glucose residues[71], which appears to be indicative of permissive relationships[22]. In future experiments, we plan to include natural *L. chagasi/infantum-Lu. longipalpis* combinations, as *L. mexicana* and *L. infantum* may differ in their development or synthesis of phosphoglycans.

Scanning electron images revealed *L. mexicana* and *L. major* promastigotes apparently trapped within the PSG[101]. However, no experiments have been performed to see whether this is actually the case and whether it is merely a physical restriction of parasite movement or a response to ligand-receptor interaction. This attachment was studied *in vitro* using three different experimental approaches: promastigotes binding to PSG coated slides, promastigote video-tracking and promastigote capillary migration.

By exploiting the ability of *Leishmania* to reproduce its promastigote developmental programme during their first passage *in vitro* from tissue derived

amastigotes, we compared stage-specific binding using a cryopreserved panel of all developmental stages (procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes). *L. mexicana* nectomonad and leptomonad promastigotes bound in considerably higher numbers than both procyclic and metacyclic promastigotes. Moreover, nectomonad binding was significantly greater than the binding of procyclic, leptomonad and metacyclic promastigotes. (**Fig.1.3, Table 1.1**). Leptomonads are intermediate forms and they may share characteristics of both nectomonad and metacyclic promastigotes, which could explain their lower binding to PSG in comparison with nectomonads. It is possible that leptomonads promastigotes may express a mosaic of nectomonad and metacyclic forms of LPG as they transform into metacyclics, although this remains to be tested. Nevertheless, our data show that stage-specific binding to PSG occurs *in vitro*. The amount of PSG used per well was equivalent to 0.5 µg, and therefore, biologically plausible, as approximately 1 µg (0.86 µg) can be found at least in experimentally infected flies[57,105].

Next, PSG binding was studied by measuring promastigote movement in microtitre plate wells and capillary tubes. These approaches allow us to overcome the loss of parasites that occurs during slide washing or fixing that could affect final parasite count. They also allow us to observe their behaviour when they are completely surrounded by PSG, as it happens in the midgut. Movement was interpreted as an indirect sign of attachment. Cues that influence parasite anterior migration in the sand fly midgut are still largely unknown. Promastigotes are believed to follow a sugar gradient (chemotaxis)[118,119] mainly from sugars delivered from the crop into the midgut[120], but migration has also been observed in absence of sugars[121]. Another possible cue may result from changes in osmotic pressures (osmotaxis) in response to components of the sand fly saliva that may form gradients within the midgut [122] [118,123] . Sand fly saliva and an acid microenvironment have been shown to induce metacyclogenesis [22,31,123,124]; therefore, we cannot exclude pH effect in late parasite migration. However, these experiments showed that low pH attributable to PSG *per se* does not influence parasite movement.

Therefore, in the absence of known cues, migration through PBS and PSG appears to be a result of capillarity, forcing promastigotes to migrate forward homogenously. In the wells, movement seemed random as it happens in culture. Within the sand fly midgut it is plausible that high densities of immobilised promastigotes within the PSG could promote quorum sensing-like communication between parasites, which may induce metacyclogenesis and consequently, anterior migration of infective forms. Quorum-sensing has been observed in bacteria that act as a group for successful colonisation of certain surfaces (i.e biofilm formation)[125]. However, our tracking time was relatively short (20 seconds). This allowed us to observe immediate biochemical interactions between the parasite and the PSG, rather than any indirect effect of signalling. Despite this, we cannot exclude that within the sand fly, communication between promastigotes exists. Biofilm formation[126] and quorum-sensing[127] have been also described in *Yersinia pestis*, causative agent of plague. Within the flea vector *Xenopsylla cheopis*, *Y. pestis* form large multicellular aggregates linked by an extracellular matrix composed by bacterial and exogenous digestive products present in the gut, preventing their loss by defecation[126]. These aggregates remain free in the midgut lumen but subsequently adhere to the proventricular spines, blocking the flea and impairing the blood intake[126,128]. Unlike for Y. *pseudotuberculosis*[127], the role of quorum-sensing in parasite motility for *Yersinia pestis* remains unclear, although it is shown to regulate several metabolic functions[129,130] and biofilm formation[131].*Y.pestis* aggregates have been proposed to favour transmission by enabling non-motile *Y.pestis* to move against the blood flow when the flea feeds[126].

In capillary migration assays, we found that nectomonad promastigote movement was severely affected in PSG compared to PBS ($p<0.05$), while metacyclic promastigote movement was unhindered in PSG, with no significant differences in their movement in PSG and PBS (**Figs. 1.6, 1.7**). This was also observed in video-tracking of wells that despite their random movement, nectomonad mean track velocity was also significantly reduced in PSG compared to PBS (**Fig. 1.10**) which did not happen with metacyclic promastigotes (**Fig.1.12**).

Analysis of residual plug in the capillary after 120 minutes revealed a higher proportion of non-migrated nectomonads that remained at the end of the capillary where parasites were introduced compared to metacyclics (**Fig.1.8**).

In the literature, there are not any previous studies that compare promastigote velocity *in vitro.* However, in 1989, Walters et al[55], studied in detail *L. chagasi*/*infantum* development within its natural vector *Lu. longipalpis* and observed that usually inthe swollen cardia region, most of the parasites were immotile whereas in the posterior midgut they moved freely. They also described free swimming slender promastigotes (<1 μm body width) in the lumen of the esophagus and pharynx. When the plug was released from the cardia, parasites were found to be strongly embedded within the gel. Later, Lawyer et al[109] and Saraiva et al[110], working *with L. major* in *P. duboscqi* and *P. papatasi* respectively, observed that from plugs containing a similar mixture of promastigote forms, metacyclics were selectively released, suggesting an unrestricted movement in PSG. Detailed analysis of the composition of promastigote developmental forms within the PSG plug by Rogers et al[46], showed that although *L. mexicana* metacyclics were originated in the middle of the plug, they eventually accumulated at the anterior and posterior poles of the plug in *Lu. longipalpis* sand flies.

Moreover, Saraiva et al[110] also characterised gut released *L. major* metacyclics using stage-specific markers. These free metacyclics were recognised by 3F12 monoclonal antibody which is a specific marker of LPG modifications occurring in *L. major* metacyclics[45]. Metacyclics were also found to express MAT-1, which is a stage specific transcript[110]. The characterisation of these parasites with unrestricted movement and the extensive research on LPG, suggests that LPG may not only be important in promastigote midgut binding but also in promastigote transit through PSG. Therefore, metacyclogenesis will not only adapt promastigotes for “survival”in the mammalian host but also for “arrival” to the mouthparts[109,110] by ensuring their free movement through the PSG plug.

Promastigote movement may also depend on substrate ultra-structure or viscosity or even on parasite size and morphology. In this case, viscosity was not determined due to technical limitations. Use of deglycosylated PSG instead of PBS as a control might be a good idea, as glycans are the most active components of fPPG[57] and candidates for binding. Nevertheless, we cannot predict the effect that deglycosylation would have in PSG mesh-work structure and viscosity as glycans seem to be responsible for their structure[49]; deglycosylated PSG might be less viscous than PSG and thus, as inaccurate as PBS for our experiments. Slide binding reduces this structural/viscosity effect.

From our results, we could not rule out the role of fPPG meshwork structure or promastigote size and morphology in promastigote movement or selection, even though slide binding experiments confirmed an active binding component of this phenomenon. Therefore, our biggest obstacle was to demonstrate that the hindered movement of nectomonads was not a mere result of their wide and long body being trapped in the fPPG network. For that purpose, we analysed *L. mexicana* phosphoglycan mutant lines for PSG-slide binding, capillary and video-tracking experiments.

Nectomonad video-tracking revealed that unlike its WT counterparts, the movement of mutant *L. mexicana lpg1*$^{-}$ nectomonads specifically lacking lipophosphoglycan (LPG) was not significantly inhibited in the well containing PSG compared to PBS (**Fig. 3.13**) .Moreover, these mutants moved considerably faster than WT nectomonads in PSG (**Fig. 3.15, Table 3.5**). In addition, *lpg1*$^{-}$ nectomonad migration in capillaries was favoured in PSG compared to PBS (**Fig. 3.8**) and was significantly superior to its WT equivalent in PSG (**Fig. 3.9**). PSG plug analysis revealed that nectomonads were still more abundant than metacyclics but not significantly (**Fig. 3.10)** and that they were less abundant compared to WT (**Fig. 3.11**). These findings support the premise that promastigote movement through PSG (and its main component fPPG) is related to their main surface glycoprotein, LPG. We are observing a ligand mediated binding and not just physical entrapment and restriction of movement.

LPG participation in PSG attachment and thus movement, was further characterised and confirmed using *lpg2*¯ mutants (deficient in all phosphoglycans) and restored *lpg1*¯/+LPG1 and *lpg2*¯/+LPG2 add-back lines. For *lpg1*¯ mutants, stage-specific analysis revealed that nectomonad and leptomonads attached more to PSG compared to metacyclics, but this difference was not significant (**Fig.3.2**) unlike for WT promastigotes. Nectomonad binding for *lpg1*¯and *lpg2*¯ lines was significantly lower than WT; and was largely restored with the re-introduction of the deleted genes (**Fig.3.4**). Therefore, we can conclude that LPG is an important ligand for promastigote binding to PSG *in vitro* and could explain not only the lack of binding of metacyclics (due to conformational changes in their LPG) but also of procyclics that express very low LPG on their surface[56]. The role for PSG-binding is biologically irrelevant for procyclics since they will never coincide in time, but reinforces the role of LPG in binding to PSG. Also time-course Western Blots of *L. mexicana* and *L. infantum* infected *Lu. longipalpis* midguts has shown that fPPG is not detectable before day 3, i.e, 48 hours, after procyclics have transformed to nectomonads[48,57].

There appears to be a minor participation from PGs other than LPG in binding, as suggested by the low binding of *lpg1*¯mutants. In our experiments, we could not assess accurately the role of non-LPG phosphoglycans in *L. mexicana* binding, due to the high default binding of *lpg2*¯ to PBS (**Fig.3.3**). Therefore, we cannot rule out binding either in cracks of PSG or PBS derived from baking the slide (56 °C) prior to binding. Interestingly, unequal binding was observed in *lpg1*¯ mutants according to promastigote stage; PPGs have been shown to vary according to *Leishmania* species and promastigote stage[83]. However, these differences were non-significant. Membrane bound PPGs (mPPGs) have been described as potential ligands, due to their similarity to LPG[71]. More experiments need to be conducted with *lpg2*¯ mutants to understand their participation in PSG binding.

This is the first time that parasite-PSG/fPPG interactions have been studied *in vitro* and in such detail. Our results reveal that promastigote selection occurs as a result of fPPG binding to the non-infective form of promastigote LPG. This selection

would favour the accumulation of metacyclics near the sand fly mouthparts and consequently, *Leishmania* transmission. Western Blot revealed that biotinylated-fPPG could attach to purified nectomonad LPG but not to metacyclic LPG, reinforcing previous biological observations. A 19 kDa band was present only in nectomonad LPG (**Fig.3.23**). When the amount of metacyclic LPG loaded was doubled, this band was still absent; although some higher molecular weight bands were observed presumably due to non-specific binding (data not shown). Interestingly, this 19 kDa band coincides with the one observed by Dillon & Lane[86] when they analysed midgut binding *in vitro. L. major* LPG was found to bind *P. papatasi* midgut microvillar proteins. They described several bands, but one the strongest was 19 kDa. In this kind of experiment, it is difficult to conclude if the higher bands are a consequence of unspecific binding or a combination of specific and unspecific binding and whether the lower bands are specific or just degradation products[86]. Nevertheless, a common band was found in our fPPG study and in the midgut protein binding by Dillon and Lane. This represents the possibility of a shared ligand that participates in the binding of nectomonads to the sand fly gut and later to the secreted fPPG, which needs further characterisation. Five years later, after Dillon and Lane[86], Kamhawi et al[80] described a 35.4 kDa lectin (PpGalec) exclusively on the luminal surface of *P. papatasi* midgut epithelium that specifically binds to the galactose side chains of *L. major* LPG. They suggested that the previous protein described by Dillon and Lane[86] co-precipitated with PpGalec. However, fPPG is not a "lectin-like" molecule and it is comprised of phosphoglycan repeats also present in LPG, therefore, a cross-linker that mediates in the binding may be necessary. Such a cross-linker is presumably acquired during promastigote development, which could explain its presence in two different parasite-sand fly combinations.

Although the structure of fPPG has only been described in a few *Leishmania* species (*L. major, L. mexicana*), it seems to show species-specific substitution of the sugar residues of the phosphoglycan repeats and the cap structure[49,71], similar to LPG. The role of these sugar residues in binding to the LPG needs to be further

characterised, as it may also demonstrate species-specific binding. Thus, future experiments should analyse *in vitro* promastigote attachment to PSG from distinct *Leishmania* species.

In conclusion, these results reveal a ligand mediated binding that selectively binds immature promastigotes and allows free movement of metacyclics, which may enrich the bite for infective forms. During transmission, *Leishmania* promastigotes are regurgitated from the PSG-obstructed gut which contains a mixed population of metacyclics and non-metacyclics (predominantly nectomonads and leptomonads), therefore, contamination with non-infective promastigote stages seems inevitable. In contrast, the composition of the infective dose is enriched for metacyclic promastigotes[57]. Recent research carried out by Dr Emilie Giraud (from Dr Rogers' research group) using quantitative reverse transcription polymerase chain reaction (qRT-PCR) to measure the relative expression of stage-specific molecular markers from parasites delivered by bite, revealed a higher proportion of metacyclics compared to nectomonad or leptomonad promastigotes (75% : 25%) in mouse ears (unpublished data). This approach will help us to study *in vivo* for the first time the proportion of metacyclics delivered by bite, without relying on forced capillary feeding[105,121] or artificial membrane feeding[57,108]. In future experiments, this technique will be instrumental in testing the hypothesis framed by the work in this study, which is that PSG enriches the bite for metacyclic forms. To test this we would compare the proportion of metacyclic and non-metacyclic promastigotes delivered by bite of wild type (WT), LPG-deficient and LPG-restored parasite lines. If our hypothesis is correct, we would predict that the proportion of contaminating, non-infective forms would increase in the bites of LPG-deficient infections compared to their WT or add-back equivalents. This may allow us to conclude that PSG/fPPG is a predictor of transmission success[49] and therefore, a transmission determinant.

2 | PSG in development

Transmission is the product of successful colonisation in the sand fly midgut and appropriate promastigote development. We propose that the ability of fPPG to bind to non-metacyclic LPG may have consequences earlier in the development of the parasite. Two events in the sand fy heavily impact the colonisation of the sand fly vector: (1) "early" parasite survival (12-48 hours) that is affected by lethal action of the digestive enzymes of the vector[22,44,53] and (2) "late" parasite survival (48-96 hours) that requires the parasites to resist being defecated from the vector after bloodmeal digestion by attachment to midgut epithelium[44,56]. In so called "restrictive or selective vectors", non-natural promastigotes are completely eliminated by defecation at an early stage[22]. In both early and late survival, parasite phosphoglycans are involved, especially, lipophosphoglycan (LPG), parasite main surface phosphoglycan. For many years, promastigote midgut binding was believed to be a result of LPG-midgut receptor binding and species-specificity between *Leishmania* and a particular vector, that was attributed to the sugar projecting along the length of the galactose-mannose phosphate back-bone[22,63,77,79-81]. Nowadays, as more sand fly-*Leishmania* combinations are studied, this dogma is more flexible[22,56]; in some vectors, a non-LPG mediated binding has been proposed[65,66]. This highlights the need to rethink the glycobiology of midgut binding [31] and thus, promastigote development. Other phosphoglycans (such as fPPG) have been suggested as competitive inhibitors which could prevent attachment of metacyclic and also facilitate detachment and avoid re-attachment of nectomonad and leptomonad promastigotes[56] . Changes in LPG structure alone are insufficient to explain detachment in those parasite-sand fly combinations in which LPG seems not to be key in attachment[56]. Modifications to LPG during metacyclogenesis are well characterised and have been used to explain the process of detachment from the midgut epithelium. However, this model does not take into account that these changes occur later than the process of detachment itself. Studies have shown that LPG modification and metacyclogenesis are tightly regulated[22,44], whereas the first forms to detach themselves from the midgut and migrate to the stomodeal valve

are nectomonad promastigotes [46,55]. Nevertheless, for the vast majority of the *Leishmania* species we do not know when these changes occur, if they occur while parasites are attached and consequently detach or if they occur once they are detached. Furthermore, we do not know either how the leptomonad form, the precursor to the metacyclic promastigote fits into this theory. There are still many questions that need to be addressed. In the light of these issues, we decided to study the participation of PSG/fPPG in midgut attachment/detachment *in vitro* using *Lu. longipalpis-L. mexicana* experimental model.

Stage-specific binding of *Leishmania* promastigotes to sand fly guts *ex vivo* has been analysed by various groups, all with slightly different methodologies. In a study by Kamhawi et al[59],the incubation times were short due to problems preserving the gut. Wilson et al[56] improved this by incubation of guts in Grace´s Insect Medium (GIM) instead of PBS to prolong their integrity. By fluorescently labelling their parasites, they allowed different promastigote forms to compete against each other. *Ex vivo* midgut binding differs greatly between samples; sizes of dissected guts vary and binding is not homogeneous within the same gut. In this study, experiments containing metacyclics were performed in GIM and the rest in M199. Fluorescent parasites were substituted for unstained parasites, recovered by gut homogenisation and Giemsa stained, allowing the enumeration of bound parasites from a standard volume of homogenate. We chose not to label our parasites to avoid any possible influence upon midgut binding or parasite motility. We dissected whole guts, opened the midguts by longitudinal scission and removed hindguts, crops and Malpighian tubules. Midguts in the assays had similar size to ensure a very similar binding surface. We also performed a minimum of 10 replicates for competition experiments and 4 for stage specificity.

We observed stage-specific binding in our midgut experiments, validating the technical approach used. Similar to Wilson et al[56] we found the hierarchy of midgut binding from highest to lowest was: nectomonad, leptomonad, procyclic and metacyclic. Nectomonad promastigote binding was significantly higher compared to metacyclic promastigote binding and leptomonad binding was

generally lower than but not significally different from nectomonads. Procyclic promastigotes did not attach either (**Fig.2.1**). Guts from flies 5 to 7 days post bloodmeal were used to match the physiological status of infected flies undergoing the process of midgut attachment[56]. This suggests the participation of LPG in *ex vivo* midgut binding; procyclics do not express enough LPG on their surface[56,110] and metacyclics have a modified LPG structure[73,81,92]. However, to date there is no literature that shows that the structure of *L. mexicana* LPG changes as a result of metacyclogenesis, although there is some indirect evidence from development experiments in flies[56,72]. In this study, we observe gross structural differences between *L. mexicana* nectomonad and metacyclic promastigotes LPG, in line with changes to LPG that accompany metacyclogenesis for other *Leishmania* species. Metacyclic *L. mexicana* LPG was found to be double the molecular weight of nectomonad LPG as shown by Western Blot probed with LT15 mAb (**Fig.3.22**). This suggests an elongation of LPG in the metacyclics, as previously observed in *L. major*[73], *L. donovani*[81], *L. chagasi/infantum* [77] and *L. braziliensis*[92]. Whether this elongation of LPG is accompanied by qualitative and/or quantitative changes of the sugar residues of the phosphoglycan repeating units needs to be further characterised. In the *L. major-P. papatasi* relationship, the loss of galactose residues by substitution with arabinose and glucose is believed to be linked to their lack of attachment to midgut[85] as shown later by Kamhawi et al[80], who described a midgut galectin in *P. papatasi* midgut (PpGalec) responsible for promastigote binding that specifically recognised poly-Gal (ß1—3) of *L. major* promastigote LPG, but not in metacyclics. In Sudanese *L. donovani* the loss of terminal galactose of the cap structure has been implicated in the loss of binding[73]. In *L. chagasi / infantum* and Indian *L. donovani* loss of binding is related to the downregulation of the glucose residues of their LPG [77,93,95]. The underlying binding mechanism of *L. mexicana* to its main natural vector *Lu. olmeca olmeca*[10,11,22] and experimental vector *Lu. longipalpis* remains unknown. To our knowledge, there are no experimental colonies of *Lutzomyia olmeca olmeca*. *Leishmania mexicana* nectomonad LPG is also partially substituted with glucose residues and the terminal cap structure is rich in mannose residues but also in galactose as in Sudanese *L. donovani* [85,91].

Nevertheless, other factors such as promastigote motility and location of receptors in the midgut epithelium have been suggested to influence midgut binding[56]. Nectomonads are highly motile forms with a long flagellum and in our recent video-tracking experiments were shown to be even faster than metacyclics in PBS (**Fig.1.13, Table 1.4**). Interestingly, one of often quoted characteristics of metacyclics is that they are the fastest or more motile forms. In light of our results, showing a stage-specific attachment to PSG, this may be an artefact caused by the accumulation of fPPG in stationary phase cultures causing the non-infectious forms to slow down as they wrap themselves in fPPG. Procyclics on the other hand, have a short flagellum and are much less motile. These forms did not bind to PSG or the *Lu. longipalpis* midgut, suggesting that either there is an incomplete convering with "procyclic" LPG and/or that the flagellum is too short to enable attachment. Cuvillier et al[132] generated *L. amazonensis* that overexpressed a mutant form of ADP-ribosylation factor-like protein 3A which lack flagella. Infection of *Lu. longipalpis* with these parasites could not persist beyond early bloodmeal phase of development, indicating that the flagellum is critical for attachment to the sand fly midgut.

As for PSG *in vitro* binding LPG deficient lines were included in our midgut binding experiments. *lpg1*$^{-}$ nectomonad binding to *Lu. longipalpis* midgut was significantly lower than WT counterparts and was largely restored when genetically complemented parasites were used (*lpg2*$^{-}$/+LPG2) (**Fig 3.16**). These findings confirm a role of LPG for midgut binding *ex vivo* in *L. mexicana-Lu. longipalpis* combination. This somewhat clashes with the LPG independent binding theory in permissive combinations, including *L. mexicana-Lu. longipalpis*[57]. Nonetheless, these findings are a result of *ex vivo* experiments and not *in vivo* studies; participation of LPG *ex vivo* does not mean that the absence of it would result in a complete lack of survival *in vivo*. LPG-independent binding has been described mostly based on mutant promastigote survival in the sand flies[52,66]. Other biological factors, parasite or sand fly dependant, may translate this low binding into

successful colonisation. We cannot fully replicate early survival *ex vivo*, which determines later events in the sand fly midgut.

These studies need to include *lpg2⁻* parasites (as well as *lpg1⁻*/+LPG1 add-back) in nectomonad binding experiments and also in stage specific experiments. However, recent work by Jecná et al[133], presented similar results for *L. mexicana* midgut binding *ex vivo*, with a lower binding of *L. mexicana lpg1⁻* . In this case, a second mutant line, *L. mexicana gpi8⁻* was included in the experiments, which was shown to bind even less than *L. mexicana lpg1⁻*. *L. mexicana gpi8⁻* is deficient in all GPI anchored molecules, including the protease gp63 or Leishmanolysin. This implicates non-LPG surface glycoconjugates in promastigote midgut binding in permissive *Lu. longipalpis* binding, as proposed by Svarovska et al[65], but does not rule out the participation of LPG.

In light of the fact that LPG binds to both the sand fly midgut and the PSG, we next decided to include PSG in our midgut binding experiments, in order to have a closer picture of what is actually happening inside the sand fly gut at the time of attachment and detachment. This three way interaction seems likely since fPPG is secreted into the infected sand fly gut lumen from day 2/3 onwards[48,57]. Three possible roles (complementary, non exclusive) were proposed: (1) inhibition of promastigote midgut binding; (2) binding prevention by midgut ligand blocking; and (3) promastigote detachment from midgut. To test these scenarios, the time of PSG addition was key for these experiments (see methods). In all of them, the addition of 0.5 µg of PSG resulted in a significant reduction of midgut binding compared to WT nectomonads alone (**Fig.2.3**).

PSG seems to interfere with midgut *ex vivo* binding. Whether this is a result of active ligand competition (as LPG is important for both) or is a result of microenvironment changes (such as acidification) remains unclear. PSG was also added in *lpg2⁻*/+LPG2-midgut combination, with a similar drop in binding (**Fig.3.17**). This interference (by competition or blocking) may stop immature parasites from re-attaching, leaving them free in the gut lumen to migrate forward. Despite 0.5 µg

being a realistic PSG amount for *ex vivo* experiments[57,105], we cannot assure that it mimicks the concentration present in the infected midgut at this relatively early phase of parasite development.

In a separate experiment, the detachment of bound nectomonads increased as PSG amount increased (**Fig. 2.4**). In the infected sand fly midgut, the fPPG (and PSG) amount increases over time. It appears that initially this promotes the detachment of parasites into the lumen to continue with their development and that later, it may define the infective dose when the non-infective forms of *Leishmania* promastigotes are retained by the gel. PSG mediated promastigote detachment would explain how nectomonads free themselves prior to LPG modification. These results potentially provide a mechanism for a previously unknown process. Detachment is an important part of *Leishmania* life-cycle as it is crucial to allow nectomonads to continue with their migration and development.

Additionally, the PSG effect on binding was analysed using DsRed fluorescent nectomonad/leptomonad promastigotes. The PSG effect was similar to the one observed with Giemsa stained parasites (**Figs. 2.5 & 2.6**).

Collectively, these results conclude that LPG influences *Leishmania mexicana-Lutzomyia longipalpis* midgut binding: (1) stage specificity is present in *ex vivo* experiments, which has been linked to LPG in other *Leishmania* species and so far unexplained in *L. mexicana*; (2) preliminary assays reveal reduced midgut binding in *lpg1¯*; and (3) PSG interferes in promastigote midgut binding and now we know that LPG is specifically recognised by PSG. Nevertheless, we need to repeat midgut binding experiments including both a panel of defined promastigote stages and PG mutants to investigate further the role that non-LPG PGs could have in binding to PSG and sand fly midguts.

3 | *L. mexicana versus L. major*

Lu. longipalpis-L. mexicana experiments so far have revealed that PSG binding is largely driven by LPG and that other phosphoglycans may also be involved in midgut binding. Stage specificity, assays involving mutant *Leishmania* and competition/detachment experiments point to participation of LPG but more research needs to be conducted.

We cannot draw conclusions based just on one experimental sand fly-parasite model, more combinations need to be tested to characterise PSG role in binding and promastigote selection. *Leishmania major* has a complex LPG and *P. duboscqi* is one of its natural vectors[9-11]. *L. major lpg1¯* and *L. infantum* cannot bind to *P. duboscqi* midguts[65], but it can support the development of *L. tropica*[64] . *P. duboscqi* lacks GalNac containing midgut glycoproteins that are linked to permissiveness[66] and expresses PpGalec, linked to specificity [80]. Therefore, it could be considered to be an intermediate vector. For our experiment, PSG from *P. duboscqi* experimentally infected with *L. major* was obtained. In this combination, WT, *lpg1¯, lpg2¯*, *lpg1¯*/+LPG1 and *lpg2¯*/+LPG2 add-backs were tested. Like *L. mexicana* in *Lu. longipalpis*, *L. major* nectomonad promastigotes do also bind to PSG *in vitro*. Mutant binding was lower, but this difference was just significant for *lpg2¯* parasites (**Fig. 3.5**) compared to WT, suggesting that for promastigotes of this species to bind to PSG, non-LPG PGs are important. There was also a drop in binding of *lpg1¯*mutants (around 50%) but it was non-significant compared to WT, however, restoration of LPG deficiency resulted in a significant increase in binding, indicating that LPG participates in binding. In the future, more experiments with mutant lines and with different promastigote stages should be conducted. There is no reference data of *L. major* binding to PSG produced in *P. duboscqi*. Nonetheless, previous midgut binding data from Svárosvká et al [65]and Secundino et al[52], found that in the *L. major-P. duboscqi* combination, both LPG and PGs are needed for survival, with a significant drop in survival of mutant parasites at day 9 and day 7, respectively. In contrast, *L. major lpg1¯* mutants were able to develop normally in permissive *P. perniciosus* and *P. argentipes*.

When compared to *L. mexicana*, WT *L. major* binding to PSG was superior. PG mutants of *L. mexicana* displayed a larger drop in binding to *Lu.longipalpis* compared to their *L. major* counterparts: 91.1% *vs*. 58.12% for *lpg1¯ and* 92.2*% vs.* 88.5*% for lpg2¯*. These differences were significant for all groups except *lpg2¯* (**Fig.3.6, Table 3.3**). The drop in binding between *lpg1¯* and *lpg2¯* was also greater for *L. mexicana*.

Unfortunately, we could not test whether PSG interferes in binding to *P. duboscqi* midguts or not. In this case, *P. duboscqi* was not available for the experiments and permissive *Lu. longipalpis* was used instead. *L. major* develops successfully in *Lu. longipalpis*[66,134] and binds in high numbers to midguts *ex vivo* , even higher than natural combinations when studied in competition. This might be explained by stronger or alternative binding of *L. major* to *Lu. longipalpis ex vivo* [41]. In our experiments**,** WT nectomonads also bound in high numbers to midguts. In this case as well, LPG and other PGs were relevant to binding as *lpg1¯* and *lpg2¯* mutants displayed decreased binding that was restored for the add-back lines. (**Fig.3.18**). Nevertheless, this drop in attachment was not significant. This coincides with Myskova et al [66] and , Secundino et al[52], who found that at day 7 in *L. major-Lu. longipalpis* combination, there was a drop in survival in *lpg1¯* promastigotes but it was not significant compared to WT. Furthermore, Secundino et al[52] also described that *lpg2¯* parasites were unable to survive in flies. Similarly, Svarovská et al[65] showed that in permissive *P. argentipes* and *P. perniciosus*, *L. major lpg1¯* survival was preserved, but *lpg2¯* mutants were eliminated.

To investigate the role of PSG in *L. major* binding to *Lu. longipalpis* midgut, we added *L. major* PSG (from *P. duboscqi*). This resulted in a 54.29% reduction in binding similar to the 78.9% reduction observed for *L. mexicana* binding. However, this reduction was found not to be statistically significant unlike for *L. mexicana.*

Next, we compared midgut binding of both *L. mexicana* and *L. major* to *Lu. longipalpis* and found that binding was much higher for *L. major* than for *L. mexicana* as observed in previous experiments by Wilson et al[56]. However, *L. major*

lpg1¯ displayed only a modest fall in binding (43.4%) to the *Lu. longipalpis* midgut compared to their *L. mexicana* equivalent (98.49%). This suggests that *L. major* employs a non-LPG mediated binding, at least to a permissive sand fly gut such as *Lu. longipalpis*. Nevertheless, these differences were not significant (**Fig. 3.19, Table 3.8**).

Because these data were not obtained by comparative binding, this suggests an alternative or complementary mode of *L. major* binding instead of higher avidity. This is also supported by the fact that PSG interferes less with *L. major* than with *L. mexicana* in midgut binding assay. A LPG independent method of binding has been suggested in permissive vectors based mostly on data obtained from *in vivo* survival. These experiments have been mainly conducted in *L. major*, that has a complex LPG structure (highly substituted). In our experiments, *L. major* promastigote binding to PSG seems to occur *via* LPG and other PGs, but it appears to be less able, compared to *L. mexicana-Lu. longipalpis* system, to detach or prevent binding of *L. major* nectomonads to *Lutzomyia longipalpis* that occurs mainly *via* non-LPG[52,66]. This indicates that non-LPG receptors might be expressed in higher numbers. Moreover, in previous studies, *L. major* WT promastigotes survival was higher in permissive *P. arabicus* and *Lu. longipalpis* than in selective *P. papatasi* at day 7[66]. Interestingly, a galectin similar to PpGalec present in both *P.papatasi* and *P. duboscqi*, named LulongGale A was recently identified by Dillon et al[135]. It is important to assess *L. major* PSG effect in *L .major-P. duboscqi* midgut and interpret it jointly with our PSG *in vitro* data.

It is interesting that two parasite species, *L. mexicana* and *L. major,* seem to display different behaviour in the same permissive vector. *L. mexicana* LPG is partially substituted, simple[71] whereas *L. major* LPG is complex and highly substituted[22]. Similarly, Jecná et al[133] compared *ex vivo* binding of *L. major* and *L. infantum* to permissive *P. perniciosus* midguts and found that the lack of LPG had different impact on *ex vivo* binding depending on the parasite species. While in *L. major,* binding was almost as efficient in *lpg1¯* as in WT, *L. infantum lpg1¯* parasites did not bind well to *P. perniciosus* midguts. Also in permissive *P. argentipes*,

L. major lpg1¯ mutants survive[65] whereas *L. donovani* R2D2 mutants (deficient in LPG only) do not survive[53]. This suggests that permissivity is both linked to the fly species and the parasite species. Does promastigote LPG complexity determine midgut binding mechanism in permissive flies? Are permissive flies able to recognise and consequently bind simple LPG while they need alternatives (other PGs) for parasites with more complex LPG?

In summary, our experiments incriminate LPG as the ligand that mediates midgut and PSG binding in *L. mexicana-Lu. longipalpis*, with some alternative, non-LPG binding that could explain *lpg1¯* stage-specificity, the lack of complete ablation of *lpg1¯* nectomonad binding and also *lpg1¯* survival *in vivo* [57]. This is also supported by Jecná et al[133], that described the role of LPG but also other surface glycoconjugates in *L. mexicana in vitro* binding to *Lu. longipalpis* . In contrast, *L. major-Lu. longipalpis* binding seems to be mainly non-LPG mediated as described in previous literature[52,66] and preliminarily observed in our experiments, with a smaller effect of LPG in midgut binding and PSG in competition. Nonetheless, these *L. major* results were from single experiments with few replicates and a high SEM (Standard Error of Mean) and not statistically significant. Interestingly, in all the natural parasite-vector combinations studied so far i.e *L. major-P. papatasi*[53], *L. major-P. duboscqi* [66], *L. tropica-P. sergenti*[61,68,79], *L. infantum-P. perniciosus*[133], *L. donovani- P. argentipes*[53], LPG seems to be important in survival regardless of their vector specificity or permissivity. LPG deficient lines of both *L. donovani* and *L. infantum* do not survive in their permissive natural vectors, *P. perniciosus*[133] and *P. argentipes*[53] which clashes with the successful survival of *L. major lpg1¯* in both of them[65,66]. This suggests that requirement for LPG for midgut binding might be linked to co-adaptation/co-evolution of parasites and vectors in natural combinations[133]. *L. infantum-Lu. longipalpis* relationship is more flexible, with different strains being able to survive in the fly[72,77], in contrast to Old World *L. tropica-P. sergenti* combination[61,68,79]; *Lu. longipalpis* permissivity might be linked to its short history as vector. Although it needs to be further characterised, non-LPG mediated midgut binding might be linked to vector adaptability[61,133] observed in

both newly emerged natural (*L. infantum-Lu. longipalpis*) and experimental (*L. major-Lu. Longipalpis*) combinations.

There are several issues regarding midgut binding and vectorial competence that remain unknwon. "Permissivity" and "specificity" principles cannot be universally applied as they depend on both specific parasite and vector species. It is difficult to interpret if our preliminary results correlate with just a decrease in binding or they are indicative of an actual binding mechanism. However, PSG seems to be useful in understanding the underlying binding mechanism in parasite-vector combinations. It would be interesting to perform these three way experiments in other natural and experimental *Leishmania* – sand fly combinations.

4 | Conclusions: PSG and Leishmaniasis

Leishmaniasis is a widely distributed disease related specially to poverty with considerable socio-economic consequences. It is far from being controlled; it is at constant risk of re-emergence due to conflicts and human/environmental changes that affect the *Leishmania* transmission cycle. Leishmaniasis control requires continuity and constant monitoring which means economic resources and strong public health structure, disrupted by conflicts or absent in poorer endemic countries. Once disease control stops, it is difficult to go back to previous disease levels, even in absence of active conflicts. Interestingly, disease outbreaks are not exclusive of conflict zones and developing countries. A recent large outbreak of human Leishmaniasis in Madrid (Spain)[136,137] related to an increase in hare population shows that disease can re-emerge any time in any of the endemic countries. Therefore, Leishmaniasis is a dynamic disease that requires constant research in surveillance, diagnosis, treatment, control and disease monitoring. Ideally it should be accompanied by an improvement in socio-economic conditions in developing countries which is currently unlikely to occur, subsequently stressing the need for scientific approach. Disease burden, lack of accurate epidemiological data, limitations in both treatment (side effects, resistance) and vector control

(implementation, sustainability, toxicity, resistance) and complex epidemiology (foci-based instead of country-based) among others, justify the need for new research and control tools. In this scenario, PSG would be a good candidate.

As mentioned before, PSG acts as a transmission determinant, enhancing transmission by behavioural manipulation and by "molecular" sieving and it actively participates in promastigote development within the sand fly. Promastigote detachment from midgut appears to be mediated by PSG and not by LPG modification, which could explain the lack of binding of metacyclics to midgut[44,92] Moreover, PSG is a well described exacerbation factor, with active participation in the establishment of the disease and it has been proposed as a target for a blocking vaccine[43] along with saliva[114].

In addition, characterisation of its involvement in both bite enrichment and midgut binding may allow us to interpret the vector-parasite relationship more accurately and consequently the transmissibility of new combinations that arise because of hybridization between parasite species[138] or changes in transmission ecotopes.

PSG seems to be key in *Leishmania* transmission and therefore its potential role in disease control should be further investigated.

Summary of conclusions	
1	In the sand fly, PSG is as a transmission determinant that acts as a sieve and retains immature *Leishmania* promastigotes in the midgut to produce a metacyclic enriched bite inoculum.
2	Immature promastigotes (nectomonads and leptomonads) are retained by a stage-specific attachment to PSG that occurs via LPG accompanied by a minor participation of other PGs.
3	PSG actively participates in *Leishmania* development by allowing immature promastigotes to detach from midgut epithelium and by preventing their re-attachment. This is essential for promastigotes to complete their development within the sand fly midgut.
4	The addition of PSG in promastigote midgut binding assays appears to be useful to elucidate underlying midgut binding mechanisms in different *Leishmania* –sand fly combinations. PSG may help us to interpret parasite-vector relationships more accurately.

5 | Future work

This thesis provides evidence of the role of PSG as a *Leishmania* transmission determinant and a key player in promastigote development in *L. mexicana-Lu. longipalpis* experimental model of infection. This study is in the line with previous works by Rogers and Bates[48,57]. Nevertheless, these transmission and midgut binding experiments need to be replicated using *L. infantum* and *Lu. longipalpis* i.e. a natural parasite-vector combination. Ideally, the *L. mexicana- L. olmeca olmeca* pairing should also be included in experiments, but currently there are no known laboratory colonies of this sand fly species. It would also be interesting to repeat the experiments with *L. infantum* and original vector *P. perniciosus* as bindingmechanism to both PSG and midgut may differ from those observed in *L. infantum-Lu. longipalpis* "newly" emerge adaptative combination.

Our results point to promastigote LPG to be essential in the selective sieving that underlies behind the enriched infective bite. However, as mentioned before, transmission experiments will be complemented with qRT-PCR measuring relative expression of stage-specific markers from *lpg1¯* parasites delivered by bite, in order to see whether decreased attachment of nectomonad and leptomonad promastigotes to PSG *in vitro* correlates with an increased proportion of immature forms in the bite. Dr Emilie Giraud from Dr Rogers´group is currently performing transmission experiments with *L. mexicana lpg1¯* and *Lu. longipalpis* sand flies. The ~19 KDa band obtained by Western Blotting is destined for sequencing by Mass Spectrometry.

To conclude *L. mexicana* promastigote binding to PSG *in vitro*, the role of non-LPG will be further characterised by new experiments with *lpg2¯* mutants, as a minor participation of non-LPG phosphoglycans has been observed with *lpg1¯* experiments. Similarly, midgut binding assays will also include *L. mexicana lpg2¯* parasites; *lpg1¯* parasites bind to midguts *ex vivo* and survive in *Lu. longipalpis*[57]. Jecná et al,[133] observed the direct participation of non-LPG glycans in *L. mexicana-Lu. longipalpis* midgut binding *in vitro*. Transmission experiments (and qRT-PCR) cannot be performed with *lpg2¯* mutants as they are eliminated early in development in the sand fly[57].

The structure of *L. mexicana* metacyclic LPG has not been characterised yet[72]. In this study we found that similarly to other species (such as *L. major*, *L. donovani* or *L. braziliensis*)[73,81,92] there is an elongation on metacyclic LPG structure that results in an increase of their molecular weight. The next step would be to characterise the sugar residues by HPLC to see whether the lack of binding of *L. mexicana* metacyclic promastigotes is associated with a loss or substitution of its side sugars or changes in its cap structure as previously described for other *Leishmania* species[77,93,95].

Interestingly, leptomonads were less able to bind to both PSG and midgut compared to nectomonad promastigotes. As intermediate forms, leptomonad LPG structure should be characterised and its binding to PSG further examined. It would be interesting to see whether binding to biotinyilated fPPG is observed by Western Blotting *in vitro* as in nectomonads or is absent as in metacyclics. Absence of leptomonad LPG binding to fPPG would further incriminate non-LPG glycans in leptomonad binding to PSG.

In the current thesis, the role of PSG in other parasite-vector combinations has been limited. We have observed that *L. major* nectomonads also bind via their LPG and other PGs to PSG *in vitro* and that PSG interferes with midgut binding, but it needs further characterisation. First, *L. major* promastigote binding to PSG from *P. duboscqi* should be completed by analysis of stage-specific binding. Secondly, although the role of PSG from *P. duboscqi* in *L. major* nectomonad binding to *Lu. longipalpis* midgut has been analysed by competitive midgut binding (addition of PSG and promastigotes to guts at the same time), detailed detachment experiments should be performed , following *L. mexicana-Lu. longipalpis* protocol. It would be interesting to study the role of PSG in *L. major* development in its natural vectors *P. duboscqi* (intermediate vector) and *P. papatasi* (restrictive vector).

Completion of PSG and midgut binding experiments in *L. infantum- Lu. longipalpis* and *L. major-P. papatasi* would be valuable to understand parasite-vector relationships, as they are examples of natural vector permissivity and specificity, respectively. Nevertheless, it would be interesting to include other combinations such as *L. tropica* and natural restrictive vector *P. sergenti* and permissive vector *P. arabicus* or *L. donovani* and permissive natural vector *P. argentipes*. Ideally, *L. donovani* behaviour in *P. martini* or other African sand fly vectors should be studied.

To conclude, midgut binding experiments should include fPPG/PSG from a different parasite species from the one tested in the sand fly. This information could be useful to observe the degree of adaptability displayed by vectors and

parasites and to evaluate hybrids that may arise in the future. It would be helpful to fully characterise fPPG produced by *Leishmania* species other than *L. major* and *L. mexicana*[49,71] as PPGs differ among different developmental stages, species and even strains [83].

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