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Leishmania development in sand flies during bloodmeal digestion

Vývoj leishmanií ve flebotomech během trávení krve

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Ph.D. Thesis / Disertační práce

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Prague, 2015

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I declare that the submitted thesis is my own work and that I properly cited all scientific literature used. Neither this thesis as a whole nor its substantial part has been submitted for the award of any other degree or diploma.

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Prague, August 18th, 2015

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I declare that Kateřina Pružinová substantially contributed to the experimental work in the three projects presented in her thesis and had a principal role in writing two of the three publications presented.

Prohlašuji, že se Kateřina Pružinová významně podílela na experimentální práci na třech projektech shrnutých v této dizertační práci a je hlavní autorkou textu dvou publikací.

Prague, August 18th, 2015

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Prof. RNDr. Petr Volf, CSc.

Poděkování

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Contents

List of abbreviations

gp63	glycoprotein 63
GIPLs	glycoinositolphospholipids
LPG	lipophosphoglycan
MAPK	mitogen-activated protein kinases
PBM	post-bloodmeal
PI	post-infection
PM	peritrophic matrix
PPG	proteophosphoglycan
PSG	promastigote secretory gel
AP	acid phosphatases

Abstract

This thesis is focused on the bloodmeal digestion of phlebotomine sand flies and its effects on *Leishmania* development within their midguts. In the first part, we studied various parameters of bloodmeal digestion in four sand fly species differing in susceptibility to *Leishmania donovani* to evaluate the effects on vector competence. Both proven vectors of *L. donovani* (*Phlebotomus orientalis* and *P. argentipes*) showed lower trypsin activity and slower formation of the peritrophic matrix (PM) than refractory species (*P. papatasi* and *Sergentomyia schwetzi*). Remarkably, *P. orientalis* and *P. argentipes* strikingly differed from each other in a time course of bloodmeal digestion. *Phlebotomus orientalis* females showed very slow bloodmeal digestion with low peaks of proteolytic activities and defecated around day five post bloodmeal. In contrast, *P. argentipes* females digested faster with a very high peak of chymotrypsin activity, their PM was present for only a short time and defecation was finished by day three post bloodmeal. We presume that the period between the degradation of the PM and defecation (i.e. time frame when *Leishmania* bind to the midgut to avoid expulsion with bloodmeal remnants), is one of crucial parameters affecting the establishment of *Leishmania* in the sand flies. In both natural vectors of *L. donovani*, this period was significantly longer than in the refractory species *S. schwetzi*.

The second aim of this study was to measure the volume of the bloodmeal ingested by sand flies and using different initial infective doses evaluate the susceptibility of *P. orientalis* and *P. argentipes* to *L. donovani*. We showed that both vectors ingest similar volume of blood (about 0.6 μl) and are similarly susceptible to experimental infection with *L. donovani*; even 1 – 2 parasites was a sufficient dose to initiate heavy mature infections in *P. argentipes* and *P. orientalis* females.

Since various descriptions of the effects of avian blood on *Leishmania* are in contradictions and different authors published divergent results, the third part of my work was aimed at the investigation of the effects of chicken blood on bloodmeal digestion and the development of *L. major* in its natural vector *P. duboscqi*. Avian blood affected the trypsin activity and oocyte development of females; however, no effects of chicken blood were detected on the development of *L. major* in *P. duboscqi*. Our study has unambiguously demonstrated that sand fly feeding on avian host, either before or after infection, is not harmful to *Leishmania* parasites within the midgut.

Abstract in Czech

Tato práce se zabývá trávením krve u flebotomů a jeho vlivem na vývoj leishmanií v přenašeči. V první části práce jsme studovali různé parametry trávení u čtyř druhů flebotomů lišících se ve vnímavosti k infekci *Leishmania donovani*, abychom posoudili jejich vliv na vektorovou kompetenci. U obou přirozených přenašečů *L. donovani* (*Phlebotomus orientalis* a *P. argentipes*) byla zaznamenána nižší trypsinová aktivita a pomalejší tvorba peritrofické matrix (PM) v porovnání s druhy, které jsou vůči *L. donovani* resistentní (*P. papatasi* a *Sergentomyia schwetzi*). Zajímavé je, že průběh trávení krve se výrazně lišil i mezi *P. orientalis* a *P. argentipes*. U *P. orientalis* bylo pozorováno velmi pomalé trávení s nízkými vrcholy proteolytických aktivit trypsinu a chymotrypsinu. K defekaci docházelo až okolo pátého dne po sání krve. Naproti tomu samice *P. argentipes* trávily krev o poznání rychleji, vrchol chymotrypsinové aktivity dosahoval vysokých hodnot, PM byla přítomna pouze krátkou dobu a defekace byla ukončena již třetí den po sání. Na základě našich výsledků předpokládáme, že jedním z klíčových faktorů, které ovlivňují uchycení leishmanií ve flebotomech, je doba mezi degradací PM a defekací (tj. čas, kdy se leishmanie přichytávají ke střevnímu epitelu, aby nebyly vyloučeny spolu se zbytky nestrávené krve). U obou přenašečů *L. donovani* byla tato perioda signifikantně delší než u resistentního druhu *S. schwetzi*.

Dalším cílem této práce bylo změřit množství krve, kterou flebotomové nasají, a pomocí různých infekčních dávek otestovat vnímavost *P. orientalis* a *P. argentipes* k experimentálním infekcím *L. donovani*. Prokázali jsme, že oba přenašeči nasají obdobné množství krve (v průměru 0,6 μl) a jsou k infekci *L. donovani* stejně vnímaví. Pro rozvoj infekce u samic *P. orientalis* a *P. argentipes* byla dostatečná infekční dávka 1–2 promastigoti.

Vliv trávení ptačí krve na vývoj leishmanií ve flebotomech je diskutabilní a různí autoři se ve svých teoriích neshodují. Proto jsme v poslední části práce studovali vliv kuřecí krve na trávení samic a vývoj *L. major* v přirozeném přenašeči *P. duboscqi*. Ptačí krev sice u samic snížila trypsinovou aktivitu a zpomalila vývoj oocytů, ale nijak neovlivnila vývoj leishmanií. V sérii různých experimentů jsme jednoznačně ukázali, že sání flebotomů na ptačích hostitelích nemá žádný vliv na průběh infekce leishmaniemi.

1 Introduction

1.1 *Leishmania* development in sand flies

Protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) have a digenetic life cycle consisting of extracellular promastigotes developing in sand fly vectors (Diptera: Phlebotominae) and intracellular amastigotes that reside and multiply in macrophages of their vertebrate hosts. *Leishmania* parasites are the etiological agents of severe mammalian diseases called leishmaniases. Over ten medically important *Leishmania* species produce a spectrum of clinical manifestation in their human hosts ranging from mild self-healing cutaneous lesions to destruction of mucous membranes to fatal visceral cases (Dostalova and Volf 2012). On account of the lack of a human vaccine and increasing resistance to the currently used drugs, the more detailed research of leishmaniases is needed (Dostalova and Volf 2012, Maroli et al. 2013, Bates et al. 2015)

Outside the mammalian hosts, the *Leishmania* life cycle is confined to the alimentary tract of insect vectors, phlebotomine sand flies. Sand flies are small haematophagous insects that are encountered in tropical and subtropical areas. Two sand fly genera, *Phlebotomus* and *Lutzomyia*, are considerable important for the public health as the only proven vectors of *Leishmania* species pathogenic for humans (Bates and Rogers 2004, Dostalova and Volf 2012, Maroli et al. 2013, Ready 2013, Bates et al. 2015). Females of the genus *Sergentomyia* can also bite human, however, they are proven vectors of reptile *Leishmania* species only, non-pathogenic to humans (Bates 2007, Sadlova et al. 2013).

In my thesis I will focus on the vectorial part of *Leishmania* life cycle, especially on the early stage of infection. Although the genus *Leishmania* is divided into two subgenera, *Leishmania* and *Viannia*, my experimental work has been focused on the Old World sand fly species and the subgenus *Leishmania*, therefore, I describe their development in more detail.

The infection of sand flies is initiated when females ingest *Leishmania* amastigotes, together with the bloodmeal, during feeding on an infected host. In the lumen of the sand fly midgut, amastigotes transform into various flagellated promastigote stages, which are characterized by morphological and functional changes targeted to enable *Leishmania* survival and transmission into the vertebrate hosts. It takes approximately 5 – 7 days to *Leishmania* to develop into infective metacyclic stages (Kamhawi 2006, Dostalova and Volf 2012).

In the first instance, amastigotes are released from macrophages and transform into first replicative forms – procyclic promastigotes with short flagella. These forms occur within the bloodmeal surrounded by the peritrophic matrix (PM) and are relatively resistant to the harmful effects of digestive enzymes (Pimenta et al. 1997). Around 48 – 72 hours post bloodmeal (PBM), procyclic forms develop into strongly motile long nectomonads that escape from the disintegrating peritrophic matrix (Sadlova nad Volf 2009). They reversibly attach to epithelial cells of the midgut and migrate forward towards the anterior gut. Afterwards, long nectomonads differentiate into short nectomonad promastigotes, according to Rogers et al. (2002) called leptomonads, which undergo the other proliferative cycle. Eventually, two other promastigote forms are found in the thoracic midgut and cardia region, haptomonads and metacyclics. Haptomonads are non-motile promastigotes that attach to the chitin lining of the stomodeal valve by hemidesmosomal structures. Metacyclic promastigotes are short and slender cells that are characterized by rapid motility and high adaptation for successful transmission into the vertebrate skin (Gossage et al. 2003, Bates and Rogers 2004, Kamhawi 2006, Dostalova and Volf 2012).

Leishmania parasites colonize the stomodeal valve of their vectors and produce promastigote secretory gel (PSG) that creates a plug mechanically blocking the gut and affecting the feeding success of the sand fly females. This phenomenon of the 'blocked valve' facilitates the regurgitation of *Leishmania* metacyclic stages into the vertebrate host (Killick-Kendrick et al. 1977, Jefferies et al. 1986, Rogers et al. 2002, 2004, Rogers and Bates 2007). In addition to this mechanical block, *Leishmania* destroys the chitin lining (Schlein et al. 1992) and degrade the unique filamentous structures on the apical end of cylindrical cells of the stomodeal valve, which probably interferes with its function and also facilitates the reflux of *Leishmania* promastigotes from the midgut (Volf et al. 2004, Rogers et al. 2008).

In order to survive and complete development in sand flies, *Leishmania* must overcome a number of documented barriers. These barriers include a proteolytic attack by digestive enzymes, which, according to some authors, can suppress the early growth of parasites (Borovsky and Schlein 1987, Pimenta et al. 1997, Schlein and Jacobson 1998); the peritrophic matrix, which can pose a physical barrier to *Leishmania* escape into ectoperitrophic space (Walters et al. 1992, Sadlova and Volf 2009, Sadlova et al. 2013); the defecation of bloodmeal remnants, which can result in the suppression of *Leishmania* infection (Sadlova et al. 2013); and the anatomy and physiology of the midgut, which can prevent *Leishmania* attachment to the midgut epithelium and anterior migration (Pimenta et al. 1994, Kamhawi et al. 2004, Myskova et al. 2007, Volf and Myskova 2007). *Leishmania* survival is also likely to be affected by sand fly immune response (lectins and antibacterial peptides; reviewed by Sacks and Kamhawi 2001, Ramalho-Ortigao et al. 2010, Dostalova and Volf 2012, Ready 2013; Fig. 1)

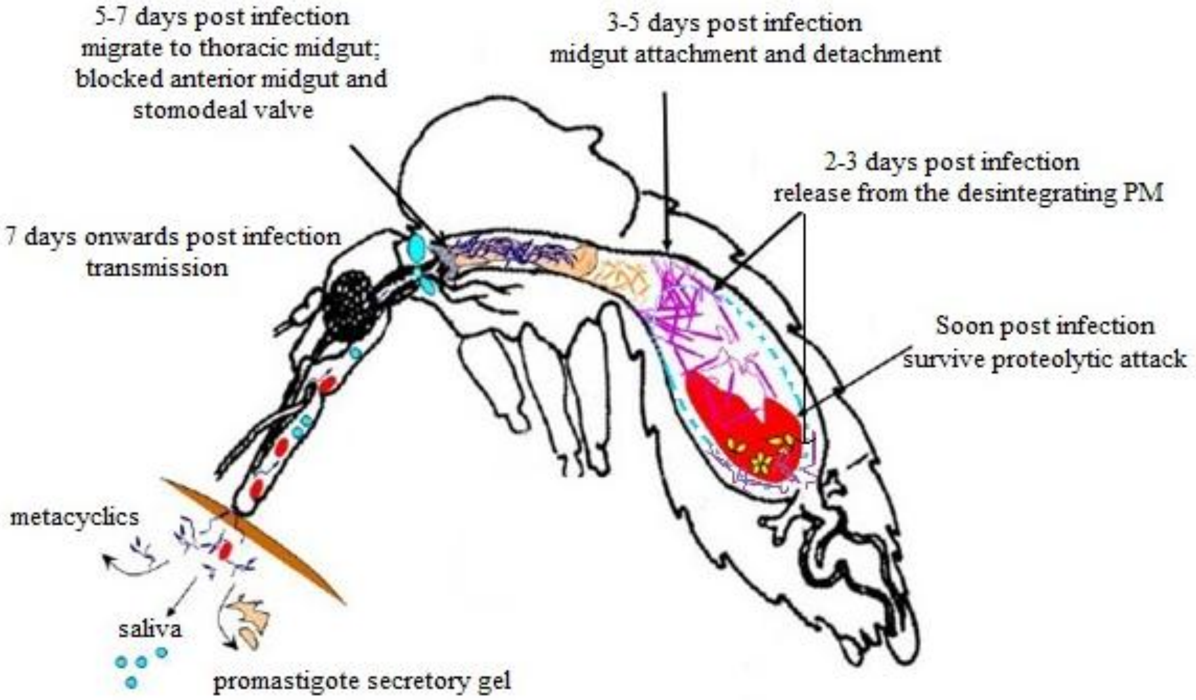

Figure 1: The life cycle of *Leishmania* within the sand fly vector (Ramalho-Ortigao et al. 2010, modified).

To survive in the hostile and diverse environment in the vectors and mammalian hosts, *Leishmania* has developed stage- and species-related molecules that promote its survival and development. Molecules which are attributed to the protection of parasites belong to the family of glycoconjugates. *Leishmania* glycoconjugates are predominantly phosphoglycans that share a conserved polymer of phosphorylated galactose-mannose disaccharide repeating units [PO4-6Gal(β1-4)Manα1)] with a oligosaccharide cap. These phosphoglycans consist of cell surface or secreted proteophosphoglycans (**PPG** or **sPPG**), lipophosphoglycan (**LPG**) and acid phosphatases (**AP**). However, glycoconjugates also include molecules that do not incorporate the nominal phosphoglycan, such as glycoinositolphospholipids (**GIPLs**) and glycoprotein 63 (**gp63**). Surface molecules (LPG, PPG, GIPLs and gp63) are attached to the cell through glycosylphosphatidylinositol (GPI) lipid anchors (Fig. 2, Sacks and Kamhawi 2001, Kamhawi 2006). A role of these glycoconjugate molecules in *Leishmania* development is briefly summarised below.

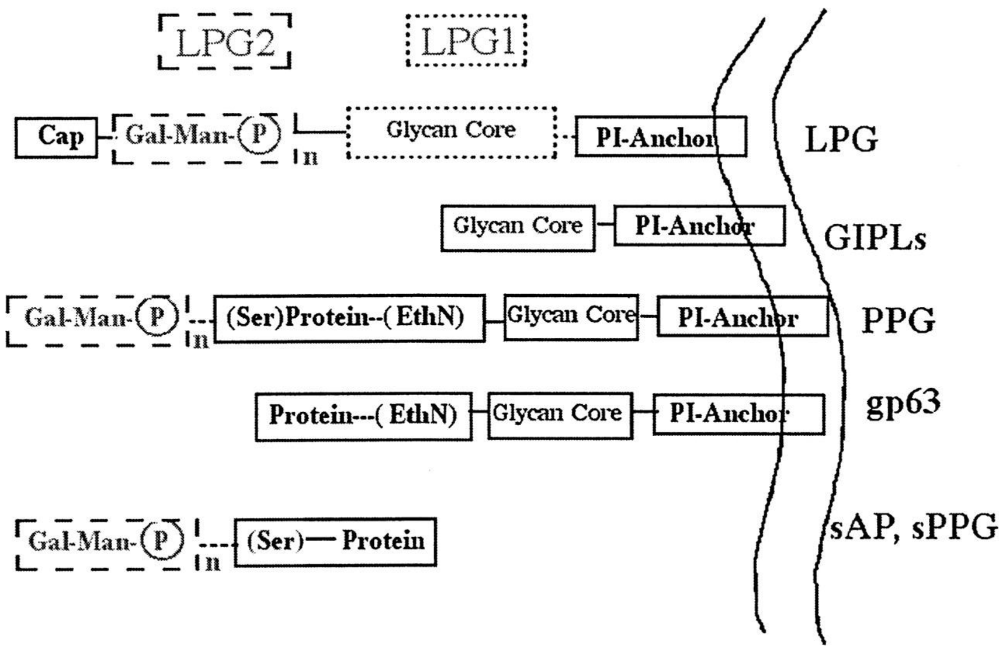

Figure 2: Schematic representation of GPI-anchored and secreted glycoconjugates from *Leishmania*. Structures depicted as either *LPG1* (dotted-lined box) or *LPG2* (dashed-lined box) designate those domains that are specifically affected by mutations in these respective genes (Sacks and Kamhawi 2001).

Lipophosphoglycan (LPG) is one of the most abundant glycoconjugates on the surface of *Leishmania* promastigotes. It has been identified in all *Leishmania* species that have been studied to date. There has been described structural polymorphism; the phosphoglycan repeating units are often modified by strain-, species-, and stage-specific side-chain sugar residues. Lipophosphoglycan is expressed on the whole surface, including the flagellum and is organised as a densely packed filamentous glycocalyx (McConville and Ferguson 1993, Sacks and Kamhawi 2001). It has been reported to be involved in various virulence activities in both mammalian hosts and sand fly vectors. In mammals, LPG plays a role in the inhibition of macrophage signal transduction pathways (Descoteaux et al. 1992, Giorgione et al. 1996), modulation of respiratory burst (Brittingham and Mosser 1996), and resistance to complement mediated lysis (Puentes et al. 1988, Spath et al. 2003). Mbuchi et al. (2006) also showed that LPG plays an important role in *Leishmania* transformation; biding of C-reactive protein (from human serum) to LPG initiated transformation of *L. donovani* and *L. mexicana* promastigotes to amastigotes *in vitro* (Mbuchi et al. 2006).

In the vectors, it was suggested that LPG promote promastigotes survival by the inhibition of midgut protease activity, and thus protects parasites from the proteolytic attack (Schlein et al. 1990). According to Sacks et al. (2000), LPG is not essential for *Leishmania* survival during the early phase within the blood fed midgut but, together with other glycoconjugates, can protect parasites from the harsh effects of the digestive enzymes. The main role of *Leishmania* LPG is attributed to the midgut attachment of promastigotes in specific vectors (*P. papatasi*, *P. duboscqi* and *P. sergenti*; Pimenta et al. 1992, 1994, Sacks et al. 2000, Kamhawi et al. 2000, 2004) in order to avoid its expulsion during defecation. On the other hand, this molecule is not necessary for the binding of *Leishmania* to the midgut epithelium in permissive vectors (Myskova et al. 2007, Volf and Myskova 2007, Jecna et al. 2013; for more details see section 1.3). Svarovska et al. (2010) and Secundino et al. (2010) confirmed a LPG-independent mechanism of *Leishmania* attachment in the permissive vectors and they also demonstrated the importance of LPG2-dependent glycoconjugates in *L. major* survival in various sand fly species. The *lpg2*$^{-}$ *L. major* mutant that is a broader mutant and lacks all phosphoglycans including LPG and proteophosphoglycans was unable to survive in *P. perniciosus*, *P. argentipes*, *P. duboscqi* and *L. longipalpis*.

Proteophosphoglycans (PPG) are a unique serine-rich class of glycoproteins secreted by promastigotes as well as amastigotes and occurring in two forms: filamentous PPG and membrane-bound PPG. Filamentous PPG is the largest secreted molecule by *Leishmania* promastigotes and the major component of the promastigote secretory gel (PSG); a mucine-like gel accumulated in sand fly guts and mouthparts (Ilg 2000, Rogers 2012). Proteophosphoglycans in the form of the PSG plug can manipulate the blood feeding behaviour of the sand fly vectors and the immune system of the vertebrate hosts in order to promote *Leishmania* transmission and infection. The gel plug obstructs and impairs the feeding ability of the vectors, which results in regurgitation of both *Leishmania* and the gel to the host as to obtain a sufficient bloodmeal (Rogers et al. 2002, 2004, Rogers and Bates 2007).

In addition, PSG has an important role in parasite transmission. Rogers et al. (2004, 2009 and 2010) showed that PSG is able to exacerbate cutaneous and visceral leishmaniases and can promote the chronicity of *Leishmania* infection. And thus, PSG is thought as an essential component of the infectious sand fly bite for the early establishment of *Leishmania* in host skin (Rogers et al. 2009). The function of membrane-bound PPG is not clearly known, but based on its structure it could constitute a potential ligand for macrophages (Ilg 2000).

In the sand fly vectors, Secundino et al. (2010) and Svarovska et al. (2010) showed that PPG plays the key role in *Leishmania* protection against the midgut digestive enzymes. The *lpg2*$^-$ mutant of *L. major* was highly susceptible to being killed following an 8 hours exposure to the midgut lysates prepared from blood fed sand flies, however, addition of purified PPG significantly prevented the killing of *lpg2*$^-$ promastigotes. The protection was not connected with the inhibition of proteolytic activities, but with the cell surface acquisition of PPG (Secundino et al. 2010).

Zinc-dependent metalloprotease **glycoprotein 63 (gp63)**, also known as leishmanolysin, major surface protease or promastigote surface protease, is predominantly expressed on the surface of *Leishmania* promastigotes rather than on amastigotes (Chaudhuri et al. 1989, Schneider et al. 1992). This enzyme with wide substrate specificity and broad pH optimum (Chaudhuri and Chang 1988, Ip et al. 1990, Schneider and Glaser 1993a) is able to degrade serum proteins as albumin, casein, fibrinogen, haemoglobin and complement components (Bouvier et al. 1990) as well as components of the extracellular matrix (McGwire et al. 2003). Glycoprotein 63 has been reported as an important virulence factor that plays multiple roles in *Leishmania* infection of the vertebrate hosts. It enables *Leishmania* parasites to evade complement-mediate lysis (Brittingham et al. 1995), helps parasites to adhere to macrophages (Russel and Wilhelm 1986, Chen et al. 2000, Lima et al. 2009), protects intracellular amastigotes from degradation in macrophage phagolysosomes (Chaudhuri et al. 1989, Seay et al. 1996) and inhibits monocyte and neutrophil chemotaxis (Sorensen et al. 1994). It is also involved in lesion formation (Joshi et al. 1998, 2002).

However, the role of gp63 in the vector is less clear. Joshi et al. (1998, 2002) described that *L. major* leishmanolysin deficient mutants showed normal development inside the sand flies *P. papatasi*, *P. duboscqi* and *P. argentipes*. On the contrary, Hajmova et al. (2004) observed slight changes in the development of gp63-deficient mutants of *L. amazonensis* during the early stage of infection in *L. longipalpis* suggesting that gp63 could be involved in the protection of promastigotes against degradation by the midgut digestive enzymes. The protease could also participate in the obtaining of nutrients by haemoglobin proteolysis (Chang and Chang 1985). In addition, Sadlova et al. (2006) described that repeated passaging of *L. major* through *P. duboscqi* led to enhanced gp63 expression.

Interestingly, surface enzyme similar to *Leishmania* gp63 was found in monoxenous trypanosomatids of the genera *Crithidia* and *Herpetomonas* (Etges 1992, Inverso et al. 1993, Schneider and Glaser 1993b). Pereira et al. (2009, 2010) observed that gp63 of *Herpetomonas samuelpessoai* and *Leptomonas* species contributes to the parasite adhesion to *Aedes aegypti* guts or *Aedes albopictus* cell line C6/36. A potential role of gp63 in the attachment of parasites was also proposed in *Leishmania*; *L. amazonensis* gp63 down-regulated transfectans bound slightly less efficiently to the midguts of *L. longipalpis* compared to controls (Jecna et al. 2013). Altogether, these results suggest that while the role of analogous enzyme in monoxenous trypanosomatids is clear, further studies are necessary to elucidate the role of gp63 during *Leishmania* development in sand flies.

Glycoinositolphospholipids (GIPLs) are present on the cell surface in all *Leishmania* stages and represent one of the most abundant glycoconjugates. Similarly to LPG, GIPLs are polymorphic in both glycan and lipid structures. Three distinct lineages of GIPLs have been identified that are expressed in considerably different levels in different species or developmental stages (McConville and Blackwell 1991, Schneider et al. 1993, Ralton and McConville 1998). Some GIPLs of the type 2 may act as biosynthetic precursors to LPG (McConville et al. 1990). It is presumed that *Leishmania* GIPLs contribute towards the survival of parasites in the hosts as they modulate many macrophages function such as release of nitric oxide (Proudfoot et al. 1995, Assis et al. 2012), cytokine production and different activation of MAPK (Assis et al. 2012). McNeely et al. (1989) and Chawla and Vishwakarma (2003) also demonstrated that GIPLs and its synthetic lipid domain of *L. major* and *L. donovani* are able to inhibit the protein kinase C-dependent signaling pathway.

Serine- and threonine-rich **acid phosphatases (AP)** have been found in the promastigotes (Bates and Dwyer 1987, Bates et al. 1989, Gottlieb and Dwyer 1982, Shakarian et al. 2002) and amastigotes (Bates et al. 1989, Doyle et al. 1991, Ellis et al. 1998, Shakarian et al. 2002) of most *Leishmania* species. These molecules are either secreted or surface membrane-bound. Early studies characterised AP from *L. donovani* as a phosphorylated, multidisperse glycoprotein (Gottlieb and Dwyer 1982, Bates et al. 1989). These phosphatases with broad substrate specificity are able to catalyze the hydrolysis of impermeable organic phosphates including glycerol phosphates, mono- and di-phosphorylated sugars, inositol phosphates and phosphorylated proteins (Gottlieb and Dwyer 1981, 1982, Das et al. 1986). On the basis of their enzymatic activities it is presumed that they play a role in the growth, development and survival of *Leishmania* in sand flies as well as in mammalian hosts (Bates and Dwyer 1987, Shakarian and Dwyer 2000, Shakarian et al. 2002). Acid ecto-phosphatase activity in protozoan cells can play a role in adaptation in acidic environment and getting nutrients from phosphorylated substrates of host cells (Cosentino-Gomes and Meyer-Fernandes et al. 2011, Gomes et al. 2011) and may participate in parasite's virulence (Remaley et al. 1985, Papadaki et al. 2015). Acid phosphatases isolated from the external surface of *L. donovani* promastigotes inhibited toxic oxidative metabolite production of human neutrophils (Remaley et al. 1985). Recently, Papadaki et al. (2015) showed that the overexpression of membrane acid phosphatases of *L. donovani* transgenic promastigotes (*Ld*MAcP) improved parasite ability to survive within macrophages in the culture at least in the first 48 – 72 hours post infection. In the infection of J774 mouse macrophages, the transfectants showed a significantly higher infectivity and virulence index compared to control *L. donovani* promastigotes, suggesting a possible similar role for the endogenous LdMAcP (Papadaki et al. 2015).

1.2 Bloodmeal digestion of sand flies

The ingested blood, taken by sand fly females as protein sources required for oocyte production and maturation, is gathered in the abdominal midgut. Excessive water is removed from the bloodmeal by diuresis (Sadlova et al. 1998, Sadlova and Volf 1999) and by an osmotic gradient between the midgut lumen, the midgut epithelial cells, and the hemolymph. Thereafter, the midgut epithelium forms the semi-permeable peritrophic matrix, which encloses the bloodmeal and compartmentalizes processes of digestion (Lehane 1997). As vertebrate blood cells contain the majority of proteins and other macromolecules, haemolysis is necessary to release these substances to be digested enzymatically (Lehane 1994). The digestive enzymes are secreted into the ectoperitrophic space and pass across the peritrophic matrix in order to digest the bloodmeal. The highest enzymatic activities occur in a thin layer between the midgut epithelium and the peritrophic matrix. At first, proteins are cleaved into large peptides by endopeptidases (e.g. trypsin and chymotrypsin), and then exopeptidases (e.g. aminopeptidases and carboxypeptidases) remove terminal dipeptides or single amino acids from each end of these polypeptides. The same midgut cells that synthesize and secrete the digestive enzymes presumably also perform absorption (summarized by Clements 2000). After digestion has been completed the peritrophic matrix is degradated and females defecate the residual substances of the bloodmeal digestion (Lehane 1997, Sadlova and Volf 2009).

As the majority of nutrients in the ingested blood is constituted by protein molecules, the proteolytic enzymes play the main role during bloodmeal digestion. Most haematophagous insects synthesise particularly serine proteases, namely trypsin- and chymotrypsin-like molecules, however, the ingestion of blood also induces the synthesis of other digestive enzymes, such as aminopeptidases, carboxypeptidases and glucosidases (Dillon et al. 2006, Ramalho-Ortigao et al. 2007, Jochim et al. 2008).

There is little basal protease activity in the midgut of unfed sand fly females. Following a few hours after the engorgement of the bloodmeal, the midgut epithelial cells start to produce trypsin- and chymotrypsin-like proteases. Significant levels of these proteases appear at 6 hours post bloodmeal (PBM) and their activity peaks at 12 – 48 hours PBM, depending on sand fly species (Dillon and Lane 1993a, Telleria et al. 2010). There is a peak of trypsin activity between 12 – 24 hours PBM in *L. longipalpis*, while in *P. papatasi* and *P. langeroni*, proteolytic activity reach its peak at 24 – 34 and 34 – 48 hours PBM, respectively. The level of protease activity drop to almost zero by 58 – 72 hours PBM (Dillon and Lane 1993a, Telleria et al. 2010).

According to Dillon and Lane (1993a), trypsin activity is confined to the midgut lumen, while aminopeptidase activity is connected principally with the midgut epithelium. The pH optimum of trypsin and aminopeptidase activity occurs in the alkaline range 7.5 – 9.5 and 8, respectively. The authors also detected chymotrypsin activity corresponding to < 2 % of trypsin activity (Dillon and Lane 1993a).

Analysing of cDNA libraries constructed from *P. papatasi* midgut of exclusively sugar or blood fed females has described the sequence of seven midgut serine proteases: four trypsin-like (PpTryp1 – PpTryp4) and three chymotrypsin-like (PpChym1 – PpChym3; Ramalho-Ortigao et al. 2003, 2007). Recently, detailed characterisation of full *P. papatasi* transcriptome has identified one other trypsin gene with five different alleles (PpTryp5a-e) and numbers of chymotrypsin-like sequences (Abrudan et al. 2013). Several transcripts coding for putative serine proteases have been also identified in *L. longipalpis* and *P. perniciosus.* The molecules are predicted as secreted proteins with molecular weight of the mature protein ranging from 25.9 to 27.9 kDa (Telleria et al. 2007, Jochim et al. 2008, Dostalova et al. 2011). In all mentioned species, some transcripts of the proteases occur in high abundance in the midgut of females fed on sugar, whereas their level drops after the engorgement of the bloodmeal. Simultaneously, the expression of other trypsin-like molecules is upregulated by blood feeding. This indicates that not all trypsins and chymotrypsins work in the same manner (Ramalho-Ortigao et al. 2003, 2007, Telleria et al. 2007, Jochim et al. 2008, Dostalova et al. 2011). In *L. longipalpis*, a midgut trypsin (LlTryp1) was confirmed as a blood feeding induced molecule by the detection of its expression at the protein level too (Telleria et al. 2010).

Furthermore, transcripts for other putative digestive proteases have been identified in the midgut of sand flies. Using an expressed sequence tag (EST) library from whole flies of *L. longipalpis*, Dillon et al. (2006) have described several putative aminopeptidases and a carboxypeptidase. Ramalho-Ortigao et al. (2007) and Jochim et al. (2008) confirmed that these molecules are midgut proteases by analysis of cDNA libraries from the midguts of *P. papatasi* and *L. longipalpis.* In the midgut of these sand fly species, the authors have also identified other carboxypeptidases, an astacin-like metalloprotease and a novel serin protease (Ramalho-Ortigao et al. 2007, Jochim et al.

2008). Similarly, exopeptidases have been identified in the midgut transcriptome of *P. perniciosus* (Dostalova et al. 2011). Recently, several new carboxypeptidases and aminopeptidases have been described in *P. papatasi* by Abrudan et al. (2013).

The peritrophic matrix (PM) is an acellular semipermeable envelope that encapsulates the ingested bloodmeal and compartmentalizes digestion between the endo- and ectoperitrophic spaces. The structure is formed of chitin, proteins and glycoproteins, and protects the midgut epithelium against pathogens and mechanical or chemical damage (Lehane 1997). In mosquitoes and other blood sucking insects, the PM plays an important role in heme detoxification (Pascoa et al. 2002) and its disruption by the adding of exogenous chitinase to the bloodmeal has an effect on fecundity of sand fly females (de Araujo et al. 2012).

Sand flies, as other nematoceran Diptera, produce the peritrophic matrix type I, which is delaminated from the entire midgut epithelium and is formed in response to blood feeding (Gemetchu 1974, Lehane 1997). The PM of sand flies is a stratified structure consisting of a thin laminar outer layer and a thick amorphous inner layer. In *P. duboscqi*, secretion of chitin is initiated instantly after the engorgement of blood and production of proteins and glycoproteins follows about six hours later. The PM is matured in less than 12 hours PBM and starts to break down from the third day PBM. The posterior opening of the PM can be observed between the third and fifth day PBM (Sadlova and Volf 2009).

Few putative proteins participating on the formation and degradation of the PM have been identified in the midgut transcriptomes of *P. papatasi*, *L. longipalpis* and *P. pernicious*. There have been described two types of peritrophin molecules containing chitin-binding domains. Peritrophins with multiple chitin-binding domains are likely necessary for cross linking the chitin fibrils that form the PM around the ingested blood, while peritrophins with single chitin-binding domain can play a role in capping the ends of chitin fibrils or sequestering free chitinous molecules within the midgut lumen (Ramalho-Ortigao 2007, Jochim et al. 2008, Dostalova et al. 2011).

The degradation of the PM is attributed to chitinolytic activity in the sand fly midgut, which is regulated by blood feeding and peaks around 48 hours PBM. Several studies have characterized cDNA from *L. longipalpis*, *P. papatasi* and *P. pernicious* that encodes a midgut specific chitinase, and have described the occurrence of a blood-induced chitinolytic system (Ramalho-Ortigao and Traub-Cseko 2003, Ramalho-Ortigao et al. 2005, Dostalova et al. 2011).

1.3 Effects of bloodmeal digestion on *Leishmania* development

One of the most significant barriers to *Leishmania* survival is posed by digestive enzymes. It has been published for a long time that proteolytic activities in the sand fly midgut affect *Leishmania* development (Borovsky and Schlein 1987, Pimenta et al. 1997, Schlein and Jacobson 1998). Adler (1938) first proposed that the products of bloodmeal digestion inhibit *Leishmania* infection within "noncompatible" sand fly species. Later studies showed that the specific components of trypsin-like activity cause the reduction of parasite numbers or even death of *L. donovani* in the "noncompatible" vector *P. papatasi*, whereas the capability to influence this factor allows *L. major* to survive and develop within its natural vector (Schlein and Romano 1986, Borovsky and Schlein 1987). It was described *in vitro* that the susceptibility of *Leishmania* to destruction by midgut proteolytic activity in the "compatible" vector is stage-specific. According to Pimenta et al. (1997), *L. major* amastigotes and fully-transformed promastigotes are relatively resistant to *P. papatasi* proteases, while the parasites transforming from the amastigote to promastigote stage are highly susceptible to be killed.

Using the exogenous suppression of midgut proteolytic activity, several other studies supported the hypothesis that the activities of midgut proteases influence the vector competence of sand flies. The addition of soybean trypsin inhibitor to the bloodmeal enabled *L. donovani* to survive and multiple during the early phase of infection in *P. papatasi* (Borovsky and Schlein 1987, Schlein and Jacobson 1998). In the study carried out by Schlein and Jacobson (1998), only 5 % of *L. donovani* promastigotes survived within *P. papatasi* fed on defibrinated blood, but this number increased to 90 % and 25 % on day 3 and 6, respectively, when females fed on blood with trypsin inhibitor. Thereafter, Volf et al. (2001) showed the enhancing effect of heparin. The addition of heparin into the infective bloodmeal enhanced *L. major* development in its natural vector *P. duboscqi*, as it increased infection rates as well as intensities of infection. However, there were no effects on the location and anterior migration of promastigotes. According to the authors, this effect of heparin could be explained by its interference with midgut proteases. Heparin decreased trypsin activity 12 and 72 hours PBM but did not affect the defecation, mortality and oviposition of females (Volf et al. 2001).

In order to survive in the natural vectors, *Leishmania* have developed mechanisms to overcome the harmful effects of midgut proteases. Several studies demonstrated that in natural vector-parasite pairs, *Leishmania* modulate the levels and timing of insect digestive enzymes at both protein and transcriptional levels (Schlein and Romano 1986, Borovsky and Schlein 1987, Dillon and Lane 1993b, Sant'Anna et al 2009, Tellleria et al. 2010, Dostalova et al. 2011). Schlein and Romano (1986) and Borovsky and Schlein (1987) described the ability of *L. major* to decrease proteolytic activity in the midgut of *P. papatasi*. The protease activity of midgut homogenates from infected females was about one-third lower then of controls. In other study, Dillon and Lane (1993b) reported that presence of *L. major* amastigotes in the bloodmeal delayed the peak levels of protease activity in the midgut of *P. papatasi*. The authors suggested that the inhibition or delay of sand fly digestion can surpass the harmful effects of proteolytic enzymes and, moreover, it may offer the parasites a nutritionally richer environment to multiple, for longer. More recently, *Leishmania* capability to modulate midgut enzymatic activity was also observed in the New World species *L. longipalpis* infected with *Leishmania infantum* and *L. mexicana* (Sant'Anna et al. 2009, Telleria et al. 2010). Santos et al. (2014) supposed that *L. infantum* modulates trypsin activity in *L. longipalpis* by decreasing the pH in the vector midgut.

Several studies described that *Leishmania* parasites are able to modulate the abundance of digestive enzymes at transcriptional level. The modulation of transcription of proteases genes was reported through the extensive EST sequencing of cDNA libraries constructed from blood fed and *Leishmania* infected sand flies. Both up- and down-regulation of some digestive enzymes transcript after blood feeding was observed in sand fly females infected with *L. infantum* or *L. major*. *Leishmania* infection can reduce the transcript abundance of chymotrypsin molecules (Ppchym2 and LuloChym1A) in the midgut of *P. papatasi* and *L. longipalpis*, respectively (Ramalho-Ortigao et al. 2007, Jochim et al. 2008), and a trypsin (PperTryp3) in the midgut of *P. perniciosus* (Dostalova et al. 2011). On the other hand, the increased abundance of midgut transcripts for other trypsin molecules (PpTryp1 and Lltryp2) was detected in infected females of *P. papatasi* and *L. longipalpis*, respectively (Ramalho-Ortigao et al. 2007, Jochim et al. 2008). Moreover, the modulation of abundance of midgut transcripts by *Leishmania* was also observed after the bloodmeal digestion has been complied (5 – 7 days PBM); *Leishmania infantum* induced the down-regulation of the trypsin LuloTryp3, whereas transcripts for the trypsin Lltryp2 were of higher abundance (Jochim et al. 2008).

The main mechanical barrier, which *Leishmania* faces, is constituted by the peritrophic matrix. The role of the PM in *Leishmania*-sand fly interactions appears to be dual: in the very early phase of infection, it can to protect parasites transforming from amastigotes to promastigotes stages against proteolytic damage (Pimenta et al. 1997); on the contrary, the PM creates the mechanical barrier that prevents escape of promastigotes from the endoperitrophic space, which may result in their defecation with the bloodmeal remnants (Walters et al. 1992, Sadlova and Volf 2009, Sadlova et al. 2013). Pimenta et al. (1997) suggested that the PM can protect *Leishmania* against the rapid diffusion of digestive enzymes during the early stage of infection. The addition of exogenous chitinase to the bloodmeal inhibited the formation of the PM in *P. papatasi*, which resulted in the reduction of *L. major* infections for 4 four hours PBM. But early parasite mortality was reversed if soybean trypsin inhibitor was added into the bloodmeal.

On the other hand, the PM may avert the release of parasites to the ectoperitrophic space. As the process of formation and disintegration of the PM in haematophagous insects is highly-species specific (Lehane 1997), its formation and persistence seems to be one of the main potential factors affecting the vector competence of sand flies. Walters et al. (1992) observed that the PM adversely affects the infection rate of *L. panamensis* in the "noncompatible" vector *P. papatasi*. *Leishmania* parasites remained entrapped in a closed peritrophic sac and were unable to escape from the endoperitrophic space, which resulted in their elimination from the midgut. Recently, Sadlova et al. (2013) suggested that one of the main parameter for the early-stage development of *Leishmania* in the midgut of *Sergentomyia schwetzi* is the duration of the period between the breakdown of the PM and defecation of the bloodmeal remnants. Moreover, according to Sadlova and Volf (2009), the anterior plug (a component of the PM secreted by the thoracic midgut and located at the junction between the anterior and posterior midgut) constitutes a temporary barrier to stop the forward migration of parasites to the thoracic midgut.

As *Leishmania* promastigotes produce chitinase (Shakarian and Dwyer 1998), it was assumed by Schlein et al. (1991) that *L. major* causes the disintegration of the anterior end of the PM in *P. papatasi* by *Leishmania* own chitinase in order to release to the ectoperitrophic space. This hypothesis was supported by Rogers et al. (2008) who reported that a chitinase over expressing strain of *L. mexicana* accelerated its escape from the PM-enclosed blood and, consequently, the parasites arrived earlier at the stomodeal valve of *L. longipalpis*. However, this overexpressing mutant developed significantly faster in the sand fly midgut than a control strain (Rogers et al. 2008) and therefore the effect might be a result of various aspects, not only due to the specific interaction of chitinase activity with the PM.

Later study conducted by Sadlova and Volf (2009) demonstrated that *L. major* in its natural vector *P. duboscqi* remains localized in the endoperitrophic space until the PM is degradated by sand fly-derived chitinases and only then colonize the ectoperitrophic space of the midgut. The detailed histological and electro-microscope study showed that *Leishmania* parasites are not able to traverse the PM before its rough disintegration, which is indicated by the release of the bloodmeal remnants to the ectoperitrophic space. In both infected and uninfected females, the disintegration of the PM begun at the posterior end and only in that place *Leishmania* promastigotes escaped from the PM (Sadlova and Volf 2009). These results were supported by another study reporting that the knockdown of *P. papatasi* chitinase (PpChit1) via injection of dsRNA led to significant reduction in the amount of PpChit1 protein, which resulted in a significant reduction of *L. major* within the midgut. The numbers of parasites were reduced by 46 % and 63 % at 48 and 120 hours PI, respectively. The injection of dsRNA also significantly affected the intensities of infection (Coutinho-Abreu et al. 2010).

Sadlova and Volf (2009) also showed that the transformation of *L. major* procyclics to long nectomonads (which are capable of attaching to the midgut epithelium in order to avoid expulsion during defecation) coincides with the degradation of the PM of *P. duboscqi*. This transformation seems to be associated with the diffusion of signal molecules (probably salivary components) from the ectoperitrophic space into the parasite neighbourhood across the broken PM (Bates and Rogers 2004). It might be concluded that *Leishmania* utilizes sand fly chitinolytic system as the main mechanism for its escape from the PM.

At the end of bloodmeal digestion, *Leishmania* must survive in the midgut during defecation. Sand fly midgut peristalsis assists in the excretion of the undigested bloodmeal remnants. According to Vaidyanathan (2004, 2005), *L. major* produces a myoinhibitory neuropeptide that is able to arrest the midgut and hidgut motility of its vector *P. papatasi* and, to a lesser degree that of *L. longipalpis*. Myoinhibitory activity was also detected in *L. braziliensis* and Sudanese strain of *L. donovani*, but Indian strain of *L. donovani* had no effects on the hidgut motility of *P. papatasi*. The inhibitory effects in the "noncompatible" vector were lower than in the natural one, which suggests that the myoinhibitory peptide is species specific to a degree. The author suggested that the inhibition of vector gut peristalsis protects *Leishmania* from being eliminated after bloodmeal digestion and decreases parasite chances to establish the infection within the sand fly digestive tract. However, these results of Vaidyanathan (2004, 2005) have never been supported by any other study.

More importantly, in order to avoid being excluded with the bloodmeal remnants, *Leishmania* attaches to the midgut via inserting its flagella between the epithelial microvilli. The midgut attachment based on receptor-ligand interaction varies among different sand fly species and have been described as strictly stage-dependent. It is a property of nectomonad and leptomonad forms, but is absent in the early and final forms; procyclics and metacyclics (Wilson et al. 2010). It has been postulated that this attachment is the crucial factor determining parasite-vector specificity (Pimenta et al. 1994).

Sand flies have been classified as permissive or specific vectors on account of their capability to support the development of wide or limited spectrum of *Leishmania* species. *Leishmania* binding to the midgut epithelium in the permissive vectors (e.g. *L. longipalpis*, *P. arabicus*, *P. perniciosus* and *P. argentipes*) is related to the occurrence of midgut glycoproteins bearing terminal *N*-acetyl-galactosamine and to the lectin-like activity of *Leishmania* surface. On the other hand, in the specific vectors (*P. papatasi*, *P. duboscqi* and *P. sergenti*), the attachment is controlled by sand fly midgut lipophosphoglycan (LPG) receptors, which bind to the terminal carbohydrates of *Leishmania* LPG (Myskova et al. 2007, Volf and Myskova 2007, Svarovska et al. 2010, Secundino et al. 2010). The important role of *Leishmania* LPG in the interaction of *L. major* with its specific vector *P. papatasi*, and the existence of a LPG-independent mechanism of attachment for *L. major* in *P. perniciosus* were also recently confirmed by Jecna et al. (2013).

The molecular principles of *Leishmania* binding in the midgut have been studied in detail in the specific parasite-vector pair *P. papatasi* and *L. major*. It was reported that purified LPG from *L. major* binds to the dissected midguts of *P. papatasi* (Lang et al. 1991) and, simultaneously, inhibits the attachment of parasites to the gut *in vitro* (Pimenta et al. 1992). According to Pimenta et al. (1992), midgut attachment and detachment is controlled by specific developmental modifications in terminally exposed saccharides on LPG. On account of increased numbers of phosphoglycan repeat units and side chain galactose residues hidden by the addition of terminal arabinose (McConville et al. 1992), the metacyclic form of *L. major* LPG does not bind to the midgut of *P. papatasi* (Pimenta et al. 1992). In more recent study, Sacks et al. (2000) confirmed the essential role of parasites LPG in the midgut attachment. Using LPG deficient mutants, the authors showed that *Leishmania* capability to maintain infection in the sand flies during defecation was completely suppressed due to inability to attach to the midgut epithelium *in vitro* (Sacks et al. 2000).

Lectin-like activities were found in the midgut of sand flies (Volf et al. 2002) and lectins or lectin-like molecules were suggested as receptors for the midgut attachment of *Leishmania* parasites. This was confirmed by Kamhawi et al. (2004) who have identified a galactose binding protein, PpGalec, from the unfed midgut cDNA library of *P. papatasi*, which has been shown to be a midgut receptor for *L. major* LPG. PpGalec, a tandem repeat galectin on the midgut epithelium, bind specifically to the LPG galactose residues of parasites. The galectin is thus identified as an important molecule affecting vector competence (Kamhawi et al. 2004). Recently, Volf et al. (2014) revealed that the LPG of *L. turanica* (other *Leishmania* species transmitted by *P. papatasi*) is similar to that of *L. major*, however, is less arabinosylated. The authors suggested that both species bind to the midgut of *P. papatasi* using the same mechanism since the high abundant galactose in *L. turanica* LPG could be recognised by the same receptor – PpGalec.

Recently, Di-Blasi et al. (2015) showed, that the *Leishmania* flagellar protein FLAG1/SMP1, characterised by Tull et al. (2004), may participate in the midgut attachment/infection of *L. major* in *P. papatasi* but not in *L. longipalpis*. Using anti-FLAG1/SMP1 monoclonal antibody, the authors showed that *ex vivo* and *in vivo* attachment/infection of *L. major* were significantly inhibited when sand fly females were pretreated with this antibody. On the other hand, there were no effects of this monoclonal antibody on attachment/infection process in the permissive vector

L. longipalpis infected with *L. infantum*. The authors hypothesised that the initial interaction of parasites with *P. papatasi* midgut epithelium through the flagellum, via FLAG1/SMP1, may be essential for further and stronger interaction via LPG (Di-Blasi et al. 2015)

1.4 Effects of avian blood on *Leishmania* development

For several times, bloodmeal sources from different animals have been reported to affect the digestion and reproductive potential of sand fly females (Ready 1979, Benito-De Martin et al. 1994, Hanafi et al. 1999, Noguera et al. 2006). With regard to nutritional value, chicken blood is potentially less convenient for sand flies as it has a haematocrit value lower than that of other domestic animals and contains less haemoglobin and proteins than dog, human or rabbit blood (Sant'Anna et al. 2010). Less nutrition in host blood could be compensated by a mechanism of prediuresis that was described in *Phlebotomus* species (Sadlova et al. 1998, Sadlova and Volf 1999). Partial compensation for the lower protein content in the chicken blood source was also observed in *L. longipalpis* (Sant'Anna et al. 2010).

Chickens attract both females and males of sand flies but, as other birds, do not support *Leishmania* infection as a result of their higher natural body temperature or biological differences from mammals such as complement or nucleated erythrocytes (Adler 1964, Schlein et al. 1983). It is questionable whether the proximity of domestic chickens or turkeys is a possible environmental risk in *Leishmania* transmission as they represent an attractive bloodmeal source and henhouses provide resting and breeding sites for sand fly females (Alexander et al. 2002), or may provide a zooprophylactic effect (Schlein et al. 1983, Schlein and Jacobson 1994), as described below. Experimentally, avian blood has been reported to affect not only digestion and the fecundity of females but also *Leishmania* development within the midgut. According to Schlein et al. (1983), the infection of *L. major* was inhibited in its natural vector *P. papatasi* if females were fed on turkey blood. The authors fed females on turkeys either before or after an infective meal containing rabbit blood with *Leishmania* promastigotes and in both experimental setups observed a suppression of infection. Infection rates in experimental females were only of 3.5 – 25.8 % with some flies harbouring only a few parasites. On the contrary, the infection rate in a "control group" of females was 85.5 % with the high intensities of infection. The low infection rates in females infected one week post feeding on turkey suggested that the reduction of vector capacity persist even after the causative turkey blood had been completely digested. The authors assumed that the suppression of *Leishmania* infection was a result of the digestive process and a relatively high DNAase level induced by nucleated erythrocytes (Schlein et al. 1983). Similar results also obtained Schlein and Jacobson (1994). Furthermore, in their study, *Leishmania* infection was 100 % inhibited in females fed on chickens one day after the infective bloodmeal. The authors concluded that sand fly feeding on turkey or chicken blood is lethal to *L. major* and, moreover, such meals reduce the vector potential of *P. papatasi* (Schlein et al. 1983, Schlein and Jacobson 1994).

On the other hand, no effects of bird blood on *Leishmania* infection was observed in the New World species. Nieves and Pimenta (2002) tested the effects of several bloodmeal sources on the development of *L. braziliensis* and *L. amazonensis* in *Lutzomyia migonei*. They observed that the type of ingested blood partly affected the infection rates of females, but none of the blood sources (including chicken blood) eliminated *Leishmania* infection. Although there was a slightly lower percentage of infected females after feeding on chicken blood with amastigotes compared to flies infected via rodent blood, infections were not suppressed and in both *L. braziliensis* and *L. amazonensis* the midgut infection was established. Similarly, Sant'Anna et al. (2010) did not observe any harmful effects of chicken blood on the development of *L. mexicana* within *Lutzomyia longipalpis*. Moreover, there was detected a trend towards higher percentage of infected flies and higher intensities of infection in females infected via chicken blood. The authors described slightly delayed transformation of *Leishmania* to the metacyclic stages in females infected via chicken blood compared to those fed on rabbit blood, however, by day 8 PI, the proportions of metacyclics was already similar in both groups of females.

These contradictory results might be interpreted that there is a difference in the effects of bird blood between the Old World species of the genus *Phlebotomus* and the New World species of the genus *Lutzomyia*. Therefore, one of the major aims of this Ph.D. thesis was to study the effects of avian blood on *Leishmania* development in *Phlebotomus* sand flies.

2 Objectives

Various sand fly species differ in vector competence to various *Leishmania*. There exists a close evolutionary fit between *Leishmania* species and their natural sand fly vectors, and the knowledge of factors affecting vector competence is crucial from an epidemiological point of view. Within the early phase of *Leishmania* development in sand flies, there are many unknown details and unanswered questions about circumstances affecting *Leishmania* infection. Therefore, my Ph.D. project was focused on various parameters of bloodmeal digestion in sand flies and their effects on *Leishmania* development.

The main objectives of this thesis were:

1. to compare various parameters of bloodmeal digestion (dynamics of proteolytic enzymes, timing of formation and degradation of the peritrophic matrix and time of defecation of the bloodmeal remnants) in four sand fly species: *Phlebotomus argentipes, Phlebotomus orientalis* (*Leishmania donovani* vectors) and *Phlebotomus papatasi, Sergentomyia schwetzi* (refractory to *L. donovani*).

2. to measure the bloodmeal volume ingested by sand fly females during blood feeding and evaluate the effect of initial infective doses on the susceptibility of *P. orientalis* and *P. argentipes* to *L. donovani*.

3. to evaluate the effects of chicken blood on bloodmeal digestion and the development of *Leishmania major* in the Old World vector *Phlebotomus duboscqi*.

3 List of publications

1. **Pruzinova K**, Sadlova J, Seblova V, Homola M, Votypka J and Volf P (2015). Comparison of bloodmeal digestion and the peritrophic matrix in four sand fly species differing in susceptibility to *Leishmania donovani*. *PLoS One*, 10, e0128203

2. Seblova V, Volfova V, Dvorak V, **Pruzinova K**, Votypka J, Kassahun A, Gebre-Michael T, Hailu A, Warburg A and Volf P (2013). *Phlebotomus orientalis* sand flies from two geographically distant Ethiopian localities: Biology, genetic analyses and susceptibility to *Leishmania donovani*. *PLoS Neglected Tropical Diseases*, 7, e2187.

3. **Pruzinova K**, Votypka J and Volf P (2013). The effect of avian blood on *Leishmania* development in *Phlebotomus duboscqi*. *Parasites and Vectors*, 6, 254.

4 Summary and conclusions

This dissertation thesis summarises the published results of three projects I was involved in during my PhD study. These projects were aimed at the development of *Leishmania* in sand flies, with the main emphasis on the bloodmeal digestion of sand flies and accompanying effects on *Leishmania* development.

In order to study the effects of bloodmeal digestion on vector competence, we compared various parameters of bloodmeal digestion in sand fly species that are either susceptible (*P. argentipes* and *P. orientalis*) or refractory (*P. papatasi and S. schwetzi*) to *L. donovani*. Despite the fact that *P. argentipes* and *P. orientalis* are both natural vectors of *L. donovani*, we found out that they strikingly differ in the time course of bloodmeal digestion, secretion of the peritrophic matrix (PM) and activities of proteolytic enzymes. Females of *P. argentipes* digested more quickly, with both trypsin and chymotrypsin activity peaking about 24 – 36 hours PMB, and dropping to almost zero by 72 hours PBM. Conversely, in the midguts of *P. orientalis*, the highest values of trypsin and chymotrypsin activities were detected between 48 and 72 hours PBM.

The maximum chymotrypsin activity in *P. orientalis* was much lower than in the other three species tested. On the contrary, there was a very high peak of chymotrypsin activity in *P. argentipes* 36 hours PBM, which indicates that these differences in chymotrypsin activity do not affect the vector competence of sand flies to *L. donovani*.

Peaks of midgut trypsin activity in refractory species, *P. papatasi* and *S. schwetzi*, were higher compared to the *L. donovani* vectors, which supports a previous hypothesis that the trypsin molecule influences *Leishmania* development in sand flies (Adler 1938, Schlein and Romano 1986, Borovsky and Schlein 1987, Schlein and Jacobson 1998, Pimenta et al. 1997).

The four sand fly species studied also differed in kinetics of the PM. Our data confirmed that the speed of PM formation and degradation is species-specific. Formation of the PM in *L. donovani* vectors was slower than in the refractory species; a fully formed PM was present in all *P. argentipes* and *P. orientalis* females by 24 hours PBM, while in *P. papatasi* and *S. schwetzi* this process lasted only 3 and 6 hours, respectively. Interestingly, both natural vectors of *L. donovani* considerably differed in the kinetics of PM formation and degradation. In *P. argentipes*, the PM was present for only a short time, first appearing at 12 hours PBM and degrading between 24 and 48 hours PBM, whereas in *P. orientalis,* the PM started to form by 6 hours PBM and degraded between 48 and 72 hours PBM. Although there were significant differences between the sand fly species, the PM was present in all species when the proteolytic activities peaked and its breakdown started several hours later.

Important differences between two natural vectors of *L. donovani* were also found in the speed of bloodmeal digestion and timing of defecation. *Phlebotomus argentipes* showed rapid defecation of the bloodmeal remnants, with all females already defecated by day three PBM, whereas *P. orientalis* females defecated from the fourth to sixth day PBM. This indicates that *L. donovani* promastigotes in *P. argentipes* had a shorter time to develop forms that are able to attach to the midgut epithelium. As the binding of *Leishmania* to the sand fly midgut is strictly stage-dependent and is a property of long and short nectomonads (Wilson et al. 2010), this suggests that procyclic promastigotes must transform to nectomonads much faster in *P. argentipes* than in *P. orientalis*. This transformation seems to be associated with the diffusion of signal molecules to the vicinity of parasites (Bates and Rogers 2004) and coincides with degradation of the PM (Sadlova and Volf 2009). The signal molecules are presumably salivary components ingested into the midgut (Bates and Rogers 2004), which are known to trigger *Leishmania* transformation *in vitro* (Charlab and Ribeiro 1993, Charlab et al. 1995).

Based on our results and the study conducted by Sadlova et al. (2013), we assume that one of the crucial parameters responsible for the establishment of *Leishmani*a infection in the sand fly midgut is the period between the breakdown of the PM and defecation of the bloodmeal remnants, i.e. the time frame when promastigotes can attach to the midgut epithelium. In *S. schwetzi*, this period was either extremely short (only 3 hours on average) or the intact PM persisted until defecation. Due to an intact PM, the transformation of *L. donovani* promastigotes to nectomonads was delayed in *S. schwetzi* compared to the permissive vector *Lutzomyia longipalpis*, which resulted in the expulsion of *Leishmania* parasites with the bloodmeal remnants during defecation (Sadlova et al. 2013). On the other hand, in *P. argentipes*, we found that despite a quite early defecation, the time between degradation of the PM and defecation lasted on average 24 hours, which provides enough time for *L. donovani* promastigotes to be released into the ectoperitrophic space and attach to the midgut epithelium. In *P. orientalis* and *P. papatasi*, this “window” was longer (48 and 38 hours, respectively). However, *P. papatasi,* as a specific vector of *L. major,* is refractory to *L. donovani* on account of the lack of a surface ligand for the parasite LPG (Volf and Myskova 2007).

To compare the susceptibility of *P. argentipes* and *P. orietalis* to *L. donovani*, we measured the size of the blood-meal taken by sand fly females during blood feeding and evaluated the effect of an initial infective dose on the development of this parasite in the vectors. Using light microscopy and real-time PCR, we showed that both vectors are comparatively highly susceptible to *L. donovani* infection. Although experimental infections were initiated with significantly different infective doses (2×10^3, 2×10^4, 1×10^5 and 5×10^5 promastigotes/ml), differences in infection rates in *P. orientalis* were observed only in the group infected with 2×10^3 promastigotes/ml. In this group, late phase infections were detected in 45 % of females, whereas infection rates in the other three groups infected with 2×10^4, 1×10^5 and 5×10^5 promastigotes/ml reached 75–95 %. However, mature infections and colonization of the stomodeal valve were observed in all groups, including the one with the lowest infective dose. Similarly, in *P. argentipes*, infection rates in groups of females infected with 2×10^3, 2×10^4 and 5×10^5 promastigotes/ml were about 45, 65 and 90 %, respectively, and even the lowest initial dose resulted in very heavy late stage infections. Taking into account the average bloodmeal size ingested by *P. orientalis* and *P. argentipes* (about 0.6 μl in both species), females infected with 2×10^3, 2×10^4 and 5×10^5 promastigotes/ml of blood ingested average 1–2, 12 and 300 promastigotes, respectively.

We can conclude that in spite of striking differences in bloodmeal digestion, both *P. orientalis* and *P. argentipes* are equally susceptible to *L. donovani*, and in both vectors even one or two parasites constitute a satisfactory dose to initiate mature infections with colonisation of the stomodeal valve in about one half of females.

Furthermore, we compared various life cycle parameters and susceptibility to *L. donovani* in two colonies of *P. orientalis* originating from two Ethiopian localities distant from each other: a non-endemic area in the lowlands (Melka Werer), and an endemic focus of human visceral leishmaniases in the highlands (Adis Zemen). We observed that in spite of marked difference in life-cycle parameters, both colonies were highly susceptible to Ethiopian *L. donovani* strains. *Leishmania* developed similarly in both of them, which suggests that the absence of visceral leishmaniases in the non-endemic area Melka Werer is not caused by the different susceptibility of the local *P. orientalis* population to *L. donovani*.

The last part of my thesis was devoted to the effects of avian and mammalian blood on sand fly digestion and *Leishmania* development within the midguts. For this, we studied protein content, trypsin activity and the development of *L. major* in the midguts of *P. duboscqi* fed on avian or mammalian blood. Chicken blood has less than half the total protein of rabbit blood, but the results of Sant'Anna et al. (2010) suggest that females of *L. longipalpis* are capable of compensating for the lower protein content in bird blood through efficient prediuresis. In our experiments, *P. duboscqi* females fed on chickens had half the protein content in their midguts in comparison with those fed on rabbits, corresponding to the concentrations measured in chicken and rabbit blood. So, it seems that *P. duboscqi* is not able to concentrate chicken blood more that rabbit blood, even though prediuresis has been demonstrated in this species (Sadlova et al. 1998, Sadlova and Volf 1999). Consequently, the lower content of proteins in ingested blood significantly affected the midgut trypsin activity and oocyte development in females fed on chickens. The largest differences between the groups of females were detected during the first 24 hours PBM, with females fed on chickens having 40 – 55 % less trypsin activity in their midguts compared to those fed on rabbits.

Some older studies proposed that avian blood is harmful to *Leishmania,* since infection with *L. major* was inhibited in *P. papatasi* females fed on turkey or chicken blood (Schlein et al. 1983, Schlein and Jacobson 1994). However, experiments with the New World sand flies *L. longipalpis* and *L. migonei* showed that chicken blood is likely to support the development of *L. mexicana, L. amazonensis* and *L. braziliensis* (Nieves and Pimenta 2002, Sant'Anna et al. 2010). Because of these discrepancies in descriptions of the effects of avian blood on *Leishmania,* the aim of our study was to test the effects of avian blood on the development of *L. major* in the natural vector *P. duboscqi.* The effects of chicken blood on parasites were studied using light microscopy as well as real-time PCR in several experimental setups where sand flies were fed on chickens or mice either before or after experimental infections. At first, *P. duboscqi* females were fed either on chickens or mice, and after oviposition were infected via experimental feeding through chick-skin membrane (nine days after the first bloodmeal). In the second setup, we studied the effect of bird blood on *L. major* already present in the gut: females infected with promastigotes in diluted (5%) blood were fed either on chickens or mice one day post infection. The decreased amount of nutrients in the diluted blood resulted in the females having to feed again without laying eggs. In addition, we evaluated the effect of avian blood during the later phase of *Leishmania* infection: females infected with promastigotes in diluted (10%) blood were fed either on chickens or mice six days post infection. We did not find any significant differences between the groups of females fed on chickens or mice in any of these experiments. *Leishmania* developed similarly in both experimental groups, leptomonads and metacyclic forms prevailed in the thoracic midgut, and no significant differences were found in infection rates and intensities of infections. We conclude that the digestion of avian blood is not harmful to *L. major* within *P. duboscqi*.

The differences between our results and those of Schlein (Schlein et al. 1983, Schlein and Jacobson 1994) cannot be explained by different techniques or the parasite-vector pair used. We used the same *Leishmania* strain (LRC-L137), and *P. duboscqi* is a sister species of *P. papatasi*, both being specific vectors of *L. major*. However, Schlein's conclusions may have been influenced by the absence of an appropriate control. For experimental infection of sand flies, they used diluted rabbit blood to decrease the amount of nutrients in the ingested bloodmeal; however, they had only one group of sand fly females fed on avian blood and did not include any control group of females fed on a mammalian host. We used the same method as Schlein et al. (1983; infection by promastigotes in 5% rabbit blood and one day later fed on avian blood), but used a control group of females fed on a mouse host. Using this experimental setup, infection rates were 50 – 60 %, which means about 40 % lower than is typical for this sand fly-*Leishmania* combination. Nevertheless, we observed this reduction in the chicken group as well as in the control mouse group of sand fly females. This indicates that the reduction in *Leishmania* infection reported by Schlein et al. (1983) and Schlein and Jacobson (1994) was not caused by the avian blood but rather by their experimental setup.

To confirm our hypothesis that Schlein's results were affected by the experimental setup, we studied the effect of diluted blood on *Leishmania* development within the sand fly midguts. Whereas females fed on 100% rabbit blood with *L. major* promastigotes were all infected and the parasite loads were high, females fed on 5% blood were infected in only 65 % of cases and the intensities of infection were light or moderate. These experiments showed that the infection of sand flies via diluted blood resulted in significant lower infection rates and intensities of infection, probably as a consequent of faster digestion. To extend our study we also considered the effects of the number of feedings and age of females on the development of *L. major* within *P. duboscqi*, but we did not find any significant differences between females infected during the first or second blood feeding; *Leishmania* developed well in both groups of females.

In conclusion, *P. duboscqi* females fed on avian blood had lower trypsin activity and slower oocyte development compared to those fed on mammalian blood; more importantly, however, various experiments showed that sand fly feeding on chickens does not inhibit *Leishmania* development within the midgut. In addition, the susceptibility of *P. duboscqi* to *L. major* is not affected by the number of feedings or female age.

Leishmania development in sand flies during bloodmeal digestion is a complex process and there are still many aspects that need to be investigated in more detail. In further work, we would like to continue studying PM and the effects of proteolytic activities on *Leishmania* development. Specifically, using *in vitro* assay we will study the effects of a midgut lysate from blood-fed females (*P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi*) to *L. donovani* amastigotes, promastigotes and parasite stages within the amastigote-promastigote transition. We would like to investigate if these transient forms are really susceptible to being killed by midgut proteases. We also plan to elucidate if disruption of the PM by exogenous chitinase may enable *Leishmania* parasites to survive in *S. schwetzi*, which should confirm or disprove our hypothesis about PM being the main factor affecting the establishment of *Leishmania* infections in *S. schwetzi*.

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Publications

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RESEARCH ARTICLE

Comparison of Bloodmeal Digestion and the Peritrophic Matrix in Four Sand Fly Species Differing in Susceptibility to *Leishmania donovani*

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Abstract

The early stage of *Leishmania* development in sand flies is closely connected with bloodmeal digestion. Here we compared various parameters of bloodmeal digestion in sand flies that are either susceptible (*Phlebotomus argentipes* and *P*. *orientalis*) or refractory (*P*. *papatasi* and *Sergentomyia schwetzi*) to *Leishmania donovani*, to study the effects on vector competence. The volume of the bloodmeal ingested, time of defecation of bloodmeal remnants, timing of formation and degradation of the peritrophic matrix (PM) and dynamics of proteolytic activities were compared in four sand fly species. Both proven vectors of *L*. *donovani* showed lower trypsin activity and slower PM formation than refractory species. Interestingly, the two natural *L*. *donovani* vectors strikingly differed from each other in secretion of the PM and midgut proteases, with *P*. *argentipes* possessing fast bloodmeal digestion with a very high peak of chymotrypsin activity and rapid degradation of the PM. Experimental infections of *P*. *argentipes* did not reveal any differences in vector competence in comparison with previously studied *P*. *orientalis*; even the very low initial dose (2×103 promastigotes/ml) led to fully developed late-stage infections with colonization of the stomodeal valve in about 40% of females. We hypothesise that the period between the breakdown of the PM and defecation of the bloodmeal remnants, i.e. the time frame when *Leishmania* attach to the midgut in order to prevent defecation, could be one of crucial parameters responsible for the establishment of *Leishmania* in the sand fly midgut. In both natural *L*. *donovani* vectors this period was significantly longer than in *S*. *schwetzi*. Both vectors are equally susceptible to *L*. *donovani*; as average bloodmeal volumes taken by females of *P*. *argentipes* and *P*. *orientalis* were 0.63 μl and 0.59 μl, respectively, an infective dose corresponding to 1–2 parasites was enough to initiate mature infections.

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Introduction

The parasitic protozoan *Leishmania donovani* (Kinetoplastida: Trypanosomatidae) causes visceral leishmaniasis, also called kala-azar, which is a serious health problem in the Indian subcontinent and East Africa. This *Leishmania* species is transmitted by sand flies of the genus *Phlebotomus*, the most important vectors being *Phlebotomus orientalis* and *P. argentipes* (reviewed by [1,2]).

In sand fly vectors, the development of *Leishmania* parasites is confined to the digestive tract. The early phase of infection is closely associated with bloodmeal digestion as parasites multiply and morphologically transform in the lumen of midgut, within the bloodmeal surrounded by the peritrophic matrix (PM). Parasites that successfully develop and survive this early stage must then attach to the midgut epithelium and establish late-stage infections (for review see [3–6]).

One of the first obstacles parasites must surpass in the sand fly midgut is the effect of midgut digestive enzymes, particularly trypsin- and chymotrypsin-like proteases. Production of these serine proteases by midgut epithelial cells starts several hours post bloodmeal (PBM) and their activities peak at 18–48 hours PBM, depending on the sand fly species [7,8].

It has been repeatedly published that proteolytic activities of midgut proteases influence *Leishmania* development and the vector competence of sand flies [9–11]. Adler [12] was the first who suggested that products of blood serum digestion destroy *Leishmania* parasites in the midguts of 'noncompatible' sand fly species. According to Schlein and Romano [13] and Borovsky and Schlein [9], specific components of the trypsin-like activity prevents the survival of *L. donovani* in the 'noncompatible' vector *P. papatasi* while the ability to modulate this factor enables *L. major* to survive in 'compatible' sand fly species. It has been shown *in vitro* that midgut lysates of blood-fed *P. papatasi* (a natural vector of *L. major*) kills *Leishmania* transforming from the amastigote to promastigote stage, and this effect was attributed to midgut proteases [11]. Several studies have also described that in natural vector-parasite combinations, *Leishmania* modulate the levels of insect digestive enzymes at both the protein and transcriptional levels [8,13,14]. In the New World sand fly *Lutrzomyia longipalpis*, Santos *et al.* [15] recently described that *L. infantum* modulates trypsin activity, supposingly by decreasing the pH in the midgut.

A second potential barrier for *Leishmania* infection is the peritrophic matrix (PM). The peritrophic matrix is an acellular semipermeable envelope that separates ingested blood from the midgut epithelium and compartmentalizes digestion between endo- and ectoperitrophic spaces. This structure is composed of chitin, proteins and glycoproteins, and plays a key role in protection of the midgut epithelium from pathogens and chemical and mechanical damage (reviewed by [16]). In mosquitoes and other hematophagous insects, the PM performs a central role in heme detoxification [17] and its disruption affects fecundity of sand fly females [18].

The role of the PM in *Leishmania*—sand fly interactions seems to be twofold: the PM has been suggested to protect parasites from proteolytic damage within amastigote-to-promastigote transition [11]; on the other hand, it creates a physical barrier that prevents the early escape of *Leishmania* parasites into the ectoperitrophic space, which may result in their defecation with bloodmeal remnants (reviewed by [3,5]). Recently, Sadlova and Volf [19] demonstrated that *Leishmania major* in its natural vector *P. duboscqi* stays in the intraperitrophic space until the PM is broken by sand fly-derived chitinases and only then colonize the ectoperitrophic space of the midgut. Finally, after successful escape into ectoperitrophic space, promastigotes must overcome a critical period of defecation by inserting flagella between microvilli of the midgut epithelium (reviewed by [4,20–22]). As the process of formation and degradation of the PM in blood sucking insects is highly species-specific (reviewed by [16]), the formation and persistence of the PM seems to be one of main potential factors involved in the vector competence of sand flies.

The main aim of the work was a thorough understanding of the factors in the sand fly midgut which *Leishmania* parasites meet during the early stage of infection. Detailed study of midgut proteolytic activity and development of the peritrophic matrix in several vector species differing in vector competence was expected to clarify which factors differ interspecifically and can cause failure of the parasite development. Therefore, we selected two sand fly species which fully support development of *L. donovani* (*P. orientalis* and *P. argentipes*, reviewed by [1,2]) and two species which are refractory to this parasite (*S. schwetzi* and *P. papatasi* [9,10,23,24]) and studied the bloodmeal volume imbibed by females, the time of defecation of bloodmeal remnants, timing of the formation and degradation of the PM and dynamics of midgut protease activities. In addition, we tested dose-dependent differences in *L. donovani* development in its natural vectors and the number of parasites required for a successful infection.

One would expect that two *L. donovani* vectors do not differ significantly each other in main aspects of bloodmeal digestion and development of the PM. However, we found the opposite; there is striking difference in all parameters studied and bloodmeal digestion of *P. orientalis* is, in some aspects, more similar to *P. papatasi* than to *P. argentipes*.

Methods

Sand fly maintenance

Colonies of *Phlebotomus argentipes*, *P. orientalis*, *P. papatasi* and *Sergentomyia schwetzi* were maintained under standard conditions as previously described [25]. Three to seven day old females were used in all experiments and were maintained at 26°C on 50% sucrose.

Haemoglobin assay for measuring the bloodmeal volume

During bloodfeeding, sand fly females expel a significant volume of water by prediuresis [26]; consequently, the classical method of weighing bloodfed females leads to an underestimation of the bloodmeal volume. Therefore, the colorimetric method by Briegel *et al.* [27], developed for measuring the haemoglobin concentration in bloodfed mosquitoes, was adopted to study the bloodmeal volume ingested by sand fly females. All studied sand fly species were fed on anesthetized BALB/c mice (Experiment A) or on heat-inactivated rabbit blood offered by membrane feeding (using a chick-skin membrane; Experiment B). Individual guts of fully bloodfed females were dissected 1h after bloodfeeding, transferred to tubes containing 200 μl 0.15 mM NaCl and homogenized. Twenty dissected guts of each sand fly species were used in two independent repetitions of each experiment. Volumes of 50 μl of the gut homogenates or diluted blood (5 μl fresh mouse blood or heat-inactivated rabbit blood in 1000 μl 0.15 mM NaCl) were mixed with 200 μl of Drabkin's reagent (Sigma) in the dark for 30 min. Absorbance was measured in 96-well plate in doublets at 540 nm. Human haemoglobin (Sigma) at concentrations from 3.1 to 100μg/well was used as a standard. Data from two independent repetitions were pooled and statistical evaluations were performed by the ANOVA and Post-Hoc Tukey HSD test using STATISTICA 12 software.

Sand fly defecation

Females of *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi* were fed on anesthetized BALB/c mice (Experiment A) or through a chick-skin membrane on heat-inactivated rabbit blood (Experiment B), and a previously described method [28] was used to compare defecation times of the sand fly females. Briefly, fully bloodfed females were individually placed in small glass vials and checked twice a day under a binocular microscope for defecation.

Timing of the formation and degradation of the PM

Bloodfed females of *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi* were dissected at ten intervals after feeding on anesthetized BALB/c mice, starting immediately PBM and at each of the following times: 1, 3, 6, 12, 24, 48, 72, 96, and 120h PBM, and checked for the formation, breakdown and degradation of the PM. Dissections were carried out in isotonic saline solution with brief washing of the gut in distilled water in order to better separate the PM [29]. For each studied sand fly species and time interval, at least 20 females were analyzed. Slides were observed under an Olympus BX51 microscope with Nomarski contrast and photographed with an Olympus D70 camera and software. The PM was classified as i) intact, when no traces of blood escape were observed, ii) broken, when blood started to escape to the ectoperitrophic space and iii) degraded, when it was disintegrated and defecated.

Fluorometric assay for quantification of proteolytic activities

Midgut trypsin and chymotrypsin activities were measured in *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi* after feeding on anesthetized BALB/c mice (Experiment A) or heat-inactivated rabbit blood (Experiment B) via a chick-skin membrane. Midguts of fully bloodfed females were dissected at 12, 24, 36, 48, 72, 80, and 96 hours PBM and transferred to 1.5 ml Eppendorf tubes. In total, 2240 midguts were dissected and used for quantification of trypsin and chymotrypsin activities; for practical reasons, the activities were measured in pools of 10 midguts in 100 μl of Tris-NaCl (0.1 M Tris, 150 mM NaCl, pH = 8.44). The samples were homogenized, and trypsin and chymotrypsin activities were measured in a black 96-well plate by a fluorometric assay with the substrates Boc-Leu-Gly-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC (40 μM; Bachem), respectively. Aminomethylcoumarin (AMC) was excited at 355 nm and the fluorescence of released AMC was measured at 460 nm by a fluorometer (Tecane infinite M200). Each point in the graphs represents a measurement of four pools (two from each experiment). The proteolytic activities are presented as rfu/μg of protein of mouse or rabbit blood. The concentration of substrate was optimised in the series of previous experiments, 40 μM concentration was found optimal, providing stable results. Each experiment was repeated twice. Total proteolytic activities were calculated as the area under the time curve.

Leishmania parasites

Leishmania donovani strain GR374 (MHOM/ET/2010/DM-1033) was maintained at 23°C on Medium 199 (Sigma) supplemented with 10% foetal calf serum (Gibco), 1% BME vitamins (Sigma), 2% human urine and amikin (250 μg/ml).

Experimental infections of *P. argentipes*: the effect of initial infective dose

The effect of different initial infective doses on *L. donovani* development was previously studied by our group in *P. orientalis* [30]. Here we tested the effect of initial infective doses on infection rates and total parasite numbers in *P. argentipes*, another major vector of *L. donovani*. In addition to the parasite dose generally used for sand fly infections (5×10^5), two lower infective doses were tested. Females of *P. argentipes* were fed for one hour through a chick-skin membrane on heat-inactivated rabbit blood containing 2×10^3, 2×10^4 or 5×10^5 promastigotes per ml and fully bloodfed females were separated. On days 2 and 6 post-infection (PI) the females were dissected and the individual guts were checked microscopically for the presence and localization of *Leishmania* promastigotes. Parasite loads were graded according to Myskova *et al.* [31] as light (< 100 parasites/gut), moderate (100 – 1000 parasites/gut), or heavy (> 1000 parasites/gut). Data were evaluated statistically by means of the Chi-square test using STATISTICA 12 software.

Experimental infections of *P. orientalis* and *P. argentipes*: comparison of susceptibility to *L. donovani*

Females of *P. argentipes* and *P. orientalis* of the same age were infected simultaneously using the same parasite culture at a dose of 2×10^3 promastigotes/ml. Females were dissected on days 6 and 8 PI and checked microscopically. Special attention was paid to colonization of the stomodeal valve as the precondition for successful transmission to a mammalian host (for review see [5]). Parasite loads and statistical evaluations were graded according to Myskova *et al.* [31] and as described above.

Real-time PCR for quantification of *Leishmania* in sand flies

On day 8 PI the numbers of *Leishmania* parasites in individual females were counted using Q-PCR as described previously [31,32]. Briefly, experimental females were stored at −20°C and total DNA extraction was performed with a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. Kinetoplast DNA was chosen as the molecular target with previously described specific primers (forward: 5´-CTTTTCTGGTCCTCCGGGT AGG-3´; reverse: 5´-CCACCCGGCCCTATTTTACACCAA-3) [32]. Q-PCR was performed by the SYBR Green detection method (iQSYBER Green Supermix, Bio-Rad, Hercules, CA) in Bio-Rad iCycler & iQ Real-Time PCR systems. Statistical evaluation was performed by the Kruskal-Wallis test using STATISTICA 12 software.

Ethical statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant EU guidelines for experimental animals. All experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under the Certificate of Competency (Registration Number: CZ 00177).

Results

Bloodmeal volume

The interspecific differences in bloodmeal volume taken during feeding for the four sand fly species were highly significant ($F_{(3,27)} = 11.62$, $P < 0.0001$; Fig 1). Species refractory to *Leishmania donovani* (*P. papatasi* and *S. schwetzi*) took more blood than *L. donovani* vectors (*P. argentipes* and *P. orientalis*; $F_{(1,29)} = 22.52$, $P < 0.0001$). In three species the volume of ingested blood was significantly conditioned by the mode of feeding ($F_{(1,20)} = 10.64$, $P < 0.005$); on average, females of *P. argentipes*, *P. papatasi* and *S. schwetzi* ingested a larger volume of blood when feeding on anaesthetized mice (0.73 μl, 0.90 μl and 0.91 μl, respectively) than on rabbit blood via the chick-skin membrane (0.63 μl, 0.66 μl and 0.82 μl, respectively). In contrast, *P. orientalis* females took a similar volume of blood during membrane feeding and feeding on mice (0.59 μl vs. 0.53 μl; $F_{(1,6)} = 1.58$, $P = 0.26$). The Tukey's Multiple Comparison Test table is provided as "Supporting information" (S1 Table). The bloodmeal volume does not necessarily correlate with body size of sand flies. For example *S. schwetzi* is smaller than *P. orientalis* but takes a bigger volume of bloodmeal.

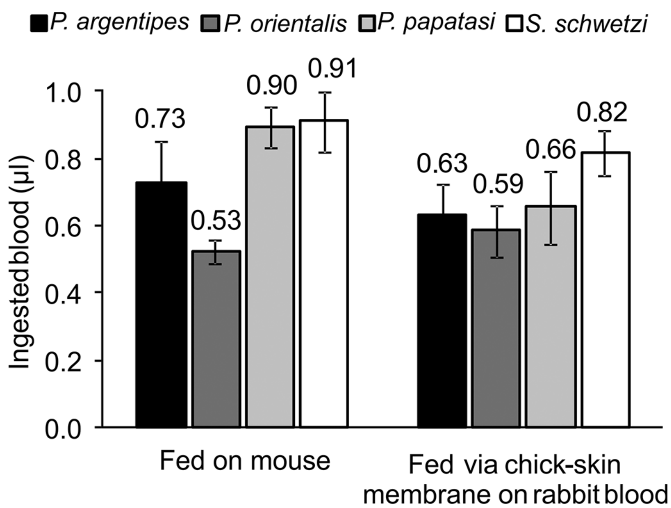

Fig 1. Bloodmeal volumes taken by *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi*. Females were fed on anesthetized BALB/c mice or through a chick-skin membrane on heat-inactivated rabbit blood and dissected 1 hour PBM. Bloodmeal volume was measured by a haemoglobin assay. Data from two independent repetitions were pooled; the bars indicate an average of forty dissected guts. The interspecific differences in bloodmeal volume taken by four sand fly species were highly significant ($F_{(3,27)} = 11.62$, $P < 0.0001$)

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Sand fly defecation

The fastest defecation was found in *P. argentipes*; all females defecated during an interval from 32 to 72 hours PBM. Conversely, the longest defecation time was found in *P. orientalis*; females started to defecate at the beginning of the fourth day PBM and 100% of females defecated by day five or six PBM. *Phlebotomus papatasi* and *S. schwetzi* females defecated from the end of the third till the fifth and sixth day PBM, respectively. In all sand fly species, the time courses of defecation did not differ between groups of females fed on mouse and those fed on rabbit blood through the chick-skin membrane (Fig 2).

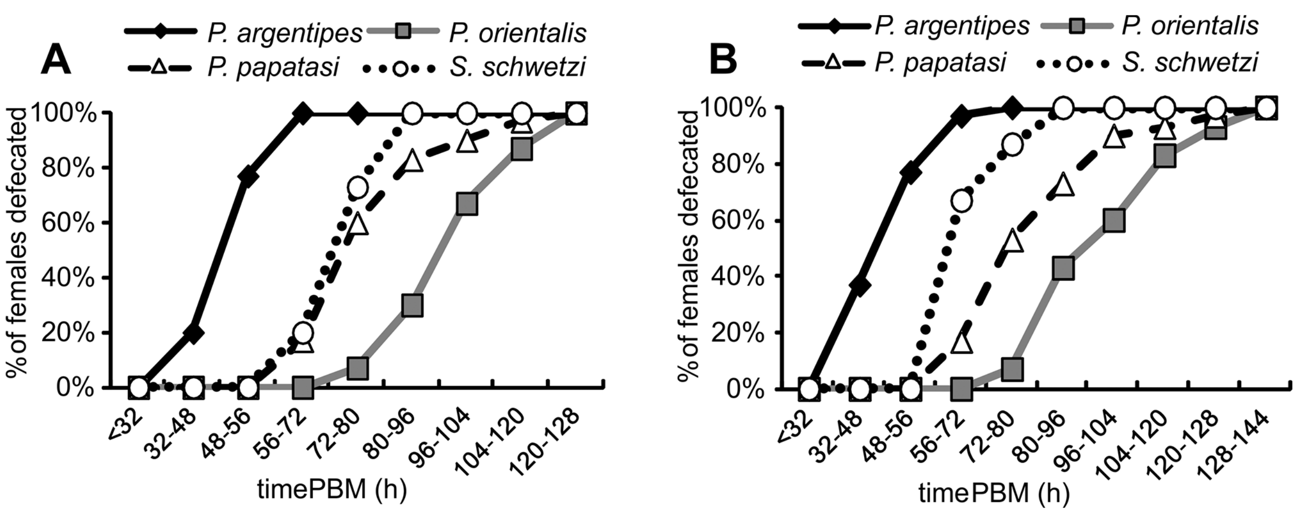

Fig 2. Defecation times of *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi*. Females were fed either on anesthetized BALB/c mice (**2A**) or on heat-inactivated rabbit blood via a chick-skin membrane (**2B**), individually placed in small glass vials and checked twice a day for defecation.

doi:10.1371/journal.pone.0128203.g002

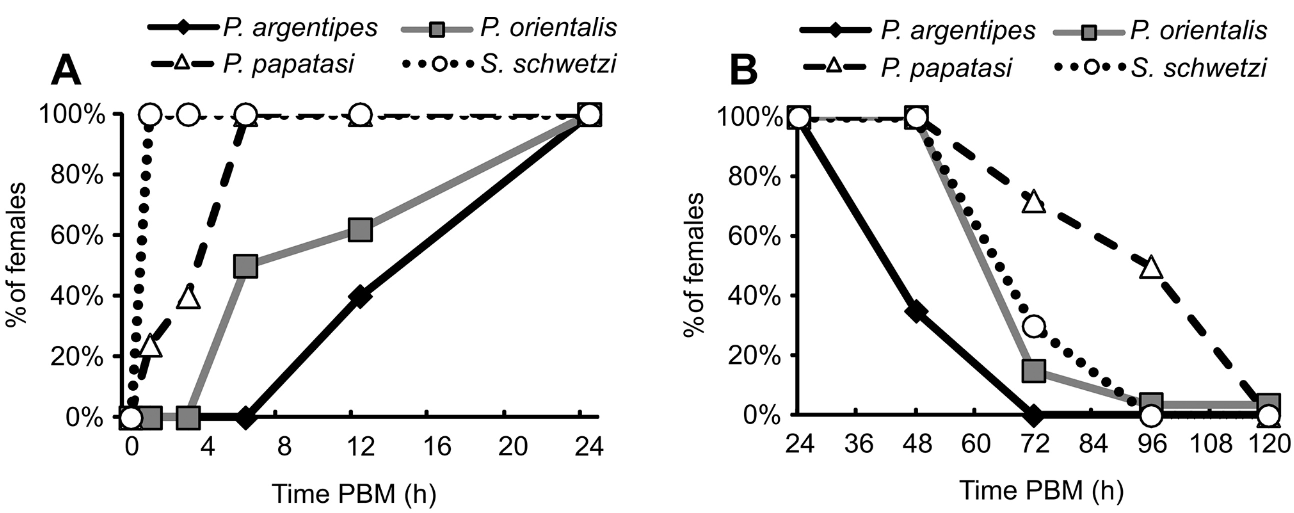

Fig 3. Timing of the peritrophic matrix (PM) formation and degradation in *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi*. Bloodfed females were dissected at 1, 3, 6, 12, 24, 48, 72, 96, and 120 hours PBM and checked for the formation (**3A**) and degradation (**3B**) of the PM.

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Timing of the formation and degradation of the PM

Formation of the PM was extremely rapid in *S. schwetzi*, where all females developed the first thin PM within the first one hour PBM (Figs 3A and 4). Rapid formation was also detected in *P. papatasi*, where the first traces of the PM were present in 24% of females by 1h PBM (Fig 4) and a fully formed PM was present in 100% of females by 6h PBM. In contrast, formation of the PM was slower in *L. donovani* vectors; the first thin PM appeared by 6 and 12h PBM in *P. orientalis* and *P. argentipes*, respectively (Fig 4), and a fully formed PM was present in all females of both species by 24h PBM (Fig 3A).

Degradation of the PM was most rapid in *P. argentipes*, where only 35% of females possessed the PM by 48h PBM (Fig 4) and the PM was completely absent in all females by 72 hrs PBM (Fig 3B). In all three other species degradation of the PM started between the second and third day PBM. In *P. orientalis* and *S. schwetzi* the PM was present in 14% and 30% of females by day three PBM, respectively, and only in a few specimens in later time intervals (Fig 4). In *P. papatasi* the PM was present in 50% of females by day four PBM and it was not present in any females by day five PBM (Figs 3B and 4).

The period between the breakdown of the PM and defecation of bloodmeal remnants, i.e., the period when *Leishmania* promastigotes attach to the midgut epithelium in order to prevent defecation, strikingly differed among the four sand fly species (Fig 5). This time lasted on average 21h, 48h and 38h in *P. argentipes*, *P. orientalis* and *P. papatasi*, respectively, while in *S. schwetzi* it was significantly shorter (3h on average).

Proteolytic activities in sand fly midguts

Phlebotomus argentipes and *P. orientalis* highly differed in the time course of trypsin and chymotrypsin activities. *Phlebotomus argentipes* females digested faster, with values of both enzymatic activities peaking about 24 – 36 hours PBM, and dropping to almost zero by 72 hours PBM. On the contrary, the highest values of proteolytic activities in *P. orientalis* females were observed between 48 and 72 hours PBM (Figs 6 and 7). In both *L. donovani* vectors, trypsin activity was significantly lower than in *S. schwetzi*. Maximum chymotrypsin activity in *P. orientalis* was very low compared to the other three species. In contrast, a very high peak of

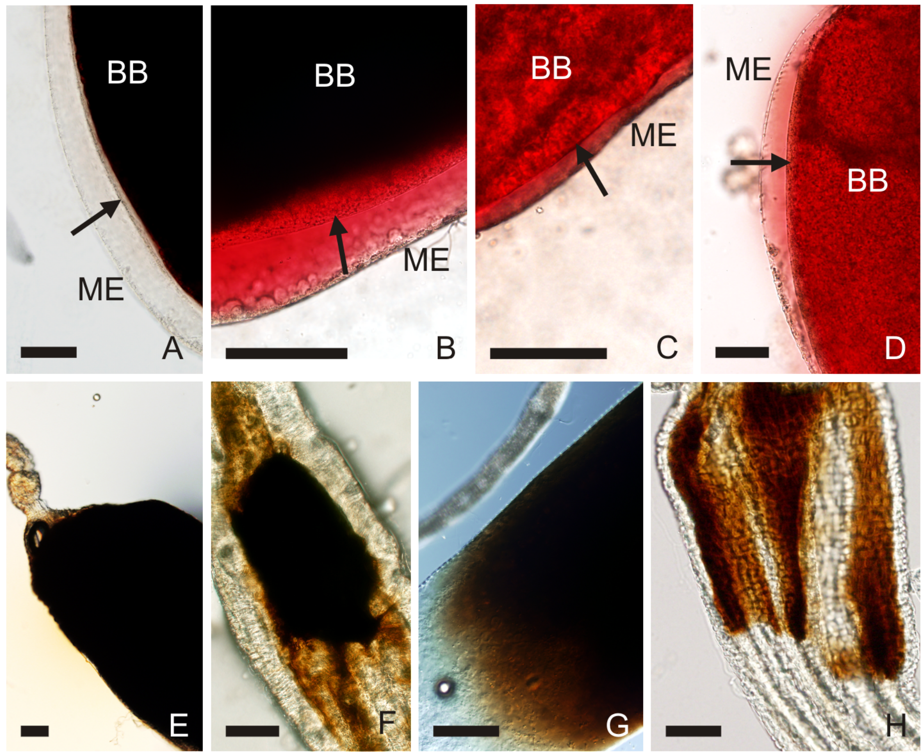

Fig 4. Formation (A-D) and degradation (E-H) of the peritrophic matrix (PM) in various sand fly species. The thin PM separating the blood bolus (BB) from the midgut epithelium (ME) is indicated by the arrow. It appears first by 1h PBM in *S. schwetzi* (4A) and *P. papatasi* (4B), 6 h PBM in *P. orientalis* (4C) and 12h PBM in *P. argentipes* (4D). At the end of digestion, the bloodmeal remnants are still enclosed by intact PM in *S. schwetzi* on day 3 PBM (4E). In contrast, in the other species studied bloodmeal remnants leak to abdominal midgut through broken PM, in *P. papatasi* typically by day 4 PBM (4F), in *P. orientalis* by day 3 PBM (4G) and in *P. argentipes* by day 2 PBM (4H). Scale bar = 50 μm.

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chymotrypsin activity was found in *P. argentipes* 36 hours PBM (Fig 7). In all sand fly species, the time courses of trypsin and chymotrypsin activities were analogous in females fed on mice and on rabbit blood through the chick-skin membrane (Figs 6 and 7).

The effect of initial infective dose on *L. donovani* development in *P. argentipes*

The effect of three initial infective doses on infection rates and total parasite numbers was tested in *P. argentipes* infected by *L. donovani* (strain GR374). Different parasite numbers taken by females affected their subsequent infection rates. In groups with lower initial infective doses (2×10^3 and 2×10^4 promastigotes/ml), the rate of late-stage infections was about 50 and 65% females, respectively, while in the group infected with 5×10^5 promastigotes/ml the infection rate reached 90% (Fig 8A). Different initial infective doses also influenced total parasite numbers in the sand fly midgut. The Q-PCR showed significant differences ($KW\text{-}H_{(1;35)} = 8.3$; $P < 0.01$) in parasite loads at late-stage infections (day 8 PI) between the groups of females with low (2×10^3 and 2×10^4 promastigotes/ml) and high (5×10^5 promastigotes/ml) infective doses. Nevertheless, the majority of positive females infected with low infective doses posed very heavy infections with more than 10 000 parasites/gut (Fig 8B).

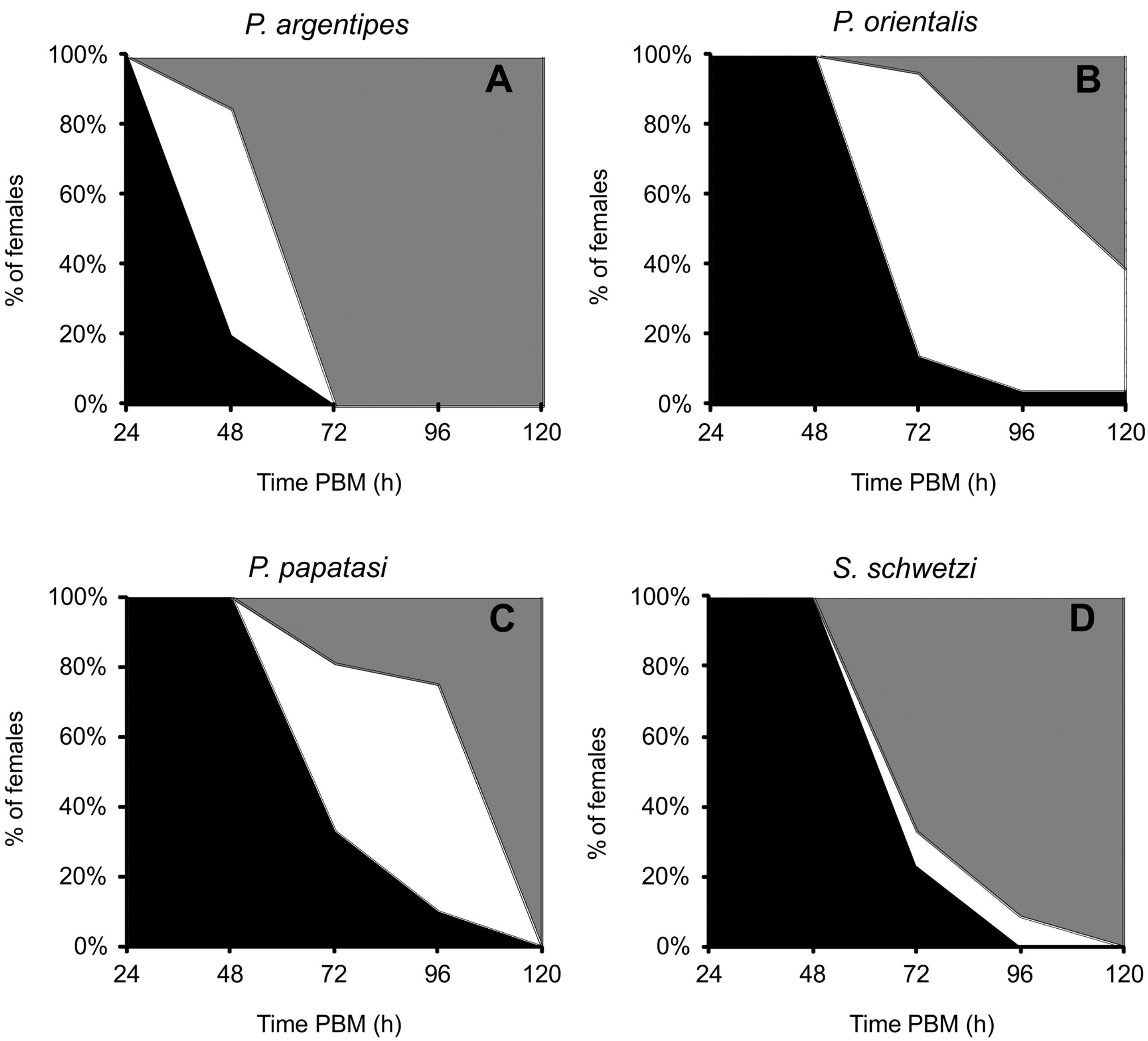

Fig 5. Timing of the peritrophic matrix (PM) breakdown and defecation of bloodmeal remnants in *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi*. Bloodfed females were dissected at 24, 48, 72, 96, and 120 hours PBM and checked for the localization of the bloodmeal and formation of the PM. **Black area:** females with bloodmeal enclosed inside the PM, **white area:** females with the PM broken or fully degraded where the bloodmeal is in contact with midgut epithelium, **grey area:** females after defecation of bloodmeal remnants. **5A:** *P. argentipes*, **5B:** *P. orientalis*, **5C:** *P. papatasi*, **5D:** *S. schwetzi*.

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Comparison of the susceptibility of *P. orientalis* and *P. argentipes* to *L. donovani* infection

Development of *L. donovani* infections initiated with 2×10^3 promastigotes/ml was compared in *P. argentipes* and *P. orientalis*. Parasites developed similarly in both groups of females: at the late stage of infection (day 6 – 8 PI) infection rates were about 44 and 35% in *P. argentipes* and *P. orientalis*, respectively, and a majority of infected females had high parasite loads (Fig 9A). Similarly, Q-PCR revealed no significant differences ($KW\text{-}H_{(1;10)} = 0.05$; $P = 0.83$) in total parasite numbers in sand fly midguts on day 8 PI (Fig 9B).

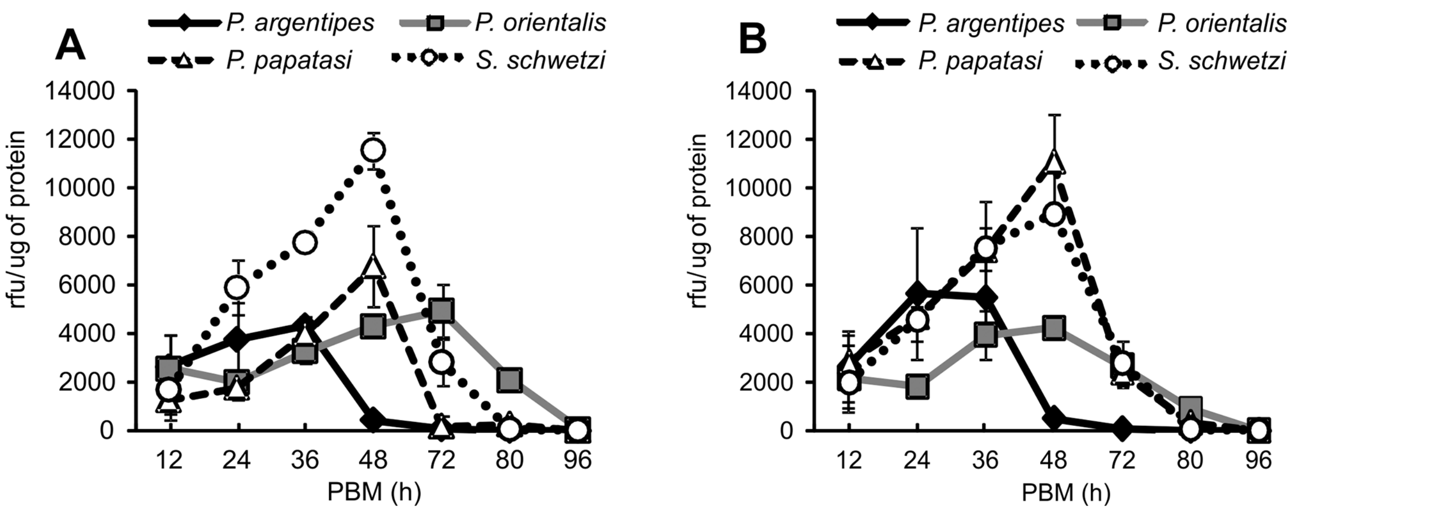

Fig 6. Trypsin activity in *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi* midguts. Trypsin activity was measured at 12, 24, 36, 48, 72, 80, and 96 hours PBM in midgut homogenates (c = 0.005 gut/ml) of bloodfed females using fluorogenic substrate Boc-Leu-Gly-Arg-AMC (40 μM). Data from two independent experiments were pooled. **6A:** Females fed on anesthetized BALB/c mice. **6B:** Females fed through a chick-skin membrane on heat-inactivated rabbit blood.

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The location of parasites during the late-stage infection was also similar in both groups of infected females, and colonization of the thoracic midgut and stomodeal valve was observed since day 6 PI. On day 8 PI, the stomodeal valve was colonized in about 80 and 70% of infected *P. argentipes* and *P. orientalis* females, respectively.

Discussion

Experiments on two *L. donovani* vectors showed that *P. argentipes* and *P. orientalis* are equally susceptible to *L. donovani* parasites; both species infected with the very low dose (2×10^3

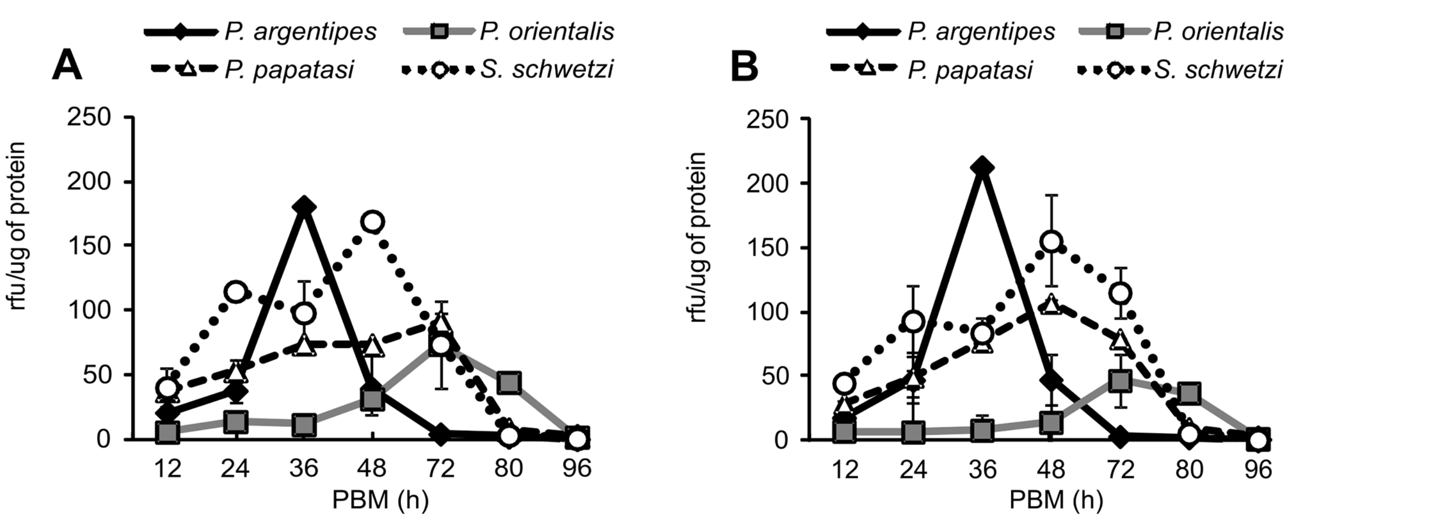

Fig 7. Chymotrypsin activity in *P. argentipes*, *P. orientalis*, *P. papatasi* and *S. schwetzi* midguts. Chymotrypsin activity was measured at 12, 24, 36, 48, 72, 80 and 96 hours PBM in midgut homogenates (c = 0.5 gut/ml) of bloodfed females using fluorogenic substrate Suc-Ala-Ala-Pro-Phe-AMC (40 μM). Data from two independent experiments were pooled. **7A:** Females fed on anesthetized BALB/c mice. **7B:** Females fed through a chick-skin membrane on heat-inactivated rabbit blood.

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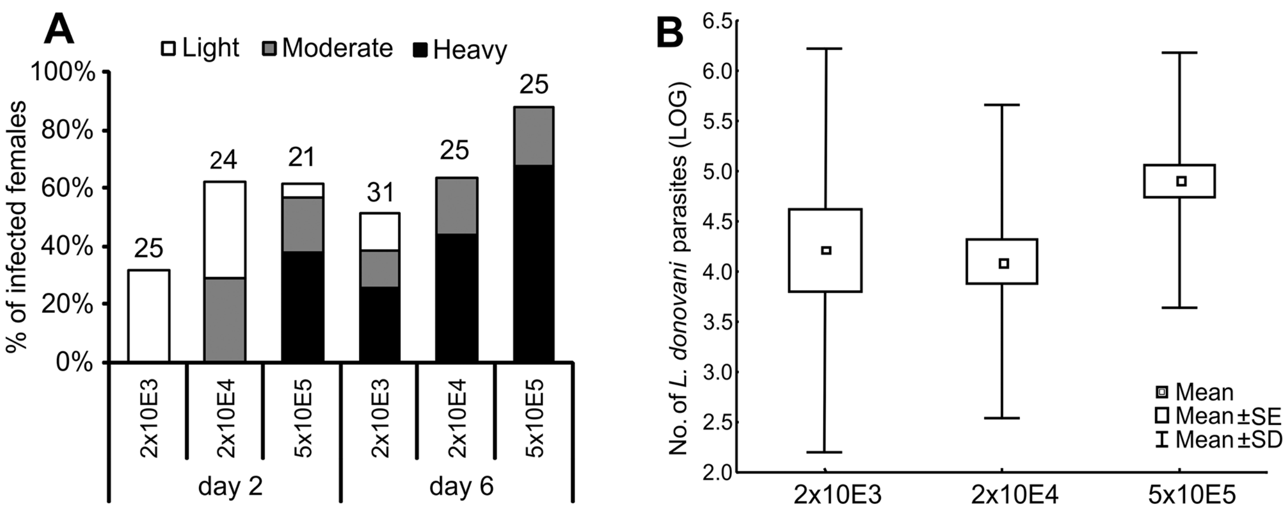

Fig 8. The effect of initial infective dose on the development of *L. donovani* in *P. argentipes*. Females of *P. argentipes* were infected by feeding on a suspension of $2{\times}10^3$, $2{\times}10^4$ or $5{\times}10^5$promastigotes/ml of blood and kept at 26°C. **8A**: Females were examined microscopically 2 and 6 days post infection. Intensities of infection were classified into three categories according to their intensity: light (< 100 parasites/gut), moderate (100 – 1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females. **8B:** The number of parasites was measured individually by Q-PCR in 20 females of each group on day 8 PBM.

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promastigotes/ml) developed fully comparable infections. Light microscopy and Q-PCR did not reveal any significant differences in the development of *L. donovani* between these two vectors.

These results are supported by experiments focused on the effect of initial infective *L. donovani* doses on total parasite numbers in late-stage infections. Our previous study [30] revealed that infection rates of *P. orientalis* females infected with $2{\times}10^3$ promastigotes/ml were 45%, while rates in three groups infected with $2{\times}10^4$, $1{\times}10^5$ or $5{\times}10^5$ promastigotes/ml reached 75–95%; however, mature infections and colonization of the stomodeal valve were observed in all

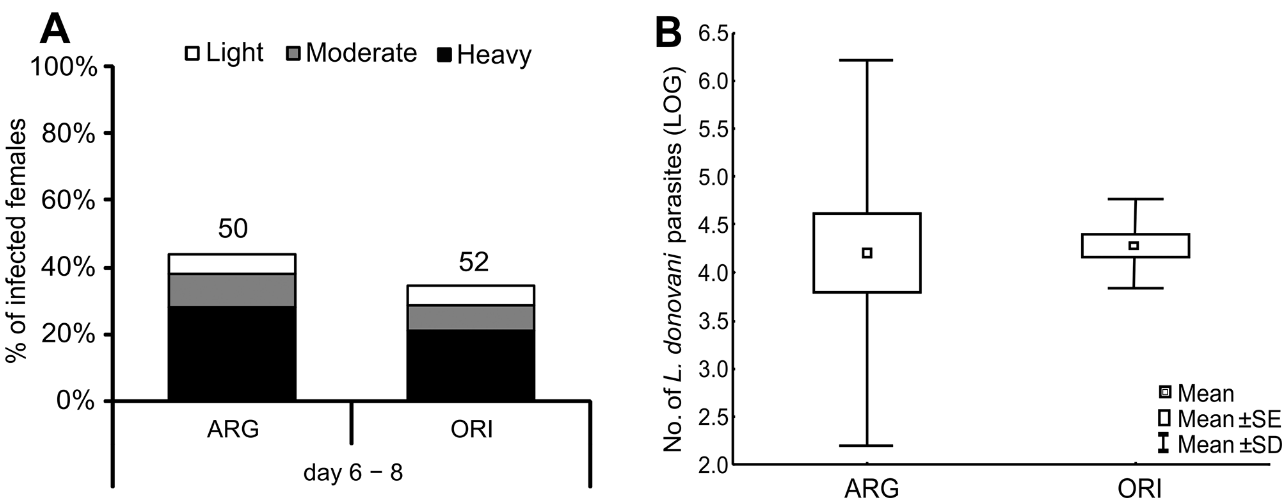

Fig 9. Comparison of the susceptibility of *P. argentipes* and *P. orientalis* to *L. donovani* Females of *P. argentipes* (ARG) and *P. orientalis* (ORI) were infected by feeding on a suspension of $2{\times}10^3$ promastigotes/ml of blood and kept at 26°C. **9A**: Females were examined microscopically on days 6 and 8 PBM. Intensities of infection were classified into three categories according to their intensity: light (< 100 parasites/gut), moderate (100 – 1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females. **9B:** The number of parasites from 20 females of each group was measured individually by Q-PCR 8 days post infection.

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groups, including the one with the lowest dose [30]. In the present study, *L. donovani* promastigotes developed similarly in *P. argentipes*. Infection rates for groups of *P. argentipes* females with initial infected dose of 2×10^3, 2×10^4 and 5×10^5 promastigotes/ml infections were about 50, 65 and 90%, respectively, and even the lowest initial dose resulted in very heavy late-stage infections.

Taking into account the average bloodmeal volume taken by *P. argentipes* and *P. orientalis* (0.63 μl and 0.59 μl, respectively), this initial dose corresponds to only one or two promastigotes ingested by females during bloodfeeding. This means that in both *L. donovani* vectors, even 1 or 2 parasites represent a sufficient dose for the initiation of mature infections with colonization of the stomodeal valve in about one half of females.

Despite the same susceptibility to *L. donovani*, the two natural vectors of this parasite showed several striking differences in bloodmeal digestion and secretion of the PM and digestive enzymes. Rapid defecation of bloodmeal remnants was observed in *P. argentipes*, where all females were defecated by day three PBM, while females of *P. orientalis* defecated until the fourth to sixth day PBM. This means that *L. donovani* promastigotes in *P. argentipes* had a shorter time to develop forms capable of attaching to the midgut epithelium. Wilson *et al.* [33] showed that in *Leishmania*, including the *L. donovani* complex, binding to the sand fly midgut is strictly stage-dependent and is a property of those forms found in the middle phase of development, long nectomonads and short nectomonads (leptomonads), but is absent in the earlier procyclic promastigote stage. This suggests that in *P. argentipes*, procyclic *Leishmania* parasites must transform to nectomonads much faster in than in *P. orientalis*.

The two principal *L. donovani* vectors also differ in the time course of trypsin and chymotrypsin activities. The proteolytic activities in *P. argentipes* peaked around 24 – 36 hours PBM, while in midguts of *P. orientalis* the highest proteolytic activities were found between 48 and 72 hours PBM. Peaks of trypsin activity in both refractory species *P. papatasi* and *S. schwetzi* were higher than in either of the *L. donovani* vectors. This finding supports previous hypotheses about trypsin as a molecule affecting *Leishmania* development in sand flies [7–9,13,14,34]. On the other hand, the peak of chymotrypsin activity in *P. argentipes* was much higher than in *P. orientalis*, which suggests that these differences in chymotrypsin activity do not play such a significant role in the vector competence of sand flies to *L. donovani*.

The peritrophic matrix plays several important roles during bloodmeal digestion and may also act as a barrier preventing the release of *Leishmania* from the bloodmeal to the ectoperitrophic space (reviewed by [4,20]) where they attach to the midgut epithelium during defecation [21,22,33]. *Leishmania* parasites are not able to traverse the PM before its rough disintegration which is indicated by escape of bloodmeal remnants into the ectoperitrophic space.[19] Although there were significant differences in the PM kinetics among the four sand fly species tested, the PM was present in all sand fly species during the peak of proteolytic activities and its degradation started several hours later. Interestingly, both proven vectors of *L. donovani* strikingly differed in the formation and degradation of the PM. In *P. argentipes* the PM was present only for a short time, firstly forming at 12 hours PBM and degrading between 24 and 48 hours PBM, while in *P. orientalis* the PM appeared by 6 hours PBM and degraded between 48 and 72 hours PBM.

Formation of the PM was more rapid in *P. papatasi* and *S. schwetzi* than in both *L. donovani* vectors. In all females the PM was present by 3 and 6 hours PBM in *S. schwetzi* and *P. papatasi*, respectively, while this process lasted 24 hours PBM in *P. orientalis* and *P. argentipes*. According to Pimenta *et al.* [11] the PM can protect parasites against the rapid diffusion of digestive enzymes during the early phase of infections. The addition of exogenous chitinase to the bloodmeal stopped formation of the PM in *P. papatasi*, which resulted in the suppression of *L. major* infections for 4 hours PBM [11]. However, our results show that natural *L. donovani* vectors form the PM later, i.e. several hours after this critical period (see above). Our data confirmed that speed of the PM formation is species-specific.

Sadlova *et al.* [24] concluded that the crucial parameter for the early-stages development of *Leishmania* in the midgut of *S. schwetzi* is the duration of the period between the degradation of the PM and defecation [24]. Using amastigotes-initiated infections, Sadlova and Volf [19] demonstrated that breakdown of the PM in *P. duboscqi* coincides with the transformation of *L. major* procyclic promastigotes to long nectomonads [19], which are able to attach to the midgut epithelium to avoid defecation with bloodmeal remnants. This transformation seems to be associated with the diffusion of signal molecules from the ectoperitrophic space to the parasite surroundings through a broken PM (reviewed by [3]). In the present study, there was a very short time period between the breakdown of the PM and defecation in *S. schwetzi* (or the intact PM was present until defecation). Previously, using promastigote-initiated infections, Sadlova *et al.* [24] described the delayed transformation of *L. donovani* promastigotes in *S. schwetzi* in comparison with the permissive vector *Lutzomyia longipalpis* on day 2 PBM. Parasites remained in the procyclic promastigote stage because of an intact PM and were defecated [24]. On the other hand, here we found that despite a quite early defecation, in *P. argentipes* there is about a 24 hour period on average between the breakdown of the PM and defecation. This "window" clearly provides enough time for *L. donovani* promastigotes to bind to the midgut epithelium. In *P. orientalis* and *P. papatasi* this period is longer (48 and 38 hours, respectively); however, *P. papatasi* is refractory to *L. donovani* due to the lack of a surface ligand for parasite LPG (reviewed by [9,10,23]).

Conclusions

Differences found in most parameters of bloodmeal digestion of various sand fly species appear not relate to the known differences in vectorial competence. In spite of the fact that *P. argentipes* and *P. orientalis* are both natural vectors of *L. donovani*, they have completely different time courses of bloodmeal digestion. Females of *P. argentipes* possess fast bloodmeal digestion with a very high peak of chymotrypsin activity, rapid degradation of the PM and defecation finishing already on day three PBM, while *P. orientalis* females digest considerably slower, have low peaks of proteolytic activities and defecate around day five PBM. However, our results indicate that both vectors are similarly susceptible to experimental infection with *L. donovani* (GR374) and even one or two *Leishmania* parasites are sufficient for the establishment of mature late-stage infections in *P. argentipes* and *P. orientalis*.

Supporting Information

S1 Table. Tukey´s Multiple Comparison Test table for bloodmeal volume.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KP JS PV. Performed the experiments: KP JS VS MH. Analyzed the data: KP JS VS JV PV. Contributed reagents/materials/analysis tools: KP JS VS JV PV. Wrote the paper: KP JS PV.

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PLOS | NEGLECTED TROPICAL DISEASES

Phlebotomus orientalis Sand Flies from Two Geographically Distant Ethiopian Localities: Biology, Genetic Analyses and Susceptibility to *Leishmania donovani*

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Abstract

Background: *Phlebotomus orientalis* Parrot (Diptera: Psychodidae) is the main vector of visceral leishmaniasis (VL) caused by *Leishmania donovani* in East Africa. Here we report on life cycle parameters and susceptibility to *L. donovani* of two *P. orientalis* colonies originating from different sites in Ethiopia: a non-endemic site in the lowlands - Melka Werer (MW), and an endemic focus of human VL in the highlands - Addis Zemen (AZ).

Methodology/Principal Findings: Marked differences in life-cycle parameters between the two colonies included distinct requirements for larval food and humidity during pupation. However, analyses using Random Amplified Polymorphic DNA (RAPD) PCR and DNA sequencing of cytB and COI mitochondrial genes did not reveal any genetic differences. F1 hybrids developed successfully with higher fecundity than the parental colonies. Susceptibility of *P. orientalis* to *L. donovani* was studied by experimental infections. Even the lowest infective dose tested (2×10^3 per ml) was sufficient for successful establishment of *L. donovani* infections in about 50% of the *P. orientalis* females. Using higher infective doses, the infection rates were around 90% for both colonies. *Leishmania* development in *P. orientalis* was fast, the presence of metacyclic promastigotes in the thoracic midgut and the colonization of the stomodeal valve by haptomonads were recorded in most *P. orientalis* females by day five post-blood feeding.

Conclusions: Both MW and AZ colonies of *P. orientalis* were highly susceptible to Ethiopian *L. donovani* strains. As the average volume of blood-meals taken by *P. orientalis* females are about 0.7 µl, the infective dose at the lowest concentration was one or two *L. donovani* promastigotes per sand fly blood-meal. The development of *L. donovani* was similar in both *P. orientalis* colonies; hence, the absence of visceral leishmaniasis in non-endemic area Melka Werer cannot be attributed to different susceptibility of local *P. orientalis* populations to *L. donovani*.

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Introduction

Visceral leishmaniasis (VL, kala-azar) caused by the protozoan parasite *Leishmania donovani* is a deadly disease occurring mainly in the Indian subcontinent and Africa. In Africa, VL is endemic in the eastern part of the continent; the Horn of Africa and adjacent countries, namely Sudan, South Sudan, Kenya, Somalia, Uganda, Erithrea and Ethiopia. In Ethiopia, the main endemic areas are located in the lowlands of the southwestern Ethiopia (e.g. Omo river plains and Segen/Woito valleys) and Metema-Humera plains in the northwest [1]. Three sand fly species, *Phlebotomus orientalis, P. celiae* and *P. martini* have been implicated as vectors [2,3]. *Phlebotomus celiae* Minter and *Phlebotomus martini* Parrot (both belonging to the subgenus *Synphlebotomus*) are limited to the south of the country, often being associated with termite hills, which provide suitable breeding sites. In the rest of Ethiopia, however, *P. (Larroussius) orientalis* seems to be the only vector.

Most biological information regarding habitat, seasonality and feeding preferences of *P. orientalis* was acquired thanks to demanding field studies in Sudan [4,5,6].The distribution of this species seems to be affected by the vegetation type, with preference for Acacia – Balanites forests and cracks of black cotton clay soil

Author Summary

Phlebotomus orientalis is the main vector of *Leishmania donovani* in East Africa and is, therefore, a sand fly species of high importance. We studied various properties of *P. orientalis* populations from both endemic (Addis Zemen) and non-endemic (Melka Werer) areas in Ethiopia. We successfully demonstrated the ability of laboratory colonies arising from these populations to crossbreed by obtaining 1st and 2nd generation hybrid progeny. Hybrids had similar or even higher fecundity than parental colonies. Comparison of the populations by sequencing of two genes (cytB and COI) and by RAPD (a multilocus method) revealed no genetic differences. We demonstrated that both populations are highly susceptible to experimental infection with *L. donovani* and even small numbers of parasites are able to initiate heavy infections in *P. orientalis* females. As the development pattern of *L. donovani* was similar for females from both colonies, we deduce that the absence of visceral leishmaniasis in the non-endemic area of Melka Werer cannot be attributed to different susceptibility of local *P. orientalis* populations to *L. donovani*.

[7,8,9]. Additional important information, like actual breeding sites of this species, remains unknown. Despite several attempts of colonization of this species [10,11] the life cycle and behaviour of *P. orientalis* in laboratory colonies has not been reported in detail and *P. orientalis* has a reputation of being difficult to colonize and maintain.

In this study, we focused on *P. orientalis* from two geographically distant Ethiopian localities, Addis Zemen (AZ) and Melka Werer (MW). Addis Zemen is located in the highlands of the Amhara Region in northwestern Ethiopia at altitude of 1800–2000 m where in 2005 and 2008, outbreaks of VL resulted in 2,500 cases and initially a very high mortality [12]. On the other hand, Melka Werer is a non-endemic area situated in Awash National game reserve in Rift Valley at an altitude of approximately 800 m, 200 km East of Addis Ababa.

Here, we compare individuals of both colonies by Random Amplification of Polymorphic DNA (RAPD) and sequencing analysis. The two populations were also tested for ability to produce viable hybrids in cross-mating studies. Different biological aspects of the two colonies found during the study allowed us to optimize the conditions for laboratory maintenance of both *P. orientalis* colonies, which appeared to be a fundamental prerequisite for the major goal of this work: experimental infections and comparison of susceptibility of both colonies to infections with *L. donovani*.

Materials and Methods

Ethical statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments (including sand fly feeding) were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under the Certificate of Competency (Registration Number: CZU 327/99, CZ 00179). All samples were anonymized.

Rearing sand fly colonies and life-cycle analysis

Both of *P. orientalis* colonies Addis Zemen (AZ) and Melka Werer (MW) were established in 2008 and reared for about ten generations at the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia. For larval food, dried and ground hyrax faeces were used, females were fed on rabbits. Both the larvae and the adults were kept at 26°C. After transfer to Prague the sand flies were adapted to the conditions and the larval food routinely used in our laboratory [13]. Briefly, the larvae of both colonies were fed on a composted mixture of rabbit faeces and rabbit pellets. The suitability of autoclaved and non-autoclaved larval food was tested and compared. Adult sand flies were maintained on 50% sugar solution at 26–27°C. In the first generation after arrival to Prague, females were offered a blood-meal on rabbit or human arm (co-author PV served as volunteer), and within several generations they were adapted to feeding on anesthetized mice. The life-cycle details (length of egg development, each larval instar etc.) were collected from 12168 (AZ) and 8751 (MW) ovipositing females and recorded for over 20 months. Data monitoring the effect of nutrition on the life cycle of two *P. orientalis* colonies originate from the offspring of about 4,600 ovipositing females (2,200 MW and 2,400 AZ) during a three month period.

Hemoglobin assay for measuring the blood-meal size

Due to massive prediuresis during bloodfeeding the classical weighing of bloodfed sand fly females leads to underestimation of the volumes of bloodmeals [14]. Therefore, the colorimetric method developed by Briegel *et al.* [15] for measuring the hemoglobin concentration in blood-fed mosquitoes was adopted. Females of *P. orientalis*, 3–6 days old, were fed through a chick-skin membrane on rabbit blood. Individual midguts of blood-fed females were dissected 1 h after blood-feeding, transferred to tubes containing 200 µl 0.15 mM NaCl and homogenized. Gut homogenates (50 µl) or diluted rabbit blood (5 µl rabbit blood/1000 µl 0.15 mM NaCl) were mixed with 200 µl of Drabkin's reagent (Sigma) in the dark for 30 min. Absorbance was measured in 96-well plates in doublets at 540 nm. Human hemoglobin (Sigma) in concentrations from 3.1 to 100 µg/well was used as standard. The bloodmeal volume was calculated from 40 midguts of fully bloodfed *P. orientalis* (MW) females.

Cross-mating study

For the cross-mating study we slightly modified the method described by Dvorak *et al.* [16]. Briefly, individual pupae from each parental colony were separated into glass vials to obtain virgin adult flies. Virgin females from one colony were grouped with virgin males from the other colony (MW male/AZ female = Hybrids 1, AZ male/MW female = Hybrids 2) in an approximate 1:1 ratio of sexes and allowed to feed on a human arm (PV served as a volunteer). Blood-fed females were separated and five days post blood-meal (PBM) transferred to moist oviposition pots to lay eggs. The egg production of hybrids was compared with both parental colonies (20 ovipositing females in each group). The parental and hybrid colonies were reared under identical conditions and their developmental life cycles were recorded (see Table 1). Adult F1 hybrids were used for F2 brother-sister mating to verify that F2 progeny were viable and develop similarly to parental lines.

Genetic analyses

The two *P. orientalis* colonies were compared by RAPD and by DNA sequencing of two mitochondrial genes, cytochrome B (cytB)

Table 1. Life-cycle of two Ethiopian *P. orientalis* colonies and their hybrid F1 and F2 progeny.

			Life cycle in days PBM*							Egg production**		
			Eggs	Larvae			Pupae	Adults		Host	Eggs	
				L1	L2	L4		From	To		Total	Mean per female
Parental colonies***	AZ	mean	6.5	13.5	19.1	28.4	36.9	46.6	105.3	mouse	544	27.2
		range	5–9	11–19	16–29	23–34	31–47	39–69	61–147	human	975	48.75
	MW	mean	7.9	14.9	20.6	28.3	35.3	45.5	83.9	mouse	641	32.05
		range	4–12	12–20	18–24	24–32	29–41	40–52	54–110	human	693	34.65
Hybrids 1 ♂MW/♀AZ	F1		7	14	18	25	30	39	91	human	852	42.6
	F2		7	14	18	25	31	42	nd	human	846	42.3
Hybrids 2 ♂AZ/♀MW	F1		7	14	18	25	30	39	91	human	806	40.3
	F2		7	14	18	25	31	42	nd	human	812	40.6

*Days represent an interval between the female took a bloodmeal and the first offspring reached the respective instar.
**In the egg production study 20 ovipositing females were used in each group.
***In the parental colonies the life cycle data were collected from 12,168 (Addis Zemen, AZ) and 8,751 (Melka Werer, MW) ovipositing females within the period from VIII/2010 to IV/2012. Each cell contains the mean and the range of values.
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and cytochrome oxidase I (COI). For RAPD analysis, eight specimens from each colony (four males and four unfed females) were selected randomly. Two other sand fly species were added into the analysis as outgroups: *Phlebotomus* (*Larroussius*) *tobbi* Adler and P*hlebotomus* (*Phlebotomus*) *bergeroti* Parrot. DNA was extracted using High Pure PCR Template Preparation Kit (Roche, France). Of 60 decamer random primers previously tested (OPA 1–20, OPD 1–20, OPF 1–20, by Operon Technologies Inc, USA), five were used: OPE16, OPI 12, 13, OPL5, OPO20. The PCR reaction was subjected to 45 amplification cycles in 25 µl volumes, with a temperature profile: 94°C for 1 min, 35°C for 2 min and 72°C for 3 min. An initial denaturation step of 94°C for 4 min and a final extension step of 72°C for 10 min were added. After PCR amplification, electophoretic bands were transformed into a binary matrix and genetic distances were computed from Nei-Li's coefficient of similarity [17]. Phylogenetic trees were constructed by the unweighted pair-grouping analysis (UPGMA) [18]. PC program FreeTree [19] was used for computations of genetic distances and construction of trees.

For sequencing analysis COI and a part of cytB genes were chosen. Templates for direct sequencing were amplified by PCR in a 50-µl volume using primers and conditions previously published [20,21]. PCR products were sequenced in both directions using the same primers as for the DNA amplification on 3100 Avant Genetic Analyser (Applied Biosystems, USA). All PCR products were cleaned by QIAquick PCR Purrification Kit (Qiangen, Germany) prior to the sequencing. Obtained DNA sequence data were compared with those in the GenBank database. The sequences were aligned using ClustalX 1.81 and the resulting alignment was manually edited by BioEdit.

Experimental infection of *P. orientalis*

Two *L. donovani* strains, GEBRE-1 (MHOM/ET/72/GEBRE1) and GR374 (MHOM/ET/2010/DM-1033) originating from VL patients in northern Ethiopia and kept in cryobank of the Department of Parasitology, Charles University were used for experimental infection of *P. orientalis*. Parasite strains were maintained at 23°C on medium 199 (Sigma) supplemented with 10% fetal calf serum (Gibco), 1% BME vitamins (Sigma), 2% human urine and amikin (250 µg/ml). Females of both colonies (∼–7 day old) were fed through a chick-skin membrane on a suspension of promastigotes (from 4-days-old *Leishmania* culture) mixed 1:10 with heat-inactivated rabbit blood (Bioveta, Ivanovice na Hane, Czech Rep.). If not stated otherwise, an infective dose of 10^5 promastigotes per ml of blood was used. To test dose-dependent differences in *Leishmania* development, GR374 cultures were used at the following concentrations: 2×10^3, 2×10^4, 10^5 and 5×10^5 promastigotes/ml of blood. Furthermore, the accurate number of parasites ingested by individual females (N = 8) was determined using Q-PCR immediately after the experimental feeding (details below).

Blood-fed females were separated immediately after feeding and kept at 26°C with free access to 50% sugar solution. One group of females was dissected for microscopical observations at different intervals PBM, the second group was placed into the plastic tubes filled with 100 µl of elution tissue buffer (from DNA isolation kit) on day 0 and 10 PBM and stored at −20°C for the following *Leishmania* DNA extraction, see below.

On days 2, 5–6, 8–11 PBM females were dissected in drops of saline solution. The individual guts were checked for presence and localization of *Leishmania* promastigotes under the light microscope, special emphasis was given to colonization of the stomodeal valve as the prerequisite for successful transmission [for review see 22]. Levels of *Leishmania* infections were graded into four categories according to Myskova *et al.* [23]: negative, light (<100 parasites/gut), moderate (100–1000 parasites/gut) and heavy (>1000 parasites/gut). Data were evaluated statistically by means of $\chi2$ test using the S-PLUS 2000 program.

The number of *Leishmania* promastigotes in individual females was estimated by Q-PCR the SYBR Green detection method (iQ SYBR Green Supermix, Biorad, CA). The total DNA was isolated using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according manufacturer's instruction. Kinetoplast DNA was chosen as the molecular target with primers described by Mary *et al.* [24]. Q-PCR was calibrated using serial dilutions of *L. donovani* DNA extracted from known number of promastigotes. Two microliters of eluted DNA was used per individual PCR reaction - 3 min at 95°C followed by 45 cycles of: 10 s at 95°C, 10 s at 56°C, and 10 s at 72°C. Results from Q-PCR were statistically evaluated using Kruskal-Wallis H-test.

Results

Life cycle of *P. orientalis* and differences between colonies

The developmental data of both *P. orientalis* colonies are summarized in Tables 1 and Figure 1. The life cycle beginning with egg development in blood-fed females to eclosion of the adult sand fly (including egg, larval and pupal stages) ranged from seven to sixteen weeks in MW and from seven to twenty-one weeks in AZ (Figures 1A, B). In contrast to most other sand flies maintained in our laboratory *P. orientalis* larvae and adults (including blood-fed females) prefer relatively high humidity. However, AZ and MW colonies differ in humidity demands during pupation: while MW pupae concentrated close to the upper edge of the rearing pot, the AZ larvae pupated mainly in the substrate on the bottom of the pot. Different pupation strategies might reflect dissimilar humidity demands of the two *P. orientalis* populations adapted to different microclimatic conditions.

Development of both colonies was affected considerably by the quality of larval food. On non-autoclaved food the emerging adults peaked at eight and nine weeks PBM for MW and AZ, respectively, and most of the adults (>90% in MW and >60% in AZ) emerged within ten weeks (Figure 1A). On autoclaved food the differences between colonies were more obvious as the development of AZ colony was significantly delayed. Peak of emerging offspring was nine and thirteen weeks PBM for MW and AZ colony, respectively. Only 16% of individuals of AZ colony achieved the adult stage within ten weeks PBM (Figure 1B). The quality of food affected mainly the fourth instar larvae where significant proportion of larvae stopped feeding and went into dormant phase, while the early larval stages were unaffected. In AZ colony, the non-synchronized larval development and tendency to diapause (predictive dormancy) occurred even on the non-autoclaved food. The growth of the L4 larvae was slightly improved by supplementation with TetraMin (aquarium fish food) (data not shown).

Cross-mating study

Reciprocal hybridization crosses of both colonies resulted in successful mating and insemination, and produced viable F1 and F2 progeny. Hybrids had very high fecundity and developed successfully. In the F1 generation, the mean number of eggs per female was 42.6 and 40.3 for hybrids 1 (MW male/AZ female) and hybrids 2 (AZ male/MW female), respectively, and 42.3 and 40.6 in F2 generation. This egg production was even higher than in parental colonies (see Table 1). Immature larval stages of hybrids developed similarly or even faster than the parents. In both hybrid colonies egg development took 7 days and the whole life cycle from egg laying to eclosion from pupae lasted 32 days and 35 days in F1 and F2 generations, respectively (Table 1).

Genetic analyses

No morphological differences were found between *P. orientalis* colonies. Five decamer random primers were used for the RAPD analysis (Figure 2). A total number of 58 fragments, ranged from 100 to 1000 bp, were amplified. The band pattern given by amplification with each primer was reproducible and stable. The UPGMA analysis of these data revealed a position of two distinct clades, each containing specimens exclusively from one colony. None of the specimens fell into a clade of the other colony. A similar grouping pattern was also obtained by the neighbor-joining method (data not shown).

All analyzed CytB and CO-I sequences of several specimens belonging to both colonies were identical and no differences were observed. Sequences were submitted to GenBank (Accession numbers KC204965-KC204968).

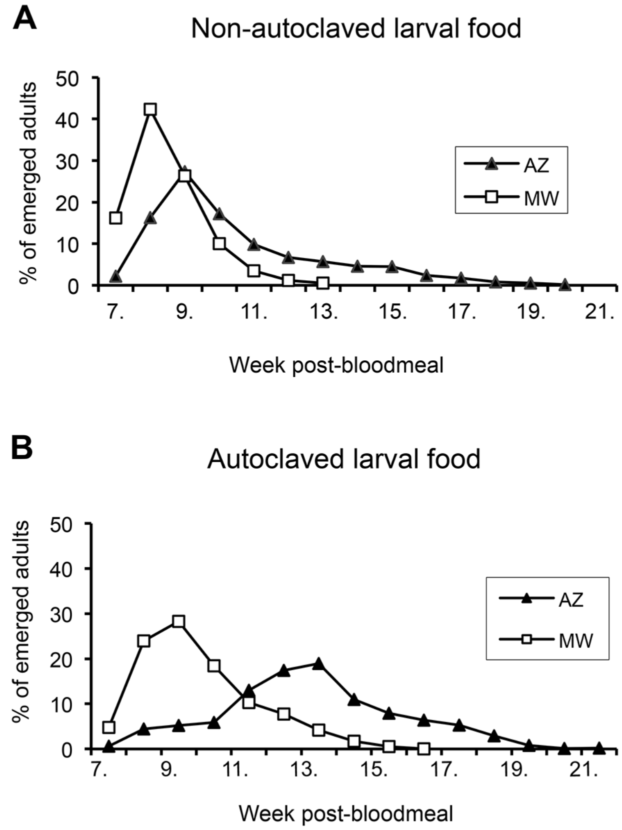

Figure 1. Effect of nutrition on the life cycle of two *P. orientalis* colonies. Data originate from the offspring of about 4,600 ovipositing females (2,200 MW and 2,400 AZ) during a 3 month period. **1A**: On the non-autoclaved food the number of adults emerging from pupae peaked on week 8 PBM in MW, and week 9 PBM in AZ. All individuals completed the life cycle within 13 and 20 weeks for MW and AZ, respectively. **1B**: On the autoclaved food the life cycle was prolonged and the larval growth appeared less synchronized in both colonies. The impact was more significant in the AZ colony: emergence of AZ adults peaked on week 13 (four weeks later than on non-autoclaved food).
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Development of *L. donovani* in *P. orientalis*

The susceptibility of both *P. orientalis* colonies to *L. donovani* was demonstrated first using GEBRE-1 strain. On day 2 PBM, parasites were located inside the intact peritrophic matrix as procyclic promastigotes and showing high intensity of infection in 75% of females. On day 6 PBM, all females had defecated and the infection rate was 78%. Elongate nectomonads were located mainly in the abdominal midgut while short promastigotes and metacyclic forms migrated forward to the thoracic midgut; in 62% of the infected females promastigotes colonized the stomodeal valve. Subsequently, on day 9 PBM, mature infection with high parasite burdens and colonization of the stomodeal valve were found in the majority (84%) of females (data not shown).

Accurate determination of potential differences in vector competence of the two *P. orientalis* colonies was assessed by infections with *L. donovani* strain GR374. In the early stage of infection (on day 2 PBM) parasites developed similarly in both *P. orientalis* colonies (P>0.05). On day 5–6 PBM, the infection rates

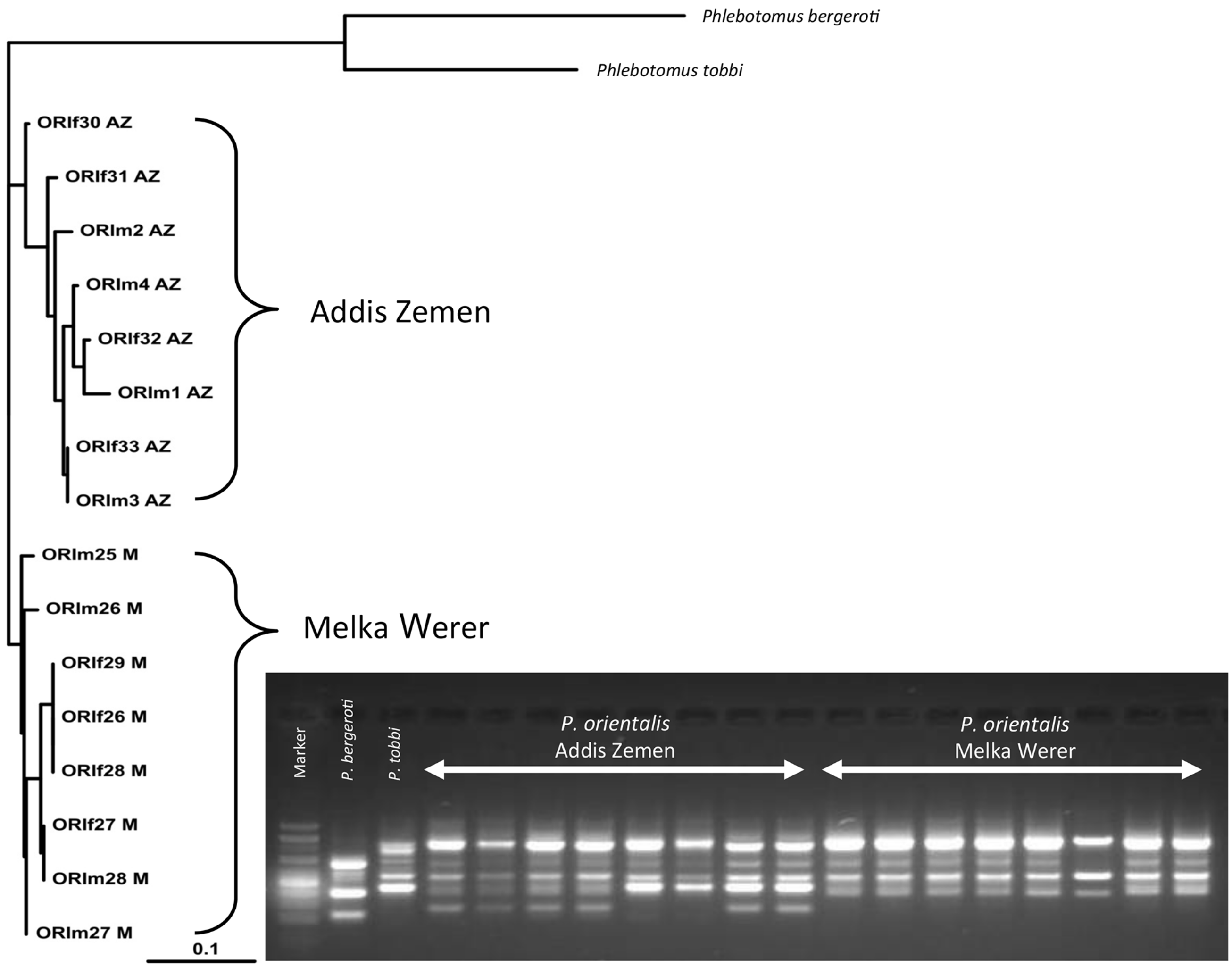

Figure 2. RAPD analysis of two *P. orientalis* colonies. RAPD analysis was based upon PCR results using five random primers (OPI12, 13, OPO20, OPE16, OPL5; in total 58 characters), electrophoretogram for OPL5 is shown as an example. Dendrogram was constructed by the Neighbor-joining method.
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were high (around 90%) in both colonies and the intensity of infection was slightly higher in AZ colony (P = 0.048). Abundant metacyclic promastigotes (more than 50%) and colonized stomodeal valves were observed as early as 5 days PBM. On day 8–11 PBM, high infection rates (94% for MW and 86% for AZ) and similar intensities of infection were found in both colonies ($P>0.05$) (Figure 3A). Similarly, the Q-PCR revealed no significant differences ($P>0.05$) in total parasite numbers in sand fly midguts on day 10 PBM (MW vs. AZ; N = 50 engorged females) (Figure 3B).

The effect of initial infective dose on total parasite numbers in sand fly gut during late stage infection was tested in *P. orientalis* (MW) infected by *L. donovani* (GR374) (Figure 4A,B). In fully bloodfed females of *P. orientalis* the average bloodmeal volume was 0.69 µl (SD = 0.1) ranging from 0.43 to 0.99 µl. It indicates that females infected of 5×10^5, 10^5, 2×10^4 and 2×10^3 promastigotes/ml of blood took on average 350, 70, 14 and 1–2 promastigotes, respectively. These results were confirmed by Q-PCR detecting accurate numbers of parasites from individual females immediately after blood feeding (data not shown). Despite the fact, that infection of sand flies was initiated with significantly different numbers of ingested promastigotes, the differences in infection rates were found only in group infected with 2×10^3 promastigotes/ml. In this group the late stage infections (on days 6 and 10 PBM) were found only in 30–45% of females while in other three groups the positivity of females reached 75–95% (Figure 4A). However, the location of parasites during late stage infections was similar in all four groups tested and colonization of the thoracic midgut and the stomodeal valve was observed as early as on day 5 PBM. Even in the group infected with the lowest dose (2×10^3 promastigotes/ml) numerous parasites colonizing the stomodeal valve were found in the majority (71%) of positive females on day 10 PBM.

The Q-PCR showed no significant differences in parasite loads at late stage infections (day 10 PBM) between groups of females infected with 5×10^5, 10^5 and 2×10^4 promastigotes. In contrast, the significantly lower parasite loads ($P<0.05$) were found in group infected with 2×10^3 promastigotes/ml of blood (Figure 4B); however, even this lowest dose was high enough to infect about 50% of females.

Discussion

Sequencing analysis of cytB and COI genes as well as RAPD confirmed the high degree of similarity between the MW and AZ

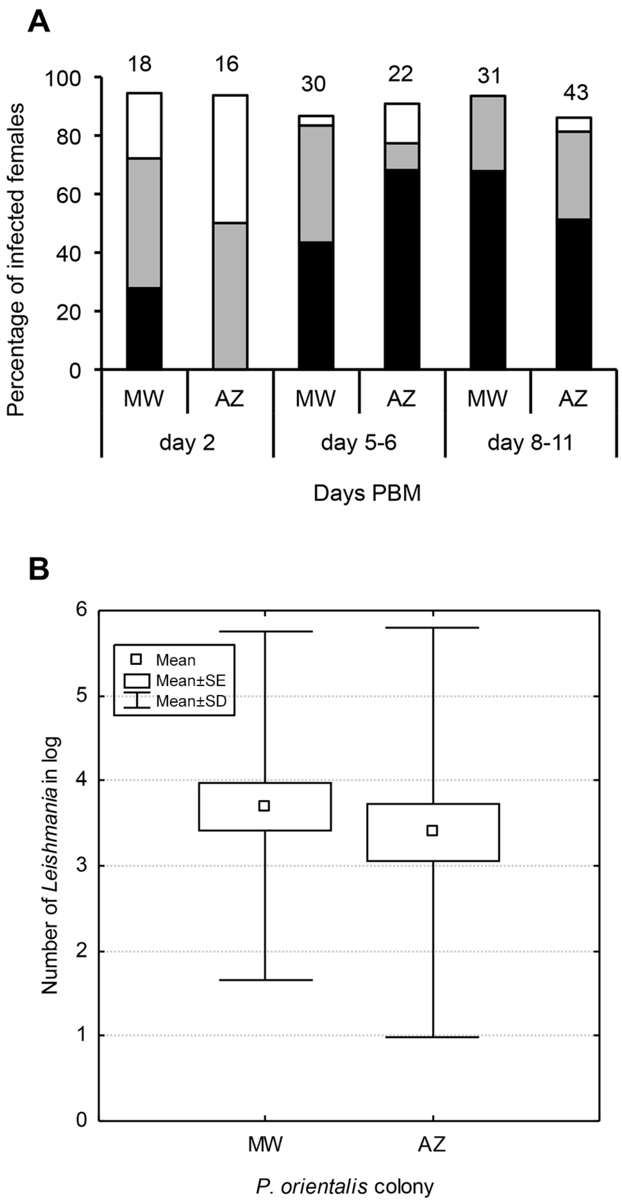

Figure 3. Development of *L. donovani* (GR 374) in females of two *P. orientalis* colonies. Sand flies were infected by feeding on a suspension of 10^5 promastigotes/ml of blood and kept at 26°C. **3A**: Infected females of *P. orientalis* were examined microscopically 2, 5–6 and 8–11 days post-bloodmeal (PBM). The infection intensities were classified into three categories according to their intensity: heavy (more than 1,000 parasites per gut [black]), moderate (100–1,000 parasites [grey]) and light (1–100 parasites [white]). Numbers above the bars indicate the number of dissected females. **3B**: Parasite numbers from 40–50 individual females were quantified by Q-PCR targeted on amplification of *Leishmania* kDNA 10 days PBM.
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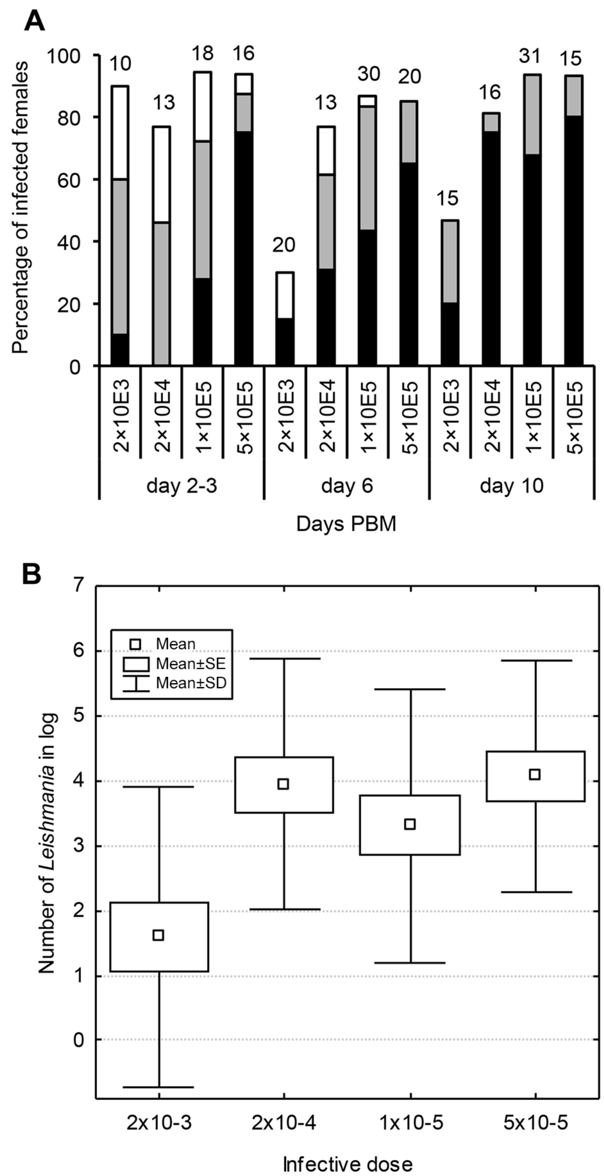

Figure 4. Effect of initial infective dose on development of *L. donovani* (GR 374) in *P. orientalis*. 4A: Infected females of *P. orientalis* (MW colony) were examined microscopically 2–3, 6 and 10 days post-bloodmeal (PBM). The infection intensity was classified as described in Fig. 3. **4B**: Parasite numbers were determined using Q-PCR at 10 days PBM. Twenty females were used per group.
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colonies originating in geographically distant areas and different altitudes. Despite this fact, obvious differences were found in certain life-cycle parameters of these populations.

The critical factor affecting larval development was the quality of larval food; autoclaved food resulted in a high proportion of dormant larvae and prolonged the generation time with the AZ colony being more sensitive to this change. Diapause of 4^{th} instar larvae has been described in some Palaearctic species, whereas species from warmer, wetter habitats are expected to diapause at the egg stage [25]. Our findings, as well study by Schmidt [26], proved the presence of diapause in the fourth larval stage in *P. orientalis* populations. The diapause and the non-synchronized larval development in the AZ population might be explained as an adaptation to more challenging natural conditions of the highland area, and probably assure that at least some of the population will survive through periods with challenging climatic conditions. A significant proportion of fourth instar AZ larvae diapaused despite of being maintained under a constant temperature of 27°C. This finding is in contrast with observations on other sand fly species where higher temperatures decreased the tendency of larvae to diapause [27].

The results of blood-meal analysis in females from endemic sites in Ethiopia showed bovines as preferred hosts of *P. orientalis* in natural conditions (about 92% of tested females) with a low proportion of females fed on humans [28]. In laboratory conditions an alternative bloodmeal source has to be adopted for the long term colonization. The AZ colony was less adaptable for substituting of blood-meal source than the MW colony. After arrival to the laboratory in Prague, females of both colonies were bloodfed on rabbits. MW females fed readily despite the initial small size of the colony and were adapted to anesthetized mice relatively easily within two or three generations (about six months). On the other hand, AZ females originally refused feeding even on rabbits and had to be offered a human arm. Adaptation for feeding on mice took more than ten generations (almost two years). To date, adaptation has not been 100% successful yet, and AZ females must be fed alternatively on rabbits and mice. Differences between the two colonies were also noted during experimental membrane feeding: AZ females were more reluctant to feed through a chick-skin membrane. Data on egg production seem to be in accord with requirements of AZ for blood source; AZ females fed on mouse produced less than 60% of eggs than those fed on human arm (see Table 1). For more robust conclusions a study on a larger sample would be needed.

The susceptibility of *P. orientalis* to *L. donovani* is the crucial factor for the epidemiology of visceral leishmaniases. Natural infections of *P. orientalis* with *L. donovani* were repeatedly reported from various foci in East Africa [1,4,11,29], but only once in the south-west Ethiopia [30]. In Sudan, the susceptibility of *P. orientalis* to *L. donovani* has also been demonstrated by feeding on patients with kala-azar [10,31] or by feeding infected blood through mouse-skin membranes [11]. These pioneering studies were, however, done using a limited number of *P. orientalis*.

In our study both tested strains of *L. donovani* developed very well in *P. orientalis* females and colonized anterior parts of the midgut and the stomodeal valve. Parasite development at 26°C was relatively fast as the presence of metacyclic promastigotes and colonization of stomodeal valve by haptomonads was observed already on day 5 PBM. On day 10 PBM, the infection rates in both colonies were very high (93% [MW] and 81% [AZ]) and the Q-PCR revealed that females from the two colonies did not differ in total numbers of parasites in their midguts.

The volume of *P. orientalis* blood-meals measured by hemoglobinometry was on average 0.7 µl of blood. This is about one half of the volume reported for *L. longipalpis* using the same technique [32]; the difference can be easily explained by body size as *P. orientalis* is a smaller sand fly.

Experimental infections revealed that even the lowest infective dose tested (2×10^3 *L. donovani* promastigotes per ml of blood) was sufficient for high infection rates and successful establishment of late stage midgut development of this parasite in about 50% of females. Taking into account the average bloodmeal size of *P. orientalis* this concentrations is equivalent to infective dose between one and two *L. donovani* promastigotes per fly. This finding suggests extremely high susceptibility of *P. orientalis* for *L. donovani*; at present, the similar study using amastigotes is underway in our laboratory. Due to technical difficulties similar studies using amastigotes have not been performed yet in *P. orientalis*, however, in *L. longipalpis* Freitas *et al.* [33] demonstrated that promastigote-initiated *L. infantum* infections are fully comparable to amastigote-initiated ones.

In summary, this study describes in details behavioural and life-cycle parameters of two laboratory colonies of *P. orientalis* originating from Ethiopia and advances the knowledge of *P. orientalis* biology. We showed that demands for laboratory maintenance may significantly differ between two sand fly colonies of the same species. Therefore, the conditions of sand fly rearing should not be considered uniform and have to be optimized individually for each colony. Importantly, the study brings the first detailed description of *L donovani* development in *P. orientalis* under laboratory conditions. It proves that *P. orientalis* is a highly susceptible vector and only very low parasites are needed for establishment of experimental infections in this sand fly species. In view of our findings, we deduce that non-endemicity of visceral leishmaniases in Melka Werer cannot be explained by low susceptibility of local *P. orientalis* to *L. donovani*.

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Author Contributions

Conceived and designed the experiments: VS VV PV. Performed the experiments: VS VV VD KP JV. Analyzed the data: VS VV VD KP JV PV. Contributed reagents/materials/analysis tools: AK TGM. Wrote the paper: VS VV VD AH AW PV.

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The effect of avian blood on *Leishmania* development in *Phlebotomus duboscqi*

Katerina Pruzinova*, Jan Votypka and Petr Volf

Abstract

Background: The development of pathogens transmitted by haematophagous invertebrate vectors is closely connected with the digestion of bloodmeals and is thus affected by midgut enzymatic activity. Some studies have demonstrated that avian blood inhibits *Leishmania major* infection in the Old World vector *Phlebotomus papatasi*; however, this effect has never been observed in the New World vectors of the genus *Lutzomyia* infected by other *Leishmania* species. Therefore, our study was focused on the effect of chicken blood on bloodmeal digestion and the development of *Leishmania major* in its natural vector *Phlebotomus duboscqi*, i.e. in a vector-parasite combination where the effect of blood is assumed. In addition, we tested the effect of avian blood on midgut trypsin activity and the influence of repeated feedings on the susceptibility of sand flies to *Leishmania* infection.

Methods: *Phlebotomus duboscqi* females were infected by rabbit blood containing *L. major* and either before or after the infection fed on chickens or mice. The individual guts were checked microscopically for presence and localization of *Leishmania*, parasite numbers were detected by Q-PCR. In addition, midgut trypsin activity was studied.

Results: Sand fly females fed on chicken blood had significantly lower midgut trypsin activity and delayed egg development compared to those fed on rabbits. On the other hand, there was no effect detected of avian blood on parasite development within the sand fly gut: similar infection rates and parasite loads were observed in *P. duboscqi* females infected by *L. major* and fed on chickens or mouse one or six days later. Similarly, previous blood feeding of sand flies on chickens or mice did not show any differences in subsequent *Leishmania* infections, and there was equal susceptibility of *P. duboscqi* to *L. major* infection during the first and second bloodmeals.

Conclusion: In spite of the fact that avian blood affects trypsin activity and the oocyte development of sand flies, no effect of chicken blood was observed on the development of *L. major* in *P. duboscqi*. Our study unambiguously shows that sand fly feeding on avian hosts is not harmful to *Leishmania* parasites within the sand fly midgut.

Keywords: Leishmaniases, Vectors, Bloodmeal digestion, Trypsin, Chicken blood

Background

Digenetic parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) alternate between intracellular amastigotes in mammalian hosts and extracellular promastigotes in sand fly vectors (Diptera: Phlebotominae). In the sand fly vector, development is confined to the digestive tract and is closely connected with bloodmeal digestion (reviewed by [1,2]).

The source of the bloodmeal influences the digestion and fecundity of females [3-7]. Proteolytic activity in the midgut of haematophagous insects is activated by ingested proteins and the consequent rate of trypsin activity is correlated with the protein content in the bloodmeal [8,9]. Thus, the reproductive potential of sand fly females partly depends on the type of bloodmeal and amount of ingested nutrients [4].

Ingested blood affects not only the digestion and fecundity of sand flies but also can affect *Leishmania* development. Sand fly midgut proteases influence *Leishmania* development and are one of the obstacles that parasites must overcome to establish an infection in the midgut (reviewed by [1,2]). Adler [10] first suggested that products of blood serum digestion destroy *Leishmania*

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parasites in the midguts of 'noncompatible' sand fly species. According to Schlein and Romano [11] and Borovsky and Schlein [12], a specific component of the trypsin-like activity prevents the survival of *L. donovani* in the 'noncompatible' vector *Phlebotomus papatasi* while the ability to modulate this factor enables *L. major* to survive in 'compatible' sand fly species. Pimenta et al. [13] described the susceptibility of *Leishmania* to midgut digestion in the 'compatible' vector *P. papatasi* as stage-specific: *L. major* amastigotes and fully transformed promastigotes were relatively resistant to *P. papatasi* proteolytic activity, whereas parasites within the amastigote-to-promastigote transition were highly susceptible being killed.

However, even in 'compatible' vectors the bloodmeal from different animals has been described as having different effects on *Leishmania* [14]. Schlein et al. [15] reported that *Leishmania* infection is inhibited in its natural vector *P. papatasi* if the sand fly females were fed on turkeys before or after the infection. According to the authors, the parasite reduction is caused by the digestive process and a relatively high DNAase level is induced by nucleated avian erythrocytes. On the other hand, Nieves and Pimenta [16] tested the effect of nine different sources of blood (human, dog, horse, opossum, rodent, chick, chicken, mouse and hamster) on the development of *L. braziliensis* and *L. amazonensis* in *Lutzomyia migonei.* The bloodmeal source influenced the infection rates of the females, but none of the bloodmeal types (including avian blood) eliminated *Leishmania* parasites. Similarly, Sant'Anna et al. [17] noted that chicken blood supports the development of *L. mexicana* in *Lutzomyia longipalpis*. Moreover, in late-stage infections they found similar numbers of metacyclic promastigotes in females infected via rabbit blood or chicken blood [17]. These findings raised the hypotheses that there might be a difference in the effect of avian blood between the New World vectors of the genus *Lutzomyia* and Old World vectors of the genus *Phlebotomus*.

Since descriptions of the effects of avian blood on sand fly digestion and *Leishmania* development are contradictory, we studied the effect of mammalian and avian blood on the trypsin activity and oocyte development of *P. duboscqi*. In parallel experiments we tested whether the digestion of avian blood is harmful to the development of *L. major* in its natural vector: first we repeated the experiments done by Schlein et al. [15] but included proper control groups. Then, to explain our results we compared the susceptibility of *P. duboscqi* to *L. major* infection acquired in the first or second bloodmeal and in 100% versus 5% blood.

Methods

Sand fly maintenance

The colony of *P. duboscqi* was maintained under standard conditions as previously described [18].

Fluorometric measurements of trypsin activity

Trypsin has been reported to affect *Leishmania* infections in sand flies [11,12]. Therefore, we measured trypsin activities after feeding on avian blood. Midguts of *P. duboscqi* females fed on rabbits or chickens were dissected at 18, 24, 30, 48, and 72 hours post blood meal (PBM) and transferred to 1.5 ml Eppendorf tubes. Each sample contained 10 midguts in 100 μl of Tris-NaCl (0.1 M Tris, 150 mM NaCl, pH = 8.44). The samples were homogenised and trypsin activity was measured in 96-well plate by a fluorometric assay with the substrate Boc-Leu-Gly-Arg-AMC (Bachem). Aminomethylcoumarin (AMC) was excited at 355 nm and fluorescence of released AMC was measured at 460 nm by a fluorometer (Tecane infinite M200). Data were evaluated statistically using main effect ANOVA (in STATISTICA 6.1 and StatSoft software).

Protein assays of sand fly midgut homogenates

In bloodsucking insects, levels of proteolytic activity are known to correspond to the quantity and quality of proteins ingested during the bloodmeal [8,9]. To explain differences in midgut trypsin activities present after feeding on different blood sources, we measured the protein content of *P. duboscsqi* females. Midguts of *P. duboscqi* females fed on rabbits or chickens were dissected 4 hours PBM and transferred to 1.5 ml Eppendorf tubes. Each sample contained 15 midguts in 250 μl of Tris-NaCl (0.1 M Tris, 150 mM NaCl, pH 7.8).The samples were homogenised and total amounts of midgut protein were quantified according to the Bradford [19] method adapted to 96-well plates. Ten μl of midgut homogenates were mixed with 200 μl of the Bio-Rad protein assay reagent diluted 5× in distilled, deionised water. Absorbance was measured in 96-well plate at 595 nm by the Tecan infinite M200. Bovine serum albumin (Sigma, concentration 1 to 10 μg/well) was used as a standard.

Experimental infections of sand flies

The *Leishmania major* strain LV561 (LRC-L137; MHOM/IL/1967/Jericho-II), the same strain as used by Schlein et. al [15], was maintained at 23°C on Medium 199 (Sigma) supplemented with 10% foetal calf serum (Gibco), 1% BME vitamins (Sigma), 2% human urine and gentamicin (80 μg/ml).

Females of *P. duboscqi* were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 10^6 promastigotes per ml. If not stated otherwise 100% rabbit blood was used. Blood-engorged females were separated and maintained on 50% sucrose. Bloodfed females were always maintained at constant temperature (26°C) because it is known that ambient temperature affects the digestion and *Leishmania* development within sand flies [20]. At various intervals post-infection (PI) the individual guts were checked microscopically for the presence and localization of *Leishmania* promastigotes. Parasite loads were graded according to Myskova et al. [21] as light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Data were evaluated statistically by means of the χ^2 test using S-PLUS 2000 software.

The number of *Leishmania* parasites in individual females was counted using Q-PCR as described previously [21,22]. Briefly, experimental females were stored at −20°C and total DNA extraction was performed with a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. Q-PCR using *Leishmania*-specific primers (forward: 5′-CTTTTCTGGTCCTCCG GGTAGG-3′; reverse: 5′-CCACCCGGCCCTATTTTAC ACCAA-3′ [22]) was performed by the SYBR Green detection method (iQSYBER Green Supermix, Bio-Rad, Hercules, CA) in Bio-Rad iCycler & iQ Real-Time PCR systems. Statistical evaluation was performed by the Kruskal-Wallis test and Mann–Whitney U-test using STATISTICA 6.1.

The effects of sand fly feeding on avian blood before and after infection

To evaluate the effect of avian blood on *Leishmania* infection we followed three different experimental feeding schemes (the first two done according to Schlein et al. [15]): (i) We evaluated the effect of chicken blood taken before infection. Sand fly females fed either on chickens or mice were given a chance to lay eggs in breeding pots and then, after oviposition, were fed an infective bloodmeal (nine days after the first bloodmeal). (ii) In the second scheme we evaluated the effect of avian blood on parasites already present in the gut: females infected with promastigotes in diluted (5%) blood were fed either on chickens or mice one day PI. The decreased amount of nutrients in the diluted blood resulted in the females having to feed again without laying eggs. (iii) In addition, we evaluated the effect of avian blood during the later phase of *Leishmania* infection: females infected with promastigotes in diluted (5% or 10%) blood were fed either on chickens or mice six days PI. Initial experiments showed that 10% blood resulted in higher infection rates, and therefore in repeated experiments we used only this blood concentration.

A comparison of sand fly susceptibility to *L. major* during the first and second bloodmeal

One group of *P. duboscqi* females was fed first on non-infected mice, allowed to lay eggs in a breeding pot and then (9 days post first blood feeding) infected experimentally, while the control group (one week younger) was maintained without a bloodmeal until the experimental infection. Both groups were infected simultaneously with the same parasite culture.

The effect of 5% or 100% blood in the infective bloodmeal on parasite establishment in the sand fly midgut

To explain the results of infections done using diluted (5%) blood (experiment (ii)), we compared the infection rates and intensities of infection after feeding on 5% or 100% blood. Females were infected with promastigotes in diluted or undiluted rabbit blood and checked on days 1 and 2 PI; by this time infected females had fed on chickens or mice in the previous experiment.

Differences in the digestion of 10% and 100% blood

To explain the results of infections done using diluted (10%) blood (experiment (iii)), we compared the trypsin activity after feeding on 10% and 100% blood. Midguts of females fed on 10% or 100% rabbit blood through a chick-skin membrane were dissected at 24, 30, 48, and 72 hours PBM and transferred to 1.5 ml Eppendorf tubes. Each sample contained a mixture of 10 midguts in 100 μl of Tris-NaCl (0.1 M Tris, 150 mM NaCl, pH = 8.44). Trypsin activity was measured by the fluorometric method described above.

In both groups of females, the time of defecation was also measured. Thirty fully blood-fed females from both groups were individually placed in small glass vials, maintained at 26°C and checked twice daily under a binocular microscope for defecation.

Ethical statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments (including sand fly feeding) were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under the Certificate of Competency (Registration Number: CZU 246/1992, CZ 00177).

Results

The effect of blood source on midgut trypsin activity

Phlebotomus duboscqi females were fed either on rabbits or chickens and dissected at 4, 18, 24, 30, 48, and 72 hours PBM, and protein absorbance and trypsin activity was measured. Data from two independent experiments were pooled. Sand flies fed on chickens had half the midgut protein content compared to those fed on rabbits (103 μg/gut versus 202 μg/gut, respectively). Similarly, midgut trypsin activity in females fed on chickens was significantly lower ($F_{(4,50)}$ = 5.26, P < 0.01). The highest differences between groups were observed during the first 24 hours post bloodmeal, with females fed on chickens having 40 – 55% less trypsin activity in their midguts compared to those fed on rabbits (Figure 1).

The effect of chicken and mice blood on *Leishmania* development in sand fly midguts

(i) 9 days before infection: *Phlebotomus duboscqi* females, previously fed either on chickens or mice, were given an infective bloodmeal nine days later and then dissected on days 2 and 6 PI. Data from three independent experiments were pooled. In both groups of females (fed on chicken versus mouse) similar *Leishmania* development was observed (Figure 2): no significant differences were found in infection rates or intensities of infection on days 2 and 6 PI (day 2: $\chi^2 = 0.89$, P = 0.64; day 6: $\chi^2 = 0.10$, P = 0.76). On day 6 PI, all females from both groups were infected and a majority of them had high parasite loads (over 80% had heavy infections); *Leishmania* promastigotes colonized the stomodeal valve in almost all females (99%). Various promastigote forms, mainly leptomonads and metacyclic promastigotes were present in thoracic midgut.

(ii) 1 day after infection: Females infected with *Leishmania* promastigotes in 5% rabbit blood were fed one day later on chickens or mice. Data from three independent experiments were pooled. On days 2, 6, and 9 after the second bloodmeal, no significant differences were observed in *Leishmania* development: females fed on chickens or mice did not differ in infection rates ($\chi^2 = 1.37$, P = 0.24) or intensities of infection on any of the compared days PI (day 2: $\chi^2 = 2.81$, P = 0.42; day 6: $\chi^2 = 0.19$, P = 0.76; day 9 $\chi^2 = 3.07$, P = 0.38). On day 9 after the second bloodmeal, 50% of females from both groups were infected; the intensities of infection were high in most of them and

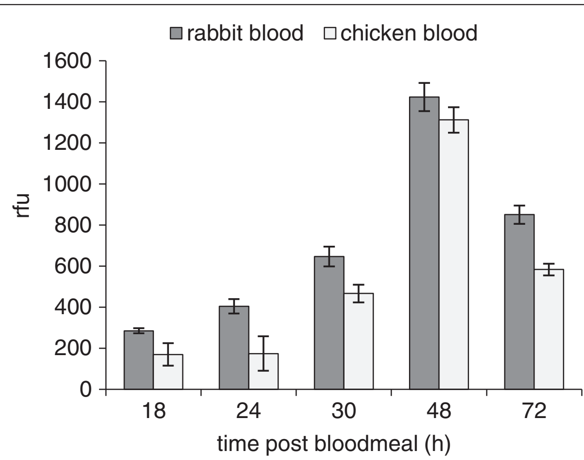

Figure 1 Trypsin activity in the midguts of *P. duboscqi* females fed on rabbits or chickens. Trypsin activity was measured at 18, 24, 30, 48, and 72 hours PBM in the homogenates of ten midguts of females fed on chickens or rabbits.

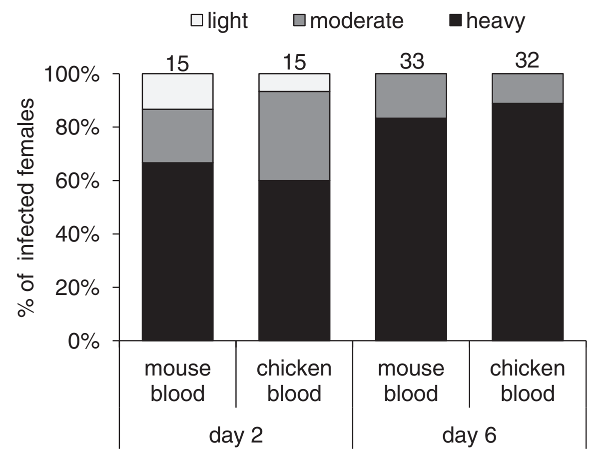

Figure 2 The effect of chicken blood on *Leishmania* development in sand fly midguts: 9 days before infection. *P. duboscqi* females fed either on chickens or mice and nine days later were infected by 10^6 promastigotes per ml of bloodmeal. Infection rates and intensities of infection were evaluated microscopically in sand fly midguts on days 2 and 6 PI, and infections were classified into three categories: light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females.

promastigotes colonized the stomodeal valve in 100% of infected females (Figure 3A). Various promastigote forms including metacyclics were observed. Similarly, Q-PCR revealed no significant differences ($KW\text{-}H_{(1;100)} = 1.03$, P = 0.31) in total parasite numbers in sand fly midguts on day 9 after the second bloodmeal (Figure 3B).

(iii) 6 days after infection: Females infected by *Leishmania* were fed on chickens or mice six days after an infective meal containing 10% rabbit blood with promastigotes. Data from two independent experiments were pooled. Females from both groups were dissected on days 2 and 6 after the second bloodmeal, and no significant differences were observed between experimental groups (chicken vs. mouse) in infection rates ($\chi^2 = 0.05$, P = 0.82) or intensities of infection (day 2: $\chi^2 = 5.72$, P = 0.13; day 6: $\chi^2 = 0.08$, P = 0.96). *Leishmania* developed similarly in both female groups: infection rates were about 80 – 85% and parasite loads were high in a majority of infected females (50 – 80%) (Figure 4A). Promastigotes colonized the stomodeal valve from day 6 after the second bloodmeal in 100% of infected females. Leptomonad and metacyclic forms prevailed in thoracic midgut. Similarly, Q-PCR revealed no significant differences ($KW\text{-}H_{(1;100)} = 1.20$, P = 0.27) in total parasite numbers in sand fly midguts on day 6 after the second bloodmeal (Figure 4B).

Sand fly susceptibility to *L. major* infection during the first and second bloodmeals

Leishmania development in females infected by rabbit blood with promastigotes during the first or second

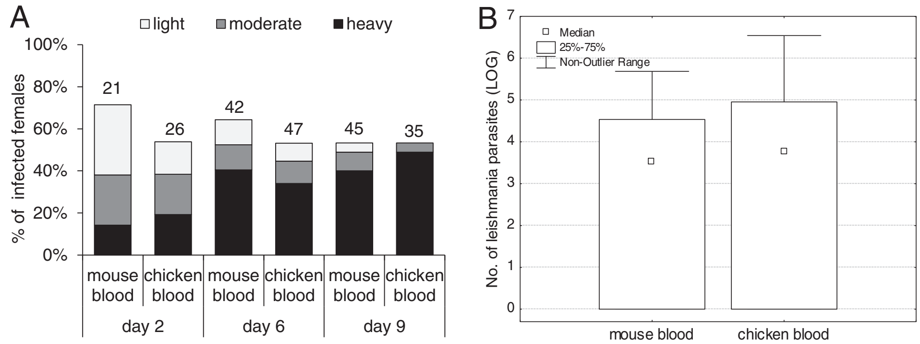

Figure 3 The effect of chicken blood on *Leishmania* development in sand fly midguts: 1 day after infection. One day after the infection by promastigotes in 5% rabbit blood (10^6 parasites/ml) *P. duboscqi* females were allowed to feed on chickens or mice. Infection rates and intensities of infection were evaluated in sand fly midguts on days 2, 6, and 9 after the second bloodmeal. **A**: Intensities of infection were microscopically classified into three categories: light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females. **B**: The precise number of parasites from 50 females of both groups was measured by Q-PCR 6 days after the second bloodmeal.

bloodmeal was compared on days 2, 6, and 9 PI. Data from two independent experiments were pooled. No significant differences were observed between experimental groups (1st vs. 2nd bloodmeal) in infection rates (χ^2 = 0.0002, P = 0.99) or intensities of infection for any of the compared days PI (day 2: χ^2 = 3.74, P = 0.15; day 6: χ^2 = 3.41, P = 0.33). In late-stage infections, on days 6 and 9 PI, *P. duboscqi* females of both groups showed very high infection rates (almost 100%), high parasite loads (about 80% of heavy infections with metacyclic forms present in thoracic midgut) and a majority of females had the stomodeal valve colonized by promastigotes (Figure 5).

The effect of blood concentration (5% vs. 100%) on parasite establishment in sand fly midguts

In experiments using *Leishmania* promastigotes in 5% rabbit blood, infection rates and parasite loads of infected females after the second bloodmeal were lower in comparison with females infected by feeding on 100% blood. Therefore, we decided to test if the infection rates and parasite loads differ already in the early stage of infection (day 1 and 2), thus before the time of the second feeding (as described in a previous experiment).

Two groups of females were infected by feeding on 5% or 100% rabbit blood with promastigotes and were dissected on days 1 and 2 PI. Data from two independent experiments were pooled and significant differences in infection rates (χ^2 = 32.48, P < 0.001) and intensities of infection in both of compared days PI (day 1: χ^2 = 41.92, P < 0.001; day 2: χ^2 = 51.97, P < 0.001) were observed between the groups (5% vs. 100% blood). While only 65% of females fed on 5% blood with promastigotes were infected and the intensities of their infection were usually light or moderate, females fed on 100% blood were all infected and the intensities of their infections were higher (Figure 6).

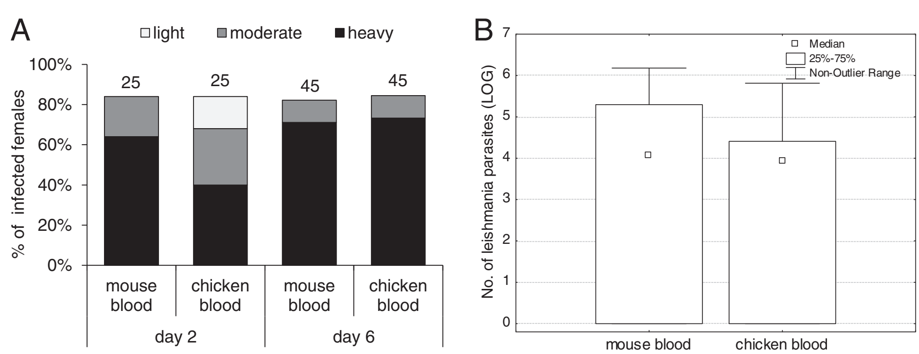

Figure 4 The effect of chicken blood on *Leishmania* development in sand fly midguts: 6 days after infection. Six days after the infection by promastigotes in 10% rabbit blood (10^6 parasites/ml) *P. duboscqi* females were allowed to feed on chickens or mice. Infection rates and intensities of infection were evaluated in sand fly midguts on days 2 and 6 after the second bloodmeal. **A**: Intensities of infection were microscopically classified into three categories: light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females. **B**: The precise number of parasites from 50 females of both groups was measured by Q-PCR 6 days after the second bloodmeal.

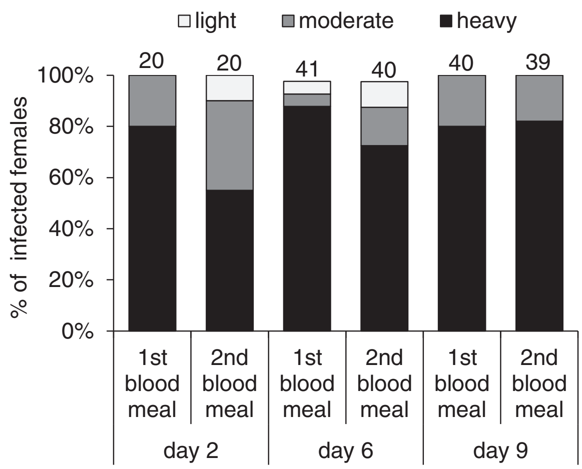

Figure 5 Susceptibility to *L. major* infection during the first and second bloodmeal. *P. duboscqi* females were infected (10^6 promastigotes/ml) during the first or second bloodmeal. Infection rates and intensities of infection were evaluated microscopically in sand fly midguts on days 2, 6, and 9 PI, and infections were classified into three categories: light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females.

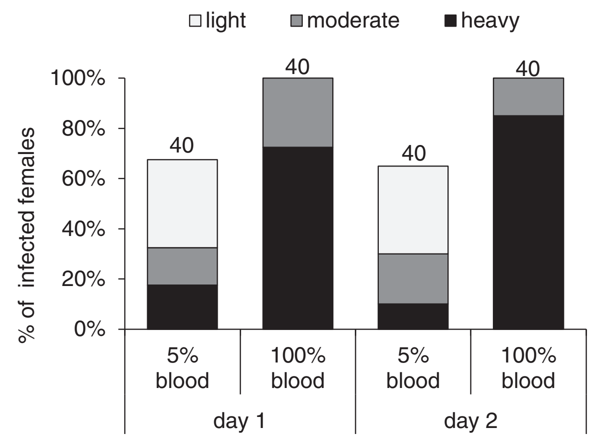

Figure 6 The effect of 5% or 100% blood in infective bloodmeals on parasite establishment in the sand fly midgut. *P. duboscqi* females were infected (10^6 promastigotes/ml) by feeding on 5% or 100% blood. Sand fly midguts of infected females were investigated microscopically on days 1 and 2 PI, and intensities of infections were classified into three categories: light (< 100 parasites/gut), moderate (100–1000 parasites/gut), or heavy (> 1000 parasites/gut). Numbers above the bars indicate the number of dissected females.

Differences in digestion of 10% and 100% blood

In experiments using promastigotes in 10% rabbit blood, infection rates and parasite loads of infected females after the second bloodmeal were slightly lower in comparison with females infected by feeding on 100% blood. Therefore, to explain this observation we decided to examine course of trypsin activity and the time of defecation after feeding on diluted or undiluted blood. Data from two independent experiments were pooled. Midgut trypsin activity in females fed on 10% blood was considerably lower and peaked earlier (Figure 7). The highest values were measured at 30 hours PBM, and by 72 hours PBM was almost zero. In contrast, midgut trypsin activity in females fed on 100% blood was the highest at 48 hours PMB and at 72 hours PMB was still relatively high. The maximum trypsin activity in females fed on 100% blood was ten times higher than those fed on 10% blood (Figure 7).

In addition, we compared the time of defecation: females fed on 10% blood defecated one or two days earlier compared to those fed on 100% blood. Females fed on full blood defecated on days 4 – 5 post bloodmeal while those fed on 10% blood defecated on day 3 post bloodmeal. Consequently, this provides considerably less time for *Leishmania* to escape from the peritrophic matrix and to establish an infection within the sand fly midgut.

Discussion

Bloodmeals from different animal sources has been reported to affect the digestion, reproductive potential of females and development of *Leishmania* parasites in the midgut [3-7,15,23]. In this study, the effect of chicken blood on digestion, oocyte development and *Leishmania* infection within the sand fly gut was evaluated.

According to Sant'Anna et al. [17], chicken blood has less than half the total protein of rabbit blood, but the midgut protein content of fully engorged *L. longipalpis* females fed on rabbit blood was only slightly lower than that of females fed on chicken blood. Clearly, *L. longipalpis* females were able to partially compensate for the lower protein content in the avian blood source through efficient

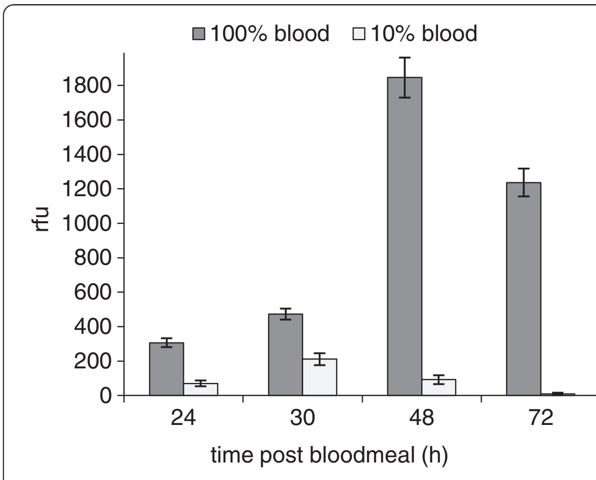

Figure 7 Differences in digestion of 10% and 100% blood. Trypsin activity was measured at 24, 30, 48, and 72 hours PBM in the homogenates of ten midguts of females fed on diluted (10%) or undiluted (100%) blood.

prediuresis [17]. In the experiments presented here *P. duboscqi* females fed on chickens had half the midgut protein content compared to those fed on rabbits, which corresponds to the concentrations measured in chicken and rabbit blood. As prediuresis has also repeatedly been described in *P. duboscqi* females [24,25], it seems that this species is not able to concentrate avian blood more than rabbit blood.

The lower protein content in the avian blood source influenced the midgut trypsin activity and oocyte development of *P. duboscqi*. Females fed on chickens had significantly lower trypsin activity in the midgut (18, 24, 30, and 72 hours PBM) and slower oocyte development (data not shown) in comparison with females fed on rabbits. These results are consistent with studies on the dependence of enzymatic activity on bloodmeal protein content in mosquitoes, where the proteolytic activity is activated by ingested proteins and that rate of proteolytic activity correlates with protein concentration in the bloodmeal [8,9].

In sand flies, proteins from the bloodmeal are digested for 48–96 hours [26-28], and it is during this time when *Leishmania* parasites encounter sand fly digestive enzymes. Some authors have shown that the digestion of blood from some hosts may adversely affect the development of *Leishmania* [14,15,23]. On the other hand, *Leishmania* was shown to modulate trypsin secretion of the sand fly vector to its own benefit. This effect has been described in the New World (*L. longipalpis* and *L. mexicana*) [29] as well as in the Old World parasite-vector pairs (*P. pernicious* and *L. infantum*) [30].

According to Schlein et al. [15] and Schlein and Jacobson [23] digestion of avian blood is harmful to *Leishmania* parasites within the sand fly midgut. They fed *P. papatasi* females on turkeys or chickens either before or after an infective meal containing rabbit blood with *Leishmania* promastigotes and in both experimental schemes described a reduction of *Leishmania* infection [15,23]. In contrast, chicken blood did not reduce the infection of *L. braziliensis*, *L. amazonensis* and *L. mexicana* in the New World sand fly species *L. longipalpis* and *L. migonei* [16,17]. Although Nieves and Pimenta [16] noted a slightly lower percentage of infected females after feeding an amastigote-infected chicken bloodmeal compared to females infected via rodent blood (*Cercomys* sp.), infections were not eliminated, and *L. braziliensis* and *L. amazonensis* established midgut infections. Sant'Anna et al. [17] also did not detect any negative effect of avian blood on *L. mexicana* infection in the midgut of *L. longipalpis*; on the contrary, in females infected via chicken blood they reported a trend towards higher infection rates and higher parasite loads in comparison with controls fed on infective rabbit blood [17]. While Sant'Anna et al. [17] used amastigote-initiated infections, in our study the infections were promastigote-initiated.

In the present work the effect of avian blood on the development of *L. major* in *P. duboscqi* was studied using light microscopy and Q-PCR in several experiment schemes where sand fly females were fed on chickens or mice either before or after infection. No significant differences were observed in any of these experiments and we can conclude that digestion of avian blood is not harmful to *L. major* development either before or after infection. The differences between our and Schlein's results cannot be explained by different techniques or parasite vector pairs. We used the same *Leishmania* strain (LRC-L137), and *P. duboscqi* is the sister species of *P. papatasi* within the subgenus *Phlebotomus*, both being natural vectors of *L. major* [31,32].

Phlebotomus duboscqi females infected using the method of Schlein et al. [15] (infection by promastigotes in 5% rabbit blood and one day later fed on avian blood) had a relatively low (about 60%) infection rate in both groups, regardless of whether fed on chickens or mice. However, Schlein et al. [15] tested only the group fed on turkeys before or after infection and did not include any control group fed on a mammalian host. Therefore, their conclusions may have been influenced by the absence of appropriate controls. To confirm this assumption, we studied the effect of diluted blood on *Leishmania* development in the early stage of infection within the sand fly midgut. While females infected via 100% blood were all infected with high intensities of infection, females fed on 5% blood with promastigotes were infected in only 65% and parasite loads were light or moderate. This experiment revealed that diluted blood in infective meal leads to significantly lower infection rates and parasite loads, probably as a consequence of faster digestion. The peritrophic matrix of *P. duboscqi* females fed on full blood matures in about 12 hours PBM, and its disintegration started only at the third day PBM [33] and females defecated on days 4 – 5 PBM. On the other hand, in females infected via diluted blood *Leishmania* promastigotes have a very limited time to escape the peritrophic matrix and establish an infection within the midgut.

To complete the study on the influence of avian blood on *Leishmania* development we considered the effect of number of feedings and age of females on parasite development within the sand flies. Such effects have previously been described in other bloodsucking arthropods: tsetse flies (*Glossina* spp.) given trypanosomes in their first bloodmeal were found to be more susceptible to infection compared to flies given trypanosomes in a later bloodmeal [34]. More recent studies by Walshe et al. [35] and Kubi et al. [36] showed that it is rather the age (hours after eclosion) of the flies when they take the first infective bloodmeal or nutritional stress that determines the susceptibility to infection. It seems that a higher susceptibility to infection is caused by the physiological immaturity and imperfect immune response of teneral (newly emergent and unfed) tsetse flies [36]. Based on this knowledge, we decided to test the susceptibility of *P. duboscqi* to *L. major* infection during the first or the second bloodmeal; however, no differences between these two experimental groups of females were observed. This finding corresponds with the lack of any information regarding significant differences in infection rate after the first or the second blood feeding in Nematocera. Such a contrast between brachyceran and nematoceran flies (tsetse and sand flies, respectively) could be explained by differences in bloodmeal digestion mode and the type of peritrophic matrix (PM). Sand flies as well as mosquitoes and other nematoceran haematophagous insects have discontinuous bloodmeal digestion and form PM type 1, while tsetse flies digest blood continuously and form PM type 2 (reviewed by [37]).

Conclusions

Phlebotomus duboscqi females fed on chicken had lower trypsin activity and slower oocyte development in comparison to those fed on mouse. Importantly, various experiments showed that the feeding of *Phlebotomus* sand flies on avian blood is not harmful to *Leishmania* development within their midgut. These experiments indicated that the reduction in *Leishmania* infection reported by Schlein et al. [15] and Schlein and Jacobson [23] was probably not caused by the inclusion of avian blood but by the experimental scheme using diluted blood.

In addition, the susceptibility of *P. duboscqi* females to *L. major* infection is equal during the first or the second bloodmeal; the number of feedings or female age did not affect the development of *Leishmania*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KP performed the experimental work, analysed the data and wrote the manuscript. JV performed and analysed Q-PCR data, performed statistical analysis. PV designed the study, contributed to interpretation and wrote the manuscript. All authors read and approved the final version of the manuscript.

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