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Development of *Leishmania* from *L. donovani* complex in various vectors

Vývoj leishmanií z komplexu *L. donovani* v různých přenašečích

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PH.D. THESIS/DIZERTAČNÍ PRÁCE

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Author´s declaration / Prohlášení autorky:

I declare that the submitted thesis summarizes the results of experimental work done by my own or in collaboration with co-authors of the presented original papers and that I properly cited all scientific literature used. Neither this thesis as whole nor it substantial part has been submited for the award of any other degree or diploma.

Prohlašuji, že předkládaná práce je souhrnem výsledků, kterých jsem dosáhla samostatně nebo ve spolupráci se spoluatory přiložených článků 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, February 27, 2013

Mgr. Veronika Hrobáriková (Šeblová)

Supervisor´s declaration / Prohlášení školitele:

I declare that Veronika Hrobáriková (Šeblová) substantially contributed to the experimental work in the five projects presented here in her thesis and had a principal role in writing two of five publications presented.

Prohlašuji, že Veronika Hrobáriková (Šeblová) se významně podílela na experimentální práci na pěti projektech shrnutých v této dizertační práci a je hlavní autorkou textu dvou publikací.

Prague, February 27, 2013

Prof. RNDr. Petr Volf, CSc.

Poděkování

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Contents

List of Abbreviations

PM peritrophic matrix

PBM post-bloodmeal

CL cutaneous leishmaniasis

VL visceral leishmaniasis

CVL canine visceral leishmaniasis

PKDL post kala-azar dermal leishmaniasis

PSG promastigote secretory gel

kDNA kinetoplast DNA

MW Melka Werer - non-endemic locality for VL in Ethiopian lowlands

AZ Addis Zemen - endemic locality for VL in Ethiopian highland

NAD+ nicotinamide adenine dinucleotide

Trp tryptophan

Asp aspartic acid

Nac nicotinic acid

NAm nicotinamide

NR nicotinamide riboside

NaMN nicotinic acid mononucleotide

NaAD nicotinic acid adenine dinucleotide

NMN nicotinamide mononucleotide

PNC nicotinamidase enzyme

LiPNC *L. infantum* nicotinamidase enzyme

SIR2 silent information regulators

Abstract

This thesis focuses on the development of protozoan parasites from *Leishmania donovani* complex in their insect vectors and summarizes results of five parts of the project I participated in during my Ph.D. studies.

Sand flies of genera *Phlebotomus* and *Lutzomyia* are the only proven vectors of leishmaniasis, however, the role of alternative vectors, like ticks, fleas and biting midges is frequently discussed in the literature. In this work, we showed that Eurasian species of biting midge *Culicoides nubeculosus* does not support late stage infections of *L. major* and *L. infantum*. We also demonstrated that microscopical observation of *Leishmania* promastigotes in the digestive tract of bloodfeeding arthropods remains a crucial method for any conclusion about the vector competence of the suspected insect.

In the second part of our study were compared the life-cycle parameters and vector competence of two Ethiopian *P. orientalis* colonies for *L. donovani*. Marked differences between colonies were found in life-cycle parametes, however, molecular analyses did not reveal any genetic differences. Experimental infections showed that both *P. orientalis* colonies are very susceptible to *L. donovani* infection and even the lowest infective dose tested (2×10^3 promastigotes/ml; corresponding to 1–2 promastigotes) was sufficient to establish *L. donovani* infection in about 50% of females.

Furthermore, we demonstrated that three *Leishmania* species tested, *L. major*, *L. infantum* and *L. donovani*, are not able to produce late-stage infections in *Sergentomyia schwetzi*, which is considered as a potential vector of human leishmaniasis in some areas. We observed that crucial parameter for complete development of *Leishmania* in *S. schwetzi* seems to be the short period between degradation of peritrophic matrix (PM) and defecation. The persistence of PM till the end of digestion leads to expulsion of parasites from the sand fly midgut together with bloodmeal remnants.

Finding of accurate natural infective dose is crucial for development of the ideal experimental model of visceral leishmaniasis. Therefore, we quantified number of transmitted dermotropic and viscerotropic *L. infantum* parasites delivered to the mouse skin by individual sand flies, *P. perniciosus* and *L. longipalpis*, during feeding. From total number of biting females, 15% to 65% of them inoculated promastigotes to the mouse skin and the parasite number transmitted by individual sand flies was very heterogenous, ranging from 4 up to 4.2×10^4 promastigotes. Surprisingly, in both sand fly species the parasite load transmitted was higher for the strain with dermal tropism; 29% of *L. longipalpis* and 14% of *P. perniciosus* transmitted more than 1000 parasites.

Finally, we explored the role of *L. infantum* nicotinamidase enzyme (PNC1) during intravectorial development. We showed that the null *L. infantum* mutants in PNC1 gene are not able to establish the late stage infection in *P. perniciosus* and our data indicate that the nicotinamidase enzyme is essential for the completion of the *L. infantum* development in the sand flies.

Abstrakt

Tato práce se zabývá vývojem parazitických prvoků z komplexu *Leishmania donovani* v jejich hmyzích přenašečích a shrnuje výsledky pěti částí mého doktorského projektu.

Jedinými potvrzenými přenašeči leishmaniózy jsou dva rody flebotomů, *Phlebotomus* a *Lutzomyia*, i když otázka alternativních přenašečů jako jsou klíšťata, blechy a tiplíci, je v současnosti v literatuře velmi intenzivně diskutována. V této práci jsme prokázali, že v eurasijském druhu tiplíka *Culicoides nubeculosus* se není schopna vyvíjet *L. major* ani *L. infantum*. Názorně jsme demonstrovali, že mikroskopie zůstává i nadále zásadní metodou pro posuzování vektorové kompetence daného krevsajícího přenašeče.

Ve druhé studii byly porovnávány případné odlišnosti v biologii, životním cyklu a vektorové kompetenci dvou kolonií *P. orientalis* pocházejících z Etiopie. Přestože molekulární metody neodhalily žádné genetické odlišnosti, pozorovali jsme markantní rozdíly v biologii a životním cyklu těchto dvou kolonií. Experimentální infekce prokázaly, že druh *P. orientalis* je velice náchylný k infekci parazity *L. donovani* a že i nejmenší použitá infekční dávka (odpovídající 1–2 promastigotům) je schopna vyvolat infekci přibližně u 50% samic.

V dalších pokusech bylo prokázáno, že *Sergentomyia schwetzi* považovaná v některých oblastech za možného přenašeče lidské leishmaniózy, není vnímavá k infekci *L. major*, *L. infantum* a *L. donovani*. Bylo pozorováno, že zásadním kritériem omezujícím vývoj leishmanie v tomto přenašeči je doba mezi degradací peritrofické matrix a defekací flebotoma. Přetrvávání peritrofické matrix do konce trávicího procesu vede k vypuzení parazitů ze střeva flebotoma spolu s nestrávenými zbytky krve.

Znalost přirozené infekční dávky je nutná k tomu, aby mohl být navržen ideální experimentální model pro viscerální leishmaniózu, který co nejvíce napodobuje přirozené podmínky. Proto jsme kvantifikovali množství promastigotů *L. infatum* přenesených do kůže myši během sání flebotomů, *P. perniciosus* a *L. longipalpis*. Prokázali jsme, že 15%–65% sajících samic je schopno přenést infekci do hostitele a že množství přenesených parazitů je velmi heterogenní v rozmezí od 4 do $4,2 \times 10^4$. Překvapivě nejvíce parazitů přenesly u obou druhů flebotomů samice infikované dermotropní variantou *L. infantum*; 29% samic *L. longipalpis* a 14% samic *P. perniciosus* přeneslo více než 1000 parazitů.

V poslední části jsme se zabývali významem enzymu nikotinamidázy u leishmanií a její funkcí během vývoje ve flebotomech. Mutantní linie *L. infantum* s knockoutovaným genem pro nikotinamidázu se nebyly schopny ve flebotomech vyvíjet do pozdějších stádií, čímž jsme prokázali nezastupitelnou důležitost tohoto enzymu pro vývoj leishmanie v přenašeči.

1 Introduction

1.1 Visceral leishmaniasis

Visceral leishmaniasis (VL) is one of the most important protozoan vector-borne diseases affecting humans and animals. It is caused by parasites of the *Leishmania donovani* complex which are transmitted by sand flies of genera *Phlebotomus* and *Lutzomyia*. According to molecular analysis this complex includes two *Leishmania* species, *L. donovani* and *L. infantum*. In Latin America the etiological agent of VL was known as *L. chagasi*, however, RAPD and sequence analyses revealed identity to *L. infantum,* which was probably introduced to America by European colonists and their dogs (Maurício et al., 2000).

In total, 58 000 new VL cases per year are reported according to official counts (Alvar et al., 2012), however, this number is supposed to be underestimated and the real yearly incidence is supposed to be 10-fold higher (Dujardin et al., 2008). Approximately, 90% of VL cases occur in rural and suburban areas of five countries, namely Bangladesh, India, Nepal, Sudan and Brazil (WHO, 2013). In humans, the infection with *L. infantum* and *L. donovani* usually manifests as visceral disease targgeting macrophages and resulting in generalised signs and symptoms like fever, loosing of weight, splenomegaly, and pancytopenia. If untreated it may leads to death.

Importantly, *L. infantum* and *L. donovani* evoke also only milder cutaneous form of leishmaniasis. In such cases, lesions occuring in patiens are relatively small, non-ulcerating with tendency to self-healing. They affect area of body exposed to sand fly bites, mainly limbs and face. This form of leishmaniasis is reported worldwide, including countries in Mediterranean basin (Rioux et al., 1980; Gramiccia et al., 1987; Gradoni et al., 1991; Zeledón, 1991; Elamin et al., 2008; Svobodová et al., 2009). The *L. infantum* tropism appears to be influenced by parasite genotype and host immune status (Gradoni and Gramiccia, 1994), however, this subject is not yet fully understood and need further investigation.

Epidemiologically, the diseases occur in two different forms as antroponotic and zoonotic leishmaniasis. The antroponotic leishmaniasis is caused by *L. donovani* and parasites circulate between sand flies and humans, who are the only natural reservoir. This antroponotic form of *L. donovani* is restricted to northern India, Nepal and Bangladesh and *P. argentipes* is a principal vector (reviewed by Hati, 1991; Swaminath et al., 1942). In East Africa (Sudan, Ethiopia, Kenya, Uganda and Somalia) and Arabian peninsula the distribution is associated with *P. orientalis* or/and *P. martini* (reviewed by Maroli et al., 2012). However, in several areas, such as eastern Sudan, *L. donovani* transmission cycle is considered to be zoonotic (Dereure et al., 2003; Elnaiem et al., 2001).

Zoonotic form of VL leishmaniasis caused by *L. infatum* has wide spectrum of animal reservoirs and occurs in several countries of Central and South America, where eight *Lutzomyia* species serve as vectors. In the Old World, in the countries of Mediterranean basin and central Asia, twenty *Phlebotomus* species are implicated in

transmission of *L. infantum* (reviewed by Maroli et al., 2012). In Europe, dogs, with seroprevalence up to 25% in some areas (Miró et al., 2012) are considered to be the main domestic and peridomestic reservoir of *L. infantum* with veterinary importance (Baneth et al., 2008). As demonstrated by Molina et al. (1994), both symptomatic and asymptomatic dogs can act as reservoir of infection for phlebotomine vectors. The role of cats as a leishmaniasis reservoir is still unclear. There is much evidence that domestic cats from endemic areas are very often infected by *L. infantum* (reviewed by Maia and Campino, 2011) and studies performed by Maroli et al. (2007) and da Silva et al. (2010) showed that chronically infected cats were infectious to laboratory-bred *P. perniciosus* and *L. longipalpis*. However, there is no data regarding the infectiousness of cats to vectors from nature and only scanty recording cases throughout geographic regions are available. Therefore, participation of cats in transmission cycle of *L. infantum* deserves more attention.

In contrast, the sylvatic reservoirs of *L. infantum* represent wild carnivores (jackals, wolves, foxes, genets, lynxes etc.) and rodents (summarized in Quinnell and Courtenay, 2009). More recently, Molina et al. (2012) carried out xenodiagnosis study and showed the existence of potential sylvatic transmission cycle of *L. infantum* in hares (*Lepus granatensis*).

In Europe, visceral leishmaniasis was previously restricted to the countries around the Mediterranean Sea. However, global warming and other environmental and social factors like deforestation, urbanization or travelling of humans accompanied by dogs on long distance (Mettler et al., 2005) are responsible for spreading of susceptible vectors and *Leishmania* infection to new areas. This may contribute to the increasing incidence and northward spreading of visceral leishmaniasis (reviewed by Ready, 2010), as shown by reports from Italy (Maroli et al., 2008), southern Germany (Bogdan et al., 2001; Naucke et al., 2008) and Hungary (Farkas et al., 2011), where autochthonous cases of human, canine, feline and equine leishmaniasis are repeatedly reported (reviewed by Gramiccia and Gradoni, 2005).

Due to international projects has our group an opportunity to participate in studies dealing with problematic situation of VL in Ethiopia. Therefore, one part of my project was specifically focused on sand flies transmitting VL in this country.

1.2 Visceral leishmaniasis in Ethiopia and its vectors

Some of the most important foci of VL in Africa are situated in East Africa, mainly in Sudan and Ethiopia. In Ethiopia both visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) are endemic. CL in Ethiopia is diagnosed annually in about 50 000 patients (Alvar et al., 2012). It has a wide spectrum of manifestations including localized nodular cutaneous leishmaniasis (LCL) with a tendency to self-healing, mucocutaneous (MCL) and diffuse cutaneous leishmaniasis (DCL) leading to disseminated lesions spreading into the nasal and oral mucosa. Three *Leishmania* species are responsible for infection of cutaneous leishmaniasis in Ethiopia. The most severe form of DCL occuring mainly in the Ethiopian highlands is caused by *L. aethiopica.* These parasites are transmitted by *P. longipes*, *P. pedifer* and *P. sergenti* and hyraxes, *Procavia capensis* and *Heterohyrax brucei*, serve as reservoir (Ashford et al., 1973a). In the lowlands, humans are affected rather by *L. major* transmitted by *P. duboscqi* (Gebre-Michael et al., 1993) and by *L. tropica* (Hailu et al., 2006), where *P. sergenti* and *P. saevus* serve as vectors (Gebre-Michael et al., 2004).

More importantly, Ethiopia has the second largest number of VL cases in East Africa with an estimated 4500 to 5000 new cases every year. VL is in this country associated with high mortality and morbidity, and is worsened by malnutrition, isolated location of VL endemic areas, unavailability of treatment and medicine, presence of resistant strains and co-infections with HIV (reviewed by Cruz et al., 2006; Alvar et al., 2008).

In Ethiopia VL is reported from ten main foci (Fig. 1) (Malaria Consortium, 2010). The north-western focus covers the semi-arid Metema (region Tigray) and Humera plains (region Amhara) (area 1) bordering with Sudan at altitude of 500–700 m (Ashford et al., 1973a; Haile and Anderson, 2006). VL in this area predominantly affects male migrant workers and disease is in 40% cases associated with HIV coinfection leading to high mortality. Endemic areas occur stably in highlands at altitude of 1500–2000 m in Libo Kemken (Addis Zemen is capital of Libo) and Fogera Woredas (area 2) (region Amhara) (Herrero et al., 2009). VL was probably introduced to this area with seasonal workers returning from Metema-Humera (Bashaye et al., 2009). In 2010, 30 cases of VL were reported from Sheraro locality on the border with Eritrea (region Tigray) at altitude of 1200 m (area 3).

The southwest foci includes the Omo plains (area 4), Aba Roba plains and Segen and Woyto Valey (area 5) in basin of the Chew Bahir lake near Konso Woreda in region SNNPR (Southern Nations Nationalities and People's Region) at altitude of 375–1000 m. All areas are located in lowland savannah with low rainfall (Fuller et al., 1979; Lindtjorn and Olafsson, 1983; Hailu et al., 2009).

In the northeast, VL cases are recorded in the Awash Valley (area 6) (region Amhara and Afar) at altitude of 700–850 m (Fuller et al., 1976). This area is characterized by higher temperatures and very suitable conditions for sand flies. Positivity of humans was assessed in a survey conducted in 1994–1995 using a cross-sectional leishmanin skin test. The positivity ranged from 16% to 65% with the over all prevalence of 33% in the study area. Despite of highest number of positive leishmanin skin tests in Melka Werer (up 63%) population, paradoxically, no confirmed cases of VL were reported and this area is supposed to be non-endemic (Ali, 1997).

VL cases are also reported in the southeast Ethiopia from Liban Woreda (area 7) situated on the border between Kenya, Somalia and southeast Ethiopia (Marlet et al., 2003) and from Gode (area 8) and Afder (area 9) zones from region Somali (reviewed by Malaria Consortium, 2010). Additionally, the VL focus was reported in Oromia region around city Negele Borena (area 10) (Hailu, 2008; Gelanew et al., 2010).

VL in East Africa is frequently accompanied by occurrence of post kala-azar dermal leishmaniasis (PKDL) characterized by a macular, maculopapular, and nodular rash. If occurs in patients who recovered from VL usually starts to appear around the mouth (Zijlstra and El-Hassan, 2001). PKDL patients and perhaps asymptomatic carriers harbour parasites in their skin where they are accesible to biting sand flies (Sharma et al., 2000b,a). This is also why all VL foci in these areas were originally supposed to be antroponotic. More recently the antroponotic cycle is belived to be important during the period of VL epidemics. In contrast, zoonotic cycle probably maintains infection in nature constantly in animals like Aegyptian mangoose, genets or wild rodents (Hoogstraal and Heyneman, 1969; Elnaiem et al., 2001). Nevertheless, the role of dog as well as the presence of the animal reservoir in East Africa are not entirely understood and need further investigation (Dereure et al., 2003).

Multilocus microsatellite typing demonstrated high genetic diversity among the East African strains of *L. donovani* and showed two main populations in Ethiopia, ge-

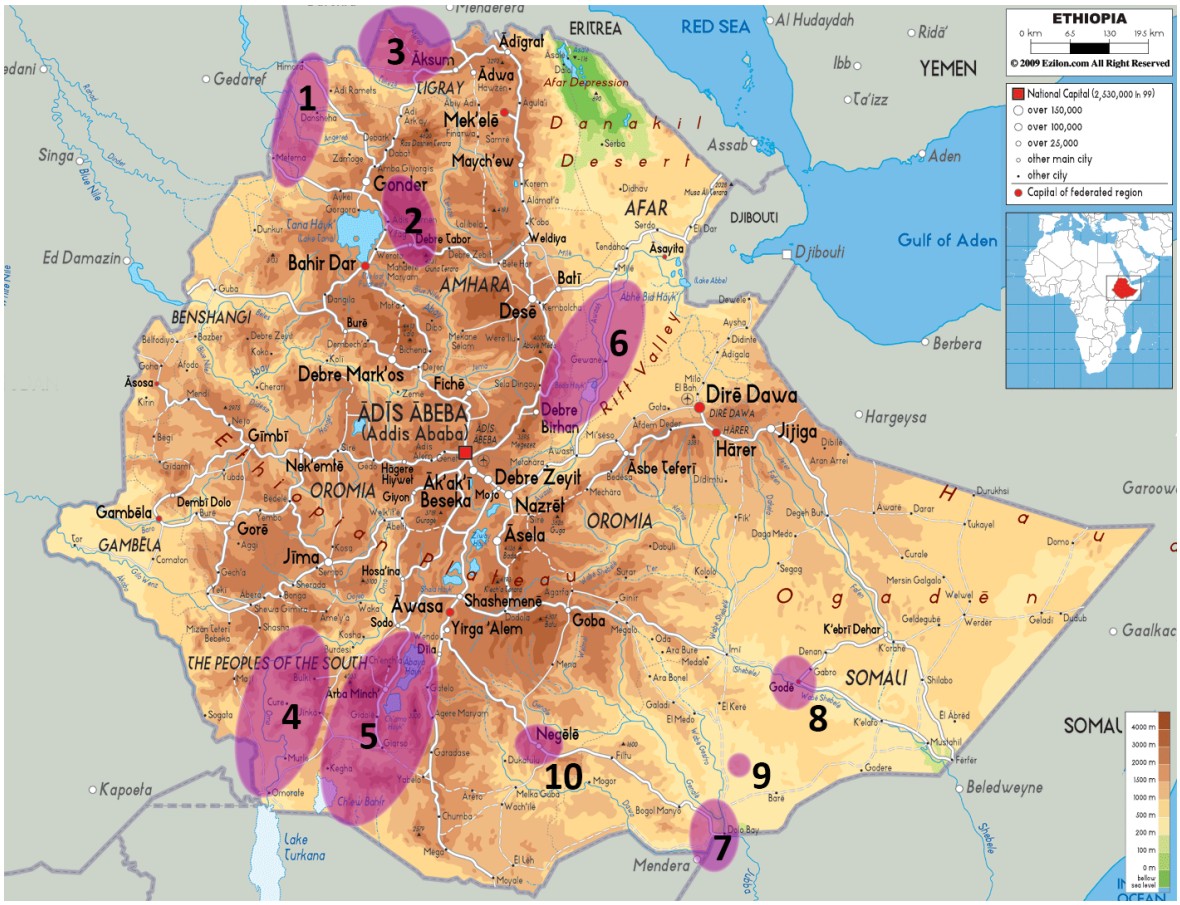

Figure 1: Distribution of the main VL foci in Ethiopia.

netically and geographically separated. The first genetically isolated group includes strains from northern Ethiopia and Sudan, while the second group comprises strains from the south Ethiopia and Kenya (Gelanew et al., 2010). The distribution of both genetically different groups of *L. donovani* correlates with sand fly species involved in transmission cycle in the given area. While in the semi-arid regions in the north Ethiopia and Sudan is VL transmitted mainly by *Phlebotomus orientalis* (Hoogstraal and Heyneman, 1969; Elnaiem et al., 1998; Gebre-Michael et al., 2007), in savanna and forest areas in the south Ethiopia and Kenya *L. donovani* transmission is ensured rather by *P. martini* and *P. celiae* (Gebre-Michael and Lane, 1996; Ngumbi et al., 2010). Moreover, Elnaiem et al. (2011) reported *P. rodhaini* as a possible zoonotic vector involved in sylvatic *L. donovani* transmission cycle in eastern Sudan.

However, frequent findings of infected *P. orientalis* females with high infection rate of *L. donovani* (up to 8%), especially in areas the neighbouring Sudan (Hoogstraal and Heyneman, 1969; Elnaiem et al., 1998; Hassan et al., 2008), makes form this sand fly the vector of a high importance responsible for the transmission of VL in majority of cases. In Ethiopia, the capture of infected *P. orientalis* females is reported very rarely; only Hailu et al. (1995) found one positive *P. orientalis* in southwest foci in the lower Omo plains. Further investigations focused on determination of *L. donovani* infection rate in *P. orientalis* from Libo-Kemkem (Ashford et al., 1973b; Gebre-Michael et al., 2007) and Metema-Humera foci (Gebre-Michael et al., 2010) did not find any positive female.

The susceptibility of *P. orientalis* to *L. donovani* was confirmed under laboratory

conditions by feeding on post kala-azar patients or their blood through mouse-skin membranes (Kirk and Lewis, 1955; Hoogstraal and Heyneman, 1969); wild-caught Sudanese *P. orientalis* were able to develop mature *L. donovani* infection. Parasites showed signs of suprapylarian development pattern including anterior migration of promastigotes to SV and production of PSG plug (Kirk and Lewis, 1955; Hoogstraal and Heyneman, 1969; McConnell, 1964). However, these pioneering studies were done using a limited number of *P. orientalis*.

Most biological information regarding habitat, feeding preferences and seasonality of *P. orientalis* was acquired thanks to demanding field studies conducted in Sudan (reviewed in Elnaiem, 2011). It was proved, that occurence of *P. orientalis* is very closely associated with vegetation type. *Phlebotomus orientalis* was observed particularly in areas rich with *Acacia seyal – Balanites aegyptica* that are groowing on black cotton soil (Quate, 1964; Ashford, 1974; Schorscher and Goris, 1992; Elnaiem et al., 1999) . This type of vegetation provides appropriate temperature and humidity as well as enough sugar sources like a fruit sugar from *Balanites aegyptiaca* and *Combretum kordofanum* and aphid or cocid honeydew (Hamilton and Elnaiem, 2000). However, *P. orientalis* is not restricted only to forest habitat but it is also abundant in villages inside houses (Lambert et al., 2002; Widaa et al., 2012), where females infected by *L. donovani* were repeatedly caught (Elnaiem and Osman, 1998).

Phlebotomus orientalis prefers to feed on humans (Ashford, 1974), cattle and other domestic animals (Gebre-Michael et al., 2010; Mukhtar et al., 2000) starting to bite soon after sunset with gradually decreasing intensity during the night (Quate, 1964; Elnaiem et al., 1999) and increasing activity again short before sunrise. Other authors (Schorscher and Goris, 1992) observed *P. orientalis* in waves of activity throughout the all night.

In Paloich area in central Sudan *P. orientalis* was described as seasonal species beginning to appear in the first part of the dry season (January and February) and than in maximum numbers from April to June (Quate, 1964) and disappears gradually at the beginning of the rainy season. The seasonal fluctuation closely correlates with the mean monthly temperature and relative humidity of the area. In contrast, in Umsalla (eastern Sudan, near Ethiopian border) is *P. orientalis* presented in small numbers whole year (Elnaiem et al., 1997).

Only little is known about the resting and breeding sites of *P. orientalis*. Sand flies were rarely found resting in the mounds made by the termite *Macrotermes herus* (Elnaiem et al., 1997), tree cavites (Quate, 1964) or in porcupine holes (Ashford et al., 1973b) providing to sand flies relatively good microclimatic conditions with low temperature and high humidity during the hot days. Even less is known about the breeding sites of *P. orientalis*. It is supposed that larval development takes place in deep cracks of vertisols (Elnaiem et al., 1999) but this has not been confirmed. In the field, males with unrotated urogenitalia could be considered as indicator for breeding sites. However, *P. orientalis* with unrotated genitalia has never been found in nature yet. Thus the breeding sites are still unknown which complicates strategies of sand fly control in affected areas. Taken together, very little is known about *P. orientalis* biology. Despite *P. orientalis* has a reputation of being difficult to colonize and maintain (McConnell, 1964; Schmidt, 1964), laboratory colonies of this species are very useful for further studies.

Some authors hypothesized about the transmission potential of *Sergentomyia* spp. These species are widespread and predominant in many African localities (Elnaiem et al., 1997; Elnaiem, 2011), including VL foci where *P. orientalis* seems

to be absent, like in the Malakal urban area in Sudan (Hoogstraal and Heyneman, 1969). Sand flies of genus *Sergentomyia* are proven vectors of reptilian *Leishmania*. The development of reptilian *Leishmania* spp. in *Sergentomyia* is usually restricted to hindgut (hypopylarian development pattern) and transmission is not by bite but rather by predation (reptiles feed on the infected sand fly) (reviewed by Bates, 2007), however, infection of oesophagus, pharynx and proboscis also has been reported (Zhang and Leng, 1997). Some species of *Sergentomyia* feed not only on reptiles but also very often on humans and mammals (Quate, 1964; Mutinga et al., 1986a; Hoogstraal and Heyneman, 1969; Hailu et al., 1995).

Sergentomyia spp. is considered to be involved in transmission of both cutaneous and visceral leishmaniasis to human in areas where *Sergentomyia* spp. are abundant and found to harbor *Leishmania* parasites (Mutinga et al., 1994). Additional support that *Sergentomyia* spp. could be involved in transmisssion of human leishmaniasis came from study performed by Mutinga et al. (1986b) in Baringo distinct in Kenya, where females *S. ingrami* were found infected by *L. major* in high infection rates (about 1%). Moreover, parasites isolated from *S. ingrami* were capable of causing typical *L. major* lesions in BALB/c mice. Alltogether, eleven species of *Sergentomyia* (*S. garhami*, *S. squamipleuris*, *S. africanus*, *S. kirki*, *S. ingrami*, *S. antennatus*, *S. bedfordi*, *S. schwetzi*, *S. affinis*, *S. graingeri* and *S. clydei*) have been showed microscopically to be infected by *Leishmania* promastigotes in Kenya (reviewed by Kaddu et al., 1986) and in Ethiopia (Hailu et al., 1995). However, these promastigotes were not characterized biochemically or genetically and therefore are not confirmed to be mammalian parasites. Additionally, PCR detected *L. major* DNA in *S. darlingi* in Mali (Berdjane-Brouk et al., 2012) and in *S. sintoni* in Iraq (Parvizi and Amirkhani, 2008) or *L. donovani* DNA in *S. babu* in India (Mukherjee et al., 1997). However, these results do not mean that *Sergentomyia* spp. is involved in transmission of *L. major* or *L. donovani* and observations have to be precisely confirmed microscopically.

Susceptibility of *Sergentomyia* spp. to *Leishmania* infection was observed also under laboratory conditions by Kaddu et al. (1986) who observed that *L. donovani* produced light parasitemia in three out of six *Sergentomyia* species (*S. schwetzi*, *S. adleri* and *S. ingrami*), however, infection was restricted only to abdominal midgut without real colonization of thoracic midgut and stomodeal valve. Similarly, Lawyer et al. (1990) showed in *L. major* infected - *S. schwetzi* originating from Kenya, a progress in parasite development in the first 48h PBM, however, transformation to long nectomonads was very rare and within 90h PBM the early promastigote stages died.

Some authors considered also a role of other bloodsucking arthropods in *Leishmania* transmission (see the following chapter 1.3).

1.3 Alternative vectors of leishmaniasis

It is widely accepted that phlebotomine sand flies are the only proven biological vectors of *Leishmania* (Killick-Kendrick, 1999). However, other ways like veneral (Silva et al., 2009) and congenital (Rosypal et al., 2005), artificial by needle intravenous-drug users (Molina et al., 2003) or infection by transfusion (Freitas et al., 2006) assert rarely in *Leishmania* transmission. Of interest, the mechanical transmission of *L. major* by *Glossina morsitans morsitans* (Lightner and Roberts, 1984) and *L. mexicana* by *Stomoxys calcitrans* to hamsters was also reported (Lainson and Southgate, 1965).

The role of alternative vectors of leishmaniasis is extensively discussed particularly in endemic regions where sand flies are apparently absent, but the prevalence of canine visceral leishmaniasis (CVL) is high (reviewed in Baneth et al., 2008). In these areas, involvement of other bloodfeeding arthropods, like ticks and fleas, in transmission cycle is very often discused in a literature. Particularly, the vectorial role of dog brown tick, *Rhiphicephalus sanguineus*, was investigated in relation to the epidemiology of CVL caused by *L. infantum* in both the New and the Old World (Coutinho and Linardi, 2007; Paz et al., 2010; Solano-Gallego et al., 2012).

Rhiphicephalus sanguineus is worldwide distributed species found in rural and suburban areas in tropical and subtropical regions as well as in some temperate areas. This cosmopolitan, three-host tick is strongly associated with canids, mainly dogs in peridomestic areas but occassionaly was observed be attached and fed on other wild or domestic hosts, including humans (reviewed by Dantas-Torres, 2010). As summarized in Dantas-Torres (2008), *R. sanguineus* is also very effective vector of many pathogens to dogs such as *Ehrlichia canis*, *Babesia canis*, *Hepatozoon canis* and many others.

Several studies were carried out in order to clarify the role of *R. sanguineus* in *Leishmania* transmission but in our opinion, the approach was not correctly chosen. Most studies were based on collection of ticks from dogs with CVL and subsequent molecular detection of *Leishmania* DNA from tick´s bodies. Unsurprisingly, these results indicate high infection rate of *L. infantum* in blood fed ticks using PCR detection (Coutinho et al., 2005; Coutinho and Linardi, 2007; Solano-Gallego et al., 2012; Trotta et al., 2012)) because promastigotes occur surely in a fresh blood taken from infected hosts. Moreover, PCR method is very sensitive allowing detection only a small amount of *Leishmania* DNA from small number of promastigotes or from death parasites. Authors never bring any data about multiplication or description of *Leishmania* morfological stages from ticks and fleas at different time point post-bloodmeal. Occassionaly, flagellates were observed microscopically but not characterized (Sherlock, 1964) and could be mistaken for monogenetic trypanosomatids like *Crithidia* or *Trypanosoma* as found in other Ixodidae ticks (Hubálek et al., 2003; Martins et al., 2008). Exception is the study performed by Paz et al. (2010), where PCR detection was accompanied by microscopical observation. Although the samples were positive for kinetoplast DNA, any viable *Leishmania* forms were not identified. This study clearly demonstrates that PCR detection of *Leishmania* DNA alone is not sufficient method for vector identification.

Study performed by Colombo et al. (2011) investigated the viability of *L. infantum* in ticks fed on CVL dogs using extraction of RNA. The detection of *Leishmania* RNA could indicate presence of live parasites because is very rapidly degraded in death cells instead of DNA, which persists in dead cells for several days as was demonstrated by bacterial cells (Josephson et al., 1993; Nocker and Camper, 2006; Romero et al., 2010). According to RNA analysis, live *Leishmania* parasites were detected in ticks (the tick species is unknown) up to 10 days after feeding as well as in ticks after ecdysis. These results suggest that *Leishmania* could survive in tick´s digestive tract for a long time, however, more detailed investigation is required.

There is also a Ph.D. work by McKenzie (1984), repeatedly cited in recent articles, which reported that *L. infantum* parasites survive in *R. sanguineus* digestive tract for more than 20 weeks. Moreover, the author described the ability of infected ticks to transmit *L. infantum* infection to dogs. This work has never been published and is not publicly available, therefore, we can not assess its credibility and information value.

Similarly, the possibility of transovarial and transstadial passage of *L. infantum* in *R. sanguineus* is suggested using quantification of kDNA in different developmental stages of ticks (Dantas-Torres et al., 2010, 2011). *Leishmania infantum* kDNA was detected also in salivary glands of ticks but there is no microscopical confirmation that promastigotes actually migrate to this stages and sites (Dantas-Torres et al., 2010). In our opinion, the migration of *Leishmania* to salivary glands is higly unlikely and the transovarial transmission is not possible at all.

Furthermore, the role of fleas in *Leishmania* transmission is considered by some authors (Coutinho and Linardi, 2007; Ferreira et al., 2009; Colombo et al., 2011), but again only on the basis of PCR positive samples collected from dogs seropositive for CVL.

More probable alternative way is an oral transmission of *Leishmania* infection during the grooming and defensive behavior as reaction on itching caused by bite and feeding of ectoparasites on canine skin. Infected parasites are accidentally licked or bitten out from fur and host could be infected through small wounds in canine mouth. This theory is in accordance with the experiments performed by Coutinho et al. (2005) and Coutinho and Linardi (2007) showing possibility to infect hamsters orally by homogenate derived from ticks or fleas infected by feeding on symptomatic CVL dogs. The infective potential of the homogenate from *Leishmania* positive ticks was earlier shown also by Blanc and Caminopetros (1930) where the infection of ground squirrel (*Spermophilus citellus*) was initiated by intraperitoneal inoculation.

Interestingly, the role of biting midges as alternative vectors of leishmaniasis was studied less frequently. Previously, biting midges of the genus *Culicoides* were suspected vectors of *L. donovani* in India. However, experimental evidence performed by feeding on kala-azar cases and subsequent microscopical examination did not show any infected midges (Christophers et al., 1925). More recently, precise study of Dougall et al. (2011) demonstrated that day-biting midges of subgenus *Forcipomyia* (*Lasiohelea*) Kieffer are implicated in transmission of *Leishmania* from *L. enrietti* complex causing cutaneous leishmaniasis in red kangoroos in Australia (Rose et al., 2004). Field collected *Forcypomyia* were dissected and microscopically examinated for presence of *Leishmania* promastigotes. Four females were found positive for *Leishmania* infection with intensity higher than 10^5 promastigotes per gut. Surprisingly, the infection pattern was similar as recorded in sand flies; parasites showed signs of migration to the thoracic midgut, including colonization of stomodeal valve and presence of PSG plug. Moreover, morfological determination revealed the presence of metacyclic promastigotes neccessary for the successful transmission from vector to a new host. However, the ability of *Forcypomia* midges to transmit *Leishmania* parasites to other hosts is still remains to be elucidated (Dougall et al., 2011)

It is important to keep in mind widely accepted criteria under which the arthropod could be incriminated as a biological vector of leishmaniasis (Killick-Kendrick, 1990). According to these criteria, the vector must be willing to feed on reservoir hosts and humans (and present in the same enviroment), the vector have to be found infected in nature with same strain of *Leishmania* as occurs in humans (this should be confirmed using molecular analysis) and the ability of *Leishmania* to undergo complete life-cycle in suspected vector must be tested experimentally. Finally, the vector has to be capable of transmitting parasite to a susceptible host during blood feeding. All these aspects mentioned above should be considered each time we think about possibility of alternative vectors.

In conclusion, bloodfeeders like ticks, fleas, biting midges, mosquitoes and others

ectoparasites are predisposed to uptake many microorganisms, including *Leishmania*, from blood of their hosts. However, detection of parasite DNA is not sufficient for incrimination of given arthropod to be the biological vector. Direct microscopical observation of *Leishmania* promastigotes and their localization in the digestive tract remains a crucial method for any conclusion about the vector competence of the putative vector.

1.4 *Leishmania* development in the sand fly and transmission by bite

Only proven vectors of leishmaniasis are two sand fly genera: *Phlebotomus* in the Old World and *Lutzomyia* in the New World. In the Old World, 42 phlebotomine species of the genus *Phlebotomus* are suspected or proven vectors of VL of which twenty are implicated in transmission of *L. infantum* and six in transmission of *L. donovani* (reviewed by Maroli et al., 2012). In the New World, 8 species of genus *Lutzomyia* are reported as proven or probable vectors (Killick-Kendrick, 1999; WHO, 2013). Furthermore, sand flies are also known as vectors of viral diseases, such as sand fly fever (Naples and Sicilian virus), summer meningitis (Toscana virus), vesicular stomatitis (Vesiculoviruses), viral encephalitis (Chandipura virus) and bacterial infection Carrión´s disease caused by *Bartonella* spp. (reviewed by Maroli et al., 2012)

Leishmania species infective for mammals are divided according to distribution and developmental pattern in sand fly gut into two subgenera, *Leishmania (Leishmania)* and *Leishmania (Viannia).* For subgenus *Viannia,* which is found only in the New World, is typical peripylarian developmental pattern, where are parasite restricted in the hindgut following by promastigote migration to the midgut and foregut. On the other hand, suprapylarian development of subgenus *Leishmania* takes place only in midgut and foregut (Lainson and Shaw, 1987). In this work, we focused exclusively on parasite-vector interactions of suprapylarian *Leishmania* parasites.

The vectorial part of *Leishmania* life cycle begins when sand fly female takes blood with amatigotes from infective host. Amastigotes transform within the bloodmeal in the abdominal midgut into various promastigote morfological forms (Bates and Rogers, 2004). Firstly, amastigotes differentiate into small procyclic promastigotes which multiply very intensively in the bloodmeal and establish the early stage of infection.

A bloodmeal is surrounded by peritrophic matrix (PM) type 1 (Jacobs-Lorena and Oo, 1996). This acellular structure is formed in sand flies by midgut epithelium as a result of gut distension during blood feeding and is composed of chitin microfibrils, proteins and glycoproteins. The main functions of PM are the protection of the midgut epithelium against pathogen and abrasion and compartmentalization of digestion between endo- and ectoperitrophic space (Lehane, 1997). In bloodsucking insects the PM is also involved in heme detoxification and protection midgut cells from toxic reactive oxidants species generated during bloodmeal (Felton and Summers, 1995). This barrier has positive functions not only for arthropods but also for parasites protecting them against attack of proteolytic enzymes during transformation from amastigotes to promastigotes (Pimenta et al., 1997). On the other hand, PM negatively affects the rapid escape of parasites to ectoperitrophic space. The breakdown of PM

coincides with the transformation of procyclic promastigotes into long, strongly motile nectomonads promastigotes probably as a result of contact with salivary components (Charlab and Ribeiro, 1993; Sádlová and Volf, 2009). The disintegration of PM is induced by sand-flies-derived chitinase (Ramalho-Ortigão and Traub-Csekö, 2003; Ramalho-Ortigão et al., 2005) and not by the chitinase of parasites as described previously by Schlein et al. (1991); in combination *P. duboscqi* - *L. major* it was proved that the same PM disintegration pattern is presented in uninfected and infected females (Sádlová and Volf, 2009).

After successful escape from the posterior end of PM, promastigotes move anteriorly and attach to the microvilli of the midgut to evoid expulsion from the gut during defecation process (Bates, 2008). In so called specific vectors (Volf and Myskova, 2007) this attachment appears to be mediated by lipophosphoglycan (LPG) on surface of *Leishmania* and galectins in sand fly midgut (Sacks et al., 1994; Kamhawi et al., 2004). On the other hand, in so called permissive sand fly species the mechanism remains to be elucidated (reviewed by Dostálová and Volf, 2012). *In-vitro* binding assays performed by Wilson et al. (2010) showed that *Leishmania* gut binding is strictly stage-dependent; it is typical for nectomonads and leptomonads in the middle phase of development, but is absent in procyclic and metacyclic forms. Based upon experimental tests, LPG polymorphisms in *Leishmania* species is considered to be the major factor essential for completing development in sand fly vectors (reviewed by Kamhawi, 2006), however, other molecules and enzymes are neccessary (see the following chapter 1.5).

Long nectomonads transform to short nectomonad promastigotes (called leptomonads according to Bates and Rogers (2004)), which enter another proliferative cycle (Gossage et al., 2003). The accumulation of short nectomonads in the thoracic midgut and colonization of stomodeal valve are essential steps for *Leishmania* transmission. Leptomonads produce a gel-like plug, promastigote secretory gel (PSG) (Rogers et al., 2002) consisting of filamentous proteophosphoglycan (fPPG) (Ilg et al., 1996), which is concentrated in cardia of sand fly gut. The PSG, together with immunomodulatory and hemostatic properties of sand fly saliva (reviewed in Rohousová and Volf, 2006; Titus and Ribeiro, 1988), has an important feature in modification of feeding site and affects *Leishmania* transmission and infection establishment (Rogers et al., 2004). The amount of PSG expeeled to the host skin is species-specific and proportional to the size of the infection in sand fly gut (Rogers et al., 2010). It is a very effective virulence factor leading to the more serious manifestation both the cutaneous and visceral form of leishmaniasis (Rogers et al., 2004, 2010).

Some promastigotes attach by hemidesmosomes to the chitin lining of stomodeal valve as haptomonads; these forms damage the stomodeal valve by chitinase (Schlein et al., 1991, 1992; Volf et al., 2004). The last morfological form during vectorial part of the *Leishmania* life cycle is metacyclic promastigotes, stages infective for mammals. These highly motile, free-swimming and non-dividing parasites with long flagellum cumulate in cardia region (reviewed by Kamhawi, 2006; Sacks and Perkins, 1985). However, occasionally were metacyclic stages observed also in proboscis, salivary glands or urine droplets (Sádlová and Volf, 1999).

Destruction of the stomodeal valve, together with physical obstruction by PSG plug (Rogers et al., 2004) is responsible for reflux of parasites to the host skin during the next bloodmeal. This mechanism is commonly known as “blocked fly hypothesis” and sand fly females with late-stage infection frequently have difficulty to intake the

blood and engorge fully (Rogers and Bates, 2007). Kimblin et al. (2008) showed the linear trend between pre-feeding parasite load and size of bloodmeal taken. These informations suggest that *Leishmania* can manipulate the feeding behavior of sand fly. Infected flies are more relentless, they spend longer time by probing and change frequently the site of feeding (Killick-Kendrick et al., 1977; Rogers and Bates, 2007).

The success of transmission depends on number and localization of metacyclic promastigotes in the sand fly midgut at a given time-point. In *L. mexicana* infected–*L. longipalpis*, the metacyclic stages appear firstly in midgut 3 days post-bloodmeal (PBM) but the maximum was observed on day 7 PBM in the cardia and thoracic midgut (Rogers et al., 2002). The majority of metacyclics (about 75%) were clustered at the anterior pole of PSG plug. The percentage of metacyclics positively correlates with initial infective dose used for infection of sand flies and with final infection intensity in sand fly gut (Kimblin et al., 2008). On the other hand, number of generated metacyclics is not affected by promastigote-stages used for experimental feeding; similar metacyclic numbers were found in promastigote- and amastigote-initiated infections (Freitas et al., 2012). Moreover, numbers of metacyclic promastigotes in sand fly gut positively correlate with frequency of successful transmission. According to these authors, determining of the total number of metacyclics in wild caught infective sand flies could help predict the frequency of *Leishmania* transmission in studied localities (Stamper et al., 2011).

The accurate number of parasites delivered during feeding to the host skin was evaluated using several different approaches. *Leishmania major* parasites egested by microcapillary feeding of *P. papatasi* were quantified by Warburg and Schlein (1986). According to these authors, the number of transmitted parasites ranged from 1 over 1000 promastigotes. The positive transmission was observed by 11–35% females and 76% of them transmitted less than 100 promastigotes. Moreover, the percentage of transmitting females was positively affected by post-bloodmeal diets. Females maintained on mixture of sucrose supplemented with albumin transmitted parasites more frequetly in comparision with females fed only with sucrose, trehalose or albumin alone (Warburg and Schlein, 1986). The advantage of this method is assessment of parasite inoculum from individual females but it is failed for natural probing and feeding behavior of sand flies during the feeding. In addition, other authors reported much lower frequency of transmission by capillary feeding (Sádlová and Volf, 1999).

The other experiments quantified transmitted *Leishmania* parasites using the system of membrane feeding. The parasites were egested through a chick-skin membrane into culture medium and immediately collected. This approach evaluates only the average number of parasites per fly delivered through membrane as many sand flies fed together in the same time. Therefore, this technique did not allow an evaluation of the variation in numbers delivered by individual sand flies. Also, the effect of used membrane instead of mammal's skin could play an important role. Described strategy has been used by Rogers et al. (2004) in *L. longipalpis* infected by *L. mexicana*; they determined that one female egested during feeding an average 1086 promastigotes from which 86-98% corresponded to metacyclic promastigotes. When the same method was performed by the same authors in *L. longipalpis* infected by *L. infantum* the number of egested parasites was about 2-fold lower (average dose 457$\pm$122 *L. infantum* promastigotes per sand fly bite) (Rogers et al., 2010). Authors counted that only 4–5% parasites from total size of infection in sand fly gut were transmitted.

The more optimal leishmaniasis model to test number of transmitted parasites is based on quantification of parasites directly from the skin mammalian host exposured to feeding of experimentally infected sand flies. Infected females are allowed to feed on mouse ears or whole body and parasite numbers at the site of bite as well the parasite load on the corresponding sand fly female are determined by quantitative PCR. This approach, which best simulates the natural conditions, was firstly used by Kimblin et al. (2008) in *P. duboscqi* infected by *L. major.* The size of inoculum detected in this study varied widely from <10 to nearly 100 000 *Leishmania* parasites. Approximately 75% of females delivered fewer than 600 parasites. The number of transmitted parasites as well as pre-feeding parasite load in the midgut showed signs of bimodal distribution. For females transmitting more than 1000 parasites there is a strong correlation with pre-feeding midgut load. Surprisingly, this is not applicable for females transmitting less than 600 parasites because more than half tested females with infection > 30 000 promastigotes, which corresponds to heavy infection, transmitted only low number of parasites (Kimblin et al., 2008).

The study by Secundino et al. (2012) with New World sand fly species *Lutzomyia longipalpis* infected by *L. infantum chagasi* was performed similarly to Kimblin´s experiment. No differences were observed between amounts of parasites delivered to skin of mice or hamster and in single bite was transmitted up to 10 000 parasites. However, most of females (~75%) regurgitated less than 300 parasites. All available data regarding transmission experiments are summarized in the chapter Summary and conclusions in Table 1.

Taken together, all recent results clearly showed that variability of parasite transmission by single bite is very heterogenous depending on methods, sand fly species and *Leishmania* strains used. There is no doubt that determination of an accurate infective dose is crucial for finding the ideal experimental model, which would mimic natural conditions as much as possible, to evaluate new drugs and vaccine candidates.

1.5 Role of nicotinamidase in *Leishmania* development in the vector

Nicotinamide adenine dinucleotide (NAD^+) is a cofactor in many redox reactions as hydride donor (NADH and NADPH) and acceptor (NAD^+ and NADP) playing essential role in all eukaryotic cells, bacteria and archaea. It is involved in several biological processes as signaling, ageing or controlling of gene expression (Bogan and Brenner, 2008).

In general, two main pathways leading to NAD^+ production are known. In *de novo* biosynthesis pathway is L-tryptophan (Trp) in eukaryotes or aspartic acid (Asp) in prokaryotes and plants used as substrate from which nicotinic acid mononucleotide (NaMN) is produced in several steps. This product is subsequently transformed into NAD^+.

In contrast, in second pathway is NAD^+ synthetized through NAD^+ salvage pathway starting with nicotinamide (NAm), nicotinic acid (Nac) or nicotinamide riboside (NR) as precursors (Bieganowski and Brenner, 2004). According to genome studies, *Leishmania* is NAD^+ auxotroph organism lacking genes for *de novo* NAD^+ synthesis, therefore, the precursors (Nac, NAm, NR) are required from the host

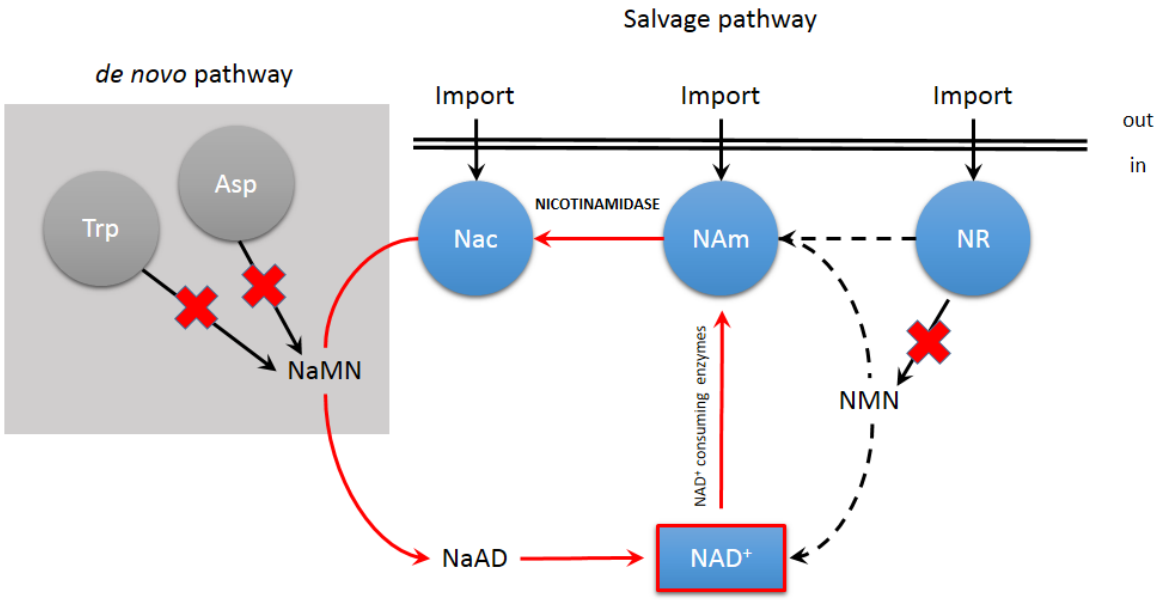

Figure 2: Schematic representation of *de novo* and salvage pathways of the NAD^+ (nicotinamide adenine dinucleotide) biosynthesis. The pathway used by *Leishmania* parasites is highlighted in red. The dashed lines showing steps that are not confirmed in *Leishmania* salvage pathway. Abbreviations: Nac-nicotinic acid; NAm-nicotinamide; NR-nicotinamide riboside; Asp-aspartic acid; Trp-tryptophan; NaMN-nicotinic acid mononucleotide; NaAD-nicotinic acid adenine dinucleotide and NMN nicotinamide mononucleotide (Michels and Avilán, 2011, modified).

environment. NAD^+ auxotrophy was confirmed experimentally by addition of Trp and Asp to *L. infantum* promastigotes but no effect on cellular NAD^+ concentration was detected (Gazanion et al., 2011a).

In *Leishmania*, Nac is directly transformed via the nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD) to NAD^+. Alternatively, NAm is converted into Na by a nicotinamidase enzyme (PNC1) which is present in lower organisms, such as bacteria or yeast, but is absent in mammals. Nicotinamidase enzyme is not localized in special cellular compartment but is freely in cytosol and for enzymatic activity is essential the catalytic cysteine in position 161 (Gazanion et al., 2011a). In *Leishmania* genome, was not found any homolog of NR kinase, responsible for NR conversion to NMN. Moreover, in LiPNC1 deficient strain, the supplementation of NR in culture medium did not restore the growth defect phenotype, thus confirming the absence of NR salvage pathway via NMN in *Leishmania*. However, several nucleosid hydrolases that are able to convert NR into NAm was detected. The nicotinamide adenine dinucleotide (NAD^+) biosynthesis pathway employed in *Leishmania* parasites is summarized in Fig. 2.

Several studies on microorganism confirmed the importance of intracellular NAD^+ levels in the interactions between pathogen and their host. For example, in *Candida glabrata* the limitation of NAD^+ precursors directly influences the expression of some EPA genes (Domergue et al., 2005), a family of adhesins, necessary for successful dissemination and colonization of host gastrointestinal and genitourinary tract (Ma et al., 2009). Purser et al. (2003) and Kim et al. (2004) revealed that nicotinamidase

is a virulence-associated factor for intracellular pathogens. They demonstrated that PncA mutant of *Brucella abortus* or *Borrelia burgdorferi* showed lower rate of intracellular replication in macrophages and reduced virulence in mice. These properties of nicotinamidase and fact that this enzyme is absent in mammals makes from nicotinamidase a promised potential drug target (Michels and Avilán, 2011).

In both *L. major* and *L. infantum* parasites are present SIR2 genes (silent information regulators) which are NAD-dependent histone deacetylases accumulated in the cytoplasm of promastigote as well as amastigotes (Zemzoumi et al., 1998). Experiments with mutants of *L infantum* overexpressed SIR2 proteins showed responsibility of this protein in cooperation with other cellular factors for promoting the survival of *Leishmania* amastigotes by preventing programmed cell death (Vergnes et al., 2002).

On the other hand, the inhibition of SIR2 genes leads to chromatin condensation and transcriptional silencing (reviewed by Gasser and Cockell, 2001). This is in agreement with experiments by Vergnes et al. (2005b) where inhibitor of SIR2 deacetylase Sirtinol inhibited the *in vitro* proliferation of *L. infantum* amastigotes. Contrastingly, the inhibitor had no effect on *in vitro* growth of promastigotes. Similarly, the disruption of *L. infantum* SIR2 gene did not affect the growth of promastigotes but markedly reduced the ability of amastigotes to multiply inside macrophages *in vitro* as well as in BALB/mice *in vivo* (Vergnes et al., 2005a). Both studies revealed that requirement of activity SIR2 proteins is stage specific.

Furthermore, Sereno et al. (2005) demonstrated the antileishmanial activity of 5 mM nicotinamide (NAm) leading to inhibition of *L. infantum* amastigotes growth. Surprisingly, the mechanism of inhibition was not dependent on presence of SIR2 proteins as they expected and probably includes other in vivo targets like two SIR2-related proteins identified in *Leishmania* genome (Sereno et al., 2005). Furthermore, Gazanion et al. (2011b) revealed that NAm significantly improves the antileishmanial activity of trivalent and pentavalent antimony drugs. Only lower cytostatic activity of NAm was observed together with amphotericin B and even slightly antagonized activity with pentamidine. They suggest the potential of NAm to be used as adjuvant to antileishmanial drugs because NAm is known as vitamin B3 and is already used in therapy of some diseases like pellagra or osteoarthritis and together with chemotherapeutics may indicate more effective treatment of visceral leishmaniasis.

Recently, the role of nicotinamidase in *L. infantum* infection was studied using LiPNC1 null mutant clones (Gazanion et al., 2011a). After cultivation of LiPNC1-/- mutants in RPMI, FACS analysis showed that deletion of gene for nicotinamidase leading to reduction of intracellular NAD^+ content and thereby to extending lag phase of growth to approximately 10 days. Growth rate was fully restored by addition of 1 µM Na, but not its substrate NAm or NR. Interestingly, increased concentration up to 100 µM NAm and NR also restored the growth. This result indicates the possibility of alternative nicotinamide deaminase that could hydrolaze excess NAm in the absence of nicotinamidase (Michels and Avilán, 2011). *In vivo* infection experiments performed using BALB/c mice demonstrated necessity of nicotinamidase enzyme for establishment of infection in mice (Gazanion et al., 2011a). Similarly, the capacity of null-mutants to proliferate inside macrophages *in vitro* was decreased.

It is clear that nicotinamidase is essential for parasite survival and multiplication *in vitro* as well as *in vivo*, however, nothing is known about the role of *Leishmania* nicotinamidase during intravectorial development. This is why we decided to study experimental infections of sand flies by *L. infantum* with knock-outed gene for nicotinamidase enzyme.

2 Objectives

The Ph.D. thesis is focused on various aspects of interaction between parasites of *L. donovani complex* and their vectors. I tested the susceptibility of various sand fly species and biting midge to parasites from *L. donovani* complex and attempted to determine the accurate number of *L. infantum* parasites delivered to the host skin during the bite. I also investigated the importance of nicotinamidase activity for the intravectorial development of *Leishmania*.

The main objectives of this thesis were:

- to determine the transmission rate and quantify the number of promastigotes transmitted into the mouse skin by individual *P. perniciosus* and *L. longipalpis* sand fly females and compare differences in transmission parameters between dermotropic and viscerotopic strain
- to describe potentional differences in life-cycles parameters of two *P. orientalis* colonies originating from Ethiopia and test their susceptibility to *L. donovani* infection and study the effect of initial infective dose on *Leishmania* development in these vectors
- to study susceptibility of *Sergentomyia schwetzi* to different *Leishmania* species
- to test the susceptibility of biting midges *Culicoides nubeculosus* to various *Leishmania* including *L. infantum*
- to investigate the importance of nicotinamidase enzyme of *L. infantum* in development in its natural vector *P. perniciosus*

3 Publications

1. Maia C, **Seblova V**, Sadlova J, Votypka J, Volf P. (2011). Experimental transmission of *Leishmania infantum* by two major vectors: a comparison between a viscerotropic and a dermotropic strain. *PLoS Neglected Tropical Diseases* 5(6): e1181. PMID: 21695108

2. **Seblova V**, Volfova V, Dvorak V, Pruzinova K, Votypka J, Kassahun A, Gebre-Michael T, Hailu A, Warburg A, Volf P. (2013). *Phlebotomus orientalis* sand flies from two distant Ethiopian localities and their susceptibility to *L. donovani*. Manuscript submitted to *PLoS Neglected Tropical Diseases*.

3. Sadlova J, Dvorak V, **Seblova V**, Warburg A, Votypka J, Volf P. (2013). *Sergentomyia schwetzi* is not a competent vector for *Leishmania donovani* and other *Leishmania* species pathogenic to human. Manuscript submitted to *Parasites & Vectors*.

4. **Seblova V**, Sadlova J, Carpenter S, Volf P. (2012). Development of *Leishmania* parasites in *Culicoides nubeculosus* (Diptera: Ceratopogonidae) and implications for screening vector competence. *Journal of Medical Entomology* 49(5): 967–970. PMID: 23025175

5. Gazanion E, **Seblova V**, Votypka J, Vergnes B, Garcia D, Volf P, Sereno D. (2012). *Leishmania infantum* nicotinamidase is required for late-stage development in its natural sand fly vector, *Phlebotomus perniciosus*. *International Journal of Parasitology* 42(4): 323–327. PMID: 22619752

4 Summary and conclusions

My project was focused on various aspects of *L. donovani* complex interaction with their vectors. The emphasis was put on transmission parameters, vector competence of different sand flies (proven biological vector) and biting midges (putative alternative vectors).

Most studies focused on pathogenesis and immune response of mammals towards leishmaniasis used the model of needle-injection, involving intradermal, intracardiac, intraperitoneal or intravenous injection of millions promastigotes into laboratory animals (reviewed by Garg and Dube, 2006). However, this method does not reflect the complexity of transmission by vector bites. During the feeding of infected sand fly are delivered to the host skin pharmacologically active saliva and promastigotes embeded in PSG. Both saliva and PSG represent immunomodulatory factors (reviewed by Gillespie et al., 2000; Rogers et al., 2004) promoting parasite establishment in host skin. Equally important for the development of an accurate experimental model is the correct natural infective dose of promastigotes. The disease caused by parasites of *L. donovani* complex manifests either as cutaneous or visceral leishmaniasis and it is not clear if sand fly saliva or parasite dose affect the outcome of disease. Therefore, on transmission of viscerotropic or dermotropic *L. infantum* strains by two sand fly species, *P. perniciosus* and *L. longipalpis*, we focused in the first part of this thesis. In our study, we followed the approach used by Kimblin et al. (2008) which better mimic the natural conditions than forced- or membrane-feeding used previously (Warburg and Schlein, 1986; Rogers et al., 2004, 2010). The emphasis was given on determination of accurate number of parasites delivered to the mouse skin by individual sand flies and transmission rate.

We observed that in most females of both sand fly species used the *L. infantum* strains developed very well producing heavy infection including colonization of stomodeal valve. Q-PCR showed that prefeeding load in sand fly midguts ranged from 2.8×10^4 to 1.15×10^5 promastigotes per fly what corresponds with numbers quantified previously by Kimblin et al. (2008). From total number of infected females, 15%–65% transmitted promastigotes to host skin depending on sand fly-*Leishmania* combination. The most frequent transmission was achieved by *P. perniciosus* females infected by dermotropic *L. infantum* where more than half of them transmitted infection to the mouse skin. This is 2-fold more than reported by Warburg and Schlein (1986) using force-feeding where the positive transmission was detected by less than 35% females depending on post-bloodmeal diet. The wide range of parasites transmitted per individual sand fly in our study (4 up to 4.2×10^4 promastigotes) is in accordance to data previously obtained with other *Leishmania*-vector combinations (Warburg and Schlein, 1986; Kimblin et al., 2008; Secundino et al., 2012). Interestingly, in both sand fly species the parasite load transmitted was higher by females infected by dermotropic strain where 29% of *L. longipalpis* and 14% of *P. perniciosus* transmitted more than 1000 parasites. On the contary, sand flies infected by viscerotropic strain transmitted less than 600 promastigotes. The significant variation in inoculum size between females infected by dermotropic and viscerotropic allows us to hypothesise

that infectious dose delivered to host skin could be determining factor for infection outcome and the resulting tropism of *Leishmania* strain. In our opinion, females infected by dermotropic *L. infantum* transmitted high-dose inoculum to host skin leading to strong local immune response and manifestation of disease only as cutaneous leishmaniasis. On the other hand, infection initiated with low-dose inoculum, as in case of viscerotropic *L. infantum*, may result in mild immune response and parasites disseminate easily to internal organs. This hypothesis is in accordance to study Kimblin et al. (2008) on the *L. major - P. duboscqi* combination. Authors compared the impact of low inoculum size (5×10^3) and high inoculum size (2×10^2) intradermally injected into the ears of mice. They showed that high-dose inoculum evoked rapid development of large skin lesion in the ears of mice. In contrast, the low-dose inoculum resulted in smaller lesions with delayed onset but a higher parasite titre during chronic phase. These observations support our hypothesis about the effect of infective dose delivered by sand fly bite to the host skin on *Leishmania* tropism. However, it should be noted that for outcome and progression of infection is, apart from inoculum size, very important the way of transmission. As shown by Aslan et al. (2013) it is marked difference in pathogenesis of VL caused by *L. infatum* and *L. donovani* initiated via vector bite or via needle injection.

In contrast to studies Kimblin et al. (2008) and Secundino et al. (2012), we allowed to feed infected females on whole mouse body which enabled us observed preferential biting place. For all sand fly-*Leishmania* combinations were ears the most preferred site of bite followed by the paws and tail. Besides the site of feeding, we also observed the feeding time, in order to elucidate difference in feeding duration of females who transmitted or non-transmitted parasites. Interestingly, only females transmitting dermotropic *L. infantum* strain need more time to feed than those that were infected but non-transmitting. No differences in feeding time were observed between transmitting and non-transmitting females with viscerotropic *L. infantum*.

In the second part of study dealing with Ethiopian vectors of VL, we focused on life cycle parameters, genetic differences and susceptibility to *Leishmania* infection of two *P. orientalis* colonies originating from a non-endemic site in lowlands – Melka Werer (MW), and an endemic focus of VL in the highlands – Addis Zemen (AZ). In spite of the high leishmanin positivity of humans from MW (around 65%), no confirmed cases are reported of either cutaneous or visceral leishmaniasis. Therefore, we decided to test susceptibility of *P. orientalis* to *L. donovani* just from this locality and compare it with susceptibility of *P. orientalis* population from clearly endemic AZ area.

For investigation of such parameters, the long term colonization of both *P. orientalis* populations was critical step (Volf and Volfova, 2011). After arrival to Prague, females of both colonies were fed on rabbit or human arms and it took relatively long time to adapt them to feed on mouse. Especially, AZ colony was less adaptable for substituting of bloodmeal source and to date, they feeding rate on mouse is much lower than on rabbit. Similarly, differences between colonies were observed during experimental feeding through chick-skin membrane; AZ colony was more reluctant to membrane feeding. Both colonies prefer relative high humidity which was surprising for the species living in relatively dry areas. Therefore, we suppose that adults spend most of the time resting in deep cracks of the soil.

The critical factor affecting larval development was the quality of larval food. On non-autoclaved food the emerging adults peaked at eight and nine weeks PBM (post-bloodmeal) for MW and AZ, respectively. Autoclaved food led to delayed

Combination sand fly - *Leishmania*	**Infective dose for sand flies**	**Used method**	**Size of total prefeeding load** (promastigotes/midgut)	**No. Promastigotes transmitted per fly**	**% of transmitting flies**
P. papatasi - L. major **(Warburg and Schlein, 1986)**	2×10^6 promastigotes/ml	forced feeding by microcapillaries	unknown	range 0–1000 80% females 1–100 20% females 100–1000 5% more than 1000	11.4–35%
L. longipalpis - L. mexicana **(Rogers et al., 2004)**	2×10^6 amastigotes/ml	artificial feeding through membrane	unknown	1086* (86-96% metacyclic promastigotes)	unknown
L. longipalpis - L. infantum **(Rogers et al., 2010)**	2×10^6 amastigotes/ml	artificial feeding through membrane	$1.06 \times 10^4 \pm 2.1 \times 10^4$ (35% metacyclic promastigotes)	457 ± 122* (95% metacyclic promastigotes)	unknown
P. duboscqi - L. major **(Kimblin et al., 2008)**	4×10^6 amastigotes/ml	natural feeding on mouse ears	$1 \times 10^4 - 1 \times 10^5$	range 0–100000 75% females 600 and less 25% females more than 1000	19.26%
L. longipalpis - L. infantum chagasi **(Secundino et al., 2012)**	4×10^6 promastigotes/ml	natural feeding on mouse ears	1×10^4	1002 range 10–10000 75% less than 300	unknown
L. longipalpis - L. infantum dermotropic **(Maia et al., 2011)**	1×10^7 promastigotes/ml	natural feeding on whole mouse	8×10^4	1127/13** range 10–11100	58%
L. longipalpis - L. infantum viscerotropic **(Maia et al., 2011)**	1×10^7 promastigotes/ml	natural feeding on whole mouse	1.5×10^5	104*/24** range 0–1250	14.5%
L. perniciosus - L. infantum dermotropic **(Maia et al., 2011)**	1×10^7 promastigotes/ml	natural feeding on whole mouse	5×10^5	2350*/29** range 0–42000	65%
L. perniciosus - L. infantum viscerotropic **(Maia et al., 2011)**	1×10^7 promastigotes/ml	natural feeding on whole mouse	6.6×10^4	88*/28** range 0–500	33%

* an average dose of transmitted parasites per sand fly
** median of transmitted parasites per sand fly

Table 1: Summary of all available data regarding transmission experiments.

non-synchronized development with tendency of the L4 larvae to diapause. The L4 larvae diapause is complying with observation reported by Schmidt (1964) in *P. orientalis* as well as in some Palaearctic species, whereas species from warmer, while, according to Killick-Kendrick (1999), wetter habitats are supposed to diapause at the egg stage. The tendency of AZ colony to diapause could be explained as an adaptation to adverse and frequently changing conditions of the highland area.

Despite obvious differences found in certain life-cycle parameters, RAPD PCR and DNA sequencing of cytB and COI mitochondrial genes demonstrated that both colonies are genetically identical Subsequently, the cross-mating study between MW male/AZ female and AZ male/MW female showed that hybrids from F1 and F2 progeny developed very successfully and had higher fecundity than parental colonies.

Besides life-cycle parameters and genetic analysis, we also tested the susceptibility of both Ethiopian colonies to *L. donovani* infection. Our results extended those reported in the studies Kirk and Lewis (1955); Hoogstraal and Heyneman (1969) and McConnell (1964) performed only with a small number of wild-caught Sudanese *P. orientalis* sand flies. The observed development patterns are in accordance with the results of these earlier studies. Both *L. donovani* strains tested developed very well in *P. orientalis* females and colonized anterior parts of the midgut and the stomodeal valve. Parasite development was relatively fast at 26°C 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% in MW and 81% in AZ) and the Q-PCR revealed that females from the two colonies did not differ in total numbers of parasites in their midguts.

In order to elucidate the minimal number of promastigotes which is capable to cause heavy late-stage infection in sand fly gut, we tested various initial infective doses of *L. donovani* in *P. orientalis*. In fully bloodfed females of *P. orientalis* the average bloodmeal volume was 0.69 µl 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. Surprisingly, experimental infections revealed that even the lowest infective dose tested (2×10^3 *L. donovani* promastigotes per ml of blood), equivalent to two promastigotes, was sufficient to establish the late stage infection in about 50% of females.

Alltogether, the study describes in details differences in behavioural and life-cycle parameters of two Ethiopian *P. orientalis* colonies. It also suggests extremely high susceptibility of both *P. orientalis* for *L. donovani* and clearly showed that only very low number of parasites (1–2 promastigotes) are needed for establishment of infection in this sand fly species. As we did not find any diffrences in susceptibility to *L. donovani* between AZ and MW colonies, we can conclude that non-endemicity of visceral leishmaniases in Melka Werer area can not be explained by low susceptibility of local *P. orientalis* to *L. donovani.*

Furthermore, we tested also susceptibility of *Sergentomyia schwetzi* originating from Sheraro in northwestern Ethiopia to three *Leishmania* species, *L. major*, *L. infantum* and *L. donovani*. During early phases of infection, when parasites were still enclose inside the endoperitrophic space, infection rates of all tested *Leishmania* species were very high (>90%) and comparable with those reached in control vectors, i.e. *L. longipalpis* and *P. duboscqi* with *L. infantum* and *L. major*, respectively. However, infection rates and intensity of infection in *S. schwetzi* rapidly decreased to 28% by day 3 PBM, 19% by day 4 PBM and 1.4% by day 9 PBM. Only in single female was

observed *L. donovani* promastigotes in the abdominal midgut and no females infected by *L. infantum* and *L. major* suvived till day 10 PBM. Similar results were reported by Kaddu et al. (1986), who also observed only accidental occurence of *L. donovani* slow-moving promastigotes in three *Sergentomyia* species (*S. schwetzi*, *S. adleri* and *S. ingrami*), however, infection was restricted only to abdominal midgut and in one case in the cardia.

To understand factors affecting refractoriness of *S. schwetzi* to *Leishmania* we attempted to observe in details the kinetic of the development of the peritrophic matrix (PM) and defecation of bloodmeal remnants. As discussed in introduction of this thesis, the role of PM is very important for both vectors and parasites. If PM is not degraded, the signal molecules, probably salivary glands (Charlab and Ribeiro, 1993; Charlab et al., 1995), do not get inside the bloodmeal bolus and procyclic promastigotes do not transform into long nectomonads. As procyclic promastigotes are not able to attach to midgut epithelium of sand flies; they are defecated with bloodmeal remnants and the infection is lost (reviewed by Kamhawi, 2006; Bates, 2008).

We observed highly significant interspecific differences in formation of the PM by days 3 and 4 PBM. While in *L. longipalpis* the PM was found in 8% and 0% on days 3 and 4 PBM, respectively, in *S. schwetzi* it persisted longer and was still present in more than 20% of females on day 4 PBM. Importantly, on day 3 PBM, the percentage of females in which the PM had already degraded but did not yet defecate the blood remnants was significantly higher in *L. longipalpis*.

Knowing that lower temperature prolongs the duration of blood digestion and defecation in sand flies (Benkova and Volf, 2007), we maintained infected females at 21°C to give more time to promastigotes to establish infection in *S. schwetzi*. As we expected, the degradation of PM was delayed; the PM presented till day 4 and 5 PBM in *L. longipalpis* and *S. schwetzi*, respectively. The difference between vector species was significant: on day 5 PBM the PM was present in 78% of *S. schwetzi* and 0% of *L. longipalpis*. In *S. schwetzi* delayed defecation resulted in higher infection rates on days 3-5 PBM and prolonged presence of *L. donovani* till day 5 PBM. However, no infected *S. schwetzi* females were found on day 9 PBM. These results are in accordance to study of Lawyer et al. (1990), who described that Kenyan *S. schwetzi* does not support the late-stage development of *L. major*: for the first 48hr, parasite development progressed but parasites were rarely seen after 48hr and never found after 90hr PBM.

In conclussion, we showed that persistence of the PM can very significantly influence *Leishmania* development. Of course, the role of other factors, like production of proteolytic enzymes during digestion of the bloodmeal,must not be neglected. However, for development of human *Leishmania* in *S. schwetzi*, the period between the degradation of the PM and defecation seems to be a crucial parameter. In *S. schwetzi*, the PM degraded simultaneously with defecation and promastigotes had no chance to leave endoperithrophic space what leads to their defecation. The persistence of the PM till the end of digestion was described also in *S. arpaklensis* (Shatova et al., 1991).

Several field studies done mainly in Kenya indicate *Sergentomyia* spp. as putative vectors of leishmaniasis (Mutinga et al., 1986b, 1994). We suggested that findings of field studies need to be interpreted with caution and PCR detection of *Leishmania* DNA or accidental observation of flagellates in sand fly gut should be accompanied by detailed molecular analyses and laboratory experiments. As we never found heavy infection with colonization of stomodeal valve in *S. schwetzi* we conclude that this species cannot serve as the vector of *L. donovani* in the VL foci of northern

Ethiopia.

Issues of positive *Leishmania* DNA findings is also often reflected in studies dealing with alternative vectors of leishmaniasis. As shown in introduction, several authors consider the ticks and fleas as vectors involved in *Leishmania* transmission, especially in areas where sand flies are less abundant or absent (Coutinho and Linardi, 2007; Ferreira et al., 2009; Paz et al., 2010; Colombo et al., 2011; Solano-Gallego et al., 2012). Recently, Dougall et al. (2011) reported, that biting midges of subgenus *Forcipomyia* (*Lasiohelea*) are implicated in transmission of *Leishmania* from *L. enrietti* complex causing cutaneous leishmaniasis in red kangoroos (Rose et al., 2004), therefore, we decided to study transmission potential of Eurasian biting midges.

We tested susceptibility of *Culicoides nubeculosus* to two major zoonotic species of *Leishmania* infecting humans in the Old World, *L. major* and *L. infantum*. Experimental infections of *C. nubeculosus* with *Leishmania* were carried out using membrane feeding, the method routinely used for phlebotomine sand flies. In the first series of experiments at 26°C, *L. major* grew rapidly in the *C. nubeculosus* midgut until day 2–3 when midges defecated, whereupon infections were completely lost. In the second series of experiments we decreased the ambient temperature and maintained engorged females with *L. infantum* at 20°C to slow down the digestion and delay the defecation (Benkova and Volf, 2007) providing *Leishmania* more time to establish midgut infections. However, by day 3 PBM, most of the females had defecated and midgut infections rates were very low; in 17% females only light infection with long nectomonads was observed in the abdominal midgut. On days 7 and 10 PBM no *Leishmania* parasites were detected in 70 examined females. These results corresponds with an old experimental study conducted by Christophers et al. (1925), who suspected *C. macrostoma* to be involved in transmission of *L. donovani* in India. *Culicoides macrostoma* midges infected by feeding on kala-azar cases were not found positive for *Leishmania* infection on days 3–4 PBM. Despite microscopical examination recorded any viable promastigotes up to and including day 7 PBM in *Culicoides* midges, the PCR detected DNA of *Leishmania*. It is important to realize that PCR might amplify *Leishmania* DNA from dead promastigotes. This illustrates that PCR positivity, especially when non-quantified, can be misleading in implication of arthropods as vectors of *Leishmania*. PCR does not detect whether parasites are viable, transformed to highly virulent metacyclic promastigotes and localized in the stomodeal valve what is prerequisites for successful transmission. Therefore, direct microscopical observation of *Leishmania* promastigotes and their localization in the digestive tract remains a crucial method for any conclusions about the vectorial competence of the insect.

As proteinase inhibitors are known to enhance the early survival of *Leishmania* in sand flies (Borovsky and Schlein, 1987) we tested their effect on development of *L. infantum* in *C. nubeculosus*. Therefore, the blood prepared for midges feeding was mixed with the soybean trypsin inhibitor or the protease inhibitor Coctail Tablets. Proteinase inhibitors did not have any significant effect upon *Leishmania* development and survival in *C. nubeculosus*. As observed during the previous experiment, heavy *Leishmania* infections were frequently present in *C. nubeculosus* on days 1 and 2 PBM with various promastigote forms including long nectomonads restricted to the abdominal midgut, but on day 3 PBM, when digestive process was completed and bloodmeal remnants defecated, infection rates and parasite loads significantly dropped down. By 11 days PBM no parasites were detected in any of the examinated

females.

Taken together, in this study we showed that *Leishmania* is not able undergo complete life cycle in *C. nubeculosus*. Promastigotes of both parasite species, *L. infantum* and *L. major*, were able to escape from PM but not to attach to *Culicoides* midgut epithelium and were defecated. Therefore, we suggest that the lack of midgut attachment is the major refractory barrier for *Leishmania* in *Culicoides* midges. In adddion, we demonstrated how important is using microscopical examination for any conclusion about the vector competence of the putative vectors.

Within the study of various factors affecting *Leishmania* development in sand flies we investigate the importance of nicotinamidase enzyme. Previously it was shown that *Leishmania* is an organism auxotroph for the cofactor NAD^+, what means that these parasites rely on presence of external precursors nicotinamide (NAm) and nicotinic acid (Nac) in host enviroment. The first step of NAm assimilation into NAD^+ is converted by enzyme nicotinamidase (PNC1). This enzyme controls the major part of NAD^+ production and, in turn, parasite growth and pathogenesis (Gazanion et al., 2011a). We tested ability of four lines of *L. infantum* to survive and complete their intravectorial development in *P. perniciosus*. We used a wild-type (WT) strain, a *Lipnc1* double knockout parasite strain (*Lipnc1-/-*), line with inactive form of enzyme *Lipnc1-/-* + LiPNC1C_{161}A and add back *Lipnc1-/-* + LiPNC1(ΔLipnc1::NEO/ΔLipnc1::HYG[pSPαBLA$\alpha-$LiPNC1]) (Gazanion et al., 2011a).

On day 2 PBM, during bloodmeal digestion and before defecation, *Leishmania* developed similarly regardless of the parasite strain. In contrast, after defecation (6 day PBM), significant differences were observed between the double knockout and WT strains ($P<0.001$) as only 1 of 15 sand flies examined was still weakly infected with knockouted parasites. At 8 and 14 days PBM, *Lipnc1-/-* null mutants were detected in sand fly midguts in only 33% and 26% of dissected females, respectively, and at very low densities, demonstrating the inability of these parasites to grow and to establish mature infections in the midgut after defecation. Similarly, parasite line with inactive form *Lipnc1-/-* + LiPNC1C_{161}A was unable to develop in *P. pernicious*. On the other hand, the infection initiated by add backs was comparable with infection by WT strain.

Finally, we tried to restore development of *Lipnc1-/-* null mutants by adding Nac or NAm to sand fly sugar meal. The results obtained with LiPNC1 double-knockouts revealed that Nac and high levels of NAm supplemented in sand fly sugar meal significantly improved *Leishmania* development, however, only immediatelly after defecation (on day 4 PBM). In our opinion, the restoration of the growth is caused by regular feeding of sugar meal by sand flies immediatelly after defecation, however, in the late stage of infection females took sugar only irregularly and it is not enough for salvaging of the parasites.

Overall, only little is known about the nutritional requirements of *Leishmania* within the sand fly midgut. It is predicted that during the early stage of *Leishmania* development the bloodmeal is probably the main extracellular source of nutrients, whereas the sugar is applied in later stages of *Leishmania* growth. Our study showed that deletion of gene for nicotinamidase leads to the unexpected growth arest of *L. infantum* parasites inside midgut and indicate that NAm might be a very important nutritional factor for *Leishmania*.

In conclusion, we proved that *P. orientalis* is a very susceptible vector to *L. donovani* infection. The experiments were done using promastigote-iniciated experimental

feeding, where the number and viability of parasites are easy to determine. In the near future, we would like to perform similar studies using amastigotes and compare detailed *L. donovani* development in *P. orientalis* initiated by amastigotes from culture and by amastigotes obtained from tissues of laboratory infected mice or hamsters. Similarly, we would like continue with studies dealing with alternative vectors of *Leishmania*. We plan to focus on susceptibility of laboratory-maintained colony *Rhiphicephalus sanguineus* to *L. infantum* infection and microscopical observation of *Leishmania* parasites development in digestive tract of these ticks.

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Experimental Transmission of *Leishmania infantum* by Two Major Vectors: A Comparison between a Viscerotropic and a Dermotropic Strain

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Abstract

We quantified *Leishmania infantum* parasites transmitted by natural vectors for the first time. Both *L. infantum* strains studied, dermotropic CUK3 and viscerotropic IMT373, developed well in *Phlebotomus perniciosus* and *Lutzomyia longipalpis*. They produced heavy late-stage infection and colonized the stomodeal valve, which is a prerequisite for successful transmission. Infected sand fly females, and especially those that transmit parasites, feed significantly longer on the host (1.5–1.8 times) than non-transmitting females. Quantitative PCR revealed that *P. perniciosus* harboured more CUK3 strain parasites, while in *L. longipalpis* the intensity of infection was higher for the IMT373 strain. However, in both sand fly species the parasite load transmitted was higher for the strain with dermal tropism (CUK3). All but one sand fly female infected by the IMT373 strain transmitted less than 600 promastigotes; in contrast, 29% of *L. longipalpis* and 14% of *P. perniciosus* infected with the CUK3 strain transmitted more than 1000 parasites. The parasite number transmitted by individual sand flies ranged from 4 up to 4.19×10^4 promastigotes; thus, the maximal natural dose found was still about 250 times lower than the experimental challenge dose used in previous studies. This finding emphasizes the importance of determining the natural infective dose for the development of an accurate experimental model useful for the evaluation of new drugs and vaccines.

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Introduction

Leishmania are intracellular protozoan parasites that establish infection in mammalian hosts following transmission through the bite of an infected phlebotomine sand fly. Visceral leishmaniasis, caused by *Leishmania donovani* in the Old World and *L. infantum* in both the Old and New World, invariably leads to death if left untreated [1]. Despite the fact that parasites from the *L. donovani* complex are mainly associated with disseminated infection of the spleen and liver, it has been shown that *L. infantum* can also cause cutaneous lesions [2–5]. A novel focus of cutaneous leishmaniasis caused by *L. infantum* was recently described in the Cukurova region in Turkey [6].

During the natural transmission of *Leishmania* into the dermis, sand flies deposit pharmacologically active saliva [7] and egest parasite-released glycoconjugates, the promastigote secretory gel [8]. Both substances modulate the immune response of the bitten host and enhance the severity of infection (reviewed by [9]).

The ideal leishmaniasis model to test therapeutics and immunoprophylaxis candidates should reproduce the biological and immunological aspects of natural infection and disease. Different approaches regarding the parasite number and route of inoculation have been tested in order to develop an accurate experimental model for the *L. donovani* complex, most of them using subcutaneous, intraperitoneal or intravenous injections of millions of axenic promastigotes or amastigotes [10–11]. Although in some studies up to 10^7 parasites have been co-inoculated into the dermis with small amounts of sand fly saliva, is not clear how well these experiments mimic natural transmission [12–13].

The number of *L. infantum* parasites inoculated by infected vectors during natural transmission was not previously known, even though a determination of the natural infective dose is crucial for the development of an accurate experimental model to evaluate new drugs and vaccine candidates. In the *L. major* - *P. duboscqi* model, it was demonstrated that the number of promastigotes inoculated by individual sand flies ranged between 10 and 1×10^5 *Leishmania* [14]. The average number of *L. infantum* parasites egested was recently reported [15], but the technique used (feeding the pool of infected *L. longipalpis* through chick skin membrane on culture medium) did not allow an evaluation of the variation in numbers delivered by individual sand flies. Thus, the main aims of this work were to determine the transmission rate and the number of promastigotes inoculated into the skin of mice by individual sand fly females. *Phlebotomus perniciosus* and *Lutzomyia longipalpis*, two main vectors of *L. infantum* in the Mediterranean basin and in the New World, respectively [16], were experimentally infected by *L. infantum* dermotropic and viscerotropic parasites.

Author Summary

Leishmaniasis is a disease caused by protozoan parasites which are transmitted through the bites of infected insects called sand flies. The World Health Organization has estimated that leishmaniases cause 1.6 million new cases annually, of which an estimated 1.1 million are cutaneous or mucocutaneous, and 500,000 are visceral, the most severe form of the disease and fatal if left untreated. The development of a more natural model is crucial for the evaluation of new drugs or vaccine candidates against leishmaniases. The main aim of this study was to quantify the number of *Leishmania infantum* parasites transmitted by a single sand fly female into the skin of a vertebrate host (mouse). Two *L. infantum* strains, viscerotropic IMT373 and dermotropic CUK3, were compared in two natural sand fly vectors: *Phlebotomus perniciosus* and *Lutzomyia longipalpis*. We found that the parasite number transmitted by individual sand flies ranged from 4 up to 4.19×10^4. The maximal natural infective dose found in our experiments was about 250 times lower than the experimental challenge dose used in most previous studies.

Results

The following results summarize the data obtained in 15 and 10 independent experiments with both vectors and *L. infantum* strain combinations: 9 with *P. perniciosus*-IMT373, 6 with *P. perniciosus*-CUK3, 6 with *L. longipalpis*-IMT373 and 4 with *L. longipalpis*-CUK3.

Experimental infections of sand flies: comparison of IMT373 and CUK3 strains

The *L. infantum* strains studied developed well in both *P. perniciosus* and *L. longipalpis*, producing heavy late-stage infection and colonizing the stomodeal valve of the vectors, which is a prerequisite for successful transmission. For both *L. infantum* strains, the average parasite load in the sand fly midgut is summarized in Table 1. Quantitative PCR revealed that in *P. perniciosus* the intensity of infection was higher for the CUK3 strain ($p = 0.01$) while *L. longipalpis* harboured more IMT373 parasites ($p<0.001$). However, in both sand fly species the number of parasites transmitted was higher for the dermotropic strain CUK3 ($p<0.001$); see below.

Transmission of the dermotropic strain CUK3

Out of 88 *P. perniciosus*, females that bit mice, 62 (70.5%) were infected with CUK3; of these, 36 (58%) delivered parasites into the skin of the mice on days 10–14 post infective blood meal (Fig. 1a). Out of 114 biting *L. longipalpis* females, 86 (75.5%) were infected and 56 (65% of those infected) inoculated parasites into the mice on days 7–14 post infective blood meal (Fig. 1b).

Despite the fact that the intensity of infection was significantly higher in *P. perniciosus* ($p<0.01$), the percentage of transmission and number of inoculated parasites was comparable for both vectors. The parasite load delivered by *P. perniciosus* and *L. longipalpis* in the skin of mice ranged between 16 and 4.19×10^4 and between 4 and 1.11×10^4, respectively. The average number of CUK3 parasites inoculated into the skin of mice and the percentages of transmission are summarized in Table 1.

In *L. longipalpis*, the feeding time was positively correlated with the number of CUK3 parasites delivered into host skin ($p<0.05$), while in *P. perniciosus* females no such correlation was observed. On the other hand, there was a significant correlation between the pre-feeding load inside both sand fly species' midguts and the number of parasites transmitted ($p = 0.0178$ for *L. longipalpis* and $p<0.001$ for *P. perniciosus*).

Transmission of the viscerotropic strain IMT373

Out of 101 *P. pernicious* females that bit mice, 73 (72%) were infected with IMT373, and of these 24 (33%) transmitted parasites into the mice's skin. *Leishmania* transmission occurred between days 9 and 16 post infective bloodmeal (Fig. 2a). From 190 biting *L. longipalpis* females, 159 (84%) were infected and 23 (14.5% on infected ones) inoculated parasites into the mice between days 7 and 14 post blood meal (Fig. 2b).

In contrast to above, the intensity of infection was significantly higher in *L. longipalpis* ($p<0.001$), but the transmission rate (i.e. percentage of transmitting females) and the number of parasites transmitted were significantly higher in *P. perniciosus* ($p<0.01$).

The number of parasites transmitted by *P. perniciosus* and *L. longipalpis* ranged from 8 to 513 and between 7 and 1240 promastigotes, respectively. The median number of IMT373 transmitted is summarized in Table 1.

For both sand fly species, there was no correlation between feeding time and the number of IMT373 parasites in each female ($p = 0.1594$), or between the time to take a blood meal and the number of parasites transmitted ($p = 0.6666$). Moreover, no correlation was observed between the pre-feeding load in each sand fly species and the number of *Leishmania* delivered ($p = 0.1340$ for *P. perniciosus*; $p = 0.6473$ for *L. longipalpis*).

Biting sites and feeding time of transmitting females

For all *Leishmania*-sand fly combinations, ears were the preferential biting place for sand flies transmitting the parasites, followed by the paws and tail. A few specimens that fed in the nose and eyes were also able to transmit parasites.

Table 2 summarizes the feeding times for both sand fly species: *L. longipalpis* transmitting IMT373 completed their bloodmeals in

Table 1. Pre-feeding and transmitted parasite load for *L. infantum* strains by both sand fly species.

	***L. infantum* IMT373**		***L. infantum* CUK3**	
	P. perniciosus	***L. longipalpis***	***P. perniciosus***	***L. longipalpis***
Parasite load in sand fly midgut* (mean/median)	65 768/52 506	154 433/79 691	499 500/114 963	79 888/27 854
Transmitted parasites (mean/median)	88/28	104/24	2 350/29	1127/13
Percentage of transmission** (mean/median)	0.47%/0.07%	0.19%/0.04%	0.5%/0.02%	2.3%/0.03%

*Parasite load was calculated as a sum of midgut parasites plus those transmitted by bite.
**Percentage of parasite load transmitted by bite.
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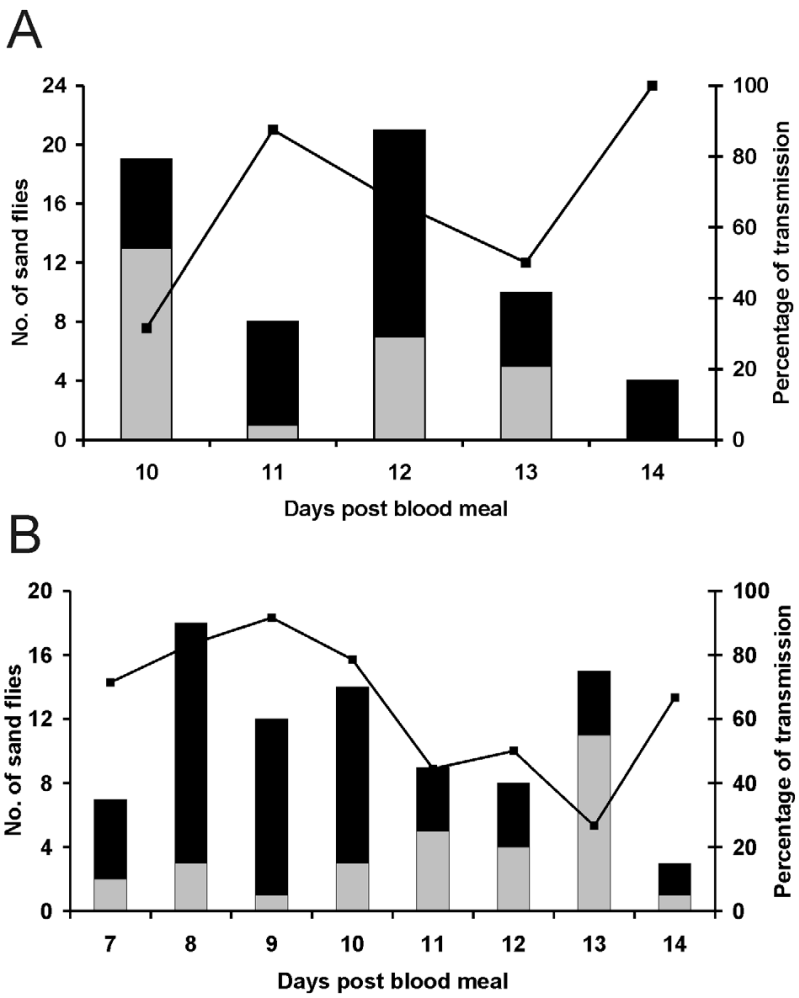

Figure 1. Transmission of *Leishmania infantum* CUK3. Percentage transmission of CUK3 strain by experimentally infected *Phlebotomus perniciosus* (A) and *Lutzomyia longipalpis* (B). Black bars, infected females that transmitted by bite; grey bars, infected females that did not transmit; line, percentage of females that transmitted parasites.
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times ranging from 2 to 27 minutes, while those transmitting CUK3 parasites needed between 3 to 55 minutes. The maximum and minimum feeding times for *P. perniciosus* transmitting CUK3 and IMT373 parasites ranged between 4–33 and 1–32 minutes, respectively. Infected sand flies transmitting CUK3 needed more time to feed than those that were infected but non-transmitting, while no differences in feeding time were observed between transmitting and non-transmitting females with IMT373 parasites.

Discussion

For the first time, we have quantified the number of parasites belonging to *L. infantum* dermotropic and viscerotropic strains transmitted to the dermis of experimental mice by individual sand fly females. The only previous attempt to calculate the number of transmitted *L. infantum* parasites was performed just recently [15], with the average number of promastigotes inoculated by 63 *L. longipalpis* into culture medium through a chicken membrane skin being 457 parasites, with 95% (431 promastigotes) of these corresponding to metacyclic parasites. However, these results do not allow us to take into consideration the individual variability of parasite transmission by a single specimen. The wide range of parasites inoculated per individual sand fly in our study (from 4 up to 4.19×10^4 promastigotes) is in accordance to data previously obtained with other *Leishmania*-vector combinations [14,17], although the approach using microcapillaries as artificial feeding systems [17] could have interfered with the normal sand fly feeding behaviour.

In our study, *Phlebotomus perniciosus* harboured more *L. infantum* dermotropic parasites of the CUK3 strain, while in *L. longipalpis* the intensity of infection was higher for the viscerotropic strain IMT373. However, in both sand fly species the parasite load transmitted was higher for the strain with dermal tropism. All but one sand fly female infected by IMT373 strain transmitted less than 600 promastigotes, the exception being a *L. longipalpis* female that inoculated 1240 parasites. On the other hand, 29% of *L.*

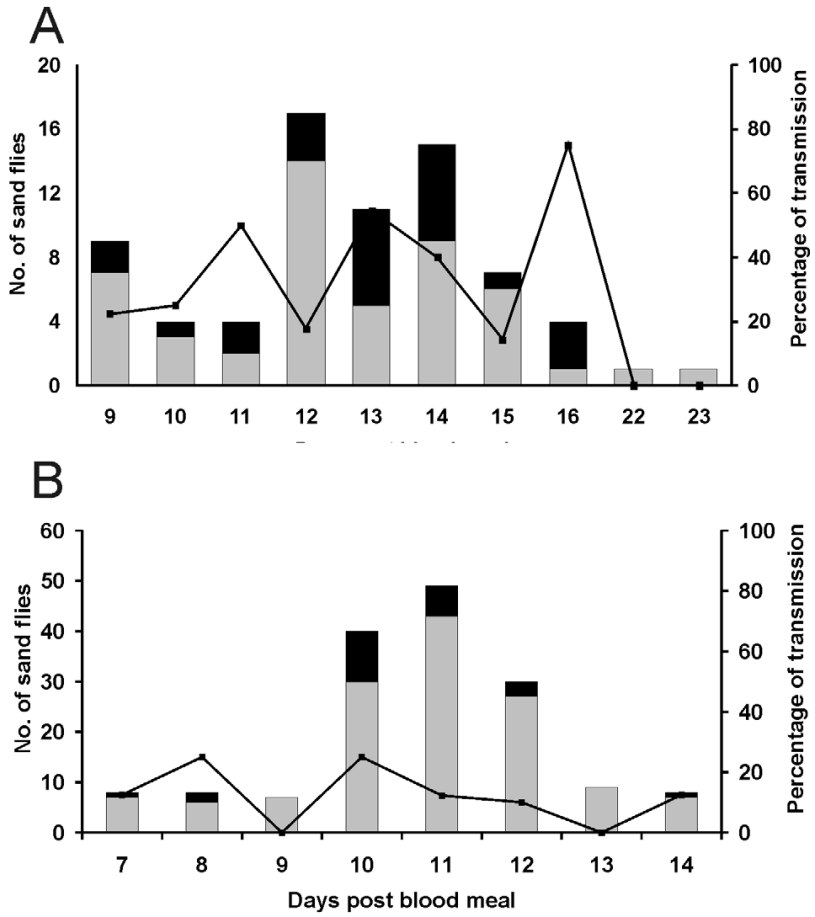

Figure 2. Transmission of *Leishmania infantum* IMT373. Percentage transmission of IMT373 strain by experimentally infected *Phlebotomus perniciosus* (A) and *Lutzomyia longipalpis* (B). Black bars, infected females that transmitted by bite; grey bars, infected females that did not transmit; line, percentage of females that transmitted parasites.
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longipalpis and 14% of *P. perniciosus* infected with the CUK3 strain transmitted more than 1000 parasites.

The majority of transmitting females inoculated less than 600 parasites. As most of these females were fully engorged by blood we may expect that their feeding pumps (the cibarial and pharyngeal pumps) and stomodeal valve were functioning normally. On the other hand, in those transmitting more than 1000 parasites there was a significant correlation between the pre-feeding load and the number of parasites transmitted. We suggest that these females with high dose deliveries regurgitated parasites because of impaired stomodeal valve function [18]. This would be consistent with previous studies [19,20] which have demonstrated

Table 2. Average feeding time of infected and noninfected *P. perniciosus* and *L. longipalpis*.

	L. infantum IMT373		*L. infantum* CUK3	
	P. perniciosus	*L. longipalpis*	*P. perniciosus*	*L. longipalpis*
Non-infected	10	11	8	10
Infected but without transmission	11	10	9	12
Infected and with transmission	12	10	12	18

Average time necessary for non-infected and infected sand flies to feed on mice is given in minutes.
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an opened stomodeal valve due to the physical presence of a parasite plug and damage of the chitin layer of the valve by *Leishmania* chitinase.

Infected sand fly females, and especially those that transmit parasites, feed longer on hosts than non-transmitting ones do. *Lutzomyia longipalpis* females transmitting dermotropic CUK3 strain parasites took an average of 1.5 times longer to complete a bloodmeal compared to specimens infected but not transmitting, and 1.8 times longer than uninfected females. Similarly, *P. perniciosus* infected by CUK3 and IMT373 take 1.5 and 1.2 times more time for a blood meal. Most of the infected sand flies exposed to anaesthetized mice did not demonstrate increased probing, but rather remained feeding for longer periods until either they were fully or partially engorged. This is in agreement with data previously published on the *L. longipalpis-L. mexicana* combination [21].

Although only one dermotropic and one viscerotropic *L. infantum* strains were evaluated, the significant variation in inoculum size between them allow us to hypothetise that the infectious dose delivered by vector sand flies may be an inherent character of each *Leishmania* strain. Moreover, the infectious dose might be a determining factor in the outcome of *Leishmania* infection. Local cutaneous lesions might result from a high-dose inoculum of dermotropic *Leishmania* resulting in a strong local immune response, whereas dissemination to internal organs might be the result of infected sand flies delivering a low number of parasites below the threshold required to produce/develop a localized and restraining immune response. This hypothesis corresponds with the data of Kimblin et al. [14] on the *L. major-P. duboscqi* combination. These authors evaluated the impact of inoculum size on infection outcome by comparing *L. major* infections with high (5×10^3) and low (1×10^2) dose intradermally inoculated by needle in the ears of C57BL/6 mice, and observed the rapid development of large lesions in the ears of mice receiving the high-dose inoculum. In contrast, the low dose resulted in only minor pathology but a higher parasite titre during the chronic phase [14]. Nevertheless, it will be necessary to evaluate more *L. infantum* strains with visceral and cutaneous tropism in order to determine if differences detected in our study were due to individual stock characteristics or if they are associated with parasite tropism in vertebrate hosts.

In conclusion, we have demonstrated that individual sand flies transmit *Leishmania* parasites in a wide dose range. However, the maximal natural dose found was still about 250 times lower than the challenge dose used for the *L. donovani* complex in most previous experimental works. This finding emphasizes the importance of determining the natural infective dose for the development of an accurate experimental model, which is crucial for the evaluation of new drugs and vaccine candidates against leishmaniasis.

Materials and Methods

Parasite strains

The viscerotropic *Leishmania infantum* strain IMT373 MON-1 (MCAN/PT/2005/IMT373) and the dermotropic *L. infantum* strain CUK3 (ITOB/TR/2005/CUK3) were used in this study. CUK3 was isolated from *Phlebotomus tobbi* from a Cukurova focus of cutaneous leishmaniasis [6] while IMT373 was isolated from a dog with leishmaniasis and passaged through mice in order to keep its virulence [13,22]. Promastigotes (with less than 12 *in vitro* passages since isolation) were cultured at +26°C in M199 medium (Sigma, USA) containing 10% heat-inactivated foetal calf serum (Gibco, USA), 50 mg/ml mikacin solution (Bristol-Myers Squibb, Czech Republic) and 1% sterile urine.

Sand flies and experimental infections

Lutzomyia longipalpis (originating from Jacobina, Brazil) and *Phlebotomus perniciosus* (originating from Murcia, Spain) colonies were maintained in an insectary under standard conditions as described by Volf and Volfova [23]. Five to six-day old female flies (200 *P. perniciosus* and 150 *L. longipalpis* females per experiment, respectively) were fed on heat inactivated rabbit blood containing promastigotes (10^7 parasites per ml of blood) through a chicken-skin membrane. Blood-engorged females were separated immediately and maintained on a 50% sucrose diet in >70% relative humidity at +26°C.

One group of females was dissected to study the development and localization of infection in the sand fly midgut two and ten days post blood meal, i.e., during early and late stage infection, respectively. Individual midguts were placed into a drop of saline buffer, and parasite numbers were estimated under a light microscope at 200X and 400X magnifications by an experienced worker. Parasite loads were graded as previously described [24] into four categories: negative, 1–100, 100–1000, and >1000 parasites per gut. A second group of females from the same batch was used for transmission experiments and parasite quantification by Real-time PCR (see below). Nine and six independent experiments were performed with *P. perniciosus*-IMT373 and *P. perniciosus*-CUK3 combinations, respectively, while six and four artificial infections were done with *L. longipalpis*-IMT373 and *L. longipalpis*-CUK3 combinations.

Mice

One hundred and eight BALB/c mice (41 for *P. perniciosus*-IMT373, 28 mice for *L. longipalpis*-IMT373, 23 for *P. perniciosus*–CUK3 and 16 for *L. longipalpis*-CUK3 combinations) older than 8 weeks of age were purchased from AnLab (Czech Republic) and housed at Charles University, Prague, under stable climatic and dietary conditions. Experiments were approved by the institutional Ethical Committee and performed in accordance with national legislation for the care and use of animals for research purposes. Mice were anaesthetized intraperitoneally with ketamine (150 mg/kg) and xylazine (15 mg/kg).

Transmission by bite and sample collection

Sand fly females were allowed to feed on whole body of anesthetised mice in a rectangular cage (20×20 cm) for about one hour at various days post infective blood meal (7–14 days for *L. longipalpis* and 9–23 days for *P. perniciosus*). Each mouse was placed individually into a cage together with about 50 *P. perniciosus* or 10 *L. longipalpis* females (the difference was due to the fact that *L. longipalpis* were more aggressive and had higher feeding rate). Two people followed each experiment; one recorded biting place and feeding time while the second ensured that each sand fly female probed in different place and then collected engorged flies by an aspirator immediately after terminating their blood meal; the site of bite and time of feeding were recorded for each female. After exposure, mice were sacrificed, biting place was inspected under a stereoscope and excised. Both samples (skin biopsies and corresponding fed sand flies) were stored at −20°C until DNA extraction.

Real-time PCR (qPCR)

Extraction of total DNA from each bite site and the corresponding sand fly were performed using a DNA tissue isolation kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µl and stored at −20°C. qPCR for detection and quantification of *Leishmania* sp.

was performed in a Rotor-Gene 2000 from Corbett Research (St. Neots, UK) using the SYBR Green detection method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA). For adequate sensitivity, kinetoplast DNA was chosen as the molecular target, with primers as previously described [25] (forward primer 5′-CTTTTCTGGTCCTCCGGGTAGG-3′ and reverse primer 5′-CCACCCGGCCCTATTTTACACCAA- 3′). Two microliters of eluted DNA was used per individual reaction. PCR amplifications were performed in duplicate wells using the conditions described previously [26]. Briefly, 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. Reaction specificities were checked for all samples by melting analysis. Quantitative results were expressed by interpolation with a standard curve included in each PCR run. Mass cultures of *L. infantum* promastigotes were used to construct a series of 10-fold dilutions ranging from 10^5 to 1 parasite per PCR reaction. Diluted parasites were co-processed with mouse tissue or sand fly females for DNA extraction. DNA from uninfected sand flies and mice were used as a negative control.

For sand fly females transmitting promastigotes into mouse skin, the pre-feeding midgut load was calculated as the sum of parasites in the midgut after feeding and the number of parasites transmitted.

Statistical analysis

Statistical analysis was performed using the software STATISTICA. For each *L. infantum* strain, a nonparametric Kruskal-Wallis test was used to compare: (i) the intensity of infection in *P. perniciosus* and *L. longipalpis*, and (ii) the number of parasites transmitted by each sand fly species into mice's skin. Correlations between feeding time and the number of parasites (i) in each sand fly female and (ii) inoculated into the skin, as well as the correlation between pre-feeding load and the number of parasites transmitted, were determined by simple linear regression analysis. Differences were considered statistically significant for p values <0.05.

Author Contributions

Conceived and designed the experiments: CM VS JS JV PV. Performed the experiments: CM VS JS JV. Analyzed the data: CM JV PV. Wrote the paper: CM PV.

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Phlebotomus orientalis sand flies from two geographically distant Ethiopian localities and their susceptibility to *L. donovani*

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Abstract

Background:

Phlebotomus orientalis (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 ($2x10^3$ 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*.

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*.

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 south-western Ethiopia

(e.g. Omo river plains and Segen/Woito valleys) and Metema-Humera plains in the northwest [1]. Three sand flies species, *Phlebotomus orientalis*, *P. celiae* and *P. martini* have been implicated as vectors [2,3]. *Phlebotomus celiae* and *P. martini* (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. 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 [4]. Additional important information, like actual breeding sites of this species, remains unknown. Despite several attempts of colonization of this species [5,6] 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 an outbreak of VL resulted in 2,500 cases and initially a very high mortality [7]. 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 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 [8]. 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 recorded for over 20 months.

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 [9]. Therefore, the colorimetric method developed by Briegel *et al.* [10] for measuring the hemoglobin concentration in blood-fed mosquitoes was adopted. Females of *P. orientalis*, 3 – 6 day old, were fed through a chick-skin membrane on rabbit blood. Individual midguts of blood-fed females were dissected 1h 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 100µg/well was used as standard.

Cross-mating study

For the cross-mating study we slightly modified the method described by Dvorak *et al*. [11]. 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. 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) and cytochrome oxidase I (COI). For RAPD analysis, 8 specimens from each colony (4 males and 4 unfed females) were selected randomly. Two other sand fly species were added into the analysis as outgroups: *P.* (*Larroussius*) *tobbi* and *P.* (*Phlebotomus*) *bergeroti*. DNA was extracted using High Pure PCR Template Preparation Kit (Roche, France). Out 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 [12]. Phylogenetic trees were constructed by the unweighted pair-grouping analysis (UPGMA) [13]. PC program FreeTree [14] 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 [15,16]. 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 North 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 (250µg/ml). Females of both colonies (~ 3-7 day old) were fed through a chick-skin membrane on a suspension of promastigotes (from 4-day-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 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 100µl 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 17]. Levels of *Leishmania* infections were graded into four categories according to Myskova *et al.* [18]: negative, light (<100 parasites/gut), moderate (100-1000 parasites/gut) and heavy (>1000 parasites/gut). Data were evaluated statistically by means of $\chi 2$ 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.* [19]. 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: 10s at 95°C, 10s at 56°C, and 10s 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 strategy 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 diapause, while the early larval stages were unaffected. In AZ colony, the non-synchronized larval development and tendency to diapause persisted 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 neighbour-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).

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 were high (around 90%) in both colonies and the intensity of infection was slightly higher in AZ colony ($P=0.048$). Abundant metacyclic promastigotes 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 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 diapausing larvae and prolonged the generation time with AZ colony being more sensitive to this change. Diapause of 4th instar larvae has been described in some Palaearctic species, whereas species from warmer, wetter habitats are expected to diapause at the egg stage [20]. Our findings, as well study by Schmidt [21], 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 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 [22].

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 [23]. In laboratory conditions an alternative blood-meal 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 low 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 [24,25,26] but only once in the south-west of Ethiopia [24]. In Sudan, the susceptibility of *P. orientalis* to *L. donovani* has also been demonstrated by feeding on patients with kala-azar [5,27] or by feeding infected blood through mouse-skin membranes [6]. 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 [28]; 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.* [29] 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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Figure legends

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).

TABLE 1. Life-cycle of *P.orientalis* colonies originating from Addis Zemen (AZ) and Melka Werer (MW) and their hybrid F1 and F2 progeny.

*Days represent an interval between the day the parental female took a blood-meal and the first offspring reached the respective stage.

**In the egg production study 20 ovipositing females were used in each group.

***In the parental colonies the life cycle data were collected from 12168 (AZ) and 8751 (MW) ovipositing females within the period from VIII/2010 to IV/2012. Each cell contains the mean and the range of values.

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 Neighbour-joining method.

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..

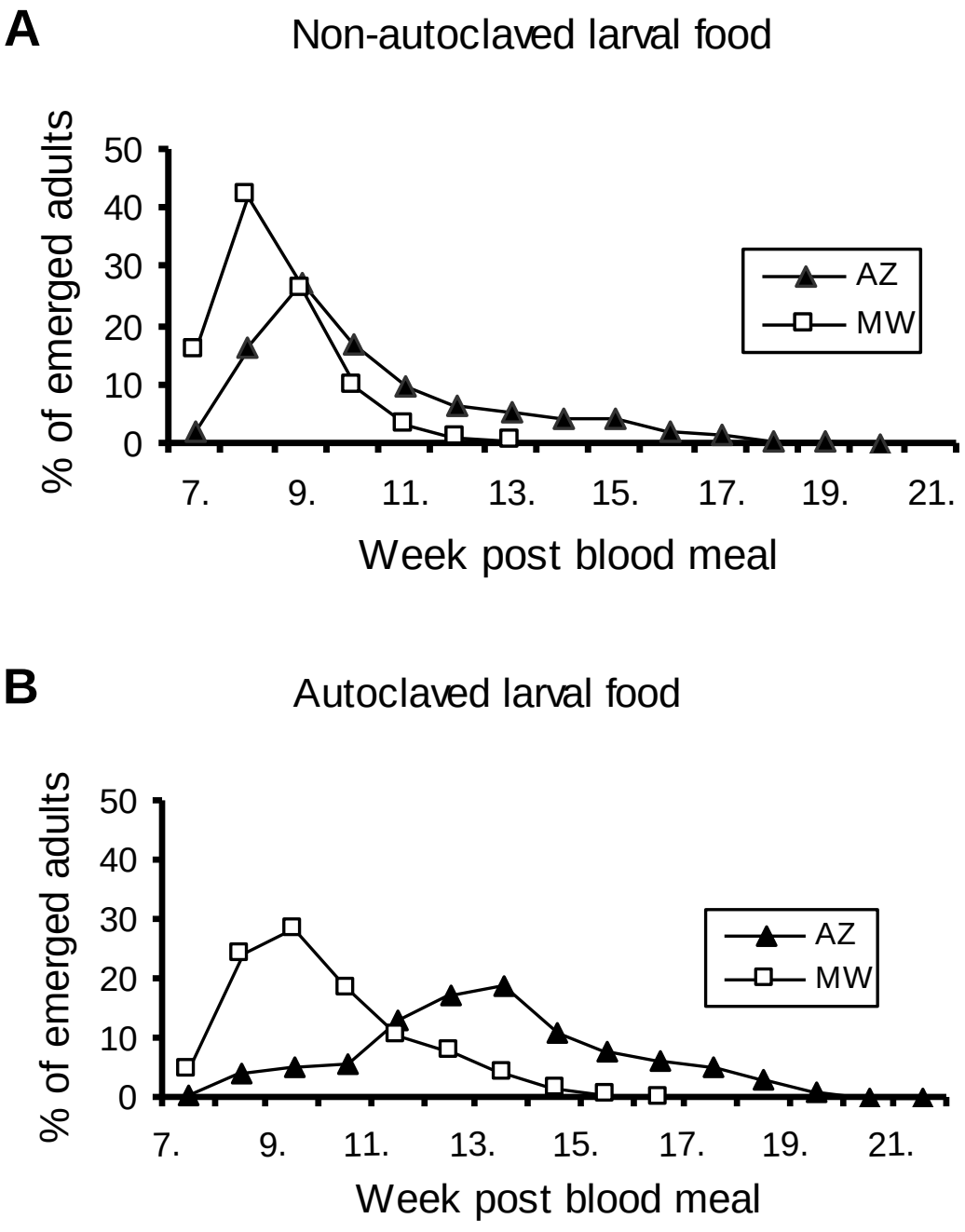
3A: Infected females of *P. orientalis* were examined microscopically 2, 5-6 and 8-11 days post-bloodmeal (PBM). The infection intensities were classified into three categories according to their intensity: heavy (more than 1,000 parasites per gut [black]), moderate (100-

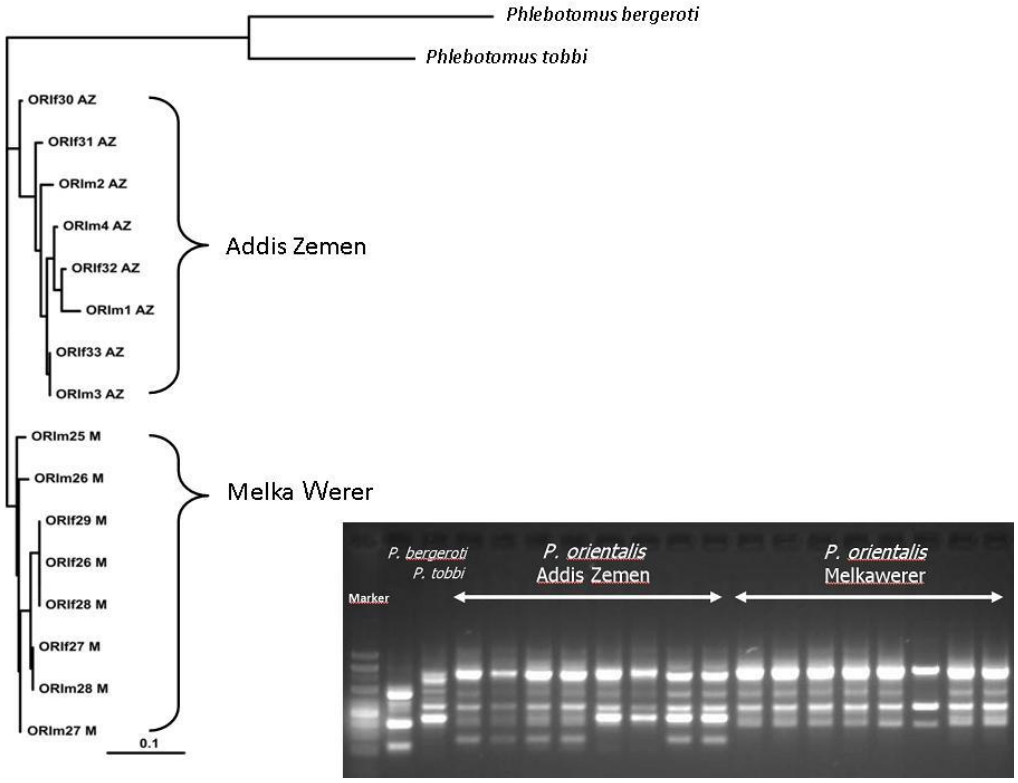
514 1,000 parasites [grey]) and light (1-100 parasites [white]). Numbers above the bars indicate
515 the number of dissected females.
516 **3B:** Parasite numbers from 40-50 individual females were quantified by Q-PCR targeted on
517 amplification of *Leishmania* kDNA 10 days PBM.
518
519 **Figure 4. Effect of initial infective dose on development of *L. donovani* (GR 374) in *P.***
520 ***orientalis*.**
521 **4A:** Infected females of *P. orientalis* (MW colony) were examined microscopically 2-3, 6 and
522 10 days post-bloodmeal (PBM). The infection intensity was classified as described in Fig.3.
523 **4B:** Parasite numbers were determined using Q-PCR at 10 days PBM. Twenty females were
524 used per group.

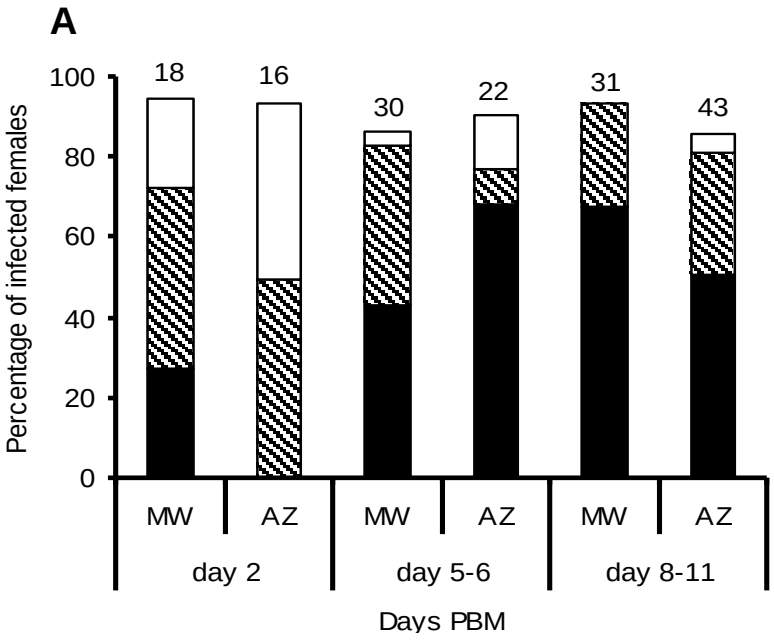
TABLE 1: **Life-cycle of *P.orientalis* colonies originating from Addis Zemen (AZ) and Melka Werer (MW) and their hybrid F1 and F2 progeny.**

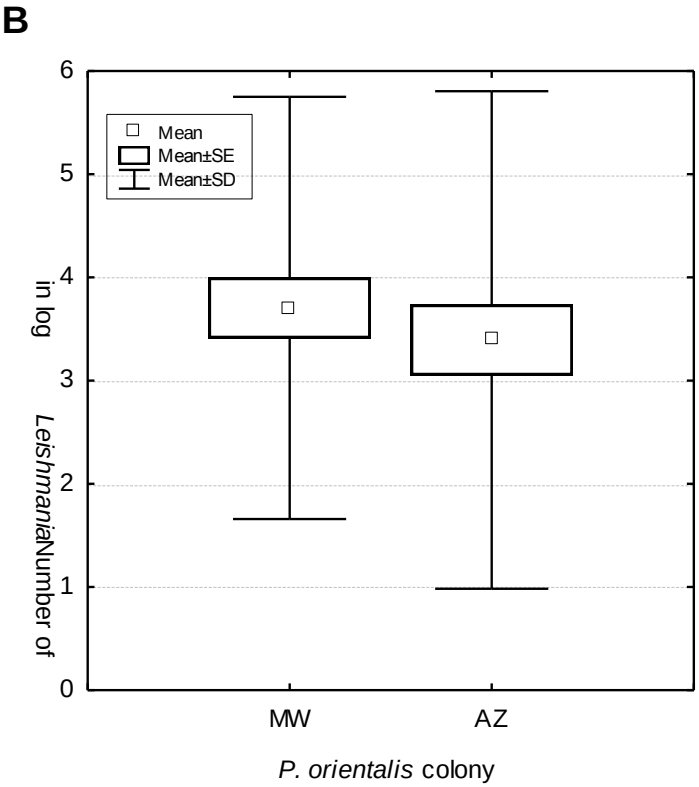
			Life cycle in days PBM*							Host	Egg production**	
			Eggs	Larvae			Pupae	Adults				Eggs
				L1	L2	L4		From	To		Total	Mean per female
Parental colonies***	AZ	mean	6.5	13.5	19.1	28.4	36.9	46.6	105.3	mouse	544	27.2
		range	5 - 9	11 - 19	16 - 29	23 - 34	31 - 47	39 - 69	61 - 147	human	975	48.75
	MW	mean	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

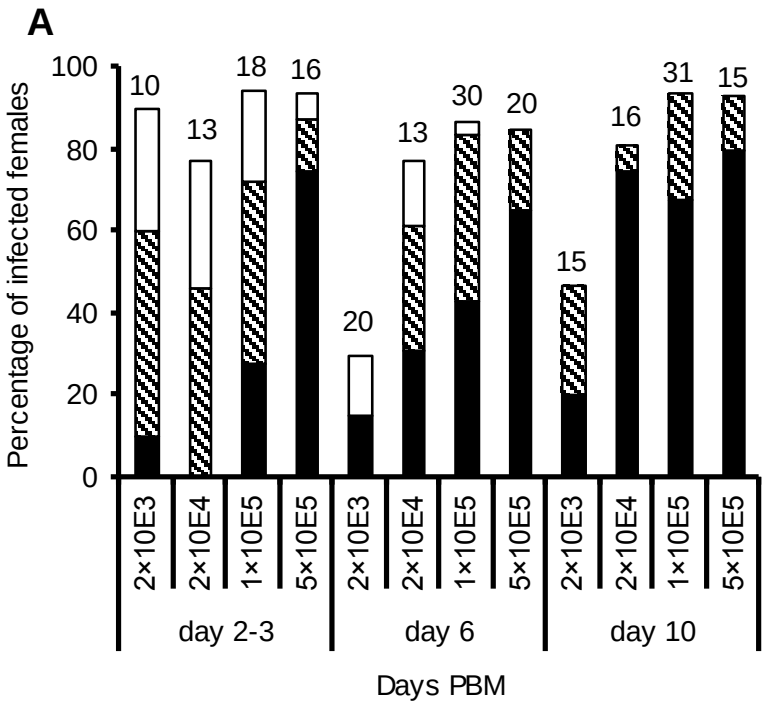
* Days represent an interval between the female took a blood-meal and the first offspring reached the respective instar.
**20 ovipositing females were used in each group.
***In the parental colonies the life cycle data were collected from 12,168 (AZ) and 8,751 (MW) ovipositing females within the period from VIII/2010 to IV/2012. Each cell contains the mean and the range of values.


A
Non-autoclaved larval food
% of emerged adults
50
40
30
20
10
0
7.
9.
11.
13.
15.
17.
19.
21.
Week post blood meal
AZ
MW
B
Autoclaved larval food
% of emerged adults
50
40
30
20
10
0
7.
9.
11.
13.
15.
17.
19.
21.
Week post blood meal
AZ
MW


Phlebotomus bergeroti
Phlebotomus tobbi
ORIf30 AZ
ORIf31 AZ
ORIm2 AZ
ORIm4 AZ
ORIf32 AZ
ORIm1 AZ
ORIf33 AZ
ORIm3 AZ
Addis Zemen
ORIm25 M
ORIm26 M
ORIf29 M
ORIf26 M
ORIf28 M
ORIf27 M
ORIm28 M
ORIm27 M
Melka Werer
0.1
P. bergeroti
P. tobbi
P. orientalis
Addis Zemen
P. orientalis
Melkawerer
Marker


A
Percentage of infected females
100
80
60
40
20
0
18
16
30
22
31
43
MW
AZ
MW
AZ
MW
AZ
day 2
day 5-6
day 8-11
Days PBM


B
Mean
Mean±SE
Mean±SD
6
5
4
3
2
1
0
in log
Leishmania Number of
MW
AZ
P. orientalis colony


A
Percentage of infected females
100
80
60
40
20
0
10
13
18
16
20
13
30
20
15
16
31
15
2×10E3
2×10E4
1×10E5
5×10E5
2×10E3
2×10E4
1×10E5
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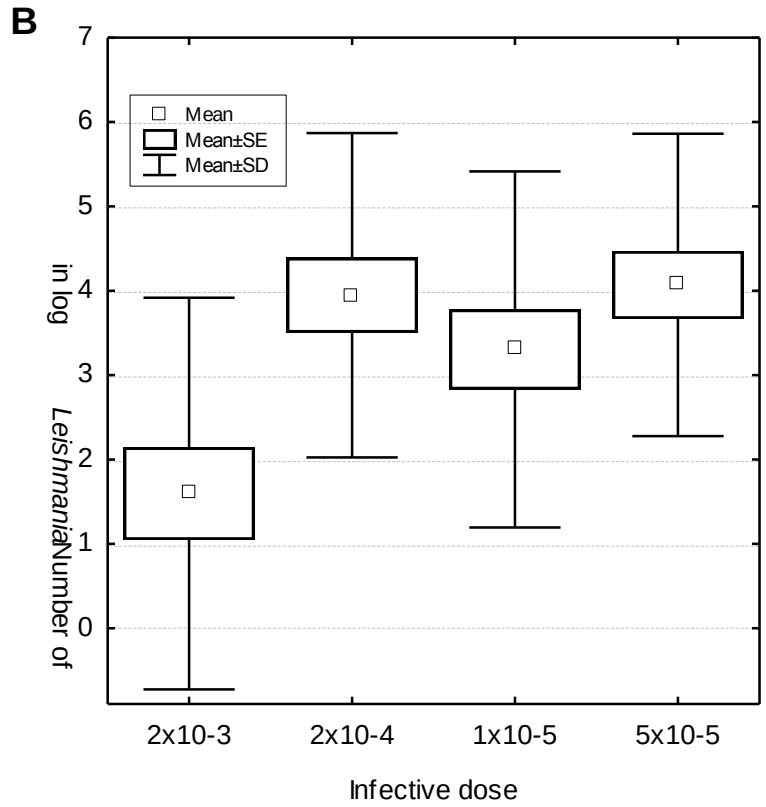
2×10E3
2×10E4
1×10E5
5×10E5
day 2-3
day 6
day 10
Days PBM


B
Mean
Mean±SE
Mean±SD
7
6
5
4
3
2
1
0
in log
Leishmania Number of
2x10-3
2x10-4
1x10-5
5x10-5
Infective dose

Sadlova J, Dvorak V, Seblova V, Warburg A, Votypka J, Volf P. (2013). *Sergentomyia schwetzi* is not a competent vector for *Leishmania donovani* and other *Leishmania* species pathogenic to human. Manuscript submitted to *Parasites & Vectors*.

SERGENTOMYIA SCHWETZI IS NOT A COMPETENT VECTOR FOR *LEISHMANIA DONOVANI* AND OTHER *LEISHMANIA* SPECIES PATHOGENIC TO HUMANS.

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Abstract.

Sand fly species of the genus *Sergentomyia* are proven vectors of reptilian *Leishmania* that are non-pathogenic to humans. However, their role in transmission of human pathogens remains unclear. Here we studied the susceptibility of colonized *Sergentomyia schwetzi* to *L. donovani* and two other *Leishmania* species pathogenic to humans: *L. infantum* and *L. major*. All three *Leishmania* species produced heavy late stage infections in *Lutzomyia longipalpis* or *Phlebotomus duboscqi* sand flies used as positive controls. In contrast, none of them completed their developmental cycle in *Sergentomyia* females; *Leishmania* promastigotes developed within the bloodmeal enclosed by the peritrophic matrix (PM) but were defecated together with bloodmeal remnants failing to establish a midgut infection. In *S. schwetzi*, the PM persisted significantly longer than in *L. longipalpis* and it was degraded almost simultaneously with defecation. Therefore, *Leishmania* transformation from procyclic to long nectomonad forms was delayed and parasites did not attach to the midgut epithelium. Data indicate that the relative timing of defecation versus PM degradation represents a crucial aspect of *Sergentomyia* refractoriness to human *Leishmania* species.

Key words: visceral leishmaniases, phlebotomine sand flies, *Phlebotomus*, peritrophic matrix

Introduction

Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a serious health problem in parts of the Indian subcontinent and in several East African countries, mainly Kenya, Ethiopia and Sudan. Three sand fly species, *P. (Larroussius) orientalis*, *P. (Synphlebotomus) martini*, and *P. (Synphlebotomus) celiae*, have been incriminated as vectors in East Africa (reviewed by[1]). *Phlebotomus martini* and *P. celiae* are associated with the presence of termite mounds, soil moisture and a prolonged wet season while *P. orientalis* prefers drier habitats and is the main man-biter in *Acacia-Balanites* forests in Sudan and Ethiopia [2–4]. It is the dominant vector in the VL endemic areas in Sudan (reviewed by [5]) and the probable vector in most VL foci in Ethiopia [6,7]. However, although *P. orientalis* is a predominant species in some VL foci in north and northwest Ethiopia, no natural infection was detected in hundreds females examined [6,8]. In addition, VL is present also in localities like the Malakal urban area in Sudan, where *P. orientalis* or other proven vectors of *L. donovani* were not found [9]. Therefore, vector competence of other sand fly species found in endemic areas has been tested. Recently, *P. rodhaini* was implicated as a possible zoonotic vector of *L. donovani* in woodlands in eastern Sudan [10]. However, *P. rodhaini* is rather a rare species with a low man-biting rate while the prerequisite of a vector of human pathogens is that it be abundant in the disease endemic areas and display man-biting behaviour.

Sergentomyia spp. are widespread in Africa, tolerate various biotopes and environments and are by far the predominant sand flies in many African ecosystems [2,11,12]. Sand flies of this genus are proven vectors of reptile *Leishmania* non-pathogenic to humans, previously separated to the genus *Sauroleishmania* [13] but following recent DNA sequence-based phylogenies included back in the genus *Leishmania* (reviewed by [14]). The development of reptilian *Leishmania* spp. in vectors is usually hypopylarian (occurring in the hind gut) with transmission by predation (lizards feed on infected sand fly) and not by bite although infections of oesophagus, pharynx and proboscis have been reported [15]. However, *Sergentomyia* species are not restricted to feeding on reptiles and at least some of them feed on humans and/or mammalian reservoirs of *Leishmania* pathogenic to humans. Therefore, they were suspected as vectors in some VL and cutaneous leishmaniasis (CL) foci where *Sergentomia* spp. were abundant and found to harbor *Leishmania* [16] or significantly

associated with leishmaniasis seroprevalence [12]. Additional support for the role of *Sergentomyia* spp. in transmission of mammalian *Leishmania* came from a study performed in a *L. major* focus in Baringo district, Kenya [17] where *P. duboscqi* was proven as primary vector. *S. ingrami* females were found to be infected in comparatively high rates (about 1%). Moreover, *Leishmania* parasites isolated from dissected *S. ingrami* guts and inoculated into BALB/c mice caused typical *L. major* lesions; smears from lesions revealed numerous amastigotes. Therefore, *S. ingrami* was considered by the authors as a secondary zoonotic vector of *L. major* in the Baringo focus[17].

Sergentomyia schwetzi has a wide range of distribution in Africa, south of the Sahara. It predominated among all sand fly species caught in Senegal [12], southern Ethiopia [18] and eastern Sudan [11,19] showing strong endophilic behaviour [9,19] and man biting tendencies [7,9]. In a focus of VL in Northern Ethiopia, *S. schwetzi* was exceptionally abundant and the only *Sergentomyia* species attracted to CO_2 (Kirstein and Faiman, personal communication).

To solve the question if *S. schwetzi* supports the full developmental cycle of *Leishmania* pathogenic to humans, we experimentally infected laboratory-reared *S. schwetzi* with *L. donovani*, *L. infantum* and *L. major*. The permissive vector species *Lutzomyia longipalpis* and the proven vector of *L. major, P. duboscqi*, were chosen as positive controls.

Methods.

Leishmania and sand flies. *Leishmania major* (MHOM/IL/81/Friedlin/VI; FVI) was cultured in M199 medium (Sigma) containing 10% heat-inactivated fetal calf serum (Gibco) and 250μg/ml amikacin (Amikin, Bristol-Myers Squibb). *L. donovani* (MHOM/ET/2010/GR374) and *L. infantum* (ITOB/TR/2005/CUK3) were cultured in the same medium supplemented by 1% BME vitamins (Sigma) and 2% sterile urine. The colony of *S. schwetzi* was established from specimens collected in Sheraro (14° 24' 09.69"N – 37° 46' 39.69"E), a town in North-Western Ethiopia, located in the Mi'irabawi Zone of the Tigray Region. Laboratory colonies of *L. longipalpis* (from Jacobina, Brazil) and *P. duboscqi* (from Senegal) served as a control. All three sand fly colonies were maintained at 26°C on 50% sucrose and 14 h light/10 h dark photoperiod as described previously [20].

Sand fly infections. Sand fly females (5-9 days old) were infected by feeding through a chick-skin membrane on heat-inactivated rabbit blood containing 10^6 promastigotes ml^{-1}. If

not otherwise stated, engorged sand flies were maintained in the same conditions as the colony. The effect of temperature was tested by comparison of parasite development at 21°C. Females were dissected at different time intervals post bloodmeal (PBM), the abundance and location of *Leishmania* infections in the sand fly digestive tract was examined by light microscopy. Parasite loads were graded according to [21] as light (< 100 parasites per gut), moderate (100–1000 parasites per gut) and heavy (> 1000 parasites per gut). Experiments with each *Leishmania* – sand fly combination were repeated twice or three times.

Morphometry of parasites. On day 2 post-blood meal (PBM) midgut smears of *S. schwetzi* and *Lu. longipalpis* infected with *L. donovani* were fixed with methanol, stained with Giemsa, examined under the light microscope with an oil- immersion objective and photographed with an Olympus D70 camera. Body length, flagellar length and body width of 300 randomly selected promastigotes from five females/smears were measured for each sand fly species using Image-J software.

Statistical analysis. Differences in intensities of infections, presence vs. absence of peritrophic matrix and remnants of blood were tested using Fisher's exact test (for 2x2 contingency tables) or Chi-square tests. Measurements of parasites were compared using Analysis of variance. All the statistical evaluations were performed with statistical software SPSS version 16.

Results.

Development of three *Leishmania* species in *S. schwetzi*. Development of *L. donovani* in *S. schwetzi* was followed from day 2 to 9 PBM and compared with development in *Lu. longipalpis*, sand fly known as highly susceptible for this *Leishmania* [22]. On day 2 PBM, heavy infections were enclosed inside the peritrophic matrix (PM) in most females of both species. However, further development differed considerably (Fig. 1). In *L. longipalpis*, parasites developed heavy infection of the abdominal midgut (AMG) and thoracic midgut (TMG) and started to colonize the stomodeal valve region by day 3 PBM already; infection rates did not fall below 80% throughout the experiment.

On the other hand, infection rates in *S. schwetzi* rapidly decreased to 28 % by day 3 PBM, 19 % by day 4 PBM and 1.4% by day 9 PBM. In all but one positive females (n=65), parasites were located within the bloodmeal and enclosed by the intact PM. In a single

female, promastigotes were observed freely-swimming in the AMG but we cannot exclude the possibility that they were released due to damage of the PM during dissection.

The morphology of *L. donovani* was studied on day 2 PBM when the bloodmeal was still enclosed inside the PM in 100% of *S. schwetzi* and 90.9% of *L. longipalpis*. Although both sand fly species were infected by the same parasite culture, the body length of *L. donovani* developing in *L. longipalpis* was significantly higher than that of parasites developing in *S. schwetzi* (Table 1).

Notably, Like *L. donovani, L. infantum* and *L. major* infections did not thrive *in S. schwetzi* either (Figs. 2, 3). During early phases of infection, when parasites were still inside the endoperitrophic space, infection rates were comparable with those reached in control vectors, i.e. *L. longipalpis* and *P. duboscqi* with *L. infantum* and *L. major*, respectively. However, on day 5 PBM, only one *L. major* and two *L. infantum* infections were found in the abdominal midgut of *S. schwetzi* and no parasites survived till day 9 or 10 PBM.

To explain the different competences of *S. schwetzi* and *L. longipalpis* for *L. donovani*, we focused on physiological differences between these two sand fly species, namely on the kinetics of the development of the PM and the defecation of bloodmeal remnants. We also tested development of *L. donovani* infections in females maintained under different ambient temperatures (see below).

Kinetics of the development of the PM and the defecation of digested blood remnants. Table 2 shows highly significant interspecific differences in formation of the PM by days 3 and 4 PBM. While in *L. longipalpis* the PM was found in 8% and 0% on days 3 and 4 PBM, respectively, in *S. schwetzi* it persisted longer and was still present in more than 20% of females on day 4 PBM. The interspecific difference was even more pronounced in infected females (Table 2). Defecation of blood meal remnants was faster in *L. longipalpis*; by day 4 PBM all but one female of this species finished defecation (Table 2). Importantly, on day 3 PBM, the percentage of females in which the PM already degraded but did not yet defecate the blood remnants was significantly higher in *L. longipalpis* (Table 3).

Effect of decreased temperature on the PM and the development of *L. donovani* in *S. schwetzi*. Lower temperature prolongs the duration of blood digestion in sand flies [20]. Therefore, we tested if lowering the temperature to 21ºC would result in enhanced development of *L. donovani* in *S. schwetzi*. At 21ºC, the degradation of PM was delayed, it

was present till day 4 and 5 PBM in *L. longipalpis* and *S. schwetzi*, respectively (Fig. 4). The difference between vector species was significant, on day 5 PBM the PM was present in 78% of *S. schwetzi* and 0% of *L. longipalpis* (Chi-square = 10.957, d.f. = 1, P = 0.001).

In *S. schwetzi* delayed defecation resulted in higher infection rates on days 3 – 5 PBM and prolonged presence of *L. donovani* till day 5 PBM (Fig. 2). However, from 22 positive females dissected on days 4 and 5 PBM, all but one had parasites still enclosed inside the PM (only in one female parasites were found free in the abdominal midgut). No infected *S. schwetzi* females were found on day 9 PBM. On the other hand, in *L. longipalpis* the lower temperature did not affect the infection rates, *L. donovani* developed well and on day 9 PBM all infected females showed heavy infections with colonization of the SV.

Discussion.

Demonstration of pathogen development under experimental conditions is one of crucial parameters for vector incrimination [23]. Our observations clearly showed that *L. donovani, L. infantum* and *L. major* promastigotes did not develop late stage infections in *S. schwetzi*. They did not survive defecation of bloodmeal remnants and did not colonize the anterior midgut, which is the prerequisite for transmission by bite. Similar results were observed by Kaddu et al. [24]; *L. donovani* promastigotes produced only scanty parasitaemia in the abdominal midgut in three out of six *Sergentomyia* species without real colonization of the thoracic midgut and cardia. Lawyer et al. [25] also described that Kenyan *S. schwetzi* does not support the development of *L. major*: for the first 48hr, parasite development progressed but parasites were rarely seen after 48hr and never after 90hr PBM.

The mechanism of the resistance of *Sergentomyia* species to *Leishmania* parasites is not clear and different hypotheses are plausible. Generally, there are several barriers in the sand fly midgut which must be overcome by the parasite to establish the infection: production of proteolytic enzymes during digestion of the bloodmeal, persistence of the peritrophic matrix and molecular characteristics of the midgut epithelium enabling or precluding the attachment of parasites. Parasites which do not overcome these midgut barriers are defecated with food remnants (for review see [26,27]).

Several authors hypothesized that main refractory barrier for *Leishmania* development in *Sergentomyia* is the fast digestion of the bloodmeal. Strelkova [28] and Reznik and Kuznecova [29] showed that destruction of erythrocytes in *S. arpaklensis* (=*S. sintoni* based on recent nomenclature) proceeded markedly faster in comparison with the

Phlebotomus spp. These authors concluded that faster digestion was due to specialization of *Sergentomyia* for feeding on reptiles and digestion of nucleated erythrocytes [28]. Similarly, Lawyer et al. [25] observed faster digestion of the bloodmeal in *S. schwetzi* than in *P. duboscqi*.

However, the speed of bloodmeal digestion alone is not critical for *Leishmania* development in the vector. In our study digested blood defecation by *S. schwetzi* females spanned a significantly longer time period than in *L. longipalpis*. In addition, prolonged time of digestion induced by decreased temperature did not enhance the development of *L. donovani* in *S. schwetzi*; parasites were eliminated due to defecation after 5 days of development within the bloodmeal. Data indicated that the crucial aspect mediating the refractoriness of *Sergentomyia* was the relative timing of defecation versus degradation of the PM.

Timing of disintegration of the PM in sand fly females may be important for the development of *Leishmania* promastigotes due to several reasons. Addition of exogenous chitinase to the bloodmeal blocked PM formation in *P. papatasi* which resulted in complete loss of *L. major* infections. These experiments showed that during early phase of infections the PM can protect the parasites against the rapid diffusion of digestive enzymes [30].

Our previous study with *L. major* and *P. duboscqi* revealed that disintegration of the PM coincides with transformation of procyclic promastigotes to long nectomonads [31]. Broken PM ceases to form a mechanical barrier for parasites and enables the diffusion of signal molecules from the ectoperitrophic space to the vicinity of parasites and leads to their transformation. These signal molecules are probably salivary components ingested into the midgut [26] which are known to trigger parasite transformation *in vitro* [32,33]. While the procyclic promastigotes lack the ability to bind to midgut epithelium [34], highly motile nectomonad forms escape from the endoperitrophic space and bind to the midgut epithelium to avoid defecation together with bloodmeal remnants [26]. In this study, measurement of promastigotes in *L. longipalpis* on day 2 PBM revealed the presence of long nectomonads simultaneously with the disintegration of the PM. On the other hand, delayed transformation or elongation of *L. donovani* promastigotes was observed in *S. schwetzi* on day 2 PBM. Promastigotes mostly remained as procyclic forms probably due to lack of the signal molecules due to intact PM.

Persistence of the PM can influence *Leishmania* development in additional ways. A crucial parameter is the duration of the period between the degradation of the PM and

defecation. On day 3 PBM, most *L. longipalpis* females had broken PMs but still retained blood remnants within the midgut as they did not defecate yet. Therefore, long nectomonads were free to leave the endoperitrophic space and attach to the *L. longipalpis* midgut wall. In *S. schwetzi*, the degradation of the PM was delayed often till defecation. Thus, there was either a very short time period between the degradation of the PM and defecation or the PM broke simultaneously with defecation. Therefore, promastigotes swimming freely in ectoperitrophic space of *Sergentomyia* midgut were extremely rare. The persistence of the PM till the end of digestion was described also in *S. arpaklensis* [29,35] where it probably excluded transmission of *L. gymnodactyli* through the bite of *S. arpaklensis* [35].

Results of laboratory experiments suggest that findings of field studies be interpreted with caution. Altogether, eleven species of *Sergentomyia* have been showed microscopically to carry *Leishmania* promastigotes in Kenya (reviewed by [36]) and Ethiopia [7]. However, these promastigotes were not characterized biochemically or genetically and therefore not confirmed to be mammalian parasites. In addition, several *Sergentomyia* species were found PCR positive for DNA of human pathogenic *Leishmania* species: *L. major* DNA was found in *S. darlingi* in Mali [37], *S. garnhami* in Kenya [16] and in *S. sintoni* in Iran [38] while *L. donovani* DNA was detected in *S. babu* in Indian VL foci [39]. These results, however, do not mean that *Sergentomyia* spp. are involved in transmission of *L. major* or *L. donovani*.PCR positivity alone should not be used for incrimination of the sand fly (or other blood-sucking arthropod) as *Leishmania* vector; PCR does not detect whether parasites are viable and transformed to virulent metacyclic promastigotes. Early phase of *Leishmania* development in the vector is non-specific and promastigotes are able to develop in various bloodsucking arthropods, even in biting midges: *Leishmania* development was demonstrated in the *Culicoides nubeculosus* midgut until day 2 PBM, but a subsequent loss of parasites occurred, although a PCR-based assay indicated their presence for up to seven days [40].

In conclusion, the vector competence of a suspected sand fly species should be confirmed by the precise microscopical observation of parasites in infected sand flies. In the case of human *Leishmania*, by finding heavy infections with metacyclic promastigotes colonizing the thoracic midgut and the stomodeal valve which is a prerequisite for successful transmission by bite [27,41,42]. As we never found such infections in *S. schwetzi* we conclude that this species cannot serve as the vector of *L. donovani* in the VL foci of Northern Ethiopia

Figure 1

(A) Rates and intensities of infections with *L. donovani* in *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.) at 26°C. Numbers of dissected females are shown above bars. Probability of differences was tested by Chi-square test.

(B) Location of *L. donovani* in infected *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.) at 26°C.

Figure 2

(A) Rates and intensities of infections with *L. infantum* in *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.). Numbers of dissected females are shown above bars. Probability of differences was tested by Chi-square test.

(B) Location of *L. infantum* in infected *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.).

Figure 3

(A) Rates and intensities of infections with *L. major* in *Sergentomyia schwetzi* (S.s.) and *Phlebotomus duboscqi* (P.d.). Numbers of dissected females are shown above bars. Probability of differences was tested by Chi-square test.

(B) Location of *L. major* in infected *Sergentomyia schwetzi* (S.s.) and *Phlebotomus duboscqi* (P.d.).

Figure 4

(A) Rates and intensities of infections with *L. donovani* in *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.) at 21°C. Numbers of dissected females are shown above bars. Probability of differences was tested by Chi-square test.

(B) Location of *L. donovani* in infected *Sergentomyia schwetzi* (S.s.) and *Lutzomyia longipalpis* (L.l.) at 21°C.

Table 1. Comparison of body length of *L. donovani* developing in *Sergentomyia schwetzi* and *Lutzomyia longipalpis.* Parasites were measured from blood smears of sand flies dissected by day 2 PBM.

	N	Mean (S.D.) (μm)	Range (μm)	Significance of difference between vector species ANOVA.
S. schwetzi	300	9,24 (3,39)	3.5-17.9	F = 180,251; d.f. = 1, P <0.0001
L. longipalpis	300	12,83 (3,15)	4.7-22.1	

Table 2. Presence of the peritrophic matrix in *Sergentomyia schwetzi* and *Lutzomyia longipalpis* females maintained at 26ºC post infective feeding on *L. donovani*.

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Day PBM	Sand fly species	All fed females			Infected females		
		Females with PM present/ Total N	Percent	Significance of interspecific difference (Fisher's exact test)	Females with PM present/ Total N	Percent	Significance of interspecific difference (Fisher's exact test)
2	*S. schwetzi*	44/44	100,0	P = 0,075	37/37	100,0	P = 0,422
	L. longipalpis	30/33	90,9		26/27	96,3	
3	*S. schwetzi*	17/58	29,3	P = 0,019	16/16	100,0	P < 0,001
	L. longipalpis	3/38	7,9		3/34	8,8	
4	*S. schwetzi*	12/58	20,7	P = 0,001	11/11	100,0	P < 0,001
	L. longipalpis	0/42	0,0		0/39	0,0	
5	*S. schwetzi*	0/34	0,0	Not computed	-	-	Not computed
	L. longipalpis	0/25	0,0		0/22	0,0	
9	*S. schwetzi*	1/73	1,4	P = 1,000	1/1	100,0	P = 0,045
	L. longipalpis	0/25	0,0		0/21	0,0	

Table 3. Blood defecation of *Sergentomyia schwetzi* and *Lutzomyia longipalpis* females maintained at 26ºC post infective feeding on *L. donovani*.

Day PBM	Sand fly sp.	Females that finished defecation/ Total N (%)	Significance of difference between vector species (Fisher's exact test).	Females with the PM intact or slightly disintegrated / Females which did not defecate (%)	Females which the PM degraded / Females which did not defecate (%)	Significance of difference between vector species (Fisher's exact test).
2	*S. schwetzi*	0/44 (0)	Not computed	44/44 (100)	0/44 (0)	P = 0.075
	L. longipalpis	0/33 (0)		30/33 (91)	3/33 (9)	
3	*S. schwetzi*	37/58 (64)	P = 1,000	17/21 (81)	4/21 (19)	P = 0.001
	L. longipalpis	25/38 (66)		3/13(23)	10/13 (77)	
4	*S. schwetzi*	45/58 (78)	P = 0,007	12/13 (92)	1/13 (8)	Not computed
	L. longipalpis	41/42 (98)		0/1 (0)	1/1 (100)	
5	*S. schwetzi*	33/34 (97)	P = 1,000	0/1 (0)	1/1(100)	Not computed
	L. longipalpis	25/25 (100)		-	-	
9	*S. schwetzi*	72/73 (99)	P = 0,447	1/1(100)	0/1 (0)	Not computed
	L. longipalpis	24/25 (96)		0/1 (0)	1/1 (100)	

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Figure 1A

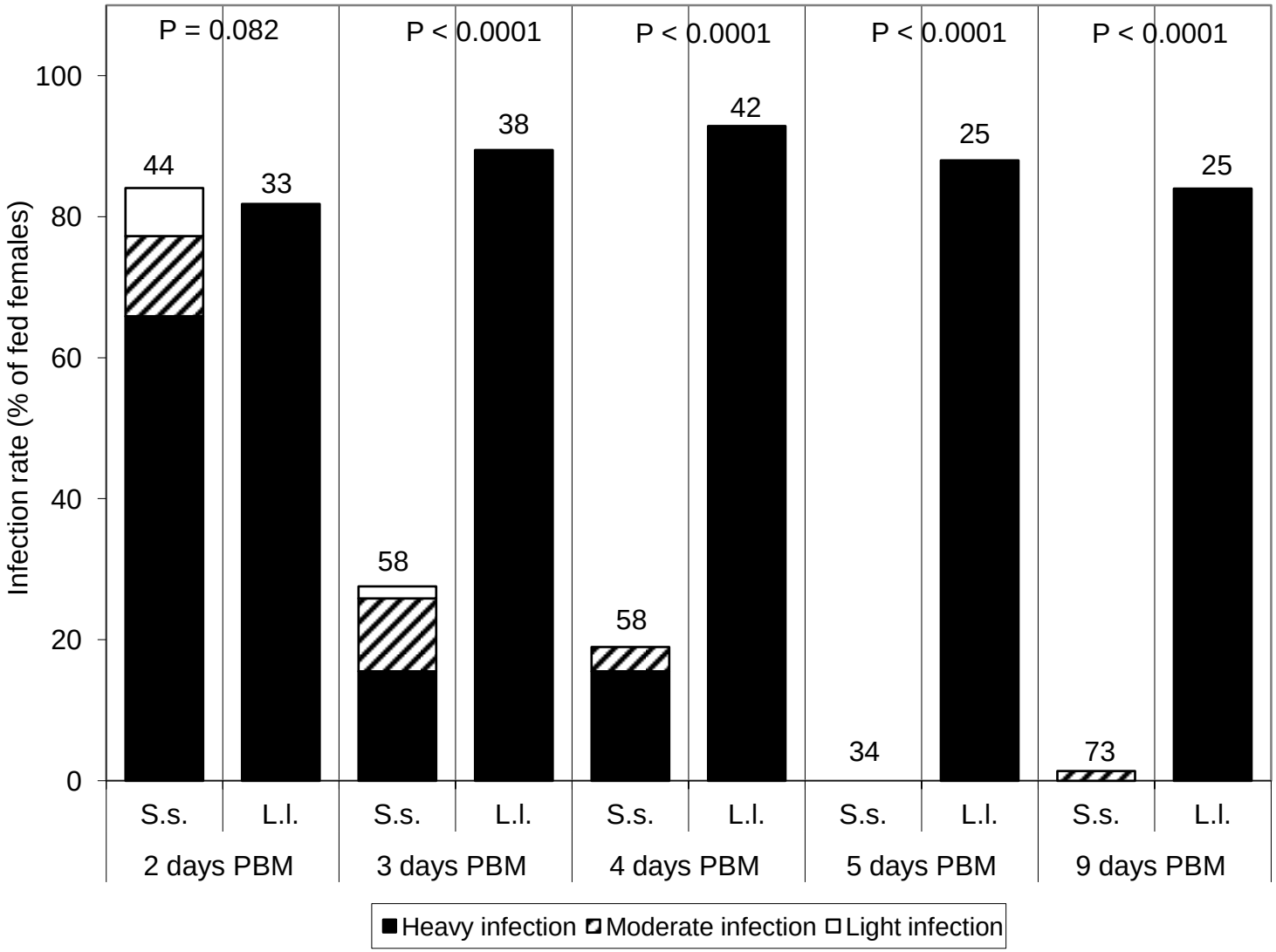

P = 0.082
P < 0.0001
P < 0.0001
P < 0.0001
P < 0.0001
Infection rate (% of fed females)
100
80
60
40
20
0
44
33
58
38
58
42
34
25
73
25
S.s.
L.l.
2 days PBM
3 days PBM
4 days PBM
5 days PBM
9 days PBM
Heavy infection
Moderate infection
Light infection

Figure 1B

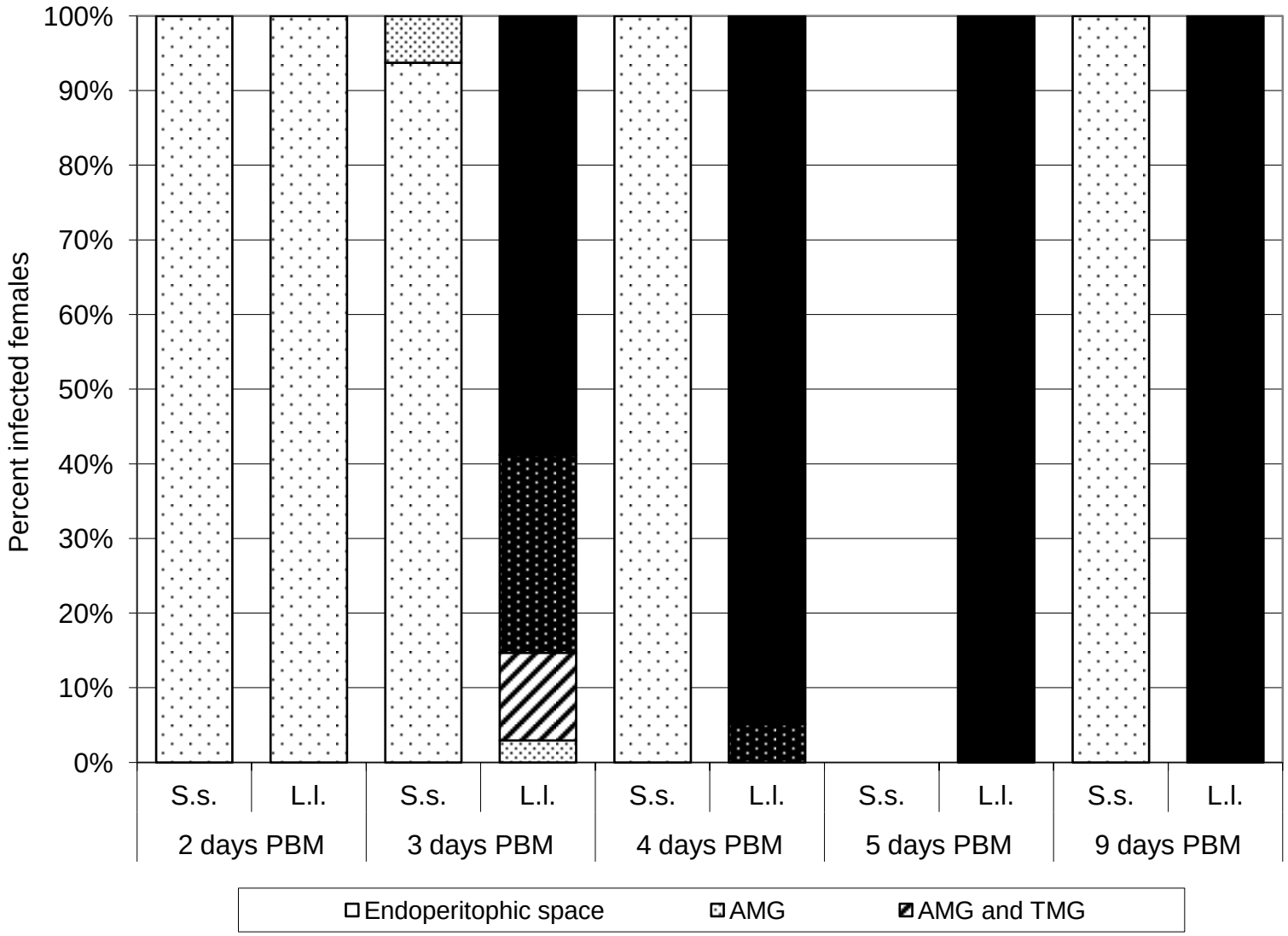

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent infected females
S.s.
L.l.
S.s.
L.l.
S.s.
L.l.
S.s.
L.l.
S.s.
L.l.
2 days PBM
3 days PBM
4 days PBM
5 days PBM
9 days PBM
Endoperitophic space
AMG
AMG and TMG

Figure 2A

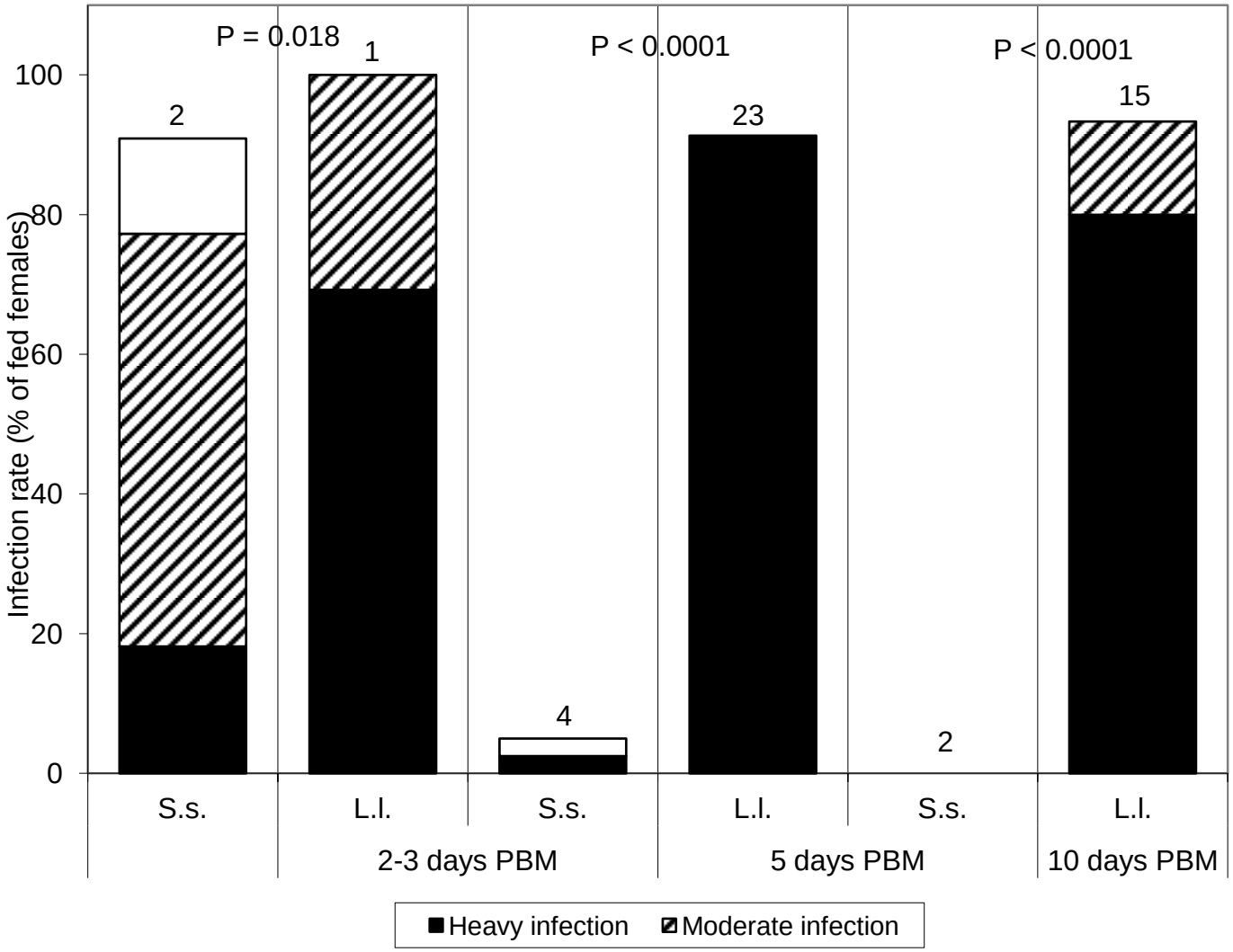

P = 0.018
P < 0.0001
P < 0.0001
2
1
4
23
2
15
100
80
60
40
20
0
Infection rate (% of fed females)
S.s.
L.l.
S.s.
L.l.
S.s.
L.l.
2-3 days PBM
5 days PBM
10 days PBM
Heavy infection
Moderate infection

Figure 2B

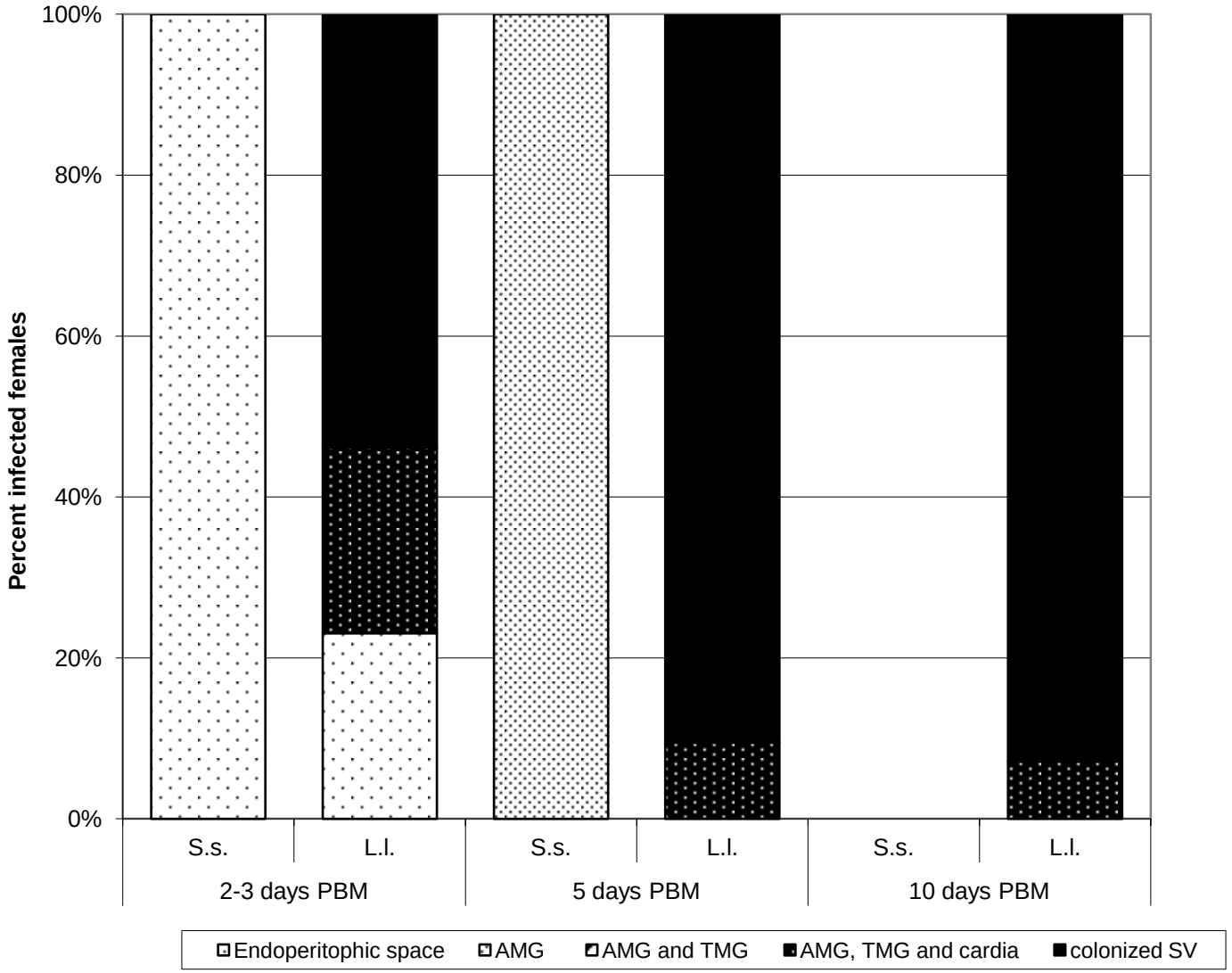

100%
80%
60%
40%
20%
0%
Percent infected females
S.s.
L.I.
S.s.
L.I.
S.s.
L.I.
2-3 days PBM
5 days PBM
10 days PBM
Endoperitophic space
AMG
AMG and TMG
AMG, TMG and cardia
colonized SV

Figure 3A

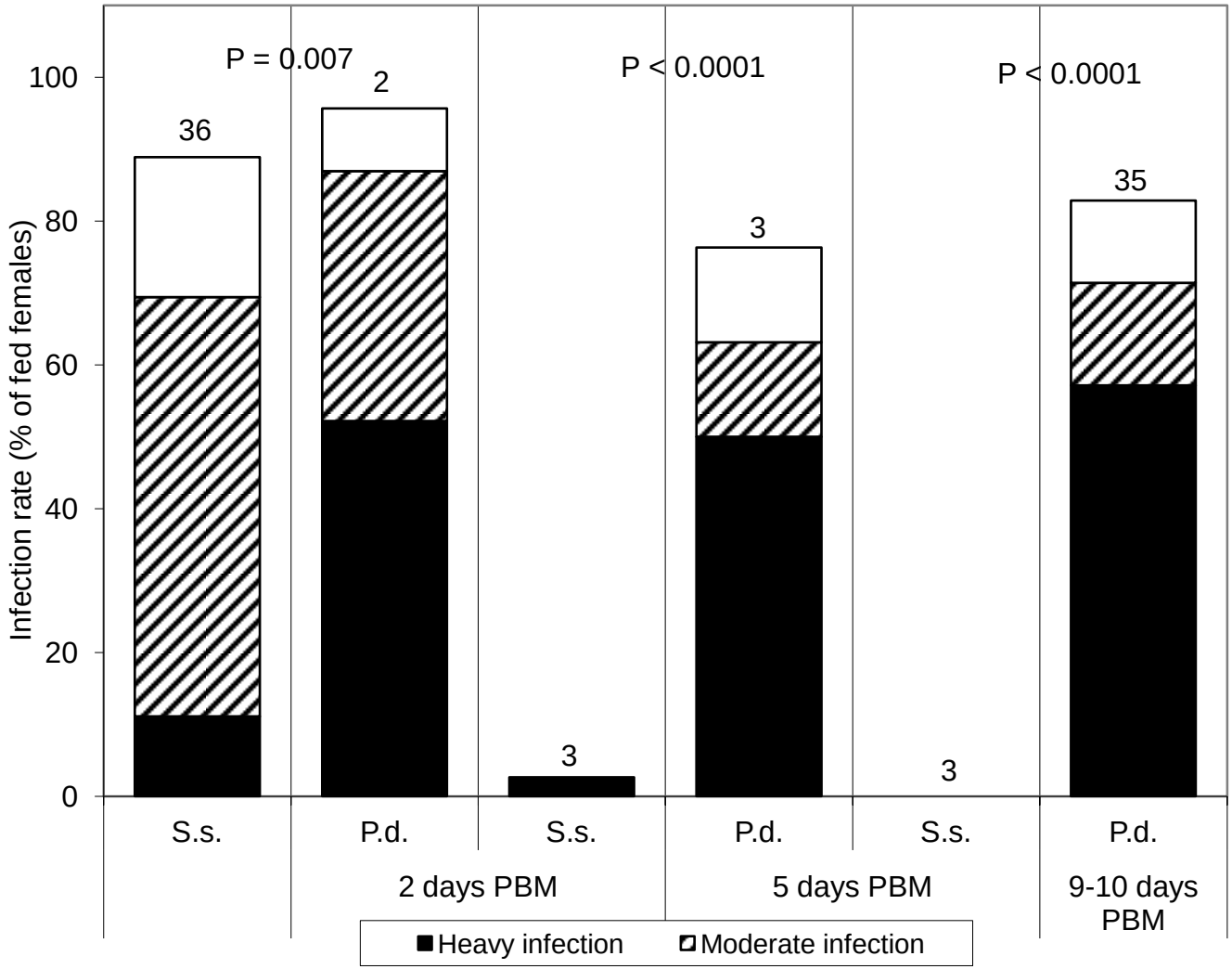

P = 0.007
P < 0.0001
P < 0.0001
36
2
3
3
3
35
100
80
60
40
20
0
Infection rate (% of fed females)
S.s.
P.d.
S.s.
P.d.
S.s.
P.d.
2 days PBM
5 days PBM
9-10 days PBM
Heavy infection
Moderate infection

Figure 3B

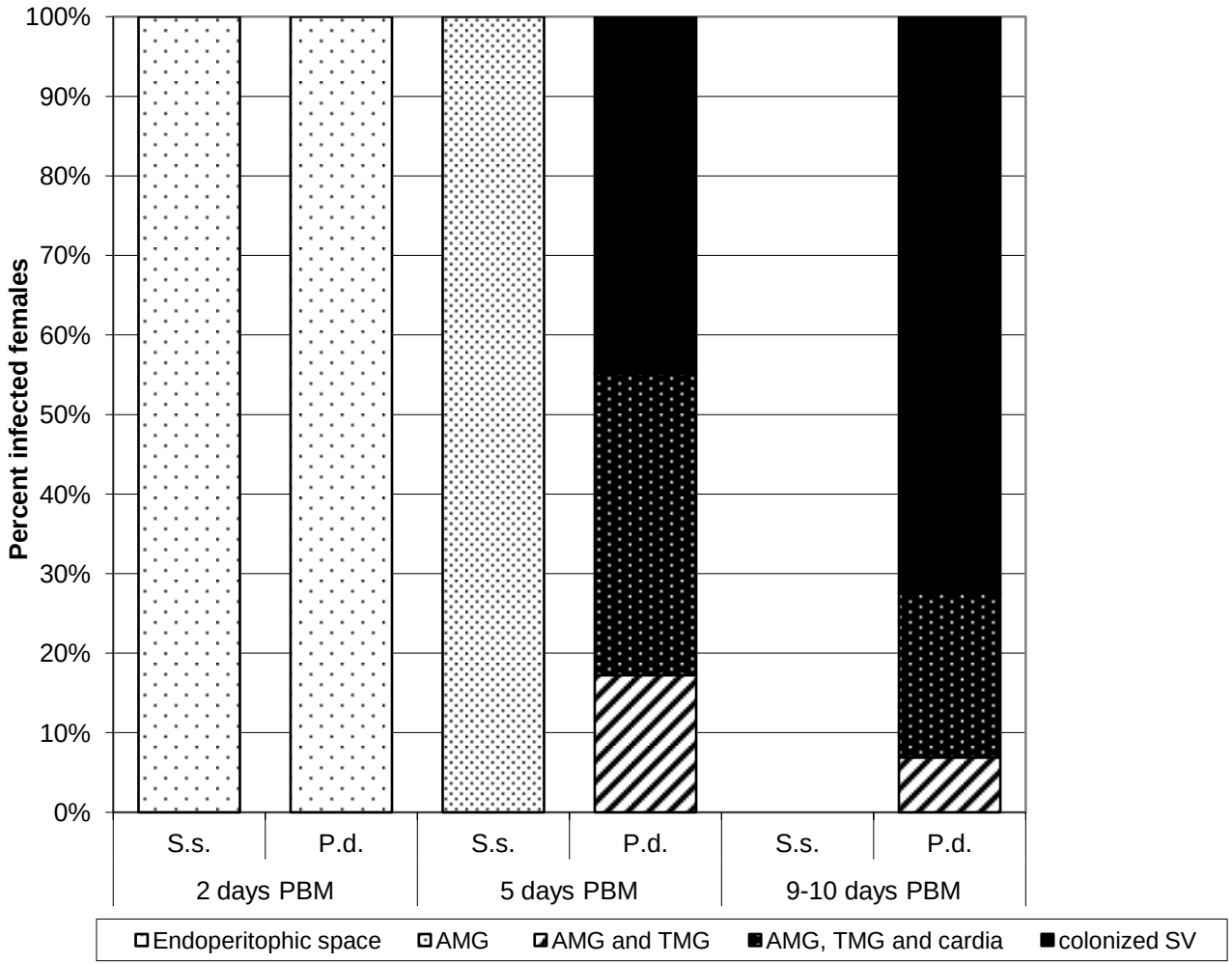

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent infected females
S.s.
P.d.
S.s.
P.d.
S.s.
P.d.
2 days PBM
5 days PBM
9-10 days PBM
Endoperitophic space
AMG
AMG and TMG
AMG, TMG and cardia
colonized SV

Figure 4A

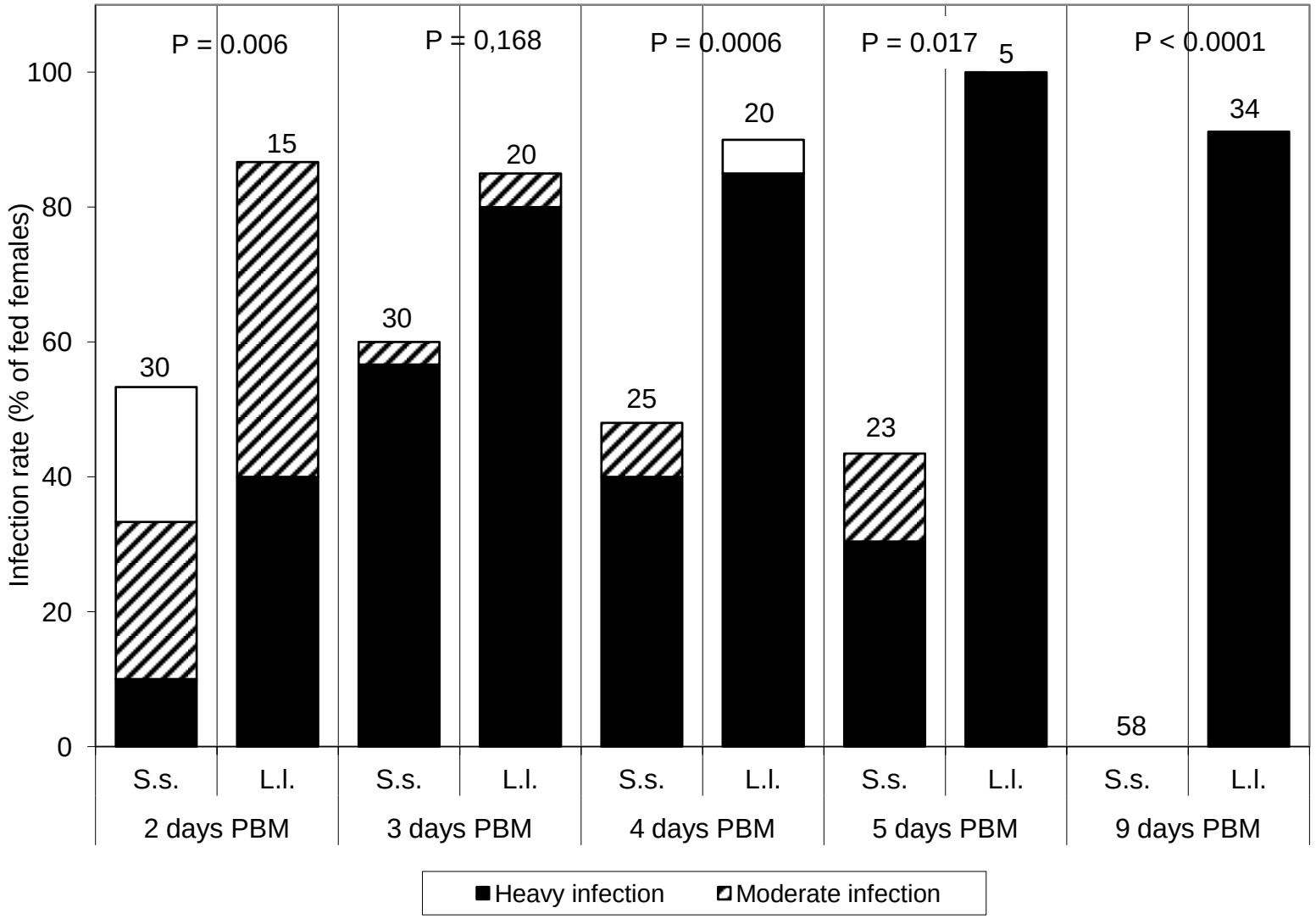
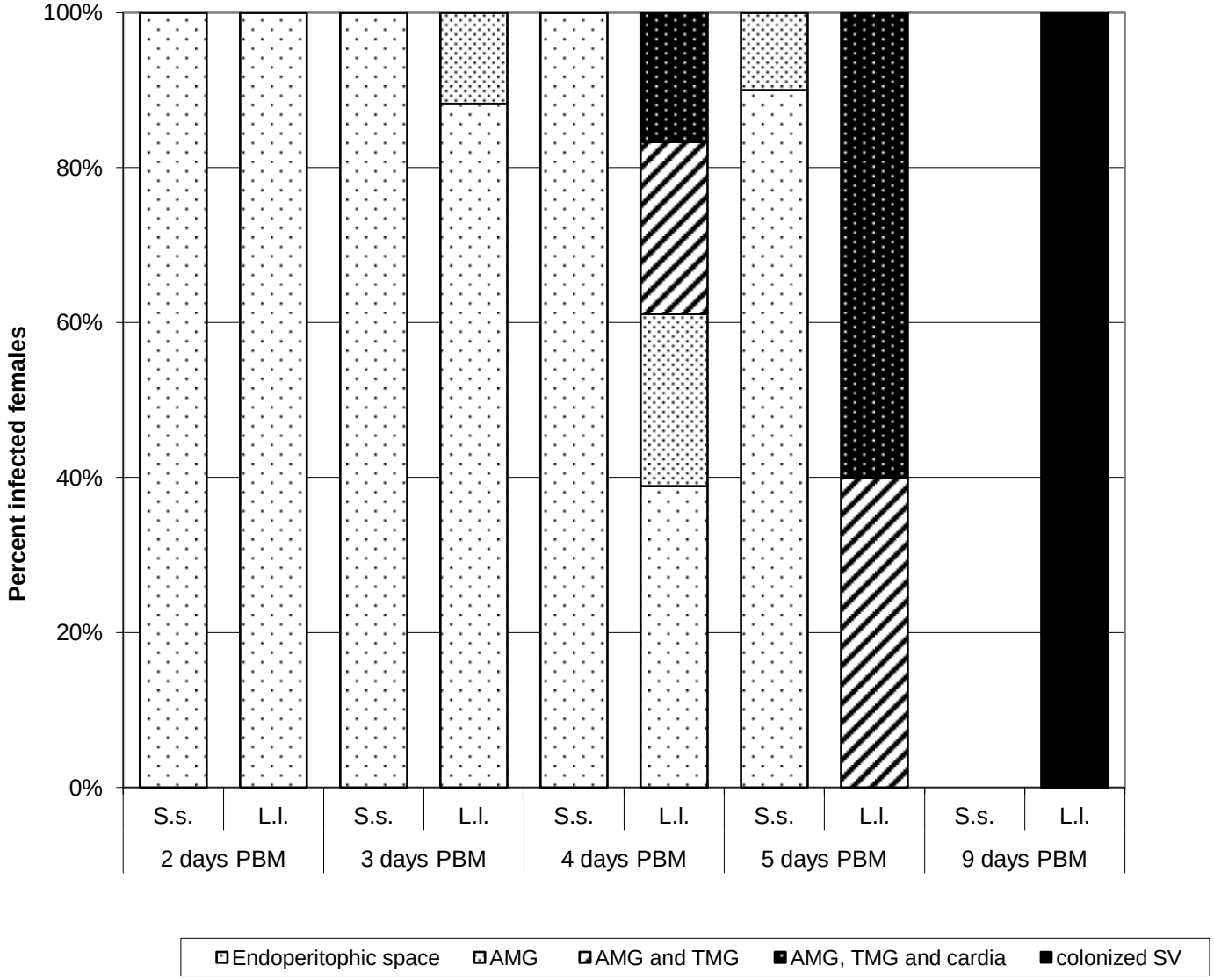

P = 0.006
P = 0,168
P = 0.0006
P = 0.017
P < 0.0001
Infection rate (% of fed females)
100
80
60
40
20
0
30
15
30
20
25
20
23
5
58
34
S.s.
L.l.
2 days PBM
3 days PBM
4 days PBM
5 days PBM
9 days PBM
Heavy infection
Moderate infection

Figure 4B


100%
80%
60%
40%
20%
0%
Percent infected females
S.s.
L.l.
2 days PBM
3 days PBM
4 days PBM
5 days PBM
9 days PBM
Endoperitophic space
AMG
AMG and TMG
AMG, TMG and cardia
colonized SV

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RAPID COMMUNICATION

Development of *Leishmania* Parasites in *Culicoides nubeculosus* (Diptera: Ceratopogonidae) and Implications for Screening Vector Competence

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ABSTRACT Biting midges of the genus *Forcipomyia* (Diptera: Ceratopogonidae) have recently been implicated as vectors of kinetoplastid parasites in the *Leishmania enrietti* complex. This study assesses susceptibility of one of the few successfully colonized Ceratopogonidae, *Culicoides nubeculosus* Meigen, to infection with *Leishmania* parasites infecting humans. While *Leishmania infantum* initially developed in the midgut of *C. nubeculosus* until 2 d postfeeding, parasite populations on day 3 were considerably reduced. Despite this, a polymerase chain reaction-based assay continued to indicate presence of *L. infantum* for up to 7 d after the bloodmeal. These findings are discussed within the wider context of implicating arthropods as vectors of *Leishmania* and it is suggested that conventional polymerase chain reaction use in vector-competence studies should be accompanied by direct microscopical observations.

KEY WORDS vector competence, biting midge, *Leishmania* transmission

Leishmaniases are vector-borne diseases whose etiological agents are protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). *Leishmania* are reported worldwide, including part of temperate zones of Europe and North America and are largely transmitted by phlebotomine sand flies of the genus *Phlebotomus* in the Old World or *Lutzomyia* in the New World (reviewed by Killick–Kendrick 1990). In Australia, however, day-biting midges of the subgenus *Forcipomyia* (*Lasiohelea*) Kieffer were recently demonstrated to act as vectors of the *Leishmania enrietti* complex between red kangaroos (Dougall et al. 2011). In field-collected *Forcipomyia* midges, *L. enrietti* complex parasites were found to exhibit infection patterns similar to those recorded in the generalized *Phlebotomus–Leishmania* interaction. After ingestion of a bloodmeal various promastigote forms, including those morphologically identical to metacyclics, migrated anteriorly to the thoracic region of the midgut and colonized the stomodeal valve of the vector (Dougall et al. 2011).

During early research on *Leishmania* transmission, biting midges of the genus *Culicoides* were suspected vectors of visceral *Leishmania donovani* in India, but little direct experimental evidence is available (Christopher et al. 1925). In later studies, *Culicoides* were implicated as the vectors of a wide range of pathogens, most notably arboviruses (reviewed by Mellor et al. 2000), but also monoxenous trypanosomatids (Podlipaev et al. 2004). Recent studies have also demonstrated infection of domestic dogs (reservoirs of *L. infantum*) with bluetongue virus (BTV) in Morocco (Oura and Harrak 2011), which could imply the feeding of *Culicoides* on canine hosts and a link to reservoirs of *L. infantum.* Hence, while direct evidence linking *Culicoides* populations with blood feeding on dogs is limited and equivocal (e.g., Blackwell et al. 1995), it appears likely that *Culicoides* use canine hosts at least occasionally.

The vector competence of *Culicoides* for *Leishmania* has not been recorded under laboratory conditions. The current study therefore examines the competence of *Culicoides nubeculosus*, one of the few colonized species of this genus, for two major *Leishmania* species infecting humans in the Old World.

Materials and Methods

The *C. nubeculosus* used were derived from those maintained in the Institute for Animal Health (IAH), Pirbright Laboratory, United Kingdom (Boorman 1974). Two *Leishmania* species, *L. infantum* (ITOB/TR/2005/CUK3) and *L. major* Friendlin (MHOM/IL/81/Friendlin/VI; FVI), were used. Parasites were cultured at 23°C in M199 medium containing 10% heat-inactivated fetal calf serum, 50 mg/ml amikacin, solution of BME vitamins (all Sigma, St. Louis, MO) and 1% sterile urine. Infection of natural sand fly vectors, *Phlebotomus perniciosus* and *Lutzomyia longipalpis*, by *L. infantum* CUK3 strain (at cell density

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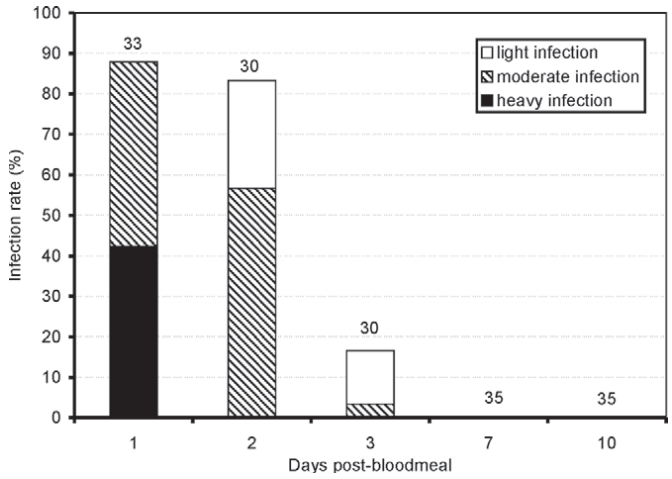

Fig. 1. Development of *L. infantum* in *C. nubeculosus*. *C. nubeculosus* were infected by feeding on a suspension of 10^7 parasites/ml of blood and dissected at 1, 2, 3, 7, and 10 d PBM. Day 1 and 2, females before defecation; day 3, most of the females after defecation; days 7 and 10, all females defecated. *L. infantum* infections were classified into three categories according to intensity: heavy (>1,000 parasites per gut), moderate (100–1,000 parasites) and light (1–100 parasites). Numbers above the bars indicated the number of dissected females.

1×10^6 promastigotes/ml) routinely results in 75% late-stage infections (Maia et al. 2011).

In each experiment, ≈200–500 *Culicoides* females (3–8 d old) were fed through a chick-skin membrane on a suspension of promastigotes (at cell density 1×10^7 promastigotes/ml) mixed with heat-inactivated rabbit blood. Blood-engorged females were given access to cotton wool soaked by sugar solution and maintained either at 26°C (*L. major*) or 20°C (*L. infantum*) under a photoperiod of 14:10 (L:D) h. As the sugar source may determine *Leishmania* development in sand flies (Young et al. 1980), approximately half of females were offered 50% sucrose and half 50% honey. On days 1, 2, 3, 7, and 10 postbloodmeal (PBM) females were dissected and their guts were examined by light microscopy at 200 and 400× magnification for presence and localization of *Leishmania* promastigotes. Levels of *Leishmania* infection were graded into four categories as negative, lightly infected (<100 parasites/gut), moderately infected (100–1,000 parasites/gut), and heavily infected (>1,000 parasites/gut) (Myskova et al. 2008). Data were evaluated statistically by means of χ^2 test using the S-PLUS 2000 program.

Twenty engorged females were pooled for each time interval and extraction of the total DNA was performed using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The small subunit (SSU) rRNA was amplified by using universal eukaryotic primers Medlin B in combination with SSU-1-B (van Eys et al. 1992). The polymerase chain reaction (PCR) reaction was performed in 15 μl total volume of reaction mix (Combi PPP Master Mix, Top Bio) using the following conditions: denaturation at 95°C for 5 min followed by 40 amplification cycles (94°C for 60 s, 55°C for 90 s, and 72°C for 90 s) and 72°C for 5 min. The PCR products were visualized on agarose gel by using Sybr Green fluorescent probe.

In another series of experiments, two different protease inhibitors were tested for effect on *Leishmania* development in the midge midgut. In two groups of females *C. nubeculosus* the bloodmeal was supplemented by soybean trypsin inhibitor from *Glycine max* (1 mg/ml) (Sigma) or the protease inhibitor Cocktail Tablets (0.5 tablet for 3 ml of blood) (Roche), with a third group fed on blood without inhibitors acting as a control. Blood engorged females were maintained at 20°C and on days 1, 2, 3, 6, and 11 PBM parasite density and localization were evaluated as described above.

Results and Discussion

Detailed studies with *L. infantum*, carried out at 20°C to slow down digestion in *C. nubeculosus* and enhance the probability of infection (Benkova and Volf 2007), demonstrated that by day 1 PBM *L. infantum* had moderately to heavily infected 88% (29/33) of females examined (Fig. 1). The abundance of parasites then decreased on day 2 PBM and moderate infections were detected in only 57% (17/30) females. At this stage, active examples of both long nectomonads and round stages forming rosettes were observed within the bloodmeal bolus surrounded by the intact peritrophic matrix. By day 3 PBM, most of the females had defecated and midgut infection rates were very low. In 17% of females (5/30), only light infection with long nectomonads was observed in the abdominal midgut. By days 7 and 10 PBM *Leishmania* parasites were not observed in any of 70 examined females. The sugar source (sucrose vs. honey) had no significant effect ($P > 0.05$) on *Leishmania* development and data from these two groups are pooled in Fig. 1. At 26°C, attempted infection with *L. major* demonstrated a very similar pattern, with rapid growth of parasite abundance in the *C. nubeculosus* midgut until days 2–3 when midges defecated, whereupon infections were

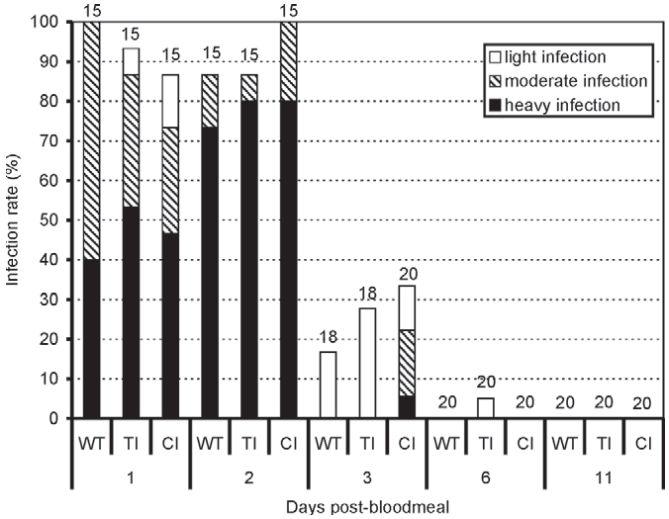

Fig. 2. Effect of protease inhibitors on *L. infantum* development in *C. nubeculosus*. *C. nubeculosus* were infected and intensities of infection were evaluated as described in Fig. 1. In two groups a trypsin inhibitor (TI) or a cocktail of protease inhibitors (CI) was added into bloodmeal, the third group without inhibitors served as the control (WT). No significant differences were observed between the groups ($P = 0.702$, df = 6, Pearson $\chi^2 = 3.812825$). The only positive female on day 6 PBM did not defecate and *Leishmania* were detected in bloodmeal remnants.

entirely cleared by all individuals ($N = 52$) examined by light microscopy dissection (data not shown).

In sand flies, there are two major barriers preventing establishment of *Leishmania* infection in unnatural parasite-vector combinations: the killing effect of midgut proteinases and an inability of the parasite to attach to the midgut epithelium (reviewed by Sacks and Kamhawi 2001). As proteinase inhibitors are known to enhance the early survival of *Leishmania* in sand flies (Borovsky and Schlein 1987) we tested their effect on development of *L. infantum* in *C. nubeculosus*. Proteinase inhibitors did not have any significant effect ($P > 0.05$) upon *Leishmania* development and survival in *C. nubeculosus*. Heavy *Leishmania* infections were frequently present in *C. nubeculosus* on day 1 and 2 PBM, but on day 3 PBM when bloodmeal remnants defecated, infection rates and parasite loads significantly dropped in all three groups. By 11 d PBM no parasites were detected in any of the three treatments (Fig. 2). It is clear that factors other than midgut proteinases are responsible for infection resistance in *C. nubeculosus*.

In Phlebotomine sand flies, the ability of *Leishmania* to survive defecation and to attach to midgut epithelium is a primary determinant of vector competence (Sacks and Kamhawi 2001, Myskova et al. 2007). In the current study, promastigotes of both parasite species, *L. infantum* and *L. major* were not able to attach to *Culicoides* midgut epithelium and were defecated. Therefore we suggest that the lack of midgut attachment is the major refractory barrier for *Leishmania* in *Culicoides* midges.

Detection of *Leishmania* parasites using PCR has been reported from ticks (Coutinho et al. 2005) and fleas (Coutinho and Linardi 2007) that has led to speculation on the role of alternative vectors. In infected *C. nubeculosus*, the PCR assay detected parasite infections up to and including 7 d PBM despite clearance of the parasite being recorded at day 3 PBM by microscopy. The weak positive signal recorded on day 7 could be a result of very low number of living or dead *L. infantum*. This illustrates that PCR positivity alone, especially when unquantified, can be misleading in implication of arthropods as vectors of *Leishmania*. Therefore, it is important that future studies use some degree of quantification, such as real-time PCR assays (Myskova et al. 2008, Ranasinghe et al. 2008, Dougall et al. 2011). Moreover, PCR positivity alone should not be used for incrimination of the sand fly (or other blood-sucking arthropod) as this technique cannot be used to infer whether parasites are viable and transformed to highly virulent metacyclic promastigotes localized in the stomodeal valve (a prerequisite for successful transmission of *Leishmania*). Therefore, direct microscopical observation of *Leishmania* promastigotes and their localization in the digestive tract remains a crucial method for any conclusions about the vector competence of the putative vector.

Acknowledgments

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Rapid Communication

Leishmania infantum nicotinamidase is required for late-stage development in its natural sand fly vector, *Phlebotomus perniciosus*

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ABSTRACT

Leishmania infantum nicotinamidase, encoded by the *Lipnc1* gene, converts nicotinamide into nicotinic acid to ensure Nicotinamide–Adenine–Dinucleotide (NAD^+) biosynthesis. We were curious to explore the role of this enzyme during *L. infantum* development in its natural sand fly vector, *Phlebotomus perniciosus* (Diptera, Phlebotominae), using null mutants with a deleted *Lipnc1* gene. The null mutants developed as well as the wild type *L. infantum* at the early time points post their ingestion within the blood meal. In contrast, once the blood meal digestion was completed, the null mutants were unable to develop further and establish late-stage infections. Data highlight the importance of the nicotinamide degradation pathway for *Leishmania* development in sand flies. They indicate that the endogenous nicotinamidase is essential for *Leishmania* development in the sand fly after the blood meal has been digested and the remnants defecated.

Female phlebotomine sand flies are haematophagous dipterans that are able to support *Leishmania* development and transmit this parasite to vertebrate hosts. *Leishmania* are responsible for various forms of disease, ranging from cutaneous to visceral leishmaniasis. The parasite exists in two main morphological forms: non-motile amastigotes that actively divide within mononuclear phagocytes of the mammalian host and motile flagellated promastigotes within the sand fly midgut. Amastigotes, which are ingested during the blood meal, transform into procyclic promastigotes that multiply within the blood meal, surrounded by the peritrophic matrix in the midgut of the sand fly. After completion of the blood meal digestion, the promastigotes can escape through the broken peritrophic matrix (Sádlová and Volf, 2009) and attach to the midgut epithelium. They then undergo a complex series of transformations that leads to the production of mammal-infective metacyclic stages that are located in the anterior part of the gut (reviewed in Bates and Rogers, 2004; Bates, 2007).

During its developmental life cycle, *Leishmania* must adapt to various environments that differ in their available nutritional resources. Interestingly, analysis of the *Leishmania* genome revealed the lack of numerous enzymes involved in the de novo synthesis of several essential metabolites (Berriman et al., 2005), supporting the hypothesis of a correlation between the nutritional requirements of *Leishmania* parasites and the cellular compartments in which they reside (McConville et al., 2007). Although the metabolic interactions of amastigotes within phagolysosomes have been well documented, little is known about the nutritional factors that support *Leishmania* development within the sand fly midgut.

We recently demonstrated that *Leishmania* is auxotroph for the cofactor Nicotinamide Adenine Dinucleotide (NAD^+) (Gazanion et al., 2011), meaning that its biosynthesis relies solely on the presence of external precursors, such as vitamin B3 nicotinamide (NAm) or its acid derivative nicotinic acid (Nac). In *Leishmania*, the conversion of both precursors into NAD^+ involved a shared three-step pathway with a supplementary step for NAm that is first converted into Nac by a nicotinamidase (LiPNC1) (Fig. 1). This enzyme controls the major part of NAD^+ production and, in turn, parasite growth and pathogenesis (Gazanion et al., 2011). The LiPNC1 enzyme thus represents a valuable candidate for rational drug design (Michels and Avilán, 2011). Additionally, previous studies have demonstrated that nicotinamidase activity is essential for several NAD^+-auxotroph pathogens, not only during the infection of mammals (Purser et al., 2003; Kim et al., 2004; Ma et al., 2007) but also for survival and replication in their arthropod vectors such as the spirochaete, *Borrelia burgdorferi*, in the tick midgut (Grimm et al., 2005). In this study, we investigated the importance of *Lipnc1* nicotinamidase activity for the intravectorial development of *Leishmania*. To this aim, we followed the development of

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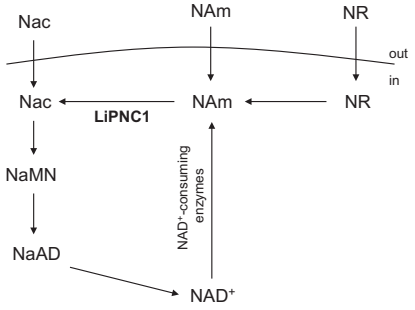

Fig. 1. Nicotinamide–Adenine–Dinucleotide (NAD^+) biosynthesis pathway in *Leishmania infantum* parasites. Targeted-gene replacement was used to delete the *Lipnc1* gene, which renders the parasites dependent on nicotinic acid (Nac) to produce NAD^+ by a set of three enzymatic reactions. NAm: nicotinamide; NR: nicotinamide riboside; NaMN: nicotinic acid mononucleotide; NaAD: nicotinic acid adenine dinucleotide.

Lipnc1−/− *L. infantum* parasites in their natural vector *Phlebotomus perniciosus*. We observed that mutants that are defective for the nicotinamidase gene are unable to grow and survive after blood meal digestion and are therefore unable to complete their intravectorial life cycle.

Four lines of *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263) were used: a wild-type (WT) strain, a *Lipnc1* double-knockout parasite strain (*Lipnc1*−/−) that was generated by targeted gene replacement (ΔLipnc1::NEO/ΔLipnc1::HYG), and two complemented strains that were generated by re-expressing either the native LiPNC1 protein, namely *Lipnc1*−/− + LiPNC1(ΔLipnc1::NEO/ΔLipnc1::HYG[pSPαBLAα-LiPNC1]), or an inactive form, namely *Lipnc1*−/− + $LiPNC1C_{161}A$. The inactive form was obtained by a substitution of the catalytic cysteine in position 161 with an alanine, which results in the abolition of enzyme activity. All of these mutant lines have been previously characterised (Gazanion et al., 2011). The parasites were maintained on SDM-79 medium (Brun and Schönenberger, 1979) that was supplemented with 10% heat-inactivated FCS (Lonza, France), 100 IU of penicillin/ml, 100 μg of streptomycin/ml and 5 mg/l of hemin. Laboratory colonies of *P. perniciosus* (Spain) were maintained at 26 °C under the conditions described by Volf and Volfová (2011).

For each experiment, approximately 150 female sand flies were fed through a chick-skin membrane with a suspension of 5-day-old promastigotes at a density of 10^6 cells/ml mixed with heat-inactivated rabbit blood. Blood-fed females were separated, kept at 26 °C and allowed to feed on a 50% sucrose solution. On days 2, 6, 8–9 and 13–14 post *L. infantum*-containing blood meal (PBM), the females were dissected and their guts were checked for the presence and localisation of parasites. The infection intensity was estimated in situ using a light microscope by scoring the infections according to defined criteria (Cihláková and Volf, 1997), i.e. weak (<100 parasites/gut), moderate (100–1000 parasites/gut) and heavy (>1000 parasites/gut) infections in the gut lumen. The experiment was repeated twice and the data were analysed using a χ^2 test and Prism software (GraphPad software, version 5).

In additional experiments, *P. perniciosus* females infected with *Lipnc1* double-knockout parasites were divided into three groups with free access either to a 50% sucrose meal or 50% sucrose supplemented with 3 μM of Nac or with 30 μM of NAm. Females were dissected on days 4 and 8–9 PBM.

As the microscopic determination of parasite density is only semi-quantitative, quantitative PCR (qPCR) was used to accurately determine the number of parasites in the sand fly midguts on day 12 PBM (Myskova et al., 2008). Briefly, for each parasite strain, 20 midguts of fed females were placed into 200 μl of lysis tissue buffer and stored at −20 °C until use. The total DNA was isolated by using a High Pure PCR Template Preparation Kit (Roche, Czech Republic) and used as a template for qPCR amplification with the primers described by Mary et al. (2004).

The capacity of *L. infantum* WT and *Lipnc1*-deficient parasites to complete their intravectorial development in *P. perniciosus* is summarised in Fig. 2. On day 2 PBM, during blood meal digestion and before defecation, *Leishmania* developed similarly regardless of the parasite strain (Fig. 2A). Living parasites were observed in the abdominal midguts in contact with partially digested blood that was located inside the endoperitrophic space. After defecation (day 6 PBM), significant differences were observed between the *Lipnc1*−/− and WT strains ($P < 0.001$) as only one of 15 sand flies examined was still weakly infected with *Lipnc1*−/− parasites. In contrast, female sand flies that were infected with the WT strain showed a high rate of infection (73%), of which 67% had high-intensity infections (Fig. 2A). On days 8 and 14 PBM, *Lipnc1*−/− parasites were detected in sand fly midguts in only 33% and 26% of dissected females, respectively, and at very low densities (Fig. 2A), demonstrating the inability of these parasites to grow and to establish mature infections in the midgut after defecation.

The introduction of an episome carrying a WT copy of the *Lipnc1* gene in null mutant parasites led to the restoration of normal parasite development in the sand fly midgut (Fig. 2A), as demonstrated by an infection rate and an intensity of infection similar to those of the control after blood meal defecation ($P = 0.67$ and $P = 0.63$, respectively). From days 8 to 14 PBM, no significant difference was observed between WT and *Lipnc1*−/− + LiPNC1 parasites with respect to their development rate ($P = 0.23$) and parasite density ($P = 0.25$) (Fig. 2A). Both strains migrated to the thoracic midgut and reached the cardia (anterior part of the thoracic midgut near the stomodeal valve), where they accumulated as elongated nectomonads. The colonisation of the stomodeal valve was observed in 23.6% (7/31) of WT-infected females and in 58.3% (7/12) of *Lipnc1*−/− + LiPNC1-infected females. Occasionally, the haptomonads were attached to the stomodeal valve. *Lipnc1* double-knockout parasites carrying an episomal copy of the *Lipnc1* gene in which the cysteine residue in position 161 was substituted with alanine (leading to an inactive nicotinamidase enzyme) were unable to complete the intravectorial cycle, similar to the *Lipnc1*−/− parasites. Therefore, the abortive development observed in sand flies infected with *Lipnc1* null mutants is clearly linked to the absence of nicotinamidase activity. These observations strongly support the notion that active nicotinamidase activity is required to ensure the complete development of *Leishmania* within its vector.

The capacity of *L. infantum* to colonise and to develop within sand flies was confirmed by qPCR analysis performed on midguts of infected females on day 12 PBM. The results revealed no significant difference in parasite loads between the WT and the complemented strains (Fig. 2B), whereas the *Lipnc1*−/− parasite development was significantly less successful (Fig. 2B).

The incapacity of *Lipnc1*-deficient parasites to establish late-stage infections could be due to deficient growth during an early stage of development, producing an insufficient amount of parasites able to colonise sand fly midgut after blood meal defecation. To test this hypothesis, we increased the inoculum of *Lipnc1*−/− + $LiPNC1C_{161}A$ parasites and followed parasite development in the sand fly midgut. A fivefold increase of inoculum (5×10^6 parasites/ml) did not restore the development of parasites in sand flies at days 9 and 13 PBM (Fig. 2C). Thus it seems likely that NAm degradation by the parasitic nicotinamidase is important during blood meal digestion and especially for establishing mature infection after blood meal defecation.

Finally, we tried to restore *Lipnc1*−/− development by adding Nac to sand fly sugar meal. Since NAm is also able to restore normal growth of *Lipnc1*−/− in in vitro-cultured promastigotes

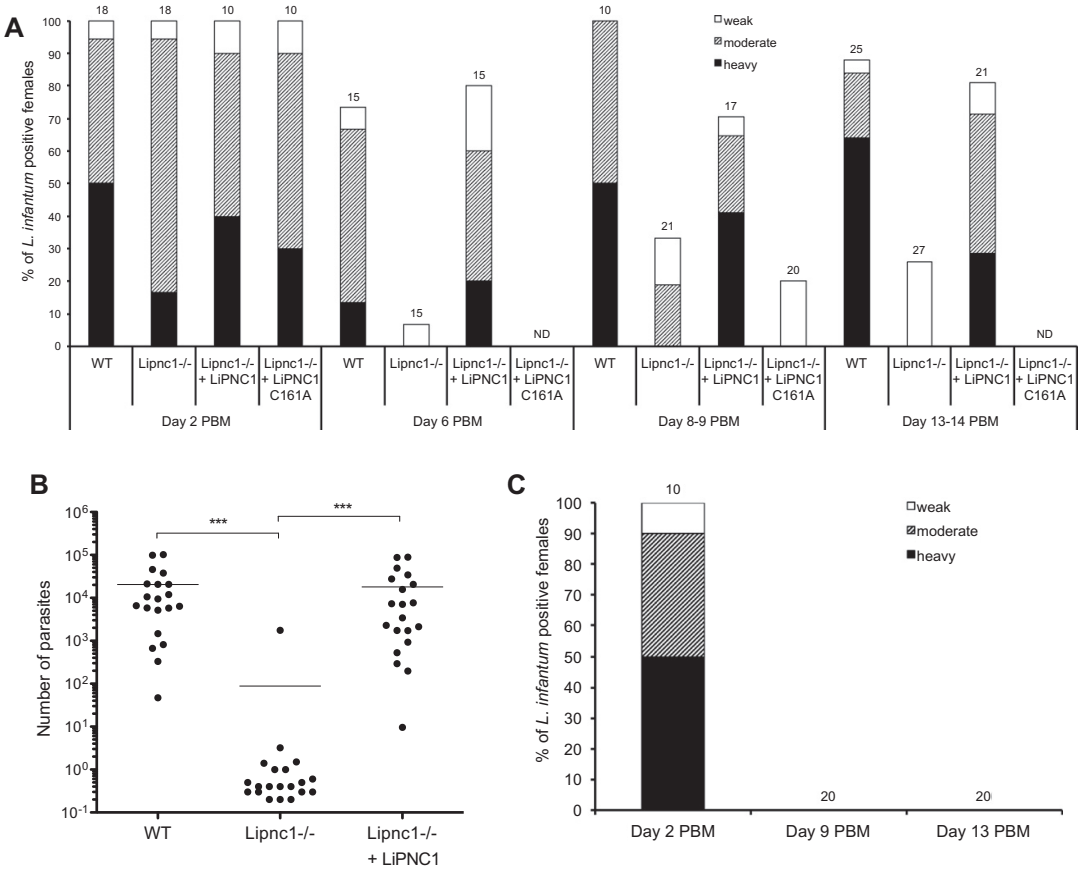

Fig. 2. Development of the *Leishmania infantum* wild type (WT) and *Lipnc1* mutant lines in *Phlebotomus perniciosus*. (A) Profile of *L. infantum*-positive *P. perniciosus* fed on rabbit blood containing either WT or mutant *L. infantum* lines (1×10^6 cells/ml). *Lipnc1*−/−: *Lipnc1* double knockout parasites; *Lipnc1*−/− + LiPNC1: complemented strain expressing a native LiPNC1 protein; *Lipnc1*−/− + LiPNC1C161A: complemented strain expressing an inactive form of LiPNC1. The rates and intensities of infection (weak, moderate, heavy) were determined on days 2, 6, 8–9 and 13–14 post *L. infantum*-containing blood meal (PBM) using light microscopy. The total number of dissected sand flies is indicated above the bars. ND: not determined. (B) Analysis of the parasite loads by quantitative PCR (qPCR) in *P. perniciosus* fed on rabbit blood containing either WT or mutant *L. infantum* lines (day 12 PBM). Twenty midguts were analysed for each line. The results were analysed using the Mann–Whitney test. $^{***}P < 0.001$. (C) Abortive development of the *L. infantum Lipnc1*−/− + LiPNC1C_{161}A strain, even after a blood meal containing a higher number of parasites (5×10^6 cells/ml). The rates and densities of infection were determined on days 2, 9 and 13 PBM using light microscopy.

but at a concentration 10-fold higher than Nac (Gazanion et al., 2011), we also included this point in our experiment. The results obtained with *Lipnc1* double-knockouts revealed that Nac and high levels of NAm supplement added into the sand fly sugar meal significantly ($P < 0.05$) improved *Leishmania* development during the early stage of infection. On day 4 PBM (soon after defecation), females with access to sugar supplemented with NAm or Nac showed high infection rates (92% and 67%, respectively) and high parasite loads (moderate and heavy infection intensities prevailed). In contrast, females with access to non-supplemented sugar had lower infection rates (42%) and low parasite loads (only weak infections occurred). In the late stage infection (days 8–9 PBM), however, the difference between groups was not significant ($P > 0.05$) as the infection rates were 29%, 40% and 49% for the control, NAm and Nac groups, respectively.

In this study, we provide the biological evidence that the LiPNC1 nicotinamidase enzyme that converts NAm into Nac, the first step in the NAD^+ synthesis pathway from a NAm precursor in *Leishmania*, is essential for the completion of the intravectorial development of *L. infantum* in its natural vector *P. perniciosus*. Whereas the WT strain developed well and colonised the stomodeal valve, *Lipnc1*−/− parasites were not able to produce mature infection in the thoracic midgut after defecation.

Currently, the nutritive view of the *Leishmania*/sand fly relationship remains largely unexplored and little is known about the nutritional factors that support *Leishmania* development within the sand fly midgut. It is predicted that the main extracellular source of nutrients available during the early stage of *Leishmania* development is provided by the blood meal, whereas the sugar meal furnishes the energy sources necessary for *Leishmania* growth in the late stages (Gontijo et al., 1996; Schlein and Jacobson, 1996; Jacobson and Schlein, 2001). In this view, glucose uptake is important, but not essential, for *Leishmania mexicana* infection of the vector host (Burchmore et al., 2003), and the genetic deletion of three glucose-transporter isoforms leads to a delayed growth of *Leishmania* in *Lutzomyia longipalpis*.

The cofactor NAD^+ is a central molecule that participates in regulation of the intracellular redox state and many biological processes such as longevity, DNA repair and transcriptional regulation (Lin et al., 2003). We previously demonstrated that *Lipnc1* gene

deletion induces large fluctuations in the NAD^+ intracellular pool that are deleterious for parasite growth and pathogenesis (Gazanion et al., 2011). In this study, we observed the unexpected growth arrest of *Lipnc1*−/− parasites in sand flies, indicating that NAm might be an important nutritional factor for *L. infantum*. These results strongly support the hypothesis that NAD^+ metabolism controls host–*Leishmania* interactions and that NAm is the primary precursor available to ensure NAD^+ biosynthesis during *Leishmania* development in both mammalian and arthropod hosts.

We demonstrated that supplementation with NAm and Nac in the sand fly sugar meal positively affected the development of *Lipnc1*−/− parasites. In early stage infection, when females took the sugar meal after defecation of blood meal remnants, the effect of NAm and Nac was pronounced. In late stage infections, when females took the sugar meal irregularly, the effect of the supplement was diminished as the NAm and Nac quantity ingested was probably not high enough to salvage the parasites. In the wild, it is known that sand flies feed on aphid honeydew, which is an important source of carbohydrate for sand flies (Moore et al., 1987) and a complex mixture of nutrients including B-vitamins (Way, 1963). However, the presence and amount of nicotinic acid and nicotinamide in aphids and in honeydew have not been defined.

Within its mammalian host, the primary NAD^+ precursor available for *Leishmania* is NAm (Gazanion et al., 2011). In humans, NAm is absorbed from the diet and circulates in the blood plasma at concentrations ranging from 0.3 μM to 2.3 mM, depending on the ingested dose (Bernier et al., 1998; Catz et al., 2005), whereas the Nac concentration is much lower (Catz et al., 2005). Differences in the blood concentrations of NAm and Nac in different mammalian hosts have been reported. Mice and rabbits have higher plasma levels of both precursors than dogs and humans (Catz et al., 2005). In the sand fly, *Leishmania* can gain access to NAD^+ precursors that are present in the blood meal only during their early developmental stage, after which the parasite must obtain these precursors from the environment present in the gut of the sand fly. Our results strongly suggest that the amount of Nac available in the blood meal is sufficient to support *Lipnc1*−/− growth, whilst subsequently NAm is the sole NAD^+ precursor present in the sand fly midgut. Whether the blood meal source and the available amount of NAm or Nac may substantially influence the outcome of the intravectorial development of *Leishmania* needs to be investigated.

In conclusion, our findings clearly highlight a new metabolic aspect of *Leishmania* promastigote development within the complex and dynamic milieu of the midgut by revealing the crucial role of the LiPNC1 nicotinamidase enzyme, which catalyses the first step of NAm assimilation into NAD^+. The apparent requirement of both promastigote and amastigote forms for a functional nicotinamidase to ensure a complete development cycle reinforces the therapeutic value of LiPNC1 and will help to delineate new transmission-blocking strategies.

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