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Proteiny slin flebotomů a imunitní aspekty přenosu leishmaniózy

Salivary proteins of sand flies and the immune aspects of *Leishmania* transmission

Dizertační práce / Ph.D. Thesis

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Praha, 2013

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Prof. RNDr. Petr Volf, CSc.
supervisor

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Table of contents

Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CD	cluster of differentiation, example given: CD80
cDNA	complementary deoxyribonucleic acid
CL	cutaneous leishmaniasis
DTH	delayed type hypersensitivity
GIPLs	glycophospahtidylinositol lipids
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
IFN	interferon, example given: IFN-γ
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin, example given: IL-8
iNOS	inducible nitric oxide synthase
L.	*Lutzomyia*
Le.	*Leishmania*
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
NK	natural killers
NO	nitric oxide
P.	*Phlebotomus*
PBMC	peripheral blood mononuclear cell
PSG	promastigote secretory gel
TGF	transforming growth factor, example given: TGF-β
Th	T helper lymphocytes
TNF	tumor necrosis factor, example given: TNF-α
UDP	uridine diphosphate
VL	visceral leishmaniasis

Abstract

Sand flies serve as the vectors of leishmaniasis and their saliva was shown to affect the outcome of *Leishmania* infection by immunomodulation of the host. On the other hand, sand fly saliva contains a large scale of farmacologically active proteins that are strongly immunogenous for bitten hosts and specific anti-saliva immunity initiated by repeated sand fly feeding provides protection against *Leishmania* infection. Specific cell-mediated immunity was shown to be the core of the protectivity; however, our data suggests that the protective immunity has certain limitations. In mice bitten by sand flies for prolonged periods, we observed the desenzitization in term of abrogation of the protective immunity. Thus, we can speculate that the protective effect of immunity is linked solely with the short-term exposure. Nevertheless, our experiments showed that this aspect is also conditioned by the immediate infection after the protective short-term immunization. Taken together, it seems that these limitations may explain the circulation of leishmaniasis in endemic areas, even though humans and animals are frequently immunized by bites of uninfected sand flies.

Repeated sand fly feeding on various hosts also promotes production of anti-saliva antibodies that reflect the intensity of exposure. We demonstrated these findings in dogs and rodents, the natural reservoir hosts of visceral and cutaneous leishmaniasis, respectively. Therefore, in endemic areas, specific humoral response might be used as the effective epidemiological tool. Moreover, our study showed remarkable relationship between the canine anti-saliva antibody response and the status of visceral leishmaniasis. As the specific IgG2 response negatively correlated with the risk of *Leishmania infantum* infection, in endemic areas these antibodies might be employed as the risk marker of *Leishmania* transmission for dogs.

For a broader use of sand fly salivary antigens in epidemiological studies it would be beneficial to replace the whole saliva with the individual salivary proteins possessing required antigenic properties. Therefore, we broaden the repertoir of salivary proteins of *P. orientalis*, *P. perniciosus*, and *P. papatasi* and we proved the feasibility of using the recombinant proteins instead of whole sand fly saliva. Furthermore, we showed that the non-endemicity of visceral leishmaniasis in certain areas in Ethiopia, albeit the presence of the vector, is not caused by the difference in composition of salivary proteins in *P. orientalis* populations.

Abstrakt

Flebotomové jsou z medicínského hlediska významní jako přenašeči leishmaniózy. Jejich sliny obsahují antihemostatické a imunomodulační složky, které podstatně ovlivňují přenos a rozvoj leishmaniózy. Proteiny slin flebotomů jsou ale zároveň i silně immunogenní a imunita vyvolaná opakovaným sáním chrání hostitele proti přenosu leishmaniózy. Mnohé studie prokázaly, že za tento protektivní efekt je zodpovědná specifická buněčná imunita. Výsledky našich pokusů však naznačují, že protektivní imunita navozená sáním flebotomů má určité limity. U myší, které byly vystaveny sání flebotomů dlouhodobě, jsme zaznamenali tvorbu specifické imunity, nicméně tato imunita neposkytovala ochranu proti leishmanióze. Můžeme se proto domnívat, že u této skupiny myší se nám v jisté míře podařilo navodit desenzitizaci. Protektivní imunita dále nebyla dosažena u skupiny myší, které byly sice imunizovány slinami flebotomů krátkodobě, avšak mezi imunizací a následnou infekcí došlo k patnácti týdennímu prodlení. Výsledky naší studie proto naznačují, že právě tyto aspekty navození protektivní imunity by v endemických oblastech mohly rozhodovat o tom, že lidé i zvířata běžně vystaveni sání neinfikovaných flebotomů nejsou proti leishmanióze chráněni.

Opakované sání flebotomů vyvolává i tvorbu humorální imunity a hladiny specifických protilátek odpovídají intenzitě pobodání. Prokázali jsme, že tuto korelaci lze vysledovat i u psů a hlodavců, kteří jsou rezervoárovými hostiteli viscerální, respektive kožní leishmaniózy. Zároveň naše pokusy prokázaly pozoruhodnou spojitost mezi protilátkami proti slinám flebotomů u psů a rizikem přenosu viscerální leishmaniózy. Protože hladiny specifických IgG2 u psů negativně korelovaly s přenosem leishmaniové infekce, tato podtřída protilátek by mohla sloužit jako ukazatel rizika přenosu psí leishmaniózy.

Nicméně pro širší využití humorální odpovědi v epidemiologických studiích by bylo vhodné nahradit sliny flebotomů jednotlivými slinnými proteiny s odpovídajícími antigenními vlastnostmi. Naše experimenty podstatně rozšířily spektrum silně antigenních proteinů, a to u druhů *Phlebotomus orientalis*, *P. perniciosus* a *P. papatasi*, a zároveň potvrdily, že rekombinantní proteiny slin mohou nahradit homogenát slinných žlaz. Navíc jsme prokázali, že se neliší složení slinných žlaz u dvou kolonií *P. orientalis* z Etiopie, které v jedné lokalitě fungují jako vektoři viscerální leishmaniózy a v jiné nikoliv.

Introduction

Leishmaniasis is the neglected protozoan disease spread among the vertebrate hosts by the bites of infected sand flies (Diptera: Phlebotominae). Two of the sand fly genera, *Phlebotomus* and *Lutzomyia*, are of big medical importance, as the sand fly species belonging to those genera transmit *Leishmania* parasites (Kinetoplastida: Trypanosomatidae) responsible for human leishmaniasis (Killick-Kendrick, 1990). While sand flies of genus *Phlebotomus* transmit leishmaniasis within the Old World with the annual incidence reaching 0.7 – 1.3 million of cases, species from the genus *Lutzomyia* serve as the vectors in the New World with 0.2 – 0.3 million of new cases per year (Alvar et al., 2012).

In my work, I focused solely on the sand fly species transmitting *Leishmania* parasites from the subgenus *Leishmania*, therefore the following paragraph deals only with the description of their life cycle. Bloodsucking female sand flies are responsible for the circulation of leishmaniasis in the endemic areas. When the host is infected, *Leishmania* parasites may be ingested within the bloodmeal during the feeding process. If so, in the abdominal midgut of the sand fly female, amastigotes are released from the ruptured phagocytic cells and transformed into the small procyclic promastigotes. These weakly motile stages are characteristic by the presence of short flagellum and by the certain resistance to attacks of digestive enzymes. Moreover, procyclic promastigotes initiate the first multiplication in sand fly midgut. After that, procyclics are transformed into the slender nectomonads, the highly motile stages that escape from the peritrophic matrix through the posterior openings (Sadlova & Volf, 2009), attach themselves to the midgut cells and subsequently migrate forward to the thoracic midgut. Later, promastigotes transform from nectomonads to leptomonads (Rogers et al., 2002) and undergo another proliferative cycle (reviewed in Kamhawi, 2006, Dostalova & Volf, 2012). Leptomonad promastigotes are potent producers of filamentous matrix identified as proteophosphoglycan which surrounds the parasites (Stierhof et al., 1999). At the stomodeal valve, the filamentous proteophosphoglycan aggregates and creates the gel plug called promastigote secretory gel (PSG) (reviewed in Rogers 2012). While producing PSG, the majority of leptomonads enters metacyclogenesis and differentiates into infective metacyclic promastigotes (Rogers et al., 2002). PSG blocks the physiological function of stomodeal valve and disable successful feeding of infected sand flies. Thus, metacyclic promastigotes, PSG as well as sand fly saliva are regurgitated into the wound during the repeated attempts to feed. Parasites deposited into the host skin are swallowed by the phagocytic cells, mainly macrophages (reviewed in Assche

et al., 2011) and inside them transform to non-flagellated amastigotes. The cycle is completed when another non-infected female sand fly feeds on the infected host and subsequently spreads the parasites to other vertebrates (reviewed in Kamhawi, 2006, Dostalova & Volf, 2012).

Various aspects of salivary glands were studied in four Old World sand fly species from the genus *Phlebotomus*; particularly in two vectors of visceral leishmaniasis, *Phlebotomus (Larroussius) perniciosus* and *P. (Larroussius) orientalis* transmitting *Leishmania infantum* and *Le. donovani*, respectively, and two vectors of cutaneous leishmaniasis caused by *Le. major*, *P. (Phlebotomus) papatasi* and *P. (Phlebotomus) duboscqi* (reviewed in Killick-Kendrick 1990). *Phlebotomus perniciosus* occurs in the European and African countries surrounding Mediterranean see (reviewed in Killick-Kendrick 1990, Killick-Kendrick 1999, Ready, 2010), but was also found in other states, such as Schwitzerland (Grimm et al., 1993), Germany (Naucke et al., 2008), or Andorra (Ballart et al., 2012). *Phlebotomus orientalis* is abundantly present in the countries of East Africa (reviewed in Elnaiem 2011) as well as in Saudi Arabia (Al-Zahrani et al., 1997, Doha & Sami, 2010) and Yemen (Daoud et al., 1989). *Phlebotomus papatasi* is distributed within the wide zone ranging from Northwest Africa, through Euroasia to India, while *Phlebotomus duboscqi* is commonly present in the northern part of Sub-Saharan Africa, including Kenya (reviewed in Killick-Kendrick 1990, Killick-Kendrick 1999).

1. Sand fly saliva

Sand fly salivary glands are sac-like structures present in pairs that are located in the thorax. The size and the composition of the salivary glands as well as the amount of salivary proteins is species specific (Volf et al., 2000, Volf & Rohousova, 2001, Černá et al., 2002, Wahba & Riera, 2006, Abdel-Badei et al., 2012, Rohousova et al., 2012a). Moreover, the variability in salivary gland composition was detected in the same sand fly species originating from different endemic foci (Lanzaro et al., 1993, Warburg et al., 1994, Volf et al., 2000, Kato et al., 2006), but also among the individual sand flies from the same colony (Elnaiem et al., 2005, Rohousova et al., 2012b). It should be noted that there are more factors that may affect the composition of saliva such as age, diet, or sex (Volf et al., 2000, Prates et al., 2008).

Sand fly males do not display haematophagy, their saliva possesses less amount of salivary proteins and the western blot analysis revealed only one major protein band (Volf et

al., 2000), therefore we can suppose that their saliva does not contain similar anti-haemostatic components known from the female saliva. The following description will hence refer to the female salivary gland compounds that are primary addapted to accomplish the bloodfeeding. Sand fly salivary proteins might be employed in the various steps of inhibition of host inflammation and haemostasis, the most important being those blocking the platelet aggregation and degranulation, interrupting the coagulation cascade or serving as the effective vasodilators (reviewed in Ribeiro et al., 2010, Fontaine et al., 2011a).

2. Fighting host haemostasis

2.1. Enzymes

Composition of sand fly salivary glands was studied in various species belonging to *Phlebotomus* (Valenzuela et al., 2001a, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012, Rohousova et al., 2012a) as well as *Lutzomyia* (Charlab et al., 1999, Valenzuela et al., 2004, Kato et al., 2012) genera. Several enzymes were detected in the salivary gland cDNA libraries when probing for the novel proteins. While some of the salivary enzymes are exclusive for a few species, the others play the key role in fighting host haemostasis and thus are present in all. For example apyrase, the principal anti-platelet aggregation enzyme with ATP and ADP hydrolyzing activity, was found in all sand fly species tested to date (Charlab et al., 1999, Valenzuela et al., 2001a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Rohousova et al., 2012a, Kato et al., 2012, Abdeladhim et al., 2012), but also in saliva of other bloodsucking insect (reviewed in Ribeiro et al., 2010, Fontaine et al., 2011a). Apyrases of the bloodsucking insect are divided into three families: GTPase/ CD-39, 5´- nucleotidase, and *Cimex* type. Apyrases of sand flies are homologous to bed bug apyrase that belongs to *Cimex* type family (Valenzuela et al., 1998) and the enzymatic activity is strictly Ca^{2+} dependent (Ribeiro et al., 1986, Ribeiro et al., 1989, Charlab et al., 1999, Valenzuela et al., 2001b, Hamasaki et al., 2009). So far, apyrase activity was demonstrated in the saliva of *L. longipalpis* (Ribeiro et al., 1986, Charlab et al., 1999), *P. papatasi* (Ribeiro et al., 1989, Valenzuela et al., 2001b), *P. perniciosus* (Ribeiro et al., 1989), *P. argentipes* (Ribeiro et al., 1989), and *P. colabaensis* (Ribeiro et al., 1989). Moreover, Hamasaki et al. (2009) proved the apyrase activity of bacterially expressed 35.2 kDa *P. duboscqi* recombinant salivary apyrase (ABI20147) (Hamasaki et al., 2009).

Presence of 5'-nucleotidase was discovered solely in the saliva of *Lutzomyia longipalpis* (Charlab et al., 1999, Ribeiro et al., 2000a, Valenzuela et al., 2004). This enzyme converts AMP to adenosine, the molecule with immunosuppressive, vasodilatory and anti-platelet aggregation effect (Edlunt et al., 1987, Urquhart & Broadley, 1991, Dionisotti et al., 1992, Katz et al., 2000), as well as catalyzes the hydrolysis of UDP-Glucose to uridine, thus acting as a phosphodiesterase (Ribeiro et al., 2000a). In parallel, transcripts coding for pyrophosphatase that contained phosphodiesterase domain were found in salivary gland cDNA libraries of *P. argentipes* (Anderson et al., 2006), *P. arabicus* (Hostomska et al., 2009), and *P. papatasi* (Abdeladhim et al., 2012). This enzyme is likely involved in the hydrolysis of dinucleotides, the important inflammatory mediators released by platelets (Gasmi et al., 1996a, Gasmi et al., 1996b, Schluter et al., 1996), and thus may act as the analogue of *L. longipalpis* 5'-nucleotidase in *Phlebotomus* species.

Adenosine deaminase was ascertained in the saliva of *L. longipalpis* (Charlab et al., 2000, Charlab et al., 2001) and *P. duboscqi* (Kato et al., 2006, Kato et al., 2007). The main role of adenosine deaminase is to hydrolyse adenosin to inosin, the another immunosuppressive purine (Hasko et al., 2000). The lack of adenosin and AMP in the *P. duboscqi* saliva indicates the certain activity of adenosine deaminase, whereas in *P. papatasi* or *P. argentipes* salivary glands large amounts of adenosin as well as AMP were detected (Ribeiro et al., 1999, Ribeiro & Modi, 2001). These findings demonstrate that various sand fly species, although closely related, like *P. papatasi* and *P. duboscqi*, possess different anti-haemostatic strategies.

Other enzymes that are probably not involved directly in fighting host haemostasis were found in saliva of different sand fly species. The most important are likely two salivary enzymes that are employed in decreasing the skin matrix viscosity around the feeding site – hyaluronidase and endonuclease. Hyaluronidase degrades hyaluronic acid and other glycosaminoglycans abundantly occurring in the vertebrate extracellular matrix and promotes spreading of other anti-haemostatic compounds of sand fly saliva within the skin (Cerna et al., 2002, Volfova et al., 2008). Hyaluronidase activity was detected in saliva of various sand fly species as well as in other bloodsucking Diptera (Charlab et al., 1999, Ribeiro et al., 2000b, Cerna et al., 2002, Volfova et al., 2008, Hostomska et al., 2009, Rohousova et al., 2012a). While positive enzymatic activity was detected in all sand fly species tested to date, transcripts coding for putative hyaluronidase were ascertained only in *L. longipalpis* (Charlab et al., 1999, Valenzuela et al., 2004), *P. arabicus* (Hostomska et al., 2009), and *P. tobbi* (Rohousova et al., 2012a). Moreover, this enzyme never appeared among the salivary proteins identified by proteom analysis. These findings suggest that transcripts coding for

hyaluronidase do not occur abundantly in the salivary glands, nevertheless this salivary protein possesses powerful enzymatic activity.

Phospholipase A2 was also ascertained in the salivary glands of *P. ariasi* (Oliveira et al., 2006), *P. argentipes* (Anderson et al., 2006), *P. perniciosus* (Anderson et al., 2006), and *P. arabicus* (Hostomska et al., 2009). Phospholipase A2 acts as the main allergen in the hymenopteran venom (reviewed in Muller, 2011), however, in sand flies the allergenic effect remains to be elucidated.

The last mentioned group is represented by the enzymes that are connected with the ingestion of sugar meal, namely α-amylase and α-glucosidase. Sand fly diet contains sugars originating from honeydews excreted by aphids (Molyneux et al., 1991, Cameron et al., 1995) and from ingested plant tissues (Schlein & Jacobson, 1994, Schlein & Muller, 1995). This meal is rich in amount of starch that is specifically hydrolyzed by α-amylase to maltose, and subsequently cleaved by α-glucosidase to glucose. Therefore it is not surprising that presence of these enzymes was observed not only in salivary glands (Charlab et al., 1999, Ribeiro et al., 2000c, Jacobson & Schlein, 2001), but also in the guts and homogenates of adult males and females, (Ribeiro et al., 2000c, Jacobson & Schlein, 2001, Dillon et al., 2006) as well as in larvae (Vale et al., 2012).

2.2. Other salivary proteins

Saliva of various sand fly species contains certain amount of other pharmacologically active proteins that assist in the fighting host haemostasis. The exact function of some of them is unknown, nevertheless, they represent the powerful antigens. The following paragraphs hence describe the most important protein families.

Members of yellow-related protein family were found in all sand fly species tested (Charlab et al., 1999, Valenzuela et al., 2001a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012, Kato et al., 2012, Rohousova et al., 2012a). In *P. duboscqi*, yellow-related protein was shown to posess lectin-like properties (Volf et al., 2002) but its role in bloodfeeding is unknown. On the other hand, some advances have been made in describing the function of *Lutzomyia* yellow-related proteins; recombinant yellow-related proteins from *Lutzomyia longipalpis* saliva (AAD32198, AAS05318) were shown to bind effectively prohaemostatic and proinflammatory biogenic amins such as serotonin, catecholamines and histamine (Xu et al., 2011). Similarly, the amino acid motif present in the ligand binding pocket of *L. longipalpis*

was also found in the yellow-related proteins of *L. ayacuchensis* (BAM69111, BAM69185, BAM69109, BAM69110) (Kato et al., 2012). These data indicates that yellow-related proteins of other sand fly species could possess similar function.

D7-related proteins and PpSP15-like proteins belong to the odorant-binding protein family. D7-related proteins were found in the saliva of various bloodfeeding dipterans including sand flies (reviewed in Valenzuela et al., 2002). These proteins own characteristic fold structure (Graham et al., 2001) that enables them to bind small ligands and therefore are supposed to trap the agonists of haemostasis. So far, this hypothesis was not confirmed in sand flies, but in mosquitoes, D7-related proteins were shown to bind biogenic amines (Calvo et al., 2006, Mans et al., 2007) as well as to serve as anticoagulants (Isawa et al., 2002, Alvarenga et al., 2010).

PpSP15-like proteins occur solely in sand flies and represent one of the most abundant group of proteins found in their salivary gland transcriptoms. High degree of variability in amino acid structure typical for these proteins (Anderson et al., 2006, Hostomska et al., 2009, Kato et al., 2012, Rohousova et al., 2012a) suggests that PpSP15-like protein of other sand fly species might be also the multicopy gene, as demonstrated in *P. papatasi* (Elnaiem et al., 2005). For the first time, SP-15 protein (AF335487) was described in the saliva of *P. papatasi* and immunization with this recombinant protein provided protective immunity against cutaneous leishmaniasis (Valenzuela et al., 2001a). Thus, it was choosen as the potential candidate for sand fly saliva-based vaccine. The detailed principle of the protective effect of various proteins is described in chapter 4.2.

Lufaxin is another salivary protein exclusively present in sand flies. Lufaxin was recently described as the 32.4 kDa *L. longipalpis* salivary protein (AAS05319) that acts as the slow-tight factor Xa inhibitor and displays anti-thrombotic and anti-inflamatory activities (Collin et al., 2012). Sequences homologous to this protein were found in saliva of other New World sand fly - *L. ayacuchensis* (Kato et al., 2012) as well as in Old World species - *P. arabicus* (Hostomska et al., 2009), *P. argentipes* and *P. perniciosus* (Anderson et al., 2006), *P. ariasi* (Oliveira et al., 2006), *P. duboscqi* (Kato et al., 2006), *P. papatasi* (Abdeladhim et al., 2012), *P. sergenti* and *P. tobbi* (Rohousova et al., 2012a).

Several groups of salivary proteins with unknown function represent powerful antigens. The most important members are antigen 5-related proteins, PpSP32-like proteins, or ParSP25-like proteins. Antigen 5-related proteins belong to the CAP family consisting of **C**ysteine-rich secretory proteins, **A**ntigen 5, and **P**athogenesis-related 1 proteins (reviewed in Gibbs et al., 2008). Antigen 5-related proteins represent important allergens in the venom of

ants, wasps, and other hymenopterans (Lu et al., 1993, Hoffman, 1993, King & Spangfort, 2000) and occur commonly in saliva of different bloodsucking insect (reviewed in Ribeiro et al., 2010). In horse flies, antigen 5-related proteins were shown to serve as the inhibitor of platelet aggregation (Xu et al., 2008) or as the inhibitor of angiogenesis (Ma et al., 2010). In stable flies, these proteins possess immunoglobulin Fc binding activity (Ameri et al., 2008) and may act as an inhibitor of the classic pathway of complement (Wang et al., 2009). PpSP32 protein (AAL11050) and ParSP25 protein (AAX55664) were described in the saliva of *P. papatasi* and *P. ariasi*, respectively (Valenzuela et al., 2001a, Oliveira et al., 2006). Both groups of proteins occur solely in sand flies, furthermore, ParSP25-like proteins are exclusive for Old World species (Oliveira et al., 2006, Anderson et al., 2006, Hostomska et al., 2009, Rohousova et al., 2012a, Abdeladhim et al., 2012).

Presence of maxadilan, the 6.5 kDa salivary peptide, was ascertained in saliva of *L. longipalpis* (Ribeiro et al.,1986).This peptide is known as the most potent salivary vasodilator (Lerner et al., 1991) and assumes the responsibility for the powerful erythema associated with the feeding of this sand fly species (Lerner & Schoemaker, 1992). Another small salivary proteins were found in the saliva of various sand fly species, however, till now such unique effect as found in maxadilan was never connented to any of these peptides.

3. Factors affecting the outcome of *Leishmania* infection

3.1. Sand fly saliva

Except for the anti-haemostatic and anti-inflammatory properties, sand fly saliva also possesses wide scale of salivary proteins with a potent immunomodulatory effect. The common aim of all these compounds is to hamper the host defence system which could otherwise alarm the host attention and potentially result into the rejection of the bloodfeeding female. The immunomodulatory properties of sand fly saliva are also of big importance in those species that serve as the vectors of pathogens. Titus & Ribeiro (1988) were the first to show that sand fly saliva exacerbates the *Leishmania* infection in the naive host. Since then, the local suppression of immune response around the feeding site caused by the sand fly saliva with the subsequent effect on the relevant pathogen transmission is called “enhancing effect”. For the first time, this phenomenon was demonstrated on the *Lutzomyia longipalpis* – *Leishmania major* model which does not occur in the nature (Titus & Ribeiro, 1988). However, in the following years, enhancing effect of sand fly saliva was documented in various sand fly species-parasite combinations including the natural ones, such as *P. papatasi*

– *Le. major* (Theodos et al., 1991), *L. longipalpis – Le. infantum chagasi* (Warburg et al., 1994), or *L. whitmani – Le. braziliensis* (Bezerra & Teixeira, 2001). Saliva of various sand fly species was shown to enlarge the size of *Leishmania* lesions as well as the amount of parasites within them (Titus & Ribeiro, 1988, Theodos et al., 1991, Bezerra & Teixeira, 2001). Moreover, sand fly saliva represented the critical factor for the successful development of *Leishmania* infection in the case, when mice were infected intradermally with 1000 metacyclic promastigotes (Belkaid et al., 1998). The importance of these data was highlighted by Maia et al. (2011), who showed that this infection dose corresponds to the real one (Maia et al., 2011).

Enhancing effect is not exclusive for sand flies, the impact of the saliva on pathogen transmission was observed also in other bloodfeeding arthropods, for example in ticks (e.g. Jones et al., 1989, Jones et al., 1992), mosquitoes (e.g. Schneider & Higgs, 2008, Schneider et al., 2010), kissing bugs (e.g. Mesquita et al., 2008), or tsetse flies (e.g. Caljon et al., 2006a). Moreover, it should be noted that enhancing effect is not resctricted to parasitic diseases, but also viral or bacterial infection are involved as well.

Due to the importance of saliva-based immunomodulation in transmission of human diseases, the immunomodulatory properties of vector saliva or of individual compounds are of big scientific interest. Leishmaniases represent one of the most intensively studied diseases in this aspect, where most of the experiments were performed particularly on *L. longipalpis* and *P. papatasi*, two sand fly species representing New World and Old World vectors, respectively. Many studies demonstrated that sand fly saliva regulates the host immunity at different level, mostly together with the impact on leishmaniasis.

During the early phases of *Leishmania* infection, the most powerful tool of host immunity are phagocytic cells. The interplay of these cells with other cells of immune system is crucial for the disease outcome. Therefore, the modulation is substantially targeted to phagocytic cells, particularly macrophages, and to the abrogation of their defence system (reviewed in Andrade et al., 2005).

Sand fly saliva attracts several immune cells to the feeding site, mainly monocytes (Anjili et al., 1995), macrophages (Zer et al., 2001), or neutrophils (Teixeira et al., 2005). However, macrophages represent the target cells for *Leishmania* parasites. Neutrophils are the first cells recruited to the wound and faced the contact with *Leishmania* parasites deposited into the host skin by needle inoculation (Beil et al., 1992) or by sand fly feeding (Peters et al., 2008). Indeed, *Leishmania* promastigotes produce a neutrophil chemotactic factor and induce IL-8 secretion by human neutrophils, thereby promote the neutrophil recruitment (van Zandbergen

et al., 2002). Therefore, neutrophils were shown to be the predominant phagocytic cells that are affected over the first few hours post infection (Peters et al., 2008, Thalhofer et al., 2011).

Neutrophils are short-lived cells, however, their longevity might be modulated by several inflammatory stimuli or pathogens. For example, saliva of *L. longipalpis* in the presence of *Le. chagasi infantum* parasites were shown to accelerate the apoptosis of murine peritoneal neutrophils by enhanced expression of Fas ligand transmembrane protein (Prates et al., 2011). Moreover, the infected neutrophils excreted higher levels of MCP-1, the chemotactic signal for macrophages (Prates et al., 2011). Similarly, the *ex vivo* experiments of Ribeiro-Gomes et al. (2012) revealed that *Le. major* parasites enhance the apoptosis of mice neutrophils evidenced by the increased expression of phosphatidylserine, the "eat me" signal on apoptotic cells for macrophages and dendritic cells (Ribeiro-Gomes et al., 2012). Infected neutrophils are subsequently engulfed by macrophages or dendritic cells, thus viable parasites are delivered to those phagocytic cells (Laskay et al., 2003, van Zandbergen et al., 2004, Gueirard et al., 2008, Ribeiro-Gomes et al., 2012).

To our knowledge, there are two possible ways how to reach the host macrophages. The first one when *Leishmania* parasites are devoured hidden inside the neutrophils as described above, the later one when individual parasites are phagocyted within the host skin. It should be noted that in the first case, the affected neutrophils provide the "protective shield" to the parasites by abrogation the contact of *Leishmania* parasite with macrophage receptors, thereby disable activation of macrophages as well as other anti-parasitic effector mechanisms (van Zandbergen et al., 2004). This immune break-out was first described by Laskay et al. 2003 and named as the theory of "Trojan horse" (Laskay et al., 2003). Conversely, in the second case, parasites are exposed directly to the host immunity, thus in effort to survive, they are forced to apply various arms.

Macrophages possess various mechanisms in order to destroy the immunogenic agens. After recognition of *Leishmania* spp., macrophages are activated and thus various cellular processes are triggered to kill the parasites. The most important are as follows: oxidative burst generation, production of nitric oxide (NO) and expression of phagolysosomal degradation enzymes such as proteases, nucleases, phosphatases, lipases, and esterases (reviewed in Assche et al., 2011). Nevertheless, sand fly saliva importantly affects the physiological function of macrophages and suppresses the killing of parasites within them (e.g. Hall & Titus, 1995, Zer et al., 2001). *Phlebotomus papatasi* saliva inhibits the expression of the gene for inducible NO synthase (iNOS) resulting into the decreased production of NO by activated macrophages (Hall & Titus, 1995, Waitumbi & Warburg, 1998, Katz et al., 2000), and thus

enables the multiplication of amastigotes within the infected macrophages. It was suggested that adenosine, that commonly occurs in *P. papatasi* saliva (Ribeiro et al., 1999), might be the molecule responsible for this effect (Katz et al., 2000). As the presence of adenosine deaminase was found in the saliva of other species such as *L. longipalpis* and *P. duboscqi*, (Charlab et al., 2000, Kato et al., 2007), other molecules might be responsible for macrophage inhibition as well. For example inosin, the product of hydrolysis catalyzed by adenosine deaminase, was shown to inhibit the peritoneal macrophages in production of proinflammatory cytokines such as TNF-α, IL-1, and IL-12, however, inosine failed to modulate the production of NO (Hasko et al., 2000).

Saliva of *L. longipalpis* was also ascertained to modulate the macrophage functions. It was shown that macrophages activated by IFN-γ are substantially inhibited in production of H_2O_2 by saliva of this sand fly species (Titus & Ribeiro, 1990). Moreover, saliva of *L. longipalpis* was demonstrated to inhibit the macrophages in presenting of antigen to T cells (Theodos & Titus, 1993) as well as to decrease the expression of CD80 costimulatory molecule on the surface of macrophages and monocytes stimulated by lipopolysaccharide (LPS) (Costa et al., 2004). Recent experiments made by Araujo-Santos et al. (2010) showed that saliva of *L. longipalpis* is capable to trigger the lipid body formation and prostaglandin E2 production in murine macrophages, thus favours the growth of *Leishmania* parasites and their replication (Araujo-Santos et al., 2010).

Morris et al. (2001) reported that maxadilan, the most powerful anti-haemostatic compound of *L. longipalpis* saliva, has strong immunomodulatory properties and exacerbate *Leishmania* infection to the same degree as whole saliva (Morris et al., 2001). Moreover, maxadilan was shown to have significant effect on production of NO and various cytokines. In macrophages activated by LPS, maxadilan down-regulated production of NO as well as IL-12 and TNF- α cytokines (Brodie et al., 2007). In contrast, cytokines such as IL-6, IL-10, and TGF-β were up-regulated (Brodie et al., 2007). These data suggest that maxadilan polarizes the immune response toward Th2 type of immune response characteristic by the inability of the hosts protect themselves against leishmaniasis. Another studies revealed that maxadilan affects various cells of immune system in similar pattern; monocytes, $CD4^+$ T cells, and dendritic cells were inhibited in production of Th1 cytokines, whereas Th2 cytokines were up-regulated (Rogers & Titus, 2003, Wheat et al., 2008). Furthermore, maxadilan reduces expression of CD80 and CD86 molecules on the surface of dendritic cells (Wheat et al., 2008), suppresses proliferation of T cells (Qureshi et al., 1996), and stimulates bone marrow

haematopoiesis through its ability to stimulate IL-6 production by bone marrow stromal cells (Guilpin et al., 2002).

3.2. PSG and *Leishmania*-surface molecules

It should be pointed out that sand fly saliva is not the sole factor affecting the outcome of *Leishmania* infection in the naive host, the others are mainly PSG or the molecules occurring on the surface of *Leishmania* parasites. As this topic was not the main aim of my PhD thesis, the other aspects will be mentioned briefly.

It was demonstrated that PSG, the gel plug secreted by *Leishmania* parasites (Rogers et al., 2002) and regurgitated into the wound along with the parasites and sand fly saliva, substantially favours the development of *Leishmania* infection (reviewed in Rogers, 2012). PSG was described to increase the success of *Leishmania* transmission by blocking the fluent feeding process of infected female sand fly (Rogers et al., 2002, Rogers & Bates, 2007), but it also acts as the virulent factor (Rogers et al., 2004). Rogers et al. (2004) showed that PSG exacerbates *Le. mexicana* infection in BALB/c as well as in CBA/Ca mice, the strains sensitive and resistant to *Leishmania* infection, respectively (Rogers et al., 2004). Later, this team explained the principle of PSG "enhancing effect". They demonstrated the powerful effect of PSG on the macrophage recruitment to the dermal site of infection and they also showed that PSG enhances arginase activity of host macrophages, thus promotes alternative activation of these cells (Rogers et al., 2009). Due to this activity, production of microbicidal NO is abrogated and the subsequent synthesis of polyamines supports the intracellular growth of *Leishmania* parasites (Rogers et al., 2009).

The second aspect which contributes to the successful development of *Leishmania* infection is presence of glycoconjugates on parasite surface. It should be noted that the surface glycoconjugates differ in *Leishmania* promastigotes and amastigotes and correspond well with the biological function of individual stages. *Leishmania* promastigotes are covered by a thick glycocalyx layer comprised of lipophosphoglycan (LPG), proteophosphoglycan (PPG), gp63 metalloproteinase, and glycophospahtidylinositol lipids (GIPLs) (reviewed in Franco et al., 2012). Due to the LPG or gp63 metalloproteinase, *Leishmania* promastigotes are resistant to the complement-mediated lysis (Puentes et al., 1990, Brittingham et al., 1995, Spath et al., 2003), however, these glycoconjugates enables the opsonization of parasites by the C3b and C3bi fragments. C3b and C3bi subunits mediate the attachment of *Leishmania* parasites to the CR1 and CR3 receptors present on macrophages and trigger their phagocytic activity (Mosser & Edelson, 1984, Mosser & Edelson, 1985, da Silva et al., 1988, Da Silva et

al., 1989). Phagocytosis of *Leishmania* via CR1 or CR3 receptor is the way how to avoid the "macrophage attention", as this entrance into the cell does not activate oxidative burst and production of IL-12 (Wright & Silverstain, 1983, Mosser & Edelson, 1987, Marth & Kelsall, 1997, Sutterwala et al.,1997). Moreover, LPG was shown to down-regulates the expression of iNOS, thus decreases the production of NO (Proudfoot et al., 1996). Similarly, GIPLs commonly present on the surface of amastigotes and promastigotes were demonstrated to supress the microbicidal activity of macrophages by inhibition of NO production (Proudfoot et al., 1995). Taken together, it seems that each of the surface glycoconjugate substantially contributes to the parasite survival and promotes the development of *Leishmania* infection.

4. Immune response to sand fly salivary antigens

4.1. Cell-mediated immunity

Apart from their inherent pharmacologic, immunomodulatory, and anti-inflammatory activities, salivary proteins are immunogenous for various hosts including humans. In fact, it has been shown that repeated exposure to sand fly bites elicits specific immunity which includes both cell-mediated and humoral immune response (reviewed in Gomes & Oliveira, 2012).

Belkaid et al. (1998) were the first to demonstrate that inoculation of the sand fly saliva into the mice skin elicited specific immunity that provided protection against *Le. major* infection (Belkaid et al., 1998). Two years later, the same outcome of *Leishmania* infection was described in mice immunized naturaly through the bites of uninfected female sand fly (Kamhawi et al., 2000). Enhancing effect of sand fly saliva, characteristic by the increased size of *Leishmania* lesions and parasite burden as well as by the polarization of immune response toward Th2 type (Belkaid et al., 1998, Mbow et al., 1998, Norsworthy et al., 2004), was abrogated in hosts pre-immunized by sand fly saliva (Belkaid et al., 1998, Kamhawi et al., 2000). Further experiments revealed that the protective effect conferred by the sand fly saliva pre-exposure is caused by the host cellular immunity, particularly by the DTH reaction (Kamhawi et al., 2000, Valenzuela et al., 2001a). In murine model, this immune reaction was characterized by the recruitment of neutrophils, eosinophils, and macrophages to the bite site and correlated with the production of IFN-γ and IL-12 (Kamhawi et al., 2000, Valenzuela et al., 2001a, Silva et al., 2005). In guinea pigs, immunization with *L. longipalpis* saliva resulted in strong eosiniphilia and weak basophilia with the peaks following each exposure (Brown &

Rosalsky, 1984). In the Th1 environment, macrophages with the engulfed parasites expose *Leishmania* antigens on their surface via MHC II receptor and produce IL-12. This cytokine is the key factor responsible for the switch of naive CD4+ lymphocytes toward Th1 cells; these cells being typical by the massive production of IFN-γ (Klein & Horejsi, 1997). Except for the Th1 cells, NK cells also represent important source of IFN-γ (Scharton & Scott, 1993), neccesary for the ”classical” activation of macrophages. Macrophages activated this way are able to fight successfully the *Leishmania* parasites, early after the infection, therefore this is supposed to be the core of the saliva-based protective effect.

Anti-saliva immunity is not directed against *Leishmania* parasites, but alters the type and activation status of macrophages and other host cells which could otherwise silently maintain the parasites. Therefore we can assume that those stimuli capable of inducing the Th1 DTH milieu around the sand fly feeding lesion might mediate the protective effect.

In the endemic areas, inhabitants are frequently bitten during the sand fly season. As the percentage of *Leishmania*-infected females is low (e.g. Anderson et al., 2011), humans and animals are thus commonly exposed to the bites of uninfected sand flies prior to confrontation with *Leishmania* parasites. Upon repeated exposure, feeding of various insect is connected with the formation of hypersensitivity reactions, sand flies being those mostly associated with the DTH, manifested by the syndrome known as “harara” (Theodor, 1935). As DTH elicited by sand fly bites is protective against leishmaniasis (Kamhawi et al., 2000), there might be several aspects contributing to the circulation of *Leishmania* infection in the endemic areas.

Mellanby (1946) was the first who described the course of immune responsiveness to mosquito bites with 4 distinct stages: naive individuals developed no reaction or DTH by the first mosquito bites, changing gradually throughout repeated exposure to combined DTH and immediate reaction, followed by the immediate reaction solely, and potentialy culminate by desensitization (Mellanby, 1946). Similar set of reaction to repeated exposure was detected also in guinea pigs bitten by fleas (Benjamini et al., 1961) and might be expected also in other bloodfeeding insect. However, to our knowledge, there is no study describing the desensitization in sand flies, thus we focused on this topic in our experiments.

In endemic areas, the individual phases of the reactivity are not clearly distinguishable and the response of each host to certain stimuli differs. This variation was well documented in humans bitten by mosquitoes (Peng et al., 1996, Peng et al., 2004), kissing bugs (Costa et al., 1981), and by sand flies (Vinhas et al., 2007, Oliveira et al., 2013). Until recently, DTH reaction to sand fly saliva was detected only in human volunteers originating from non-endemic areas when challenged by the sand fly bites (Theodor, 1935, Belkaid et al., 2000,

Vinhas et al., 2007). Nowadays, DTH reaction was also observed in inhabitants from the endemic area of cutaneous leishmaniasis in Mali that were re-exposed to sand flies under laboratory conditions (Oliveira et al., 2013). Peripheral blood mononuclear cells (PBMCs) isolated from individuals bitten by *P. duboscqi* or *L. longipalpis* mostly reacted to stimulation by homologous antigen by the production of Th1/ Th2 cytokine mixture (Vinhas et al., 2007, Oliveira et al., 2013). In contrast, PBMCs of humans bitten by *P. papatasi* produced, upon stimulation, predominantly Th2 cytokines, such as IL-4 and IL-10 (Abdeladhim et al., 2011). One of the possible explanations for this fact might be the presence of adenosin and AMP in *P. papatasi* saliva, as adenosin was reported to be responsible for polarization of immune response toward a Th2 type (Hasko et al., 1996, Hasko et al., 2000). Besides the different composition of saliva of various sand fly species, there are many factors that may alter the immune response of each host, for example genetic background, enviromental factors as well as co-infections. Moreover, it is hypothesized that the difference in composition of saliva of the same sand fly species originating from distinct foci modulates the pathology of transmitting cutaneous leishmaniasis (Warburg et al., 1994). Thus, it might be beneficial to find out, whether the same aspect is also responsible for the different epidemiology of visceral leishmaniasis.

4.2. Candidates for vaccine against leishmaniasis

The notion that the immunity achieved by the immunization with sand fly saliva is protective led to an idea that the individual salivary proteins with corresponding properties could be potentially used as the candidates for vaccine against leishmaniasis. Of course, there are several *Leishmania* antigens reported as promising candidates for the vaccine; for example membrane protein of *Leishmania* kinetoplast – KMP11 (Basu et al., 2005, da Silva et al., 2011), *Leishmania* nucleosomal histones separately (Solioz et al., 1999, Masina et al., 2003, Chenik et al., 2006, Agallou et al., 2012) or as the DNA cocktail containing H2A, H2B, H3, and H4 histones (Iborra et al., 2004, Carrion et al., 2007, Carrion et al., 2008, Carneiro et al., 2012), or the synthetic *Leishmania*-based polyproteins – Leish-110f and KSAC (Bertholet et al., 2009, Goto et al., 2011, Gomes et al., 2012a). However, these are out of scope of this short review.

As the involvement of humans in testing the potential vaccine candidates with the subsequent outcome of *Leishmania* infection is very difficult, most of the experiments were done on animal models. Thus, protection against leishmaniasis mediated by sand fly saliva or the salivary components has been well established in mice and hamsters (Belkaid et al., 1998,

Kamhawi et al., 2000, Morris et al., 2001, Valenzuela et al., 2001a, Thiakaki et al., 2005, Gomes et al., 2008, Oliveira et al., 2008, da Silva et al., 2011, Tavares et al., 2011, Xu et al., 2011, Gomes et al., 2012b). The immunogenicity of individual salivary proteins was studied in various sand fly species, some of the proteins were found to be responsible solely for stimulation of cellular or antibody response, some promoted both arms of immunity (Valenzuela et al., 2001a, Oliveira et al., 2006, Oliveira et al., 2008, Collin et al., 2009, Xu et al., 2011). The situation was markedly complicated by findings of Thiakaki et al. (2005), when they showed that the protective effect elicited by sand fly feeding is species specific (Thiakaki et al., 2005). The immunization by saliva of two Old World species, *P. papatasi* and *P. sergenti*, did not provide the protection to those mice infected by *Le. amazonensis* parasites along with *L. longipalpis* saliva (Thiakaki et al., 2005). Although the cross-protectivity between Old World and New World sand fly species seemed unlikely, different situation might be between closely related species. Later on, experiments of Tavares et al. (2011) showed that there is the cross-protectivity between two New World species – *L. longipalpis* and *L. intermedia* (Tavares et al., 2011). Moreover, similar results were also detected in the study testing two Old World species from subgenus *Phlebotomus*, *P. papatasi* and *P. duboscqi* (Kratochvilova et al., unpublished results).

Synthetic maxadilan from *L. longipalpis* saliva (AAA29288) and partially purified PpSP15 protein from *P. papatasi* saliva (AAL11047) were the first individual proteins that provided protection against *Le. major* infection in mice (Morris et al., 2001,Valenzuela et al., 2001a). Both proteins promoted cellular as well as humoral response in immunized mice and the protection was expressed by the decreased lesion size and the amount of parasite within murine tissues (Morris et al., 2001,Valenzuela et al., 2001a). Similarly as whole saliva, individual salivary proteins that provide protection against *Leishmania* infection are those promoting DTH reaction (Valenzuela et al., 2001a). However, experiments of Oliveira et al. (2008) revealed that not all DTH-inducing proteins are protective; some of them even aggravates the infection (Oliveira et al., 2008). Therefore, for the future experiments, only those proteins eliciting DTH reaction and IFN-γ production as well were employed.

When probing for the other *L. longipalpis* salivary proteins inducing strong Th1 DTH reaction, LJM19 (AAR99725), a 11 kDa salivary protein with unknown function, and two yellow-related salivary proteins, LJM11 and LJM17 (AAS05318 and AAD32198, respectively), were ascertained (Gomes et al., 2008). LJM19 was the first salivary protein successfully employed as the “protective agents” against visceral leishmaniasis (Gomes et al., 2008). Hamsters immunized by this salivary protein and infected with *Le. infantum chagasi*

plus *L. longipalpis* saliva were able to maintain a low parasite load in the spleen and liver, with high IFN-γ/TGF-β ratio and iNOS expression in both organs (Gomes et al., 2008, da Silva et al., 2011). Similar protection pattern was achieved in hamsters immunized by LJM19 after infection with *Le. braziliensis* along with *L. intermedia* saliva (Tavares et al., 2011). Members of the yellow-related protein family represent the powerful antigens (Gomes et al., 2008, Xu et al., 2011); immunization with LJM11 was connected with a robust DTH reaction and provided the protection against *Le. major* parasites deposited into the mice skin by needle inoculation (Xu et al., 2011) as well as naturally through the sand fly bites (Gomes et al., 2012b). Deeper analysis of LJM11 revealed its unique structural and functional features that might be responsible for the protective immune profile which elicits (Xu et al., 2011). Another recombinant yellow-related salivary protein from *L. longipalpis* saliva, LJM17, indicated also the feasibility of its application in protection against *Leishmania* infection; *in vitro* experiments showed that macrophages isolated from dogs immunized by this recombinant protein efficiently killed the *Le. chagasi infantum* parasites occurring within them (Collin et al., 2009).

4.3. Humoral immunity

In addition to the cellular immunity, bloodfeeding of various insect species stimulates also the production of specific antibodies. Increased levels of anti-saliva IgG, IgE, or IgM were detected in sera of hosts bitten by mosquitoes (Brummer-Korvenkontio et al., 1994, Palusuo et al., 1997, Remoue et al., 2006, Orlandi-Pradines et al., 2007, Andrade et al., 2009, Doucoure et al., 2012a, Doucoure et al., 2012b), kissing bugs (Volf et al., 1993, Nascimento et al., 2001, Schwarz et al., 2009a, Schwarz et al., 2010, Schwarz et al., 2011), tsetse flies (Caljon et al., 2006b), black flies (Cross et al., 1993), bitting midges (Wagner et al., 2006, Hellberg et al., 2006), and ticks (Ogden et al., 2002, Szabo et al., 2003). Similarly, increased levels of specific IgG and/ or IgE were detected in sera of various hosts bitten by sand flies under laboratory conditions (Ghosh & Mukhopadhyay, 1998, Volf & Rohousova, 2001, Rohousova et al., 2005, Silva et al., 2005, Thiakaki et al., 2005, de Moura et al., 2007, Hostomska et al., 2008, Martin-Martin et al., 2012) as well as in sera of hosts from endemic areas (Barral et al., 2000, Gomes et al., 2002, Rohousova et al., 2005, Bahia et al., 2007, Gomes et al., 2007, Vinhas et al., 2007, Clements et al., 2010, Marzouki et al., 2011).

The specificity of antibody response was studied using sera of mice bitten by various sand fly species and data achieved by these experiments revealed that there is no cross-reactivity between *Lutzomyia* and *Phlebotomus* and week cross-reactivity between species

within *Phlebotomus* genus (Volf & Rohousova, 2001, Rohousova et al., 2005, Thiakaki et al., 2005, Drahota et al., 2009).

The antibody isotype predominantly induced by sand fly feeding is IgG1 in mice (Silva et al., 2005, Oliveira et al., 2006), IgG1, IgG4, and IgE in humans (Vinhas et al., 2007, Marzouki et al., 2011) and IgG2 in dogs (Hostomska et al., 2008). The detailed dynamics of antibody response was described in dogs bitten by *L. longipalpis* (Hostomska et al., 2008, Collin et al., 2009); repeated exposure to sand fly bites elicited production of specific IgG, IgG1, and IgG2 and these antibodies persisted in elevated levels for more than four monts after the last exposure (Hostomska et al., 2008). In parallel, the long-lasting responsiveness to salivary antigens proven by the western blot analysis was also detected in humans bitten by *L. longipalpis* (Vinhas et al., 2007).

In contrast to IgE, specific IgG response is stimulated in all hosts biten by sand flies (Vinhas et al., 2007, Hostomska et al., 2008). Moreover, further investigation revealed that anti-sand fly saliva IgG positively correlate with the intensity of sand fly exposure, thus specific antibodies were suggested as the marker of sand fly exposure in endemic areas (Rohousova et al., 2005, Hostomska et al., 2008, Clements et al., 2010).

Similarly, in other blood sucking insects, anti-saliva antibodies were shown to reflect the intensity of exposure in chickens and guinea pigs bitten by *Triatoma infestans* (Schwarz et al., 2009a), in humans exposed to *Glossina fuscipes fuscipes* bites (Poinsignon et al., 2008a), and in humans bitten by *Culex* (Das et al., 1991), *Anopheles* (Remoue et al., 2006, Waitayakul et al., 2006, Andrade et al., 2009), and *Aedes* mosquitoes (Fontaine et al., 2011b, Doucoure et al., 2012a, Doucoure et al., 2012b).

The concept of using specific antibodies as markers of exposure was successfully employed in foci of visceral leishmaniasis in India and Nepal. Levels of anti- *P. argentipes* and anti- *P. papatasi* IgG were measured in humans that used long-lasting insecticidal nets and in controls, and although this protection was not effective, this trial proved the feasibility of utilization of specific anti-saliva antibodies in control of leishmaniasis (Gidwani et al., 2011). In other field trials, specific antibodies demonstrated that insecticide-treated nets prevent triatomine and mosquito bites, thereby descreased the chance of transmission of Chagase disease (Schwarz et al., 2011) and malaria (Drame et al., 2010a), respectively.

Apart from being the epidemiological tools, specific IgG have been linked to increased risk of cutaneous leishmaniasis (CL) caused by *Le. tropica* (Rohousova et al., 2005), *Le. braziliensis* (de Moura et al., 2007), or *Le. major* (Marzouki et al., 2011). In contrast, in the endemic areas of VL, anti-sand fly saliva antibodies were shown to correlate with human anti-

Leishmania DTH (Barral et al., 2000, Gomes et al., 2002, Aquino et al., 2010). Overall, it seems that the association of the humoral response and the risk of CL or VL, although antagonistic, is likely not random and it may have various explanations. In endemic foci of CL, the levels of anti-saliva antibodies mirror the contact with vector and thus indicate the probability of *Leishmania* transmission through the sand fly bite. Conversely, in endemic areas of VL, specific antibodies correlate with the stimulation of cell-mediated immunity, therefore reflect the protection against the potential *Leishmania* infection. Although it was shown that humoral response is not neccesary for the protection against *Leishmania* infection (Valenzuela et al., 2001a), it should be very beneficial to investigate the role of specific antibodies during the parasite transmission, establishment, and manifestation or the potential connection with the VL systemic and CL local pathophysiology.

Use of the anti-saliva antibodies as the marker of risk of pathogen transmission is not exclusive for sand flies. The field trial in the endemic area of malaria in Senegal showed that antibodies against saliva of *Anopheles gambiae* significantly differ between groups of healthy children and children with malaria. Specific IgG was higher in patients with clinical *Plasmodium falciparum* infection and it increased with the intensity of *Anopheles* exposure (Remoue et al., 2006). Similar results were achieved also in the human study, where the antibodies against saliva of *Ixodes scapularis* positively correlated with the tick infestation and with the Lyme disease seropositivity (Schwartz et al., 1991).

4.4. Sand fly salivary proteins as the markers of sand fly exposure

As one of the main aims of leishmaniasis control is to prevent the contact of sand flies with humans and animals, screening of the host specific antibody response would help to evaluate its effectiveness within the epidemiological studies. However, for broader use, the need for maintainance of sand fly colonies and the dissection of their saliva represent the big limitations. Therefore, intensive research is ongoing in effort to find suitable salivary proteins that could, in recombinant form, replace whole sand fly saliva.

First of all, the attention should be attracted to those salivary proteins that are antigenic to broad spectrum of bitten hosts, regardless of exposure under laboratory conditions or in endemic areas. The most immunogenous salivary proteins that are common for more hosts bitten by sand flies are listed in Table 1. At this point, we should definitely highlight the importance of yellow-related proteins or D7-related proteins, as these two protein families are commonly recognized by sera of humans (Rohousova et al., 2005, Teixeira et al., 2010, Marzouki et al., 2011, Nieves et al., 2012) as well as by sera of animals that might serve as

the reservoir hosts of leishmaniasis (Bahia et al., 2007, Gomes et al., 2007, Teixeira et al., 2010).

The other aspect that should be taken into account, when searching for the salivary proteins suitable as the marker of sand fly exposure, is the degree of their glycosylation. Powerful glycosylation of several salivary proteins (e.g. Hostomska et al., 2009, Rohousova et al., 2012a) suggests a question, which part of the salivary protein is responsible for its immunogenicity, whether the protein itself, or the saccharidic part, or both. The glycosylation of individual proteins should be considered when choosing the suitable expressing system.

Experiments with recombinant salivary proteins prepared in *Escherichia coli*-expressing system showed that apyrases from *P. duboscqi* (ABI20147) and *L. longipalpis* (AAD33513) saliva were recognized by specific antibodies in sera of experimentally bitten mice and by sera of humans and dogs from endemic area of VL in Brasil, respectively (Hamasaki et al., 2009, Teixeira et al., 2010). Deeper analysis revealed that also the others *L. longipalpis* salivary proteins such as yellow-related proteins (AAD32198, AAS05318), D7-related protein (AAL16051), or lufaxin (AAS05319) are strongly immunogenous for all humans and dogs tested, therefore these proteins might be the suitable candidates as the markers of exposure as well (Teixeira et al., 2010). Based on these results, Souza et al. (2010) replaced the whole sand fly saliva with the combination of LJM11 and LJM17, the two recombinant *L. longipalpis* yellow-related salivary proteins (AAS05318, AAD32198). Positive correlation was achieved between the antibody response to *L. longipalpis* saliva and the specific humoral immunity to combination of LJM11 and LJM17 salivary proteins, when utilized the sera of individuals from endemic area of VL in Brasil (Souza et al., 2010). These results indicate that yellow-related proteins from *L. longipalpis* could be used instead of whole salivary glands in the epidemiological surveys in endemic areas of VL. However, recent study showed that different situation might be in the endemic areas of cutaneous leishmaniasis, where the main target of human antibody response to *P. papatasi* saliva was PpSP32 salivary protein (Marzouki et al., 2012).

Nevertheless, the research on the recombinant proteins / peptides that could be used for screening of the specific antibodies in human or animal sera is considerably more advanced in other insect species. In mosquitoes, gSG6-P1, the salivary peptide of *Anopheles gambiae* was suggested as the potential candidate (Poinsignon et al., 2008b); further analysis showed that IgG response to this salivary peptide reflects the intensity of real exposure, including the detection of low level infestation (Poinsignon et al., 2009). Later on, the use of gSG6-P1 was broadened to detect also *An. funestus* bites (Poinsignon et al., 2010) and then successfully

employed as a biomarker of exposure of *Anopheles* bites in the endemic area of malaria in Angola (Drame et al., 2010b) and Senegal (Drame et al., 2012). In parallel, for screening of the exposure to *Aedes aegypti*, the important vector of several human arboviruses, Nterm-34 kDa salivary peptide was identified and the results of the preliminary experiments indicate the feasibility of Nterm-34 kDa peptide utilization (Elanga Ndille et al., 2012).

While in sand flies and mosquitoes, specific IgG response was shown to be the suitable marker of exposure, it may differ in other bloodsucking insect. For example in kissing bugs, 14.6 kDa salivary protein of *Triatoma infestans* (TiSP14.6) was choosen and successfully used for detection of low level infestation by four triatomine species in chickens (Schwarz et al., 2009b). However, the long-lasting IgG response was not suitable for broader use. Therefore, short-term specific IgM response corresponding well to the vector exposure was suggested instead (Schwarz et al., 2010).

Taken together, deeper analysis of the salivary proteins targeted by the antibody response of vertebrate hosts would increase our understanding of vector-host interactions. Moreover, it could also help in developing new epidemiological tools to correlate host exposure to vectors with the risk of pathogen transmission.

Table 1. The most antigenic salivary proteins recognized by sera of repeatedly bitten hosts

Salivary protein	**Sand fly species**	**Citation**
Yellow-related proteins	*L. longipalpis*	Gomes et al., 2002, Rohousova et al., 2005, Silva et al., 2005, Bahia et al., 2007, Gomes et al., 2007, Vinhas et al., 2007, Hostomska et al., 2008, Teixeira et al., 2010
	P. arabicus	Hostomska et al., 2009
	P. halepensis	Volf & Rohousova, 2001
	P. papatasi	Rohousova et al., 2005, Marzouki et al., 2011
	P. perniciosus	Volf & Rohousova, 2001, Martin-Martin et al., 2012
	P. sergenti	Rohousova et al., 2005
	P. tobbi	Rohousova et al., 2012
Apyrases	*L. longipalpis*	Silva et al., 2005, Vinhas et al., 2007
	P. halepensis	Volf & Rohousova, 2001
	P. papatasi	Rohousova et al., 2005
	P. perniciosus	Volf & Rohousova, 2001, Martin-Martin et al., 2012
	P.sergenti	Rohousova et al., 2005
D7-related proteins	*L. longipalpis*	Bahia et al., 2007, Hostomska et al., 2008
	P. papatasi	Rohousova et al., 2005, Marzouki et al., 2011
	P. perniciosus	Martin-Martin et al., 2012
	P. sergenti	Rohousova et al., 2005
Antigen 5-related proteins	*P. arabicus*	Hostomska et al., 2009
	P. sergenti	Rohousova et al., 2012
	P. tobbi	Rohousova et al., 2012
PpSP32-like proteins	*P. papatasi*	Rohousova et al., 2005, Marzouki et al., 2012
ParSP25-like proteins	*P. perniciosus*	Martin-Martin et al., 2012

Objectives

It was shown that immune response against sand fly saliva or the individual salivary proteins provides protection against various leishmaniasis. Thus, we studied the immune aspects of the protective effect achieved by sand fly pre-exposure in mice using various immunization/ infection schedules. In parallel, we tested the utilization of the specific humoral immunity as the marker of sand fly exposure and as the marker of risk of *Leishmania* transmission. Moreover, we also investigated the most immunogenous salivary proteins in various sand fly species and we analysed whether different epidemiology of visceral leishmaniasis might be caused by the composition and properties of sand fly saliva. In these experiments we used two vectors of visceral leishmaniasis – *Phlebotomus perniciosus* and *P. orientalis* and two vector of cutaneous leishmaniasis – *P. duboscqi* and *P. papatasi*, all colonies available in our animal facility.

The main objectives of this thesis were as follows:

- To study the immune aspects of protective effect in *Phlebotomus duboscqi* – *Leishmania major* – BALB/c mice model using various schedules mimicking the situations in endemic areas and to test the feasibility of desenzitization in mice exposed to sand fly bites for prolonged period

- To follow the kinetics of antibody response in dogs bitten by *Phlebotomus perniciosus* under laboratory conditions and in the field as well as in BALB/c and C57BL/6 mice bitten by *P. papatasi*, to find out whether there is any correlation between the specific humoral response and the risk of *Leishmania* transmission in dogs and to test the utilization of the recombinant proteins as markers of sand fly exposure

- To construct cDNA libraries of two *Phlebotomus orientalis* colonies originating from nonendemic – Melka Werer and endemic – Addis Zemen area of visceral leishmaniasis in Ethiopia and clarify, whether the difference in the epidemiology of visceral leishmaniasis is due to the different composition of *P. orientalis* saliva

Publications

- Rohousova I., Hostomska J., **Vlkova M.**, Kobets T., Lipoldova M., Volf P. (2011). The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure. International Journal for Parasitology 41: 481-485.

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Rapid Communication

The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure

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ABSTRACT

Under laboratory conditions, hosts exposed twice to sand fly saliva are protected against severe leishmaniasis. However, people in endemic areas are exposed to the vector over a long term and may experience sand fly-free periods. Therefore, we exposed mice long- or short-term to *Phlebotomus duboscqi* bites, followed by *Leishmania major* infection either immediately or after a sand fly-free period. We showed that protection against leishmaniasis is limited to short-term exposure to sand flies immediately before infection. Our results may explain the persistence of leishmaniasis in endemic areas and should be taken into account when designing anti-*Leishmania* vaccines based on sand fly saliva.

Leishmania spp. are intracellular protozoan parasites which infect mammalian phagocytic cells. The clinical manifestations of their multiplication and associated immunopathology depend on parasite species, genetic background and immune status of the host, ranging from cutaneous to fatal visceral disease. *Leishmania major* is a typical zoonotic species causing the cutaneous form of the disease. The transmission occurs predominantly between the vector and wild rodents (e.g. sand rats) with humans occasionally breaking into the zoonotic transmission cycle (Peters and Killick-Kendrick, 1987). The sole vectors of *Leishmania* parasites are female sand flies (Diptera: Phlebotominae), tiny bloodsucking insects widespread in the subtropics and tropics. During blood feeding, the parasites are deposited into host skin together with the fly saliva. Sand fly saliva contains antihaemostatic molecules and factors which modify the immune milieu of the skin. Not only do these components facilitate the acquisition of blood, but their presence in the infection site is also important for effective establishment of *Leishmania* parasites (Rohousova and Volf, 2006).

Individuals exposed to sand fly bites develop a specific immune response to salivary proteins (Rohousova and Volf, 2006). It was hypothesized that the enhancing effect of sand fly saliva on *Leishmania* infection could be abolished by this immune response in humans vaccinated against vector saliva, thereby preventing the establishment of infection. This concept has been demonstrated for *Leishmania* spp. causing both cutaneous and visceral leishmaniasis. Partial protection against *L. major* infection has been achieved in mice immunized by the bites of uninfected sand flies (Kamhawi et al., 2000), by salivary gland homogenate (Belkaid et al., 1998) or individual salivary components (Morris et al., 2001; Valenzuela et al., 2001; Oliveira et al., 2008). Protection has generally been associated with the production of IFN-γ and IL-12 upon challenge with *Leishmania* and saliva (Kamhawi et al., 2000; Gomes et al., 2008; Oliveira et al., 2008), suggesting that the existing anti-saliva delayed-type hypersensitivity (DTH) immune response creates an inhospitable environment for parasite survival.

While previous exposure of animals to sand fly feeding under laboratory conditions interferes with subsequent growth of transmitted *Leishmania* parasites, such protection has not been reported from the field. In endemic areas, the prevalence of *Leishmania* infection within the sand fly population is relatively low (Peters and Killick-Kendrick, 1987) and hosts are mostly exposed to the bites of uninfected sand flies. Despite this continuous exposure, leishmaniasis persists in endemic areas. Local inhabitants develop a specific antibody response to salivary antigens (Gomes et al., 2002; Rohousova et al., 2005; de Moura et al., 2007), which correlates with protection against visceral leishmaniasis (Gomes et al., 2002) but not against cutaneous leishmaniasis (Rohousova et al., 2005; de Moura et al., 2007). Clearly, the type of immune response elicited by laboratory immunization schemes and continuous exposure to uninfected sand flies occurring in the field are different.

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Thus, in our study we developed a mouse model of natural sand fly exposure to compare the outcome of infection in mice immunized using the exposure scheme previously reported (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005) and in mice exposed "naturally", for a prolonged period.

Mice were bitten by *Phlebotomus duboscqi* and subsequently infected by *L. major*, the sole *Leishmania* sp. naturally transmitted by this sand fly sp. (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990). The exposure schemes used in our study also address the seasonal dynamics of sand fly populations (Peters and Killick-Kendrick, 1987) and the persistence of the humoral immune response to sand fly saliva throughout the sand fly-free period.

All animals used in this study were maintained and handled strictly 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. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008–10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic. Mice were anaesthetized by i.p. injection of 150 mg/kg ketamine and 15 mg/kg xylazine during the exposure phase and infection. All efforts were made to minimize the number and suffering of experimental animals within the study.

BALB/c mice were maintained in the animal facility of Charles University in Prague. Preliminary experiments were carried out to determine the optimal infection dose and exposure scheme. For the main study, 30 female mice (4 weeks old) were divided into five groups and exposed to *P. duboscqi* females (colony originating from Senegal). At each exposure, 30 female sand flies were allowed to feed on whole mouse body (for exposure schemes see Fig. 1A) with an average of 27 fed sand flies per mouse. The mice were subsequently infected intradermally in the right ear pinna with 10^4 *L. major* promastigotes (MHOM/IL/67/LRC-L137 JERICHO II) together with ¼ gland pair equivalent of *P. duboscqi* salivary gland homogenate in 5 μl saline. Lesions were measured using a digital caliper. Seven weeks after the infection, mice were sacrificed and sampled for blood, infected ears and draining lymph nodes. In total, mice were followed for 37 weeks.

Specific anti-*P. duboscqi* saliva IgGs were measured by immunoblot and ELISA as previously described (Rohousova et al., 2005). Immunoblots were performed on salivary gland homogenate separated by SDS–PAGE on 12.5% gel under non-reducing conditions. An equivalent of 50 gland pairs was loaded. After transfer, the membrane was cut into strips and incubated with mouse sera diluted 1:100 and goat anti-mouse IgG peroxidase-conjugate (heavy chain-specific, Sigma–Aldrich) diluted 1:750. For ELISA tests, wells were coated with *P. duboscqi* salivary gland homogenate (1/40 gland pair equivalent), sera were diluted 1:200 and goat anti-mouse IgG peroxidase-conjugate diluted 1:1000. The same ELISA protocol was used to measure specific anti-*L. major* IgG with two minor modifications: wells were coated with crude *L. major* promastigotes (10^6 cell equivalents per well) and sera were diluted 1:400.

Parasite burdens in the infected ear and draining lymph node were quantified by PCR-ELISA using a protocol described earlier (Kobets et al., 2010). The following primers were used: digoxigenin-labeled F 5′-ATT TTA CAC CAA CCC CCA GTT-3′ and biotin-labeled R 5′-GTG GGG GAG GGG CGT TCT-3′ (VBC-Genomics Biosciences Research, Austria). The cumulative parasite load was calculated as the sum of the parasite loads in both tested tissues.

Lesion size development was analyzed by general linear models (GLM) ANOVA and Scheffe's Multiple Comparison Procedure after data transformation ($\ln_{(x+1)}$). Other data (parasite load and antibody production) were subjected to non-parametric Wilcoxon tests. For correlation tests we used the non-parametric Spearman Rank Correlation Matrix. Statistical analyses were performed using NCSS 6.0.21 software.

Leishmania infection was monitored in BALB/c mice exposed to *P. duboscqi* bites following four different exposure schemes (Fig. 1A). Groups 2 and 15 represented short- and long-term exposures during the sand fly season with subsequent *Leishmania* transmission. Schemes used for groups 2 + 0 and 15 + 0 mimicked short- and long-term exposures followed by a sand fly-free period and *Leishmania* transmission occurring during the subsequent sand fly season.

In accordance with previous reports (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005), *Leishmania* lesion size differed significantly between group 2 and control mice; in immunized mice the lesion size was smaller from week 3 p.i. onwards. Protection against *L. major* infection has previously been reported only for mice immunized by the saliva of *Phlebotomus papatasi* (Belkaid et al., 1998; Kamhawi et al., 2000). Here we report, to our knowledge for the first time, a similar protective effect in mice repeatedly bitten by *P. duboscqi*, the other proven vector of *L. major* (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990). Although *L. major* established infection in immunized mice (group 2), the skin damage remained mild and the cumulative parasite load was significantly reduced by approximately threefold at week 7 p.i. (Fig. 1E). This finding corresponds with data published previously, where the number of parasites in *P. papatasi*-immunized mice was significantly reduced up to 8 weeks p.i. (Belkaid et al., 1998). The present study provides a model for further exploration of the vector-parasite-host immune interactions that influence establishment of *L. major* infection with mild or absent skin damage. Such a controlled amastigote population is expected to act as the sustained source of *L. major* being transmissible to another sand fly.

In some regions, sand fly populations vary greatly during the year, with sand flies occurring during several months of the year (Peters and Killick-Kendrick, 1987). To mimic this situation, mice of group 2 + 0 were exposed to *P. duboscqi* bites twice, similar to group 2, but with a subsequent delay of 15 weeks before the challenge with *L. major*. Compared with group 2, these mice showed significantly larger lesion size (Fig. 1B and D) and greater parasite load (Fig. 1E). All parameters of infection were equal to the control, non-immunized mice (Fig. 1B,D,E), strongly indicating that the protective effect was lost in those mice.

Long-term exposure to sand flies with possible desensitization to salivary antigens is another scenario that is likely to occur in endemic areas and is not reflected in studies published to date. To simulate this situation, the last two groups of mice were immunized 15 times, either directly before infection (group 15) or with a subsequent delay of 15 weeks before the challenge (group 15 + 0). Similar to group 2 + 0, the protective effect against the development of infection measured by lesion size was completely abrogated in both long-term exposure groups (Fig. 1B), with parasite load being comparable with that found in the control group (Fig. 1E). In the group 15 + 0, the parasite load in the infected ear was significantly higher than in the protected group (Fig. 1E). To date, desensitization to blood feeding insect saliva has only been described in mosquitoes (Peng and Simons, 1998). Here we show that a similar phenomenon may also occur in sand fly-infested areas, but the underlying mechanism needs to be elucidated. We can hypothesize that immunization with a large antigen load tends to skew the immune system towards a Th2 response which, in the case of groups 15 + 0 and 15, could be associated with the lack of

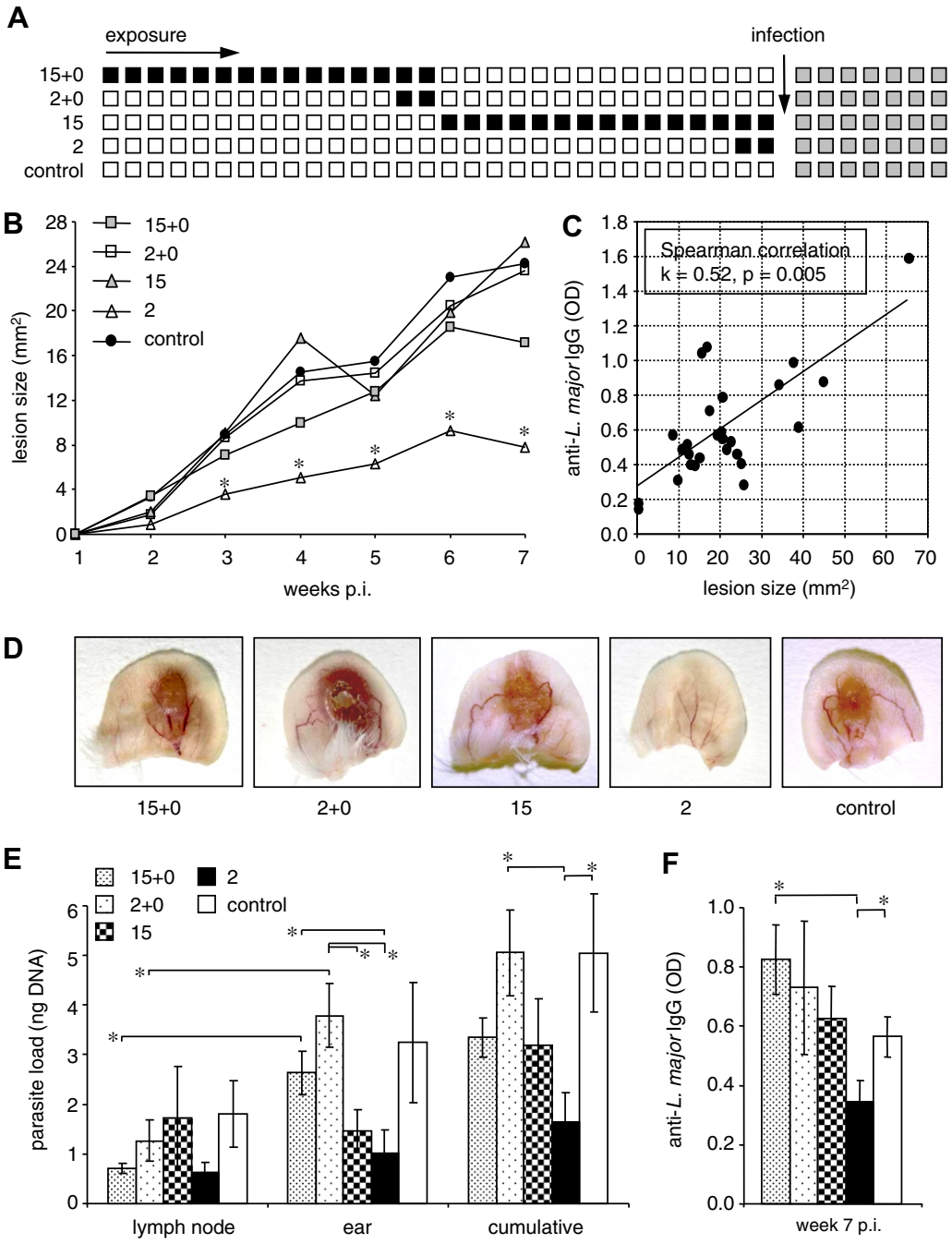

Fig. 1. Development of *Leishmania* infection. Main features of the mouse-based model set up to address the question of whether sand fly abundance and seasonality could result in different outcomes with respect to *Leishmania major* establishment and skin damage. (A) Different exposure schemes of BALB/c mice to sand flies. Each square represents 1 week when mice were exposed (■) or unexposed (□) to *Phlebotomus duboscqi* bites; mice were followed for 7 weeks p.i. with 10^4 *L. major* promastigotes plus half of a *P. duboscqi* salivary gland (■). (B) *Leishmania* lesion size was monitored weekly. Asterisks (∗) indicate the significant difference of group 2 from all other groups ($P < 0.05$). (C) Graph showing positive correlation between *Leishmania* lesion size and anti-*L. major* IgG antibodies measured at week 7 p.i. (D) Representative photographs of mouse ears from each group showing *Leishmania* lesion in infected ears at week 7 p.i. (E) At week 7 p.i., parasite load was measured in the infected ear and draining lymph node (ng of *L. major* DNA per 50 ng of total DNA). Cumulative parasite load is the sum of parasite load in both tissues. Error bars represent mean ± S.E., $^*P < 0.05$. (F) Anti-*L. major* IgG antibodies measured at week 7 p.i. Error bars represent mean ± S.E., $^*P < 0.05$.

protection. Additionally, the severity of skin damage could be due to the deposition of immune complexes in the skin, leading to an exacerbated immune response from the early stage of lesion development. These immune complexes are more likely to form between anti-saliva antibodies and salivary proteins since no binding was observed between anti-*P. duboscqi* saliva antibodies and *L. major* promastigotes (Rohousova et al., unpublished data).

All groups of mice showed elevated anti-*Leishmania* IgG at week 7 p.i. (Fig. 1F) with a positive correlation found between the levels of anti-*L. major* IgG and the lesion size (Fig. 1C). Accordingly, only the protected group (group 2) had significantly lower anti-*L. major* IgG levels compared with non-immunized mice (Fig. 1F), correlating well with the lowest parasite load in this group.

In areas where cutaneous leishmaniasis is endemic, the presence of active *Leishmania* lesions in patients is associated with elevated

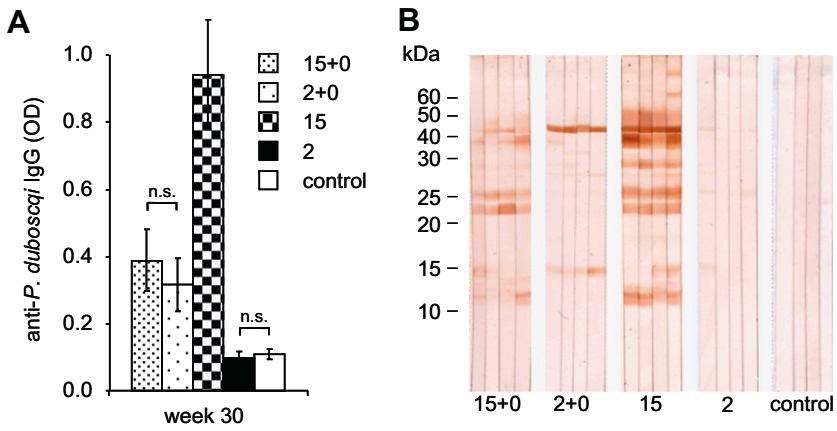

Fig. 2. Antibody response to *Phlebotomus duboscqi* salivary antigens. BALB/c mice were exposed to *P. duboscqi* bites following the scheme shown in Fig. 1A. Anti-*P. duboscqi* saliva IgG antibodies were measured at the end of immunization (week 30) in non-infected mice using ELISA (A) and immunoblot (B). Error bars represent mean ± S.E., n.s. means not significant ($P > 0.05$); the difference is significant ($P < 0.05$) between all other group combinations.

levels of anti-vector saliva IgG (Rohousova et al., 2005; de Moura et al., 2007), suggesting that these antibodies could be used as a risk marker for *Leishmania* transmission. Accordingly, we measured anti-*P. duboscqi* IgG levels and correlated those with the status of *Leishmania* infection. At the end of immunization (week 30, before infection), only groups 15 + 0, 2 + 0 and 15 had elevated levels of anti-*P. duboscqi* saliva IgG, with group 15 having the highest level (Fig. 2A) and strongly recognizing at least seven out of 12 protein bands within the broad range of 12–60 kDa (Fig. 2B). The immunoblot revealed that in group 15 + 0, the level of specific antibodies decreased similarly for all antigens. Mice in group 2 + 0 recognized only one or two antigens, with the protein band of approximately 42–45 kDa being the strongest antigen. Mice from groups 2 and control showed weak or no visible reaction with *P. duboscqi* salivary proteins (Fig. 2B). Within all tested groups, a positive correlation was found between anti-*P. duboscqi* IgG and *Leishmania* lesion parasite load ($k = 0.38$, $P = 0.04$), supporting the above mentioned hypothesis that anti-*P. duboscqi* IgG correlates with skin damage. On the other hand, no correlation was found between anti-*P. duboscqi* IgG and parasite load in the draining lymph node, indicating that pre-exposure to *P. duboscqi* bites could not alter parasite dissemination. Moreover, groups with a delay between pre-exposure and infection (groups 15 + 0 and 2 + 0) had significantly higher parasite loads in the ears than in the draining lymph nodes (Fig. 1E), indicating that in bitten hosts *L. major* preferentially multiplies in the skin tissue. The hypothesis that different immune regulatory mechanisms operate in those tissues is also supported with our previous study showing that parasite numbers in lymph nodes and development of skin lesions are under distinct genetic control (Kurey et al., 2009).

Anti-*P. duboscqi* saliva antibodies were undetectable or absent throughout the study in two groups – group 2 and unexposed control mice (Fig. 2). Taking into account the outcome of infection in these groups, we assume that one of the following applies to mice negative for anti-saliva antibodies: (i) the host is sand fly saliva-naive; therefore saliva in the infective inoculum would exacerbate the development of infection and accelerate skin damage, as in the control group, or (ii) the host has been recently immunized with a low dose of antigen, thus the antigen dose and/or the time-frame did not allow the production of detectable levels of specific anti-saliva antibodies. At the same time, the immune response resulting from this immunization scheme protects the host against leishmaniasis and although *L. major* establishes an infection, the skin damage remains mild (as in group 2). This assumption is in agreement with field results from an endemic area of cutaneous leishmaniasis caused by *Leishmania braziliensis*: low levels of anti-vector saliva IgG were found both in individuals without previous contact with *Leishmania* and in individuals positive for anti-*Leishmania* DTH, but apparently protected from lesion development (de Moura et al., 2007), possibly by short-term or low exposure to sand flies. A different situation has been reported from an endemic area of visceral leishmaniasis where anti-vector saliva antibodies in exposed individuals positively correlated with anti-parasite cell-mediated protective immunity (Gomes et al., 2002).

The concept of using sand fly salivary proteins in anti-*Leishmania* vaccines is based on reports of Belkaid et al. (1998) and Kamhawi et al. (2000), and has been reinforced by several studies testing particular salivary proteins (Morris et al., 2001; Valenzuela et al., 2001; Vinhas et al., 2007; Gomes et al., 2008; Collin et al., 2009). This concept is not rejected by our study, since proteins administered as a vaccine may prime a different immune response from those naturally deposited by sand flies (Plotkin, 2005). Moreover, the targeted host species, humans and dogs, are outbred and therefore more diverse in terms of resistance and susceptibility to leishmaniasis than one strain of inbred laboratory mice.

Our study on BALB/c mice attempts to test the limitations of the sand fly saliva-induced protective effect on *Leishmania* infection and several questions to be addressed were raised. (i) What is the kinetics of anti-saliva antibody and cellular immune responses? (ii) What is the mechanism underlying the loss of protective effect conferred by exposure to salivary antigens? Could antibodies block the protective effect as suggested by in vitro studies (Belkaid et al., 1998; Cavalcante et al., 2003)? (iii) Would saliva-induced protection be lost upon long-term exposure to sand flies even in other host species, e.g. mouse strains resistant to *Leshmania* infection or dogs?

In conclusion we described here, to our knowledge for the first time, limitations of the sand fly saliva-induced protective effect on the development of *Leishmania* infection. In previous studies, hosts protected by immunization with sand fly saliva (either by bite or by injection) were immunized twice at 1- or 2-week intervals and infected immediately thereafter (Belkaid et al., 1998; Kamhawi et al., 2000). Our results might help to explain the persistence of *Leishmania* infection in endemic areas and should be taken into account when designing and testing vaccines based on vector salivary proteins.

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Canine Antibody Response to *Phlebotomus perniciosus* Bites Negatively Correlates with the Risk of *Leishmania infantum* Transmission

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Abstract

Background: Phlebotomine sand flies are blood-sucking insects that can transmit *Leishmania* parasites. Hosts bitten by sand flies develop an immune response against sand fly salivary antigens. Specific anti-saliva IgG indicate the exposure to the vector and may also help to estimate the risk of *Leishmania* spp. transmission. In this study, we examined the canine antibody response against the saliva of *Phlebotomus perniciosus*, the main vector of *Leishmania infantum* in the Mediterranean Basin, and characterized salivary antigens of this sand fly species.

Methodology/Principal Findings: Sera of dogs bitten by *P. perniciosus* under experimental conditions and dogs naturally exposed to sand flies in a *L. infantum* focus were tested by ELISA for the presence of anti-*P. perniciosus* antibodies. Antibody levels positively correlated with the number of blood-fed *P. perniciosus* females. In naturally exposed dogs the increase of specific IgG, IgG1 and IgG2 was observed during sand fly season. Importantly, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* IgG2 compared to *Leishmania*-negative ones. Major *P. perniciosus* antigens were identified by western blot and mass spectrometry as yellow proteins, apyrases and antigen 5-related proteins.

Conclusions: Results suggest that monitoring canine antibody response to sand fly saliva in endemic foci could estimate the risk of *L. infantum* transmission. It may also help to control canine leishmaniasis by evaluating the effectiveness of anti-vector campaigns. Data from the field study where dogs from the Italian focus of *L. infantum* were naturally exposed to *P. perniciosus* bites indicates that the levels of anti-*P. perniciosus* saliva IgG2 negatively correlate with the risk of *Leishmania* transmission. Thus, specific IgG2 response is suggested as a risk marker of *L. infantum* transmission for dogs.

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Introduction

Leishmania infantum (syn. *Leishmania chagasi*) is a protozoan parasite that causes zoonotic leishmaniasis, including the life-threatening visceral form, occurring also in the Mediterranean Basin. Parasites are transmitted by the bite of infected phlebotomine sand flies to dogs, the major host and the main domestic reservoir for human visceral leishmaniasis, or to humans. The clinical forms of canine leishmaniasis range from asymptomatic to lethal (reviewed in [1,2]). Nonetheless, all seropositive infected dogs, including those without any clinical signs, can serve as a source of infection for sand flies in endemic areas [3,4]. The major vector of canine leishmaniases in Mediterranean countries, including Italy, is *Phlebotomus perniciosus* [5,6]. Control programs for human visceral leishmaniasis caused by *L. infantum* are primarily aimed at preventing sand flies from feeding on dogs to reduce *Leishmania* transmission among dogs and humans (reviewed in [1,2]).

Measuring the exposure of dogs to sand fly bites is important for estimating the risk of *L. infantum* transmission. Recently, it was demonstrated that experimental exposure of dogs to *Lutzomyia longipalpis* bites elicits the production of specific anti-saliva IgG which positively correlates with the number of blood-fed sand flies [7]. Therefore, monitoring canine IgG levels specific for sand fly saliva could indicate the intensity of exposure to sand fly bites. Such a monitoring technique would be useful for evaluating the need for, and effectiveness of, anti-vector campaigns [7,8].

Exposure to sand fly bites as well as immunization with sand fly saliva or its compounds elicits in naive hosts protection against *Leishmania* infection under laboratory conditions (reviewed in [9]). It is widely accepted that the protective effect is mediated by $CD4^+$ Th1 cellular response and characterized by increased production of IFN- γ, which activates macrophages to kill *Leishmania* parasites (reviewed in [10]). Recently, it was shown

Author Summary

Leishmania infantum is the causative agent of zoonotic visceral leishmaniasis in the Mediterranean Basin and *Phlebotomus perniciosus* serve as the major vector. In the endemic foci, *Leishmania* parasites are transmitted mostly to dogs, the main reservoir host, and to humans. We studied the canine humoral immune response to *Phlebotomus perniciosus* saliva and its potential use as a marker of sand fly exposure and consequently as a risk marker for *Leishmania* transmission. We also characterized major salivary antigens of *P. perniciosus*. We demonstrated that under laboratory conditions, the levels of anti-*P. perniciosus* saliva antibodies positively correlated with the number of blood-fed sand flies and therefore, may be used to evaluate the need for, and the effectiveness of, anti-vector campaigns. In parallel, we studied sera of dogs naturally exposed to *P. perniciosus* in highly active focus of canine leishmaniasis in Southern Italy. Specific antibodies against *P. perniciosus* saliva were significantly increased according to the ongoing sand fly season. Moreover, the levels of anti-*P. perniciosus* antibodies in naturally bitten dogs negatively correlated with anti-*Leishmania* seropositivity. Thus, for dogs living in endemic areas, specific antibody response against saliva of the vector is an important marker for estimating the risk of *Leishmania* transmission.

that protective effect elicited by inoculation of *Lutzomyia longipalpis* recombinant proteins in dogs was associated with production of IFN-γ by $CD3^+$ $CD4^+$ T cells and by dominance of IgG2 antibodies [11].

In this study we described the anti-saliva IgG response in dogs experimentally exposed to *P. perniciosus* under laboratory conditions and those naturally exposed in an endemic focus of *L. infantum*. We also tested the association between the anti-saliva IgG subclasses and the levels of IFN-γ in *Leishmania infantum*-seropositive and -seronegative dogs. Additionally, we characterized the major *P. perniciosus* salivary antigens recognized by sera of experimentally and naturally bitten dogs.

Methods

Ethical statement

Experiments with dogs exposed to sand fly bites under laboratory conditions. Husbandry of animals in the Animal Center (Germany) complies with the European Commission guidelines for the accommodation of animals used for experimental and other scientific purposes - Commission Recommendation of 18 June 2007 (2007/526/EC). The compliance to aspects of animal welfare law is regularly monitored by the BAH animal welfare commissioner and the state veterinarian. The study design and the experimental procedures were approved by the responsible authorities (LANUV - Regional Authority for Nature, Environment and Consumer protection in North Rhine-Westphalia, Germany).

Experiments with dogs naturally exposed to sand fly bites. All procedures were approved by the Animal Ethics Committee from the Faculty of Veterinary Medicine, University of Bari, Italy and authorized by the Italian Ministry of Health (Authorization number 72/2009C n°69062; 28/11/08). Adverse events were individually registered in accordance to the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and Good Clinical Practice (GCP) Guideline (GL9).

Sand flies and salivary gland dissection

A colony of *Phlebotomus perniciosus* was reared under standard conditions as described in [12]. Salivary glands were dissected from 4–6 day old female sand flies, placed into 20 mM Tris buffer with 150 mM NaCl and stored at $-20^{\circ}C$.

Experimental exposure

Twelve laboratory dogs, beagles, were housed and handled in the Bayer Animal Health GmbH animal facility (Leverkusen, Germany). Dogs were sedated and individually exposed to approximately 200 *P. perniciosus* females as described in [7,13]. Twenty hours after exposure, sand flies were collected and microscopically examined to assess the ratio of blood-fed females. In two independent experiments, two groups of three dogs each were used. Dogs in groups 2 and 4 wore insecticide-impregnated collars that were administrated 8 days before the first sand fly exposure, for a reduction of sand fly bites. In comparison, dogs in groups 1 and 3 remained without any repellent or insecticide application during the whole study. Therefore, dogs in groups 1 and 3 are hereafter defined as high-exposed (HE) and the dogs in groups 2 and 4 as low-exposed (LE). Dogs were exposed to sand fly bites once a week for five consecutive weeks. For the detailed numbers of blood-fed females see Table 1. Blood samples were collected throughout the study according to the following schedule: before the first exposure (week 0, pre-immune serum), during the sand fly sensitization (weeks 1–5), and weekly after the last exposure for 5 weeks (weeks 6–10).

Field study

Twenty nine mixed-breed young dogs (from 90 to 145 days old) and eleven laboratory reared beagles (120 days old) were enrolled in the trial. All animals were housed in a private open-air shelter in Putignano (Bari province, Apulia, Italy), where *P. perniciosus* is the most abundant phlebotomine sand fly species [14]. All dogs were vaccinated against common dog pathogens and dewormed as described in [15]. The canine antibody response against *P. perniciosus* saliva was studied at the beginning (March 2008) and at the end (November 2008) of the sand fly season. In parallel, at four intervals (March, July, November 2008 and March 2009) dogs were tested for *L. infantum* infection status by serological, cytological and molecular methods. All dogs were *L. infantum* negative at the beginning of the trial (March 2008), which was proved by all three diagnostic methods used. *Leishmania*-positive dogs were defined by positive anti-*L. infantum* serology and, in a subset of seropositive dogs (4 out of 18), the infection was confirmed by PCR or cytology. For details on the diagnostic

Table 1. Numbers of blood-fed *Phlebotomus perniciosus* females per dog.

Week	Group 1	Group 2	Group 3	Group 4
1	221±5	49±15	173±8	27±4
2	191±47	125±69	155±18	11±6
3	188±7	61±20	125±6	36±15
4	156±4	39±11	169±12	20±3
5	195±9	83±36	158±11	8±1
average	190±10	71±16	156±6	20±4

(average ± standard error; groups 1, 3 – high-exposed dogs; groups 2, 4 – low-exposed dogs).
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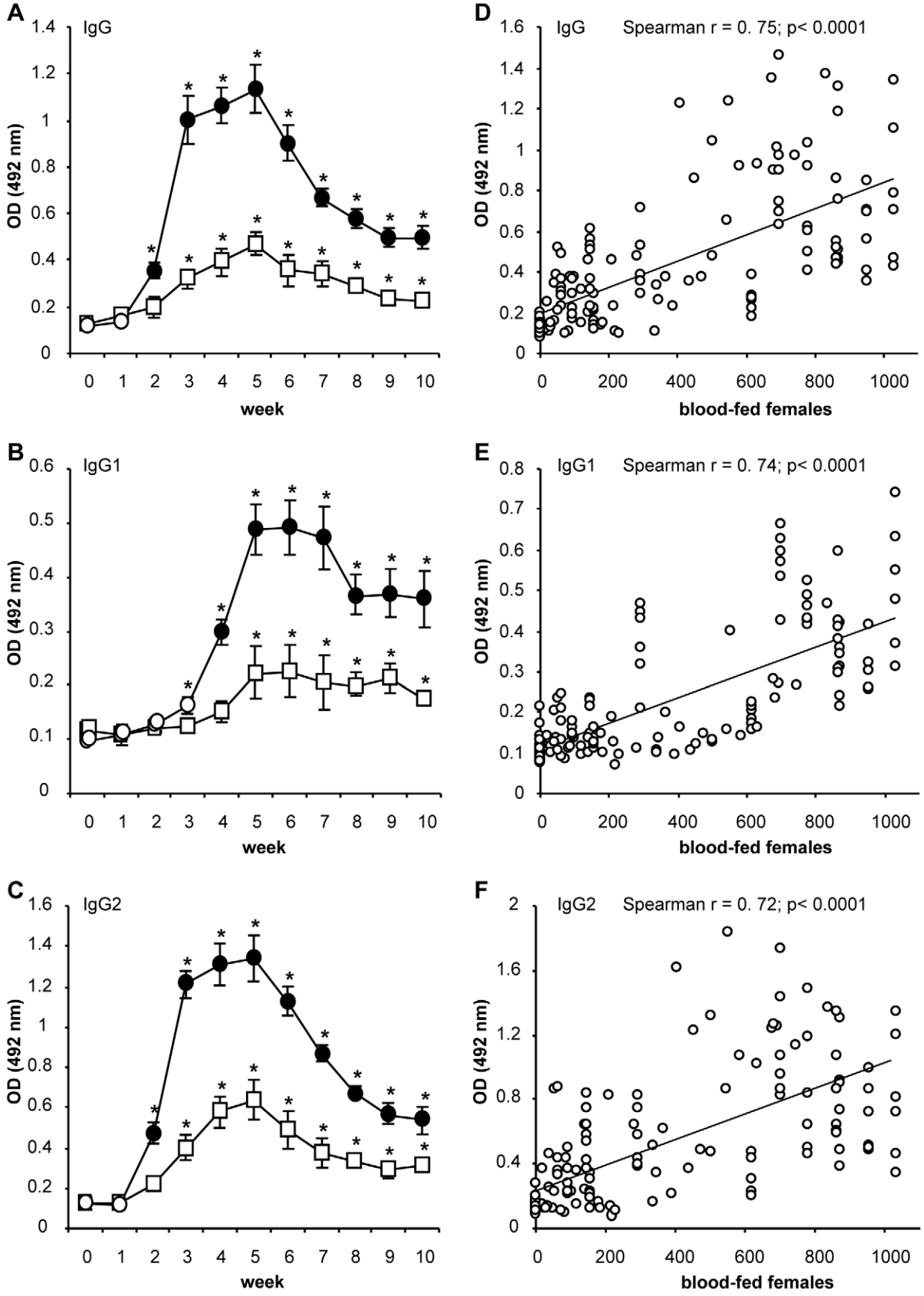
A
IgG
OD (492 nm)
week
B
IgG1
OD (492 nm)
week
C
IgG2
OD (492 nm)
week
D
IgG
Spearman r = 0. 75; p< 0.0001
OD (492 nm)
blood-fed females
E
IgG1
Spearman r = 0. 74; p< 0.0001
OD (492 nm)
blood-fed females
F
IgG2
Spearman r = 0. 72; p< 0.0001
OD (492 nm)
blood-fed females

Figure 1. Anti-sand fly saliva antibody response in dogs experimentally bitten by *Phlebotomus perniciosus*. (A–C) Beagle dogs (3 per group) were divided into low-exposed (square) and high-exposed groups (circles) and were exposed to sand fly bites in weeks 1–5. For detailed numbers of blood-fed females see Table 1. Levels of specific IgG (A); IgG1 (B); and IgG2 (C) were measured by ELISA (at 492 nm) in all canine pre-immune and immune sera. Full circles represent significant difference between high- and low-exposed dogs ($p<0.05$); asterisks indicate significant difference ($p<0.05$) compared to pre-immune sera. Data are presented as the means ± standard errors of the means from two independent studies. (D–F) Correlation between number of blood-fed sand fly females and the levels of canine anti-*P. perniciosus* IgG (D); IgG1 (E); and IgG2 (F) was performed using Spearman Rank Correlation Matrix. OD = optical density.
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methods, see [15,16]. Considering the long incubation period of canine leishmaniasis and the occurrence of sand flies exclusively during the summer season (from June to October) [14], dogs with anti-*Leishmania* seroconversion in March (2009) are presumed to have become infected during the previous season (2008). Dogs that were seronegative for *L. infantum* at all four screening intervals were included in the *Leishmania*-negative group.

Detection of anti – *P. perniciosus* saliva antibodies

Anti-*P. perniciosus* IgG, IgG1 and IgG2 were measured by enzyme-linked immunosorbent assay (ELISA) as described in [7] with some modification. Briefly, microtiter plates were incubated with 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine sera were diluted 1:200 or 1:500 in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, IgG1, or IgG2 from Bethyl laboratories) were diluted and incubated as previously described [7]. Absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). The cut-off value (IgG = 0.145; IgG1 = 0.126; IgG2 = 0.165) was determined as less than two times the standard error of the mean of the absorbance of pre-immune serum.

Western blot analysis

Phlebotomus perniciosus salivary gland homogenate from 5-day-old sand fly females were separated by SDS-PAGE on a 10% gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad). Separated proteins were blotted onto a nitrocellulose (NC) membrane by Semi-Phor equipment (Hoefer Scientific Instruments) and blocked with 5% (w/v) low fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw). Strips of NC membrane were incubated with canine sera diluted 1:50 (experimentally bitten dogs) or 1:25 (naturally bitten dogs) in TBS-Tw for 1 hour. The strips were then washed three times with TBS-Tw and incubated with peroxidase-conjugated sheep anti-dog IgG (Bethyl Laboratories) diluted 1:3000 in TBS-Tw. The chromogenic reaction was developed using a solution containing diaminobenzidine and H_2O_2.

Mass spectrometry

For mass spectrometric analysis, salivary glands from 5-day-old *P. perniciosus* females were homogenized by 3 freeze-thaw cycles. Samples were dissolved in non-reducing sample buffer and electrophoretically separated in 10% polyacrylamide SDS gel. Proteins within the gels were visualized by staining with Coomassie Blue G-250 (Bio-Rad). The individual bands were cut and incubated with 10 mM dithiothreitol (DTT) and then treated with 55 mM iodoacetamid. Washed and dried bands were digested with trypsin (5 ng Promega). The alpha-cyano-4-hydroxycinnamic acid was used as a matrix. Samples were measured using a 4800 Plus MALDI TOF/TOF analyzer (AB SCIEX). Peak list from the MS spectra was generated by 4000 Series Explorer V 3.5.3 (AB SCIEX) without smoothing. Peaks with local signal to noise ratio greater than 5 were picked and searched by local Mascot v. 2.1 (Matrix Science) against a database of putative salivary protein sequences derived from a cDNA library [17]. Database search criteria were as follows – enzyme: trypsin, taxonomy: *Phlebotomus*, fixed modification: carbamidomethylation, variable modification: methionine oxidation, peptide mass tolerance: 80 ppm, one missed cleavage allowed. Only hits that scored as significant ($p<0.05$) are included.

Statistical analysis

The data from experimentally bitten dogs obtained by ELISA were subjected to GLM ANOVA and Scheffe's Multiple Comparison procedure to analyse differences in kinetics of antibody response between HE and LE dogs at all sampling points. The non-parametric Wilcoxon rank sum test for differences in medians was used for comparison of anti-*P. perniciosus* IgG, IgG1, IgG2 and IgG1/IgG2 ratios between *Leishmania*-seropositive and -seronegative dogs. The non-parametric Wilcoxon signed-rank test for differences in medians was used for comparison of antibody increases between March and November blood samples in naturally bitten dogs. For correlation tests we used the non-parametric Spearman rank correlation matrix. For all tests statistical significance was regarded as a p-value less than or equal to 0.05. All statistical analyses were performed using NCSS 6.0.21 software.

Relative risk (the probability of the developing the disease occurring in the group exposed to the risk factor versus a non-exposed group), attributive risk (absolute effect of exposure to the risk factor) and ODDS ratio (odds of an event occurring in the exposed group to the odds of it occurring in non-exposed group) were calculated for dogs from the field study to find out the relationship between the levels of anti-*P. perniciosus* saliva antibodies and leishmaniasis incidence as described in [18]. Low level of specific antibodies (lower than the cut-off value) was determined as the risk factor and the confidence interval for relative risk was calculated as described in [19].

List of the protein accession numbers

Phlebotomus perniciosus: DQ153102; DQ154099; DQ150622; DQ150621; DQ192490; DQ192491; DQ153100; DQ153101; DQ153104; DQ150624; DQ150623; DQ150620; DQ153105.

Lutzomyia longipalpis: AF132518.

Results

Antibody response in experimentally bitten dogs

To investigate the kinetics of antibody response against anti-*P. perniciosus* saliva, two groups of experimentally bitten dogs, low-exposed (LE) and high-exposed (HE), were followed for 10 weeks. Five weekly experimental exposures to *P. perniciosus* bites led to increased levels of anti-saliva specific IgG, IgG1 and IgG2 in both LE and HE groups. No anti-saliva antibodies were detected in any pre-immune dog sera tested.

In HE dogs, anti-*P. perniciosus* antibody levels increased significantly ($p<0.05$) in comparison to the pre-immune sera after the second (IgG; IgG2) and third exposure (IgG1) (Figure 1A–C). Anti-saliva IgG and IgG2 developed with similar kinetics; rapidly increased after the third exposure, and gradual increase until week five (the last exposure), followed by a steady decrease to the end of

the study. Anti-saliva IgG1 increased rapidly between weeks three and five and persisted at elevated levels until the end of the study.

In LE dogs, anti-*P. perniciosus* antibody levels increased significantly ($p<0.05$) in comparison to the pre-immune sera after the fourth (IgG; IgG2) and sixth exposure (IgG1) (Figure 1A–C). Similar to HE dogs, kinetics of anti-*P. perniciosus* IgG and IgG2 in LE dogs was detected at peak levels on week five followed by a rapid decrease. Conversely, IgG1 was measured at peak levels on week six and persisted at elevated quantities to the end of the study (Figure 1A–C).

All HE dogs produced significantly higher levels of anti-*P. perniciosus* IgG ($p = 0.0001$), IgG1 ($p = 0.0032$) and IgG2 ($p = 0.0003$) compared to LE dogs throughout the study (Figure 1A–C). A positive correlation was detected between number of blood-fed female sand flies and the levels of canine anti-*P. perniciosus* IgG ($r = 0.75$, $p<0.0001$), IgG1 ($r = 0.74$, $p<0.0001$) and IgG2 ($r = 0.72$, $p<0.0001$) (Figure 1D–F). Overall, sera of experimentally bitten dogs produced higher concentrations of specific IgG2 compared to specific IgG1 (data not shown).

Antibody response in naturally bitten dogs

To determine the anti-*P. perniciosus* saliva antibody levels and the seasonal changes in specific antibody response, canine sera were screened at the beginning and at the end of the sand fly season, March and November, respectively. Incidence of leishmaniasis in dogs naturally exposed to sand flies was high, 18 out of 40 (45%) were found anti-*L. infantum* seropositive (0/40 in March 2008; 0/40 in July 2008; 5/40 in November 2008; 13/40 in March 2009). In March, higher levels of anti-*P. perniciosus* IgG and IgG2 (compared to cut-off value) were detected in about 55% and 10% of dog sera, respectively, while IgG1 levels were comparable to pre-immune sera (Table 2). In November, elevated levels of specific IgG were found in 87.5%, IgG2 in 72.5% and IgG1 in 45% of the 40 enrolled dogs (Table 2). In both groups of dogs, *Leishmania*-positive and *Leishmania*-negative, specific IgG, IgG1 and IgG2 levels significantly increased during the sand fly season (Figure 2A–C).

Leishmania-positive and *Leishmania*-negative dogs did not statistically differ in IgG and IgG1 production (Figure 2A, B); however, a significant difference was found in IgG2 levels (Figure 2C). Indeed, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* IgG2 at the beginning ($p = 0.047$) and at the end ($p = 0.05$) of sand fly season (Figure 2C). Negative correlation was found between the levels of anti-*P. perniciosus* saliva IgG2 and the risk of *Leishmania* transmission, supported well by epidemiological parameters: relative risk = 2.6 (95% confidence interval: 0.66; 10.63); attributive risk = 1.6; and ODDS ratio = 10. Sera of all naturally bitten dogs showed significantly higher levels of specific IgG2 compared to specific IgG1 (data not shown). Moreover, the IgG1/IgG2 ratio differed between *Leishmania*-positive and -negative dogs; *Leishmania*-positive dogs revealed higher IgG1/IgG2 ratio, although the difference was statistically significant only at the beginning of sand fly season ($p = 0.039$) (Table 2). Furthermore, higher levels of IFN-γ were detected in sera of *Leishmania*-negative dogs throughout the study but with no statistically significant difference (Figure S1).

Identification and characterization of *P. perniciosus* salivary antigens

Phlebotomus perniciosus salivary antigens were studied using sera of naturally and experimentally bitten dogs. Pre-immune sera of experimentally bitten dogs did not recognize any of the salivary proteins by Western blot analysis (Figure 3).

Sera of experimentally exposed dogs produced 11 bands on a salivary gland Western blot with approximate molecular weights of 75, 50, 42, 40, 38, 34, 33, 29, 27, 23 and 14 kDa (Figure 3). The molecular weights of salivary antigens recognized by canine sera were similar in all dogs tested with the exception of the 23 and 27 kDa protein bands (recognized only by some sera). The salivary gland antigens most intensely recognized by the sera of all experimentally bitten dogs had molecular weights of 42, 38, 33 and 29 kDa.

Sera of naturally bitten dogs with both negative and positive anti-*L. infantum* serology reacted with up to 9 protein bands of 50, 42, 38, 34, 33, 29, 27, 23 and 14 kDa. All naturally exposed dogs tested in both groups recognized similar salivary antigens and the most intensive reactions were detected with the 42 and 33 kDa salivary antigens.

Mass spectrometry revealed that the main antigens recognized by sera of bitten dogs were salivary endonuclease (50 kDa - DQ154099), yellow proteins (42 kDa - DQ150622; 40 kDa - DQ150621), apyrases (38 kDa - DQ192490; 38 kDa - DQ192491; 33 kDa - DQ192491), antigen-5 protein (29 kDa - DQ153101), D7 proteins (27 kDa - DQ153104; 23 kDa - DQ150624; 23 kDa - DQ150623, and proteins of the SP-15 like protein family (14 kDa - DQ150620; 14 kDa - DQ153105) (Table 3).

Discussion

Canine antibody response against *P. perniciosus* saliva was studied in dogs bitten by sand flies under well-defined laboratory conditions as well as in dogs from an endemic focus of visceral leishmaniasis in Italy.

In experimentally bitten dogs we observed a significant increase in production of specific IgG, IgG1 and IgG2 in the course of 10 weeks and a positive correlation was found between the levels of specific antibodies and the number of blood-fed females *P. perniciosus*. Anti-saliva specific IgG and IgG2 developed with similar

Table 2. Numbers of dogs positive for anti-*Phlebotomus perniciosus* antibodies in *Leishmania infantum*-seropositive and -seronegative dogs.

	Leishmania negative dogs (n = 22)			Leishmania positive dogs (n = 18)		
	March	November	Increase(%)	March	November	Increase (%)
IgG	14	19	144***	8	15	104**
IgG1	0	11	235***	0	7	220**
IgG2	2	20	249***	2	9	205***
IgG1/IgG2[a]	0.47*	0.54	15	0.57*	0.73	28

([a] – significant difference in IgG1/IgG2 ratio between *Leishmania*-seropositive and -seronegative groups; *** $p<0.001$; ** $p<0.01$; * $p<0.05$).

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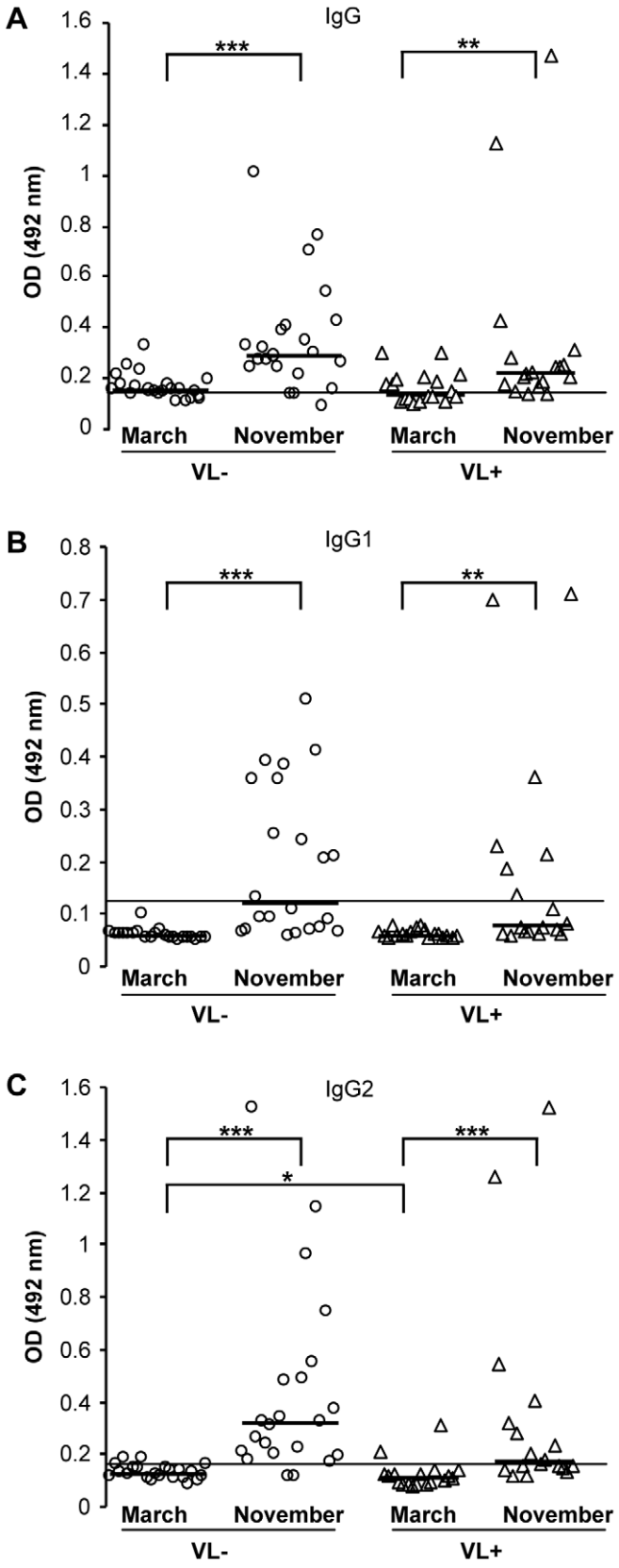

Figure 2. Anti-sand fly saliva antibody response in dogs naturally bitten by *Phlebotomus perniciosus*. Anti-*P. perniciosus* IgG (A); IgG1 (B) and IgG2 (C) response was measured in sera of naturally bitten dogs from endemic area of visceral leishmaniasis. All dogs were *Leishmania infantum* seronegative at the beginning of the trial. ELISA was performed against *P. perniciosus* salivary gland homogenate using canine sera from *Leishmania infantum*-seropositive dogs (open triangle, n = 18) and *Leishmania*-seronegative dogs (open circles, n = 22). Serum samples were taken at the

beginning (March) and at the end of the sand fly season (November). The symbols indicate results of each serum tested, bars represent median values of the groups. Lines represent cut-off values (two times the standard error of the mean of the absorbance of experimentally bitten dog pre-immune sera). Asterisks indicate statistical significance between *Leishmania*-seropositive and -seronegative dogs and significant increase of antibodies during the sand fly season within the group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). OD = optical density.
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kinetics and correspond well with previous results [7] in dogs experimentally bitten by *Lutzomyia longipalpis*. While in sera of healthy dogs, IgG1 and IgG2 usually occur in comparable concentrations [20], IgG2 prevailed in sera of bitten dogs in our study as well as in dogs experimentally bitten by *L. longipalpis* [7,11].

In our field trial, we detected the increase in number of anti-*P. perniciosus* saliva seropositive dogs as well as in the amount of specific antibodies in dog sera as the sand fly season progressed. Statistically significant increases in production of specific IgG, IgG1 and IgG2 were observed in both *Leishmania*-positive and *Leishmania*-negative dogs at the end of sand fly season. Interestingly, *Leishmania*-positive dogs revealed significantly lower anti-*P. perniciosus* saliva IgG2 compared to *Leishmania*-negative dogs and the IgG1/IgG2 ratio was significantly higher in *Leishmania*-positive dogs. These data may suggest either that dogs with low IgG2 levels were at the higher risk of becoming *Leishmania*-infected or that *Leishmania* infection decreases the production of IgG2 in bitten dogs. Considering the IFN-γ levels in canine sera, that were shown to positively correlate with the protective Th1 immune response [11], it seems that the first hypothesis is more feasible. Although, the difference in IFN- γ production between *Leishmania*-negative and *Leishmania*–positive dogs was not statistically significant.

Published data from field studies suggests that humoral immune responses against sand fly saliva vary between hosts with cutaneous and visceral forms of leishmaniases (reviewed in [9,21]). In foci of cutaneous leishmaniases caused by *L. tropica* and *L. braziliensis*, the levels of specific anti-sand fly saliva antibodies in humans positively correlated with the risk of *Leishmania* transmission [22,23]. In contrast, in foci of visceral leishmaniasis caused by *L. infantum*, levels of human anti-sand fly saliva antibodies positively correlated with anti-*Leishmania* DTH (delayed-type hypersensitivity) and thus with protection against potential infection [24,25]. So far, those studies have been performed only in humans. In canids, several studies showed presence of anti-sand fly saliva antibodies in sera from endemic areas in Brazil [8,26,27], however our study is the first describing the association with canine leishmaniasis.

Canine sera recognized more than eleven *P. perniciosus* antigenic bands by Western blot and the most intense reaction was often observed against a 42 kDa band. Mass spectrometry identified the 42 kDa band as a single protein belonging to the Yellow protein

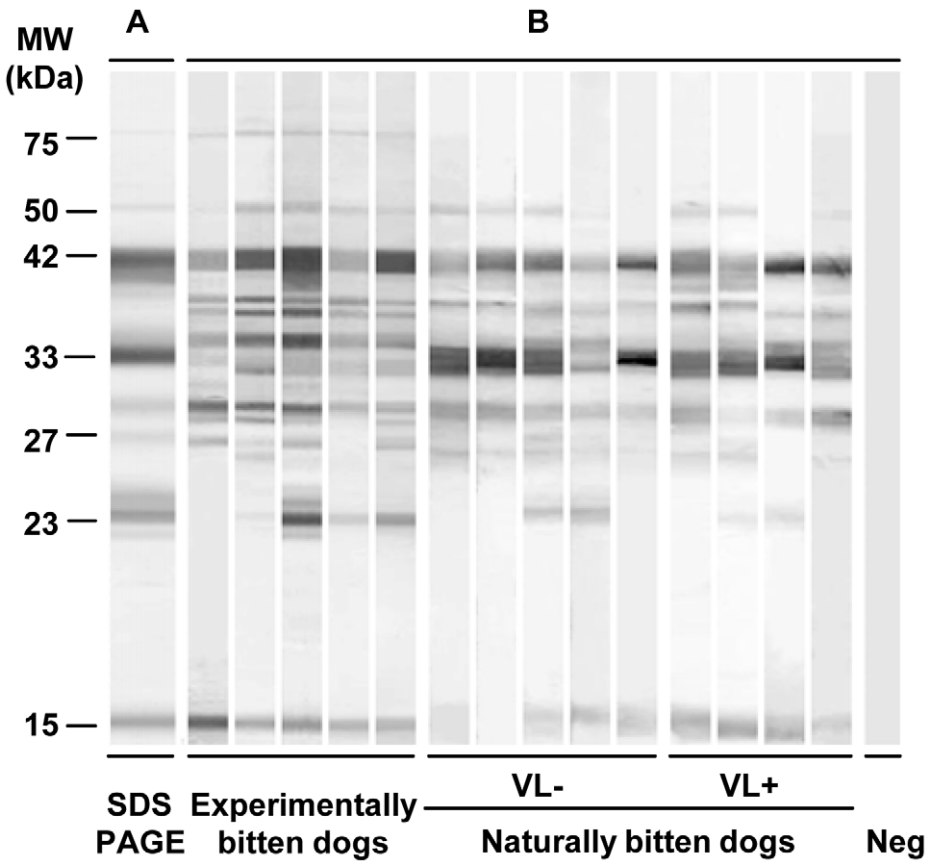

Figure 3. Anti-sand fly saliva antibody response in dogs experimentally and naturally bitten by *Phlebotomus perniciosus*. (A) Total protein profile, Commassie blue-stained SDS-PAGE gel after electrophoresis of *P. perniciosus* salivary gland homogenate. (B) Western blot of *P. perniciosus* salivary proteins recognized by sera of repeatedly bitten dogs. Western blot analysis was performed by sera of experimentally and naturally bitten dogs: *Leishmania infantum*-seronegative (VL−) and *L. infantum*-seropositive (VL+). Pre-immune serum of experimentally bitten dog was used as negative control (Neg).
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Table 3. *Phlebotomus perniciosus* salivary proteins recognized by sera of bitten dogs.

MW (kDa)	NCBI acc. number	Best match to NR protein database		
		Sequence name	E-value	Comments
75	DQ153102	29 kDa salivary protein (PpeSP08)	2.2e-6	unknown
50	DQ154099	41 kDa salivary protein (PpeSP32)	3.5e-9	endonuclease
42	DQ150622	43 kDa yellow-related salivary protein (PpeSP03B)	1.1e-68	yellow protein
40	DQ150621	42 kDa yellow-related salivary protein (PpeSP03)	4.5e-54	yellow protein
38	DQ192490	35.5 kDa salivary protein (PpeSP01)	5.6e-54	apyrase
38	DQ192491	35.3 kDa salivary protein (PpeSP01B)	0.035	apyrase
34	DQ153100	33 kDa salivary protein (PpeSP06)	2.2e-24	unknown
33	DQ192491	35.3 kDa salivary protein (PpeSP01B)	2.8e-72	apyrase
33	DQ153102	29 kDa salivary protein (PpeSP08)	0.0019	unknown
29	DQ153101	30 kDa antigen 5-related salivary protein (PpeSP07)	1.4e-12	Ag 5 protein
27	DQ153104	27 kDa D7-related salivary protein (PpeSP10)	0.0012	D7 protein
23	DQ150624	27 kDa D7-related salivary protein (PpeSP04B)	1.8e-16	D7 protein
23	DQ150623	24.5 kDa D7-related salivary protein (PpeSP04)	0.0069	D7 protein
14	DQ150620	14.8 kDa salivary protein (PpeSP02)	2.2e-13	SP15 like protein
14	DQ153105	13 kDa salivary protein (PpeSP11)	4.5e-15	SP15 like protein

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family (DQ150622). Previously, another Yellow protein of 47.3 kDa (AF132518) was reported as the major antigen recognized by sera of dogs bitten by *L. longipalpis* in the field [26]. The recombinant *L. longipalpis* Yellow proteins (rLJM11 and rLJM17) prepared in mammalian expression system kept their antigenicity and were successfully used to screen dog sera from Brazil [27], predicting similar features for Yellow protein of *P. perniciosus*. All canine sera tested recognized additional three major antigens of the 38, 33 and 29 kDa; the 38 and 33 kDa proteins are apyrases and the 29 kDa antigen represents the antigen 5-related protein family. These four antigens (42, 38, 33 and 29 kDa) are promising candidates as markers of sand fly exposure.

In conclusion, we confirmed that levels of antibodies against sand fly saliva positively correlate with the number of blood-fed sand flies and therefore, monitoring canine antibody response to specific sand fly salivary proteins may evaluate the need for, and effectiveness of, anti-vector campaigns. Moreover, this is the first study demonstrating relationship between the anti-sand fly saliva antibodies and the status of *L. infantum* infection in dogs. The levels of anti-*P. perniciosus* IgG2 in dogs naturally bitten by this sand fly species negatively correlate with the anti-*Leishmania* seropositivity. Thus, for dogs living in endemic area specific IgG2 response against saliva of the vector is suggested as a risk marker of *L. infantum* transmission.

Supporting Information

Figure S1 IFN-γ in the sera of *Leishmania infantum*-seropositive and -seronegative dogs naturally bitten by *Phlebotomus perniciosus* during the sand fly season. Concentrations of IFN-γ were measured by ELISA using the Quantikine canine IFN-γ immunoassay (R&D Systems) following the manufacturer's guidelines. Serum samples, standards and controls were added without any dilutions. Absorbance was measured at 450 nm using a Tecan Infinite M200 microplate reader (Schoeller). Data were transformed and assessed as described in manufacturer's instructions (R&D Systems).
(TIF)

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Author Contributions

Conceived and designed the experiments: PV NM DS DO. Performed the experiments: MV IR EK DS JD. Analyzed the data: MV IR PV. Contributed reagents/materials/analysis tools: PV MV IR NM DO. Wrote the paper: MV PV.

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Kinetics of Antibody Response in BALB/c and C57BL/6 Mice Bitten by *Phlebotomus papatasi*

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PLoS NEGLECTED TROPICAL DISEASES

Kinetics of Antibody Response in BALB/c and C57BL/6 Mice Bitten by *Phlebotomus papatasi*

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Abstract

Background: Phlebotomine sand flies are blood-sucking insects transmitting *Leishmania* parasites. In bitten hosts, sand fly saliva elicits specific immune response and the humoral immunity was shown to reflect the intensity of sand fly exposure. Thus, anti-saliva antibodies were suggested as the potential risk marker of *Leishmania* transmission. In this study, we examined the long-term kinetics and persistence of anti-*Phlebotomus papatasi* saliva antibody response in BALB/c and C57BL/6 mice. We also tested the reactivity of mice sera with *P. papatasi* salivary antigens and with the recombinant proteins.

Methodology/Principal Findings: Sera of BALB/c and C57BL/6 mice experimentally bitten by *Phlebotomus papatasi* were tested by ELISA for the presence of anti-saliva IgE, IgG and its subclasses. We detected a significant increase of specific IgG and IgG1 in both mice strains and IgG2b in BALB/c mice that positively correlated with the number of blood-fed *P. papatasi* females. Using western blot and mass spectrometry we identified the major *P. papatasi* antigens as Yellow-related proteins, D7-related proteins, antigen 5-related proteins and SP-15-like proteins. We therefore tested the reactivity of mice sera with four *P. papatasi* recombinant proteins coding for most of these potential antigens (PpSP44, PpSP42, PpSP30, and PpSP28). Each mouse serum reacted with at least one of the recombinant protein tested, although none of the recombinant proteins were recognized by all sera.

Conclusions: Our data confirmed the concept of using anti-sand fly saliva antibodies as a marker of sand fly exposure in *Phlebotomus papatasi*–mice model. As screening of specific antibodies is limited by the availability of salivary gland homogenate, utilization of recombinant proteins in such studies would be beneficial. Our present work demonstrates the feasibility of this implementation. A combination of recombinant salivary proteins is recommended for evaluation of intensity of sand fly exposure in endemic areas and for estimation of risk of *Leishmania* transmission.

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Introduction

Sand flies (Diptera: Phlebotominae) serve as vectors of leishmaniasis, a neglected disease with symptoms ranging from non-lethal cutaneous to life-threatening visceral form. The causative agents of the disease are protozoan parasites of the genus *Leishmania* which are transmitted to the hosts by the bites of infected sand fly females.

The percentage of infected flies in foci of leishmaniasis fluctuates and humans and animals are more frequently exposed to the bites of uninfected sand flies. Repeated exposure to sand fly saliva elicits anti-saliva antibodies that could be used as a marker of exposure to sand fly bites [1–5]. Moreover, the antibodies are sand fly species-specific. Therefore they can be utilized to differentiate between exposure to vector and non-vector species [1,4,6–9]. In several epidemiological studies, anti-sand fly saliva antibodies were already employed as a reliable tool to monitor exposure to sand fly bites, to evaluate the effectiveness of vector control programs, and in some instances to estimate the risk of *Leishmania* transmission [1,4,5,10–14].

In endemic areas sand fly population fluctuate seasonally [15], which may influence host anti-saliva antibody response. However, very little is known about the kinetics and persistence of anti-saliva antibodies in sera of hosts bitten by blood-feeding insects. Few data on antibody kinetics are available from mice, chicken and guinea pigs experimentally exposed to bites of *Triatoma infestans* [16–18], from humans bitten by mosquitoes [19–22] as well as from humans [4,23] and dogs [3,5,24] bitten by sand flies.

Screening for antibodies is, however, unsuitable for broader use in epidemiological studies until recombinant proteins could be employed instead of the crude salivary gland homogenate, which requires maintenance of sand fly colonies and laboratory

Author Summary

Leishmania major is the causative agent of zoonotic cutaneous leishmaniasis and *Phlebotomus papatasi* serve as the major vector. In endemic foci, rodents are the natural reservoirs of this disease. Thus, we studied anti-*P. papatasi* saliva antibody response in BALB/c and C57BL/6 mice that are commonly used as model organisms sensitive and resistant to cutaneous leishmaniasis, respectively. We followed the kinetics and persistence of specific antibody response in both mice strains and we characterized the main *P. papatasi* salivary antigens. We demonstrated that sand fly bites elicit production of specific IgG that reflect the intensity of sand fly exposure. In endemic areas, this could provide useful information about the effectiveness of anti-vector control programs. We also examined the reaction of mice sera with four *P. papatasi* recombinant proteins. Our data indicate that a combination of these proteins could be used instead of crude salivary gland homogenate for the monitoring of anti-sand fly saliva antibodies in natural hosts in endemic foci.

dissections of insects. So far, only recombinant salivary proteins from *Lutzomyia longipalpis* have been tested for reactivity with sera of naturally bitten humans, dogs, and foxes [8,9].

We studied mice antibody response to *P. papatasi*, the main vector of *Leishmania major*, and compared long-term kinetics and persistence of anti-saliva antibodies in BALB/c and C57BL/6 mice that are widely used as model organisms sensitive or resistant to *L. major* infection, respectively. Furthermore, we characterized and compared main *P. papatasi* salivary antigens recognized by sera of experimentally bitten BALB/c and C57BL/6 mice. The reactivity of mice sera was also tested with the four *P. papatasi* recombinant proteins; two Yellow-related proteins (PpSP44/AF335492 and PpSP42/AF335491) and two D7-related proteins (PpSP30/AF335489 and PpSP28/AF335488).

Methods

Ethical statement

BALB/c and C57BL/6 mice 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. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05; CZU 307/09) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic.

Sand flies and salivary gland dissection

A colony of *Phlebotomus papatasi* (originating from Turkey) was reared under standard conditions as described in [25]. Salivary glands were dissected from 4–6-day-old female sand flies, placed into 20 mM Tris buffer with 150 mM NaCl and stored at -20°C.

Experimental exposure

Twelve mice of BALB/c or C57BL/6 strains (6 weeks old) were divided into experimental and control groups of six mice each. Mice in the experimentally bitten groups were exposed individually to 30 *Phlebotomus papatasi* females (22 ± 0.6 (standard error) blood-fed females per mouse per exposure on BALB/C mice; 26 ± 0.7 (standard error) blood-fed females per mouse per exposure on C57BL/6 mice), once a week in a total of 5 exposures (weeks 1–5). Mice in the control groups remained without any exposure to sand flies. Animals in both groups were anaesthetized (ketamin 150 mg/kg and xylazin 15 mg/kg body weight, intraperitoneally). Blood samples were taken weekly from the tail vein of each mouse one day before exposure to sand flies from week 0 (pre-immune serum) to week 12 and than every other week till the end of the experiment (week 28 for BALB/c mice; week 27 for C57BL/6 mice). In total, mice were followed for 29 and 28 weeks, respectively. Two independent experiments were done for each mice strain.

To test the presence of memory cells, BALB/c mice were additionally exposed to *P. papatasi* bites (21 ± 0.5 (standard error) blood-fed females per mouse) in the week 27.

Preparation of recombinant proteins

Genes coding for *P. papatasi* salivary gland secreted proteins PpSP28 (AF335488), PpSP30 (AF335489), PpSP42 (AF335491) and PpSP44 (AF335492) were amplified from VR2001-TOPO vector [26] by PCR. Two specific restriction sites (*Nde* I and *Bam* HI) were incorporated into the PCR primers: PpSP28Fw (CATATGAAGTACCCTAGGAATGCCGAT), PpSP28Rev (GGATCCGTACGTTCTTGCGGATTGGTCATC), PpSP30Fw (CATATGCGATTTCCTAGGAATGGAGAC), PpSP30Rev (GGATCCGTATTTCCAAGATTCAATATCAAG), PpSP42Fw (CATATGAAAAGAGATGATGTTGGA), PpSP42Rev (GGATCCCCCTTGACACTTTTCTCC), PpSP44Fw (CATATGAAAAGAGACGATGTTGAA), and PpSP44Rev (GGATCCTTTAGGTTTTCTCACTTC). Afterwards, PCR products were ligated into *E. coli* pGEM-T Easy Vector (Promega) using TA cloning and the ligation products were transformed into *E. coli* competent cells TOP10 (Invitrogen). Vectors were replicated in bacteria and after that, genes restricted by *Nde* I and *Bam* HI enzymes and restricted *E. coli* pET-42 Expression Vectors (Novagen) were ligated. Ligation products were transformed into *E. coli* competent cells TOP10 (Invitrogen) again. Plasmids were isolated from the bacteria, and transformed into *E. coli* BL21 (DE3) gold (Agilent) for expression. *E. coli* lysates were prepared under denaturing conditions and His-tagged proteins were purified by FLPC on a Ni-NTA Superflow column with The QUIaxpressionist kit (Quiagen) according to manufacturers manual.

Detection of anti-*P. papatasi* saliva antibodies

Anti-*P. papatasi* saliva IgG antibodies and IgG subclasses were measured in sera of BALB/c and C57BL/6 mice using indirect ELISA. Microtiter plate wells were coated with *P. papatasi* salivary gland homogenate (SGH) made by three freeze-thaw cycles (about 60 ng of protein per well). To block free binding sites, washed wells were incubated with 6% low fat dry milk diluted in 20 mM phosphate-buffered saline with 0.05% Tween 20. Mice sera were diluted 1:200 in 2% low fat dry milk and incubated for 90 min at 37°C for specific IgG or overnight at 4°C for IgG subclasses. Secondary antibodies (goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3; Serotec) conjugated with horseradish peroxidase (HRP) were diluted and incubated at 37°C as described in Table S1. Orthophenylendiamine and H_2O_2 in McIlwein phosphate-citrate buffer (pH 5.5) were used as substrate solution. Absorbance was measured at 492 nm using an Infinite M200 microplate reader (Tecan). The cut-off value was determined as two standard errors of the mean of the absorbance of pre-immune serum. The

intensity of booster effect was measured by increased levels of specific antibodies in sera of bitten mice after the last sand fly exposure (comparing week 24 and 28).

Anti-*P. papatasi* IgE were measured in sera of BALB/c mice as described above with the following modifications. Microtiter plate wells were coated with *P. papatasi* SGH (about 300 ng of protein per well). To block the free binding sites, washed wells were incubated with 6% fetal calf serum. Mouse sera were diluted 1:100 in 2% fetal calf serum. Secondary antibody (rat anti-mouse IgE; BD PharMingen) was diluted and incubated as listed in Table S1.

Western blot analysis

Phlebotomus papatasi SGH (about 10 µg of protein per well) was separated on 10% SDS-PAGE gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad). Salivary proteins were blotted onto a nitrocellulose membrane by Semi-Phor equipment (Hoefer Scientific Instruments) and cut into strips. The strips were then blocked with 5% low fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw) and subsequently incubated with mice sera (week 28 for BALB/c mice; week 5 for C57BL/6 mice) diluted 1:200 for 1 hour. In the next step the strips were incubated for 1 hour with peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2b (Serotec) diluted in TBS-Tw as follows: IgG and IgG1 1:5000; IgG2b 1:2000 for BALB/c mice sera and IgG, IgG1 1:2000 for C57BL/6 mice sera. The chromogenic reaction was developed using a solution containing diaminobenzidine and H_2O_2.

Similar protocol was used for western blot analysis with *P. papatasi* recombinant proteins PpSP28, PpSP30, PpSP42, and PpSP44. Briefly, recombinant proteins were loaded on the 10% SDS-PAGE gel (3 µg protein per well) and separated under reducing conditions. BALB/c mice sera (week 28) were diluted 1:50 and secondary antibody (goat anti-mouse IgG from Serotec) was diluted 1:1000 in TBS-Tw.

Mass spectrometry

The proteins from the *P. papatasi* salivary glands used for mass spectrometric analysis were run on the same gel as salivary glands used for western blot analysis. Proteins were visualized by Coomassie Blue G-250 staining (Bio-Rad). The individual bands were cut and incubated with 10 mM dithiothreitol (DTT) and then treated with 55 mM iodoacetamid. Washed and dried bands were digested with trypsin (5 ng, Promega). Alpha-cyano-4-hydroxycinnamic acid was used as a matrix. Samples were measured using a 4800 Plus MALDI TOF/TOF analyzer (AB SCIEX). A peak list from MS spectra was generated by 4000 Series Explorer V 3.5.3 (AB SCIEX) without smoothing. Peaks with local signal to noise ratio greater than 5 were picked and searched by local Mascot v. 2.1 (Matrix Science) against a database of putative salivary protein sequences derived from GenBank. Database search criteria were as follows – enzyme: trypsin, taxonomy: *Phlebotomus*, fixed modification: carbamidomethylation, variable modification: methionine oxidation, peptide mass tolerance: 80 ppm, one missed cleavage allowed. Only hits that scored as significant ($p<0.05$) are included.

Statistical analysis

The data obtained by ELISA were subjected to GLM ANOVA and Tukey-Kramer Multiple Comparison procedure to analyze differences in kinetics of anti-*P. papatasi* saliva antibody response between experimentally bitten and control mice at all sampling points. The non-parametric Wilcoxon rank sum test for differences in medians was used for evaluation of booster effect, the comparison of antibody level between week 24 and 28. For correlation tests we used the non-parametric Spearman rank correlation matrix. For all tests statistical significance was regarded as a p-value less than 0.05. All statistical analyses were performed using NCSS 6.0.21 software.

Results

Kinetics of anti-*P. papatasi* saliva antibody response in BALB/c mice

To investigate the kinetics and persistence of anti-*P. papatasi* saliva antibody response, experimentally bitten and control mice were followed for 29 weeks. Mice exposed five times to bites of sand flies at one-week interval had significantly increased levels of specific IgG, IgG1, and IgG2b as compared to control group (Figure 1A, C, E). In contrast, specific IgG2a, IgG3, and IgE levels in sera of bitten mice were comparable to non-exposed controls with some differences only at the last data points (Figure S1). No anti-saliva antibodies were detected in any pre-immune sera tested.

In bitten mice, anti-*P. papatasi* saliva IgG and IgG1 levels increased significantly ($p<0.05$) after the fourth exposure (Figure 1A, C). IgG2b levels differed between experimental and control group from week 9 onward, with the exception of weeks 10 and 11 (Figure 1E). Anti-saliva IgG increased steadily till the end of the study, while specific IgG2b increased slowly until week 22 followed by a slight decrease at week 24. Anti-saliva IgG1 increased steadily and peaked at week 7 and persisted on this level until the end of the study.

To test the presence of putative memory cells to *P. papatasi* salivary proteins, BALB/c mice were additionally exposed to sand flies 22 weeks after the last exposure (week 27). One week after the booster (at week 28) anti-*P. papatasi* saliva antibodies increased significantly in IgG by 43%, in IgG1 by 80% and in IgG2b by 79% (Figure 1A, C, E).

Positive correlation was found between the number of blood-fed sand fly females during the individual immunization weeks (sum of the blood-fed females from the relevant week and the weeks before) and the corresponding levels of anti-*P. papatasi* IgG ($r = 0.62$, $p<0.0001$), IgG1 ($r = 0.74$, $p<0.0001$), and IgG2b ($r = 0.29$, $p<0.05$) (Figure 2A, C, E). Furthermore, positive correlation was detected between the total amount of blood-fed females and the levels of specific IgG ($r = 0.72$, $p<0.0001$) and IgG1 ($r = 0.8$, $p<0.0001$) after the fifth sand fly exposure (week 5).

Kinetics of anti-*P. papatasi* saliva antibody response in C57BL/6 mice

Experimentally bitten and control mice of C57BL/6 strain were followed in experiments lasting 28 weeks. Five exposures at one-week interval significantly increased levels of specific IgG and IgG1 in bitten mice (Figure 1B, D). In contrast, specific IgG2b, IgG2c, and IgG3 levels of bitten mice were comparable to controls. No anti-saliva antibodies were detected in any pre-immune sera tested.

Similarly to BALB/c mice, anti-*P. papatasi* IgG and IgG1 levels differed significantly between experimentally bitten and control C57BL/6 mice from week 4 onward (Figure 1B, D). Anti-saliva IgG gradually increased until week 8 and then with a slight fluctuation of antibody levels decreased until the end of the study. Specific IgG1 developed with similar kinetics to IgG, however, it peaked earlier (at week 6) and then slowly decreased till the end of

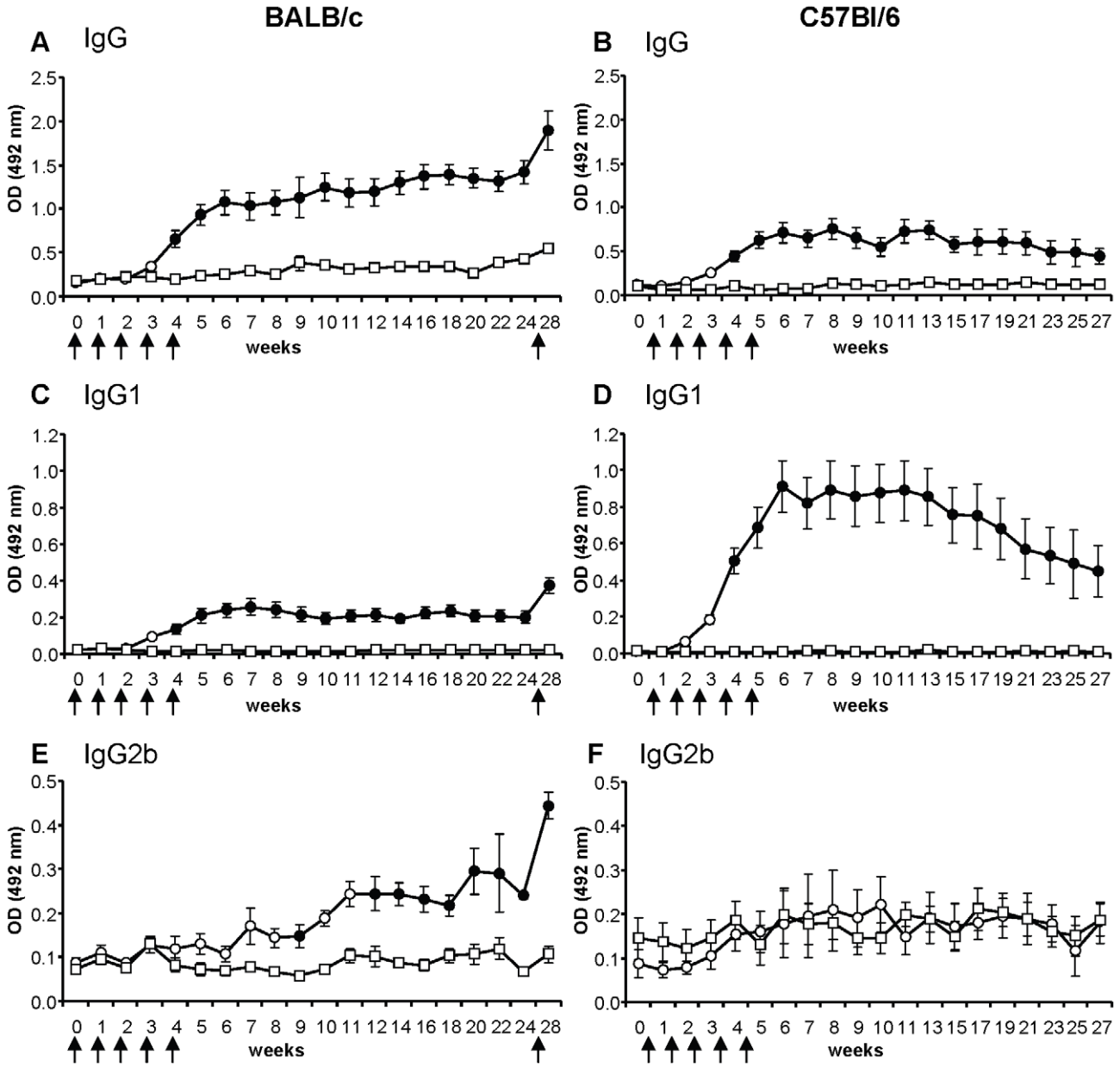

Figure 1. Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice bitten by *Phlebotomus papatasi*. BALB/c mice (A, C, E) and C57BL/6 mice (B, D, F) were divided into control (squares) and experimentally bitten groups (circles). Mice in the latter group were exposed to sand fly bites (arrows) in weeks 1–5 and additionally in the week 27 (only BALB/c mice). Levels of specific IgG (A, B); IgG1 (C, D); and IgG2b (E, F) were measured by ELISA at all sampling points. Full circles represent significant difference between control and bitten mice ($p<0.05$). Data are presented as the means ± standard errors of the means. Two independent studies were done. OD = optical density.
doi:10.1371/journal.pntd.0001719.g001

the study. Anti-saliva IgG2b, IgG2c, and IgG3 antibodies did not differ between the exposed and control group throughout the study (Figure 1F; Figure S1B, D) with the exception of week 21 for IgG3 subclass (Figure S1D).

We also detected a positive correlation between the number of blood-fed sand fly females during the individual immunization weeks (sum of the blood-fed females from the relevant week and the weeks before) and the corresponding levels of anti-*P. papatasi* IgG ($r = 0.80$, $p<0.0001$) and IgG1 ($r = 0.86$, $p<0.0001$) (Figure 2B, D). Moreover, positive correlation was detected between the total amount of blood-fed females and the levels of specific IgG ($r = 0.85$, $p<0.0001$), IgG1 ($r = 0.86$, $p<0.0001$), and IgG2c ($r = 0.5$, $p<0.05$) after the fifth sand fly exposure (week 5).

Identification and characterization of *P. papatasi* salivary antigens

Phlebotomus papatasi salivary antigens were studied using sera of experimentally bitten BALB/c and C57BL/6 mice. Only the antibody classes and subclasses shown to be produced in high titers by ELISA were tested in a western blot; specific anti-*P. papatasi* IgG and IgG1 in both mice strains and additionally specific IgG2b in BALB/c mice.

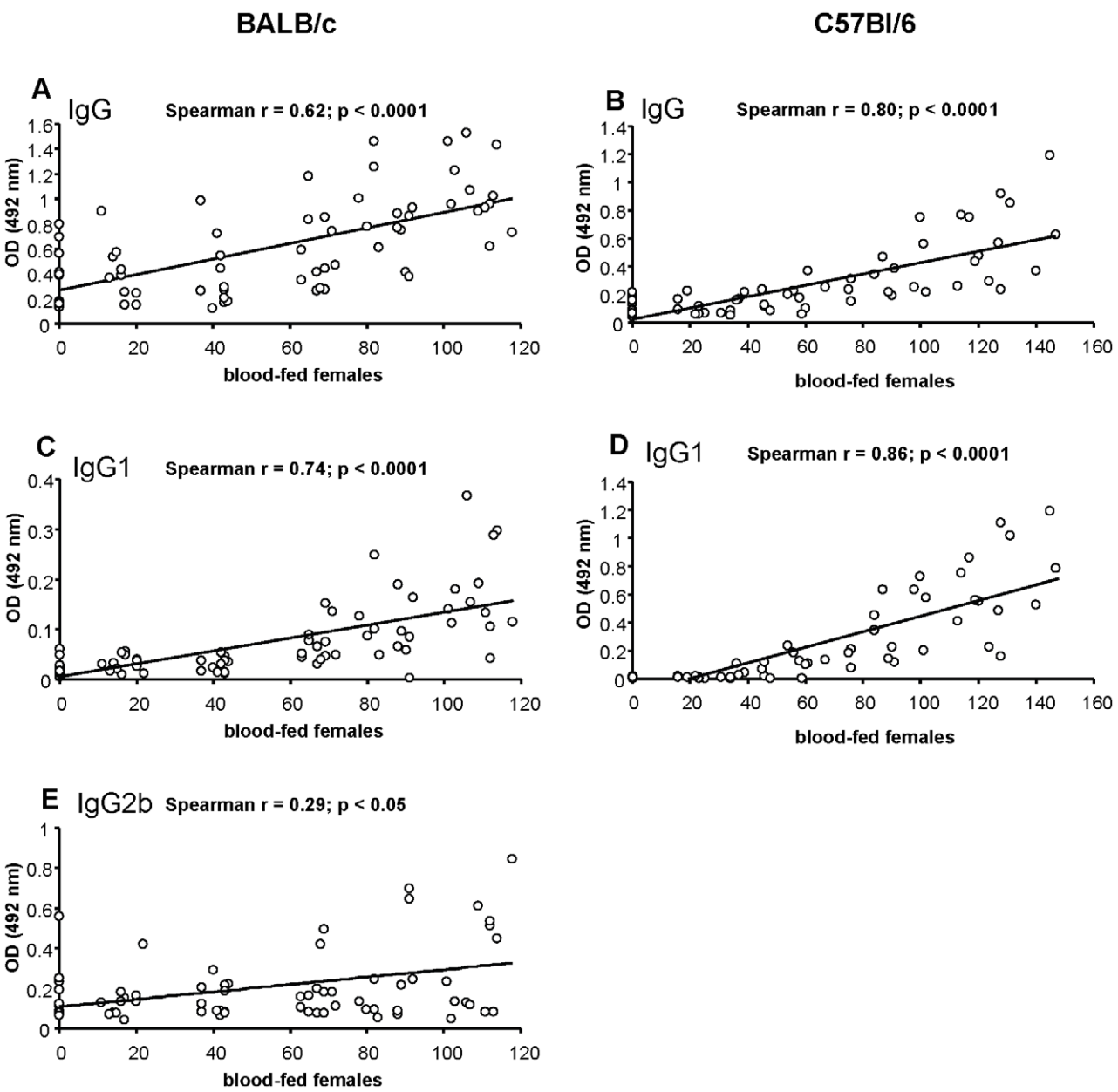

Figure 2. Correlation between the intensity of sand fly exposure and the anti-*Phlebotomus papatasi* saliva antibodies. The correlation between the number of blood-fed sand fly females and the levels of anti-saliva antibodies in experimentally bitten BALB/c (A, C, E) and C57BL/6 (B, D) mice was performed using Spearman Rank Correlation Matrix. Positive correlation was detected in specific IgG (A); IgG1 (C); and IgG2b (E) in BALB/c mice and in specific IgG (B); and IgG1 (D) in C57BL/6 mice. OD = optical density.
doi:10.1371/journal.pntd.0001719.g002

BALB/c mice sera recognized up to 10 protein bands with approximate molecular weights of 70, 65, 51, 49, 47, 35, 31, 30, 23, and 15 kDa, the last three being the most intensively recognized by all BALB/c sera in all IgG subclasses tested. Sera of C57BL/6 mice reacted additionally with the 53 kDa protein but did not recognize the 49 and 47 kDa protein bands. The most intensive reaction in all C57BL/6 mice was detected with the 65, 53, and 30 kDa protein bands in IgG as well as in IgG1 (Figure 3). Comparison of two mice strains therefore revealed an interesting difference in recognition of four protein bands of 53, 51, 49, and 47 kDa. No reaction was detected with any pre-immune mice sera tested (Figure 3).

In BALB/c mice, the 51 kDa protein was recognized only by one out of 5 sera tested in IgG and IgG1, while in C57BL/6 mice, this protein band was recognized by all mice sera tested in IgG1 and by two out of five sera tested in IgG. Anti-*P. papatasi* IgG2b antibodies reacted consistently with the 65, 35, 31, 30, 23, and 15 kDa proteins (Figure 3).

In C57BL/6 mice, 70, 65, 53, 31, and 30 kDa proteins were recognized by all mice sera tested (IgG as well as IgG1), while the 51, 35, 23, and 15 kDa antigens were recognized by some sera only (Figure 3). Specific IgG1 of C57BL/6 mice predominantly recognized the 65, 53, 51, 31, and 30 kDa antigens (Figure 3).

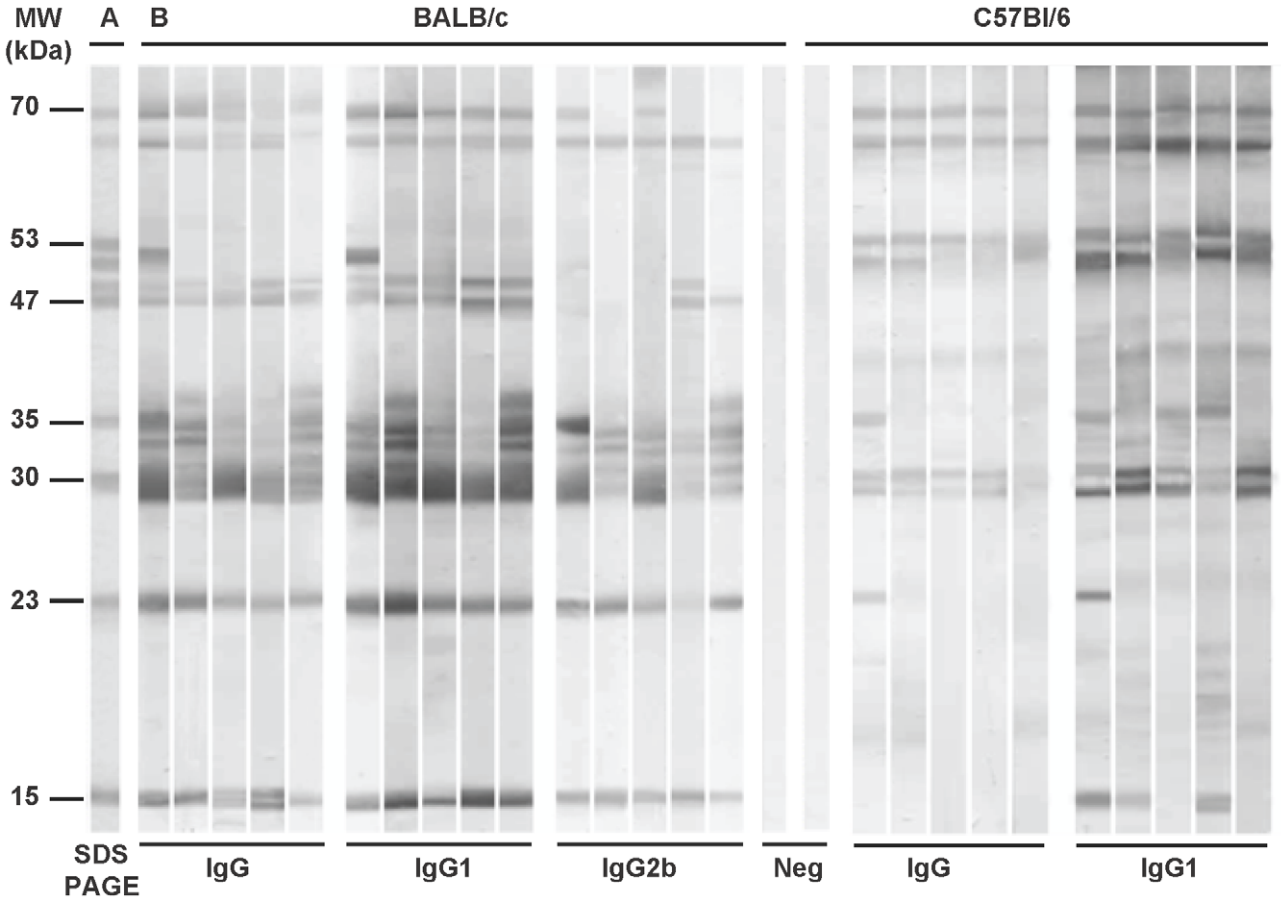

Figure 3. Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice experimentally bitten by *Phlebotomus papatasi*. (A) Total protein profile, Coomassie blue-stained SDS-PAGE gel with *P. papatasi* salivary gland homogenate. (B) Western blot of *P. papatasi* salivary proteins recognized by IgG, IgG1, or IgG2b from sera of *P. papatasi*-bitten BALB/c (week 28) and C57BL/6 mice (week 5). Pre-immune sera of BALB/c and C57BL/6 mice were used as negative controls (Neg).
doi:10.1371/journal.pntd.0001719.g003

Mass spectrometry analysis identified the salivary proteins with the same mobility in the SDS-PAGE as the proteins recognized by the sera of experimentally bitten mice as the Yellow-related proteins (GenBank acc. no. AF335492 and AF335491), apyrase (AF261768), D7-related proteins (AF335489; AF335488), antigen-5 protein (DQ205724), and proteins of the SP15 protein family (AY628879, AY628880; AF335486; AF335485) (Table 1).

Reactivity of anti-saliva antibodies with the *P. papatasi* recombinant proteins

The reactivity of PpSP44 (yellow related protein), PpSP42 (yellow related protein), PpSP30 (D7 related protein), and PpSP28 (D7 related protein) recombinant proteins was studied using sera from BALB/c mice exposed to *P. papatasi* bites and positive for anti-*P. papatasi* IgG antibodies. Sera of control mice did not recognize any of the recombinant proteins tested. The most intensive reaction was detected with the PpSP30, although, this protein was not recognized by all sera tested (4 out of 5). Three out of five mice sera reacted with the PpSP42 and PpSP44 recombinant proteins and very weak reaction was detected with the PpSP28 recombinant protein in two out of five mice sera (Figure 4).

Discussion

This study describes in detail long-term kinetics and persistence of anti-*P. papatasi* saliva antibodies in sand fly-exposed BALB/c and C57BL/6 mice strains that are widely used as model organisms sensitive or resistant to *Leishmania* infection, respectively (e.g. [27,28]).

Four IgG subtypes have been described in mice: IgG1, IgG2a, IgG2b, and IgG3. Additionally, certain strains such as C57BL/6 produce the IgG2c subclass instead of IgG2a [29]. The nomenclature of murine IgG subtypes does not correlate with the subtypes of human or canine IgG. The most abundant subclass is IgG1; it binds to Fc-receptors of mast cells and basophils, and it mediates the immediate hypersensitivity reactions. Both IgG1 and IgG2a activate the complement cascade via the alternative pathway, whereas IgG2b employs the classical pathway of complement activation [30]. Moreover, production of IgG1 is the marker of Th2 profile of immune response in mice, while IgG2a predicts Th1 type of immune response in these animals [31].

We showed that repeated exposure to sand fly bites elicits increased levels of anti-saliva IgG and IgG1 in both BALB/c and C57BL/6 strains, and additionally IgG2b in BALB/c mice. In comparison, higher levels of specific IgG were detected in BALB/c mice. This finding complies well with the fact that BALB/c mice mostly respond to repeating antigens by Th2 humoral immune response while C57BL/6 mice produce mainly Th1 cellular response [30]. It seems that *P. papatasi* saliva elicits mainly production of specific IgG1 subclass, which suggests the polariza-

Table 1. *Phlebotomus papatasi* salivary proteins recognized by sera of bitten mice.

MW	NCBI acc.number	Best match to NR protein database		
(kDa)		Sequence name	E-value	Protein family
70	N.D.	N.D.	N.D.	N.D.
65	N.D.	N.D.	N.D.	N.D.
53	N.D.	N.D.	N.D.	N.D.
51	AF335492	44 kDa yellow-related salivary protein (PpSP44)	0.00E+00	yellow-related
49	N.D.	N.D.	N.D.	N.D.
47	AF335491	42 kDa yellow-related salivary protein (PpSP42)	0.00E+00	yellow-related
35	AF261768	salivary apyrase (PpSP36)	0.00E+00	apyrase
31	AF335490	32 kDa salivary protein (PpSP32)	1.10E−05	PpSP32-like
30	AF335489	30 kDa D7-related salivary protein (PpSP30)	4.50E−12	D7-related
30	DQ205724	29 kDa antigen 5-related salivary protein	7.10E−05	antigen 5-related
23	AF335488	28 kDa D7-related salivary protein (PpSP28)	0.00E+00	D7-related
15	AY628879	SP-15 protein	1.80E−05	PpSP15-like
15	AY628880	SP-15 protein	3.50E−07	PpSP15-like
15	AF335486	14 kDa salivary protein (PpSP14)	0.00E+00	PpSP15-like
15	AF335485	12 kDa salivary protein (PpSP12)	0.00E+00	PpSP15-like

N.D. – not determined.

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tion to the Th2 type of immune response in bitten mice regardless of the strain. The production of anti-sand fly saliva IgG1 was previously described in BALB/c mice repeatedly bitten by *Lutzomyia longipalpis*, but they did not observe any production of neither IgG2a nor IgG2b [32]. As the composition of sand fly saliva varies in different sand fly species [33] and the sand fly saliva compounds elicit different profile of specific antibody response [34], this could be the feasible explanation for the production of different antibody subclasses in mice bitten by different sand fly species. To our knowledge, there are no data available about the anti-sand fly saliva antibody subclasses elicited by sand fly feeding in the C57BL/6 mice. In Swiss Webster mice immunization by *P. ariasi* saliva produced also predominantly IgG1 antibodies [34]. Production of specific IgG2b in BALB/c mice compared to the

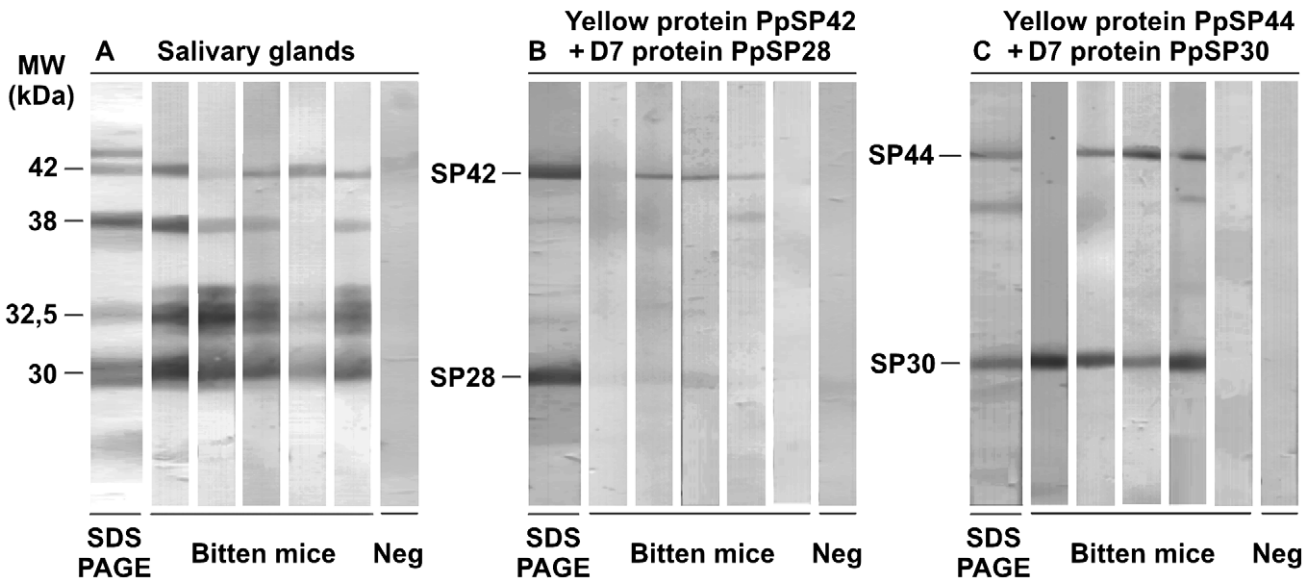

Figure 4. Reactivity of anti-*Phlebotomus papatasi* saliva IgG with *P. papatasi* recombinant proteins. Sera from BALB/c mice experimentally bitten by *P. papatasi* (Bitten mice) that were positive for anti-*P. papatasi* IgG (OD>cut-off: 0.19) were tested by Western blot analysis against (A) *P. papatasi* salivary gland homogenate, (B) a combination of bacterially-expressed PpSP42 and PpSP28 (Yellow-related protein AF335491 and D7-related protein AF335488, respectively), and (C) a combination of bacterially-expressed PpSP44 and PpSP30 (Yellow-related protein AF335492 and D7-related protein AF335489, respectively). The SDS-PAGE protein profiles of the *P. papatasi* salivary gland homogenate as well as the recombinant proteins were blotted and stained by Amido Black. Pre-immune sera of BALB/c mice were used as the controls (Neg).

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absence of this antibody subclass in C57BL/6 mice may be the result of different cytokine responses in both mice strains against sand fly saliva. The switch to IgG2b subclass is initialized by production of TGF-β [35], a suppressive cytokine that blocks the activation of lymphocytes and monocytes derived phagocytes. This could positively contribute to the susceptibility of BALB/c mice to *Leishmania* parasites.

Importantly, positive correlation was found in both mice strains between the intensity of sand fly exposure and the levels of specific antibodies in aforementioned subclasses. Our results correspond well to previously published data showing that the antibody response in dogs [3,5] as well as in humans [4] reflected the intensity and the time-course of sand fly exposure.

We found that sand fly exposure did not affect the production of IgG2a and IgG3 in BALB/c mice, and IgG2b, IgG2c, and IgG3 in C57BL/6 mice. Neither did the levels of specific IgE differ significantly between non-exposed and exposed groups of mice, and the IgE kinetics showed high variation during the study. Similarly, high fluctuation in specific IgE response was detected in humans [11,23] and dogs [3] bitten by *Lutzomyia longipalpis* in the field as well as under laboratory conditions. While some of the individuals and animals presented high levels of specific IgE, others did not mount specific IgE response at all [3,11,23].

To mimic the situation commonly occurring in endemic foci of leishmaniases, where sand fly-free periods last up to 6 months [15], BALB/c mice were exposed to *P. papatasi* bites again 23 weeks after the last sand fly exposure. This single sand fly exposure elicited statistically significant increase of anti-*P. papatasi* IgG, IgG1, IgG2b which suggests the persistence of memory cells generated during the previous round of exposures. This could be related to the ''previous sand fly season'' in the field. Furthermore, in both mice strains, the differences between non-exposed and exposed groups of mice in production of specific IgG1 and IgG2b were detectable from week four or nine, respectively, until the end of the study. Similarly, the levels of specific IgG, IgG1, and IgG2 in sera of dogs exposed to *L. longipalpis* or *P. perniciosus* bites differed significantly from pre-immune sera for more than 14 weeks after the last sand fly exposure [3,5]. In individuals repeatedly bitten by *P. argentipes*, elevated levels of specific antibodies persisted after the 30-day sand fly-free period, although anti-saliva antibodies significantly decreased throughout this time [4]. Thus, regardless the host-sand fly combination, anti-sand fly saliva antibodies can persist in sera of repeatedly bitten hosts until the next sand fly season.

We also characterized the reactivity of mice sera with *P. papatasi* salivary proteins as well as with selected recombinant proteins. Mice sera of BALB/c and C57BL/6 strains reacted with up to eleven *P. papatasi* antigenic protein bands. The 30 kDa protein band recognized by both mice strains was identified by mass spectrometry as a mixture of a D7-related (AF335489) and an antigen 5-related (DQ205724) protein. The other proteins which were intensively recognized either by BALB/c (47, 23, and 15 kDa proteins) or by C57BL/6 mice (65, 53, and 51 kDa proteins) were determined as members of the Yellow-related protein family (51 kDa - AF335492, 47 kDa - AF335491), D7-related protein family (23 kDa – AF335488), and SP-15 protein family (15 kDa – AY628879, AY628880, AF335486, AF335485). The 70, 65, 53, and 49 kDa bands were not identified by mass spectrometry. Our results correspond to previously published data, where the human and BALB/c mice IgG antibodies recognized preferentially the *P. papatasi* 30 kDa protein band [1,14].

To our knowledge, the only study describing the reactivity of specific IgG subclasses with *P. papatasi* antigens was performed on humans [14]. In accordance with our results, the 30 kDa D7-related protein was also found to be the most immunogenic antigen in all human antibody subclasses tested [14]. Taken together, our data complies well with previously published studies, where Yellow-related proteins, D7-related proteins, as well as SP-15 proteins from *P. papatasi* saliva were identified as potent antigens for mice and humans [1,14].

Sera of BALB/c mice experimentally bitten by *P. papatasi* were tested also with four bacterially expressed recombinant proteins belonging to two salivary protein families: Yellow-related proteins (PpSP44/AF335492 and PpSP42/AF335491) and D7-related proteins (PpSP30/AF335489 and PpSP28/AF335488). Within the salivary gland homogenate, sera reacted with proteins identified as PpSP42, PpSP30, and PpSP28 proteins, but no reaction was detected with PpSP44. In contrast, PpSP30 and PpSP44 recombinant proteins were strongly recognized and PpSP42 gave a weak reaction. Reaction of anti-saliva IgG with recombinant proteins may, however, differ between mouse strains. For example, the C57BL/6 mice reacted predominantly with PpSP42 and PpSP28 recombinant proteins (data not shown). Although none of the recombinant proteins were recognized by all sera. Each mouse serum tested reacted with at least one of the recombinant proteins.

Our data suggest that recombinant proteins could be used as markers of sand fly exposure instead of crude salivary gland homogenates, ideally as a mixture of several different proteins to cope with various host species and individual reactivity of each serum sample. In sand flies this concept has been demonstrated using *Lutzomyia longipalpis* recombinant proteins; the reactivity of anti-*L. longipalpis* seropositive human sera with the salivary gland sonicate was comparable to the reaction with the combination of the two *L. longipalpis* recombinant Yellow-related proteins (LJM11/AY445935 and LJM17/AF132518) [8].

In conclusion, we detected a significant increase of specific IgG and IgG1 in exposed mice of both strains, and of IgG2b in exposed BALB/c mice. The other IgG subclasses were comparable to controls. Specific IgG response was shown to reflect the intensity of sand fly exposure and furthermore, anti-*P. papatasi* saliva antibody response persisted in mice for more than 5 months. Thus, in endemic areas the antibodies could persist till the following sand fly season. The 30 kDa band recognized by sera of experimentally bitten BALB/c as well as C57BL/6 mice was identified as a mixture of D7-related and antigen 5-related proteins. Moreover, the reactivity of mice sera with PpSP44, PpSP42, PpSP30, and PpSP28 recombinant proteins suggested that their combination could substitute the salivary gland homogenate. Taken together, the kinetics, persistence and the individual variability of anti-sand fly saliva antibody response are important aspects to consider in further experiments, where anti-saliva antibodies are used as the markers of sand fly exposure.

Supporting Information

Figure S1 Anti-sand fly saliva antibody response in BALB/c and C57BL/6 mice bitten by *Phlebotomus papatasi*. BALB/c mice and C57BL/6 mice were divided into control (squares) and experimentally bitten groups (circles). Mice in the latter group were exposed to sand fly bites (arrows) (30 *P. papatasi* females per week) in weeks 1–5 and additionally in the week 27 (only BALB/c mice). Anti-*P. papatasi* saliva antibodies - IgG2a (A), IgG3 (C), and IgE (E) in BALB/c mice and IgG2c (B) and IgG3 (D) in C57BL/6 mice - were measured using ELISA as described in Methods. Data are presented as the means ± standard errors of the means. Two independent studies were done. OD = optical density.
(TIF)

Table S1 Dilution and incubation time of secondary antibodies.
(DOC)

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Author Contributions

Conceived and designed the experiments: IR JH PV. Performed the experiments: MV JH LP LZ JD. Analyzed the data: MV IR JH LP PV. Contributed reagents/materials/analysis tools: MV IR JGV PV. Wrote the paper: MV IR PV.

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Comparative analysis of salivary gland transcriptomes of *Phlebotomus orientalis* colonies from endemic and non-endemic foci of visceral leishmaniasis

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Abstract

Background: In Ethiopia**,** *Phlebotomus orientalis* serves as the main vector of *Leishmania donovani*, the causative agent of visceral leishmaniasis. *P. orientalis* was found in two areas of Ethiopia, Addis Zemen and Melka Werer, but cases of visceral leishmaniasis occur only in Addis Zemen. To find out whether the difference in the epidemiology of visceral leishmaniasis is due to different compositions of *P. orientalis* salivary glands we established colonies from Addis Zemen and Melka Werer and analyzed and compared the transcriptomes, proteomes and antigenicity of the salivary glands.

Methodology/Principal Findings: Colonies of *P. orientalis* were established from Addis Zemen and Melka Werer, Ethiopia. Two cDNA libraries were constructed from the salivary glands of female *P. orientalis* of each colony and randomly selected clones were sequenced and analyzed. In both colonies, we identified members of 13 main protein families: apyrase, yellow-related protein, antigen 5-related protein, odorant-binding proteins, hyaluronidase, endonuclease, phospholipase, pyrophosphatase, amylase, PpSP32-like protein, ParSP25-like protein, SP16-like protein, and lufaxin (SP34-like protein). Phylogenetic analysis and multiple sequence alignments were performed to evaluate differences between the *P. orientalis* colonies and to show the relationship with other sand fly species from the subgenus *Larroussius*. To further compare both colonies, we investigated the humoral antigenicity and cross-reactivity of the salivary proteins and the activity of salivary apyrase and hyaluronidase.

Conclusions: This is the first report of the salivary components of *P. orientalis*, an important vector sand fly. Our study expanded the knowledge of salivary gland compounds of sand fly species in the subgenus *Larroussius*. Based on the phylogenetic analysis, we showed that *P. orientalis* is closely related to *P. tobbi* and *P. perniciosus*, whereas *P. ariasi* is probably evolutionarily more distinct species. We also demonstrated that there is no difference between the transcriptomes, proteomes or antigenic properties of the salivary components of Addis

Zemen (endemic area) and Melka Werer (non-endemic area) *P. orientalis* colonies. Our data provides further insights into the evolution of sand fly salivary proteins and broadens the repertoire of salivary proteins potentially applicable as candidates for anti-*Leishmania* vaccines.

Author Summary

Phlebotomus orientalis is the vector of visceral leishmaniasis caused by *Leishmania donovani* in North East Africa and South West Asia. Immunization with sand fly saliva, as well as with individual salivary proteins, was shown to protect against leishmaniasis in different hosts, warranting the intensive study of salivary proteins of various sand fly species. Thus, we decided to broaden the repertoire of the salivary compounds of the sand fly species of the *Larroussius* subgenus and find out whether there is any difference between saliva of *P. orientalis* colonies originating from endemic or non-endemic areas. We characterized the transcriptomes, proteomes, and the main salivary antigens in both colonies and measured the apyrase and hyaluronidase activity of saliva. Overall, we did not detect any difference between the Addis Zemen (endemic area) and Melka Werer (non-endemic area) colonies in any of the aspects tested. Based on the phylogenetic analysis, we showed that *P. orientalis* is closely related to *P. tobbi* and *P. perniciosus*, whereas *P. ariasi* is probably evolutionarily more distinct species. Identifying the sand fly salivary gland compounds will be useful for future research to find suitable salivary proteins as anti-*Leishmania* vaccine candidates and as substrates for epidemiological surveys.

Introduction

Phlebotomine sand flies can be vectors of leishmaniasis, the parasitic disease caused by protozoans from the genus *Leishmania*. Leishmaniasis is endemic in 98 countries

worldwide and more than 350 million people are at risk (Alvar et al., 2012). The clinical forms of this disease range from a self-healing cutaneous form to the life-threatening visceral forms, primarily caused by parasites in the *Leishmania donovani* species complex. The incidence of leishmaniasis is approximately 2 million cases per year, where 400,000 of the cases are caused by parasites from the *Leishmania donovani* complex and thus, are potentially lethal (Alvar et al., 2012).

Leishmaniasis is spread among vertebrate hosts during the feeding process of infected females; parasites are co-inoculated with the sand fly saliva into the bite. Sand fly saliva plays a crucial role in the parasite-host interaction, as salivary compounds possess anti-hemostatic and immunomodulatory properties (reviewed in Fontaine et al., 2011). Sand fly saliva promotes the exacerbation of *Leishmania* infection in naive hosts (Titus and Ribeiro, 1988, Belkaid et al., 1998); however, specific immune responses to sand fly saliva can provide protection against *Leishmania* infection (Belkaid et al., 1998, Kamhawi et al., 2000, Thiakaki et al., 2005, Rohousova et al., 2011). While cellular anti-saliva immune responses are mainly involved in anti- *Leishmania* protection (Valenzuela et al., 2001a, Gomes et al., 2008, Oliveira et al., 2008, da Silva et al., 2011, Tavares et al., 2011, Xu et al., 2011), specific humoral anti-saliva immune response was proven as a reliable marker of sand fly exposure (Rohousova et al., 2005, Hostomska et al., 2008, Clements et al., 2010, Vlkova et al., 2011, Vlkova et al., 2012). Moreover, anti-sand fly saliva antibodies were successfully employed as the *Leishmania* transmission risk marker in epidemiological studies (Barral et al., 2000, Gomes et al., 2002, Rohousova et al., 2005, de Moura et al., 2007, Aquino et al., 2010, Marzouki et al., 2011, Vlkova et al., 2011) and also as a tool for vector control surveillance (Gidwani et al., 2011, Marzouki et al., 2011).

Salivary proteins from two sand fly species - *Lutzomyia logipalpis* and *Phlebotomus papatasi*, the vectors of *Leishmania infantum chagasi* and *Leishmania major*, respectively –

100 are most intensively studied. Importantly, single salivary proteins from these two sand fly
101 species, prepared in recombinant form, were shown to elicit protective immunity against
102 cutaneous (Valenzuela et al., 2001a, Oliveira et al., 2008, Tavares et al., 2011, Xu et al.,
103 2011) and visceral leishmaniasis (Gomes et al., 2008, da Silva et al., 2011). Furthermore, two
104 *L. longipalpis* recombinant salivary proteins were proven as the efficient marker of sand fly
105 exposure (Souza et al., 2010, Teixeira et al., 2010). Although the composition of sand fly
106 saliva differs among sand fly species (Volf et al., 2001) and the anti-sand fly saliva immunity
107 is mostly species specific (Rohousova et al., 2005, Thiakaki et al., 2005, Drahota et al., 2009,
108 Teixeira et al., 2010), there is still some level of cross-reactivity. Recently published data
109 showed that mice immunized by plasmids from *L. longipalpis* saliva were protected against
110 the challenge of *L. brasiliensis* with heterologous antigen (Tavares et al., 2011). Also,
111 vaccination with salivary antigen from *L. longipalpis*, the natural vector of visceral
112 leishmaniasis, provided protection against *L. major* infection (Xu et al., 2011). Therefore, it is
113 crucial to study the degree of diversity and conservancy among different sand fly species.
114 Consideration of all these aspects could facilitate the effort to develop a general anti-
115 *Leishmania* vaccine.
116 In the present study, we explore the salivary antigens from two *P. orientalis* colonies
117 originating from a non-endemic area and from an endemic focus of visceral leishmaniasis in
118 Ethiopia, where this sand fly species serves as the principal vector of *Leishmania donovani*
119 (reviewed in Killick-Kendrick, 1999). We studied the transcriptomes and proteomes of both
120 colonies to find out if there is any difference in the composition of salivary molecules or
121 apyrase and hyaluronidase activity. We also compared our data with other sand fly species
122 from the subgenus *Larroussius*, whose cDNA libraries have already been constructed
123 (Anderson et al., 2006, Oliveira et al., 2006, Rohousova et al., 2012). Additionally, we
124 determined the level of glycosylation of *P. orientalis* salivary proteins.

Methods

Sand flies and salivary gland dissections

Two colonies of *Phlebotomus orientalis* were established; one from non-endemic lowland area in central Ethiopia, Melka Werer (MW), the later one from an endemic focus of visceral leishmaniasis in highland of Northwest Ethiopia, Addis Zemen (AZ), were analyzed. Both sand fly colonies were kept in the insectary of Charles University in Prague and were reared under standard conditions as described in Volf and Volfova, 2011. For mRNA extraction salivary glands of 1-day-old adult females were dissected and stored in RNA later (Ambion). For proteome analysis, western blot, affinity blot, and hyaluronidase assay, salivary glands from 5- to 7-day old *P. orientalis* adult females were dissected and stored in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.7). For the apyrase assay 8-day old adult females were dissected intoTris buffer containing 0.005% Triton X-100 and stored at -80°C.

Construction of salivary gland cDNA libraries

Salivary gland mRNA was isolated separately from 45 pairs each of MW and AZ glands using Micro-FastTrack mRNA isolation kit (Invitrogen). Both cDNA libraries were constructed following the manufacturer's instructions for SMART^{TM} cDNA Library Construction Kit (BD Clontech) with some modifications as described in Chmelar et al., 2008. Each library was fractionated into large, medium, and small cDNA fragments. Gigapack III Gold Packaging Extract (Stratagene) was used for packaging the phage. Both libraries were then plated by infecting log-phase XL-1 blue *Escherichia coli* (Clontech). Transfected plaques were randomly selected and a PCR reaction with vector primers flanking the inserted cDNA was made. The presence of recombinants was checked by visualization the PCR products on 1.1 % agarose gel with SYBR Safe (Invitrogen). Inserts were sequenced as

previously described (Hostomska et al., 2009) using a ABI 3730XL DNA Sequencer (Applied Biosystems).

Bioinformatics

Detailed description of the bioinformatics analysis can be found elsewhere (Rohousova et al., 2012). Briefly, expression sequence tags (ESTs) were analyzed using a customized program based on the Phred algorithm (Ewing et al., 1998, Ewing & Green, 1998). Sequences with Phred quality scores lower than 25 were removed, as well as vector sequences and primers. Resulting sequences were grouped based on nucleotide homology of 90% identity over 100 residues and aligned into consensus transcript sequences (contigs) using the CAP3 sequence assembly program. BLAST programs were used to compare contigs and singletons (contigs with a single sequence) to the non-redundant protein database of the NCBI, the Gene Ontology database (GO) (Ashburner et al., 2000), to COG conserved domains database (Tatusov et al., 2003), Protein Family database (Pfam) (Bateman et al, 2000), SimpleModular Architecture Tool database (SMART) (Schultz et al., 2000), and to rRNA Nucleotide Sequences, and Mitochondrial and Plastid Sequence (MITPLA) databases available from NCBI. The three frame translations of each dataset were submitted to the SignalP server (Bendtsen et al., 2004) to find signal sequences. The grouped and assembled sequences, BLAST results, and SignalP results, combined by dCAS software in an Excel spreadsheet, were manually verified and annotated. N- and O-Glycosylation sites on the proteins were predicted using NetNGlyc 1.0 and NetOGlyc 3.1 software (www.cbs.dtu.dk/services/NetNGlyc, www.cbs.dtu.dk/services/NetOGlyc) (Julenius et al., 2005). C-mannosylation sites prediction was performed using NetCGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetCGlyc) (Julenius, 2007).

Phylogenetic analysis

Protein sequences were aligned using ClustalX (version 2.0) (Larkin et al., 2007) and manually refined in BioEdit 7.1.3.0 editing software. For each alignment, best substitution matrix was determined by ProtTest software 2.0 (Abascal et al., 2005). This matrix was subsequently used by TREE-PUZZLE 5.2 (Schmidt et al., 2002) to reconstruct maximum likelihood phylogenetic trees from the protein alignments using quartet puzzling with 1000 puzzling steps in each phylogenetic analysis. Resulting trees were visualized in MEGA 4 (Tamura et al., 2007).

Proteome analysis

For mass spectrometry analysis, salivary glands of both AZ and MW *P. orientalis* colonies were dissolved in non-reducing sample buffer and electrophoretically separated in 12.5% SDS gel. Proteins within the gel were visualized by staining with Coomassie Brilliant Blue R-250 (Serva). The individual bands were cut and incubated with 10 mM dithiothreitol (Sigma) and then treated with 55 mM iodoacetamide (Sigma). Washed and dried bands were digested with trypsin (Promega). The tryptic peptides were separated by liquid chromatography using an Ultimate 3000 HPLC system (Dionex). The peptide samples diluted in 0.3% trichloroacetic acid (TCA) with 10% acetonitrile (ACN) were loaded onto a PepMap 100 C18 RP column (Dionex) at a flow rate of 300 nl per minute. The peptides were eluted by a 45-min linear gradient of 5-80% (v/v) ACN in 0.1% (v/v) TCA over a period of 20 min. The eluate was mixed 1:3 with matrix solution (20 mg/ml a-cyano-4-hydroxycinnamic acid in 80% ACN) and subsequently spotted onto MALDI target plates using a Probot microfraction collector (Dionex). Spectra were acquired on 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) equipped with a Nd: YAG laser (355 nm, firing rate 200 Hz) as described in detail in (Rohousova et al., 2012).

Hyaluronidase activity analysis

Hyaluronidase activity in salivary glands of both *P. orientalis* colonies was quantified using a sensitive assay in microtitration plates coupled with biotinylated hyaluronic acid (bHA). Salivary glands were homogenized by three freeze-thaw cycles and salivary gland extract (SGE) was obtained by centrifugation at 17.000g (5 min, 2°C). Biotinylated HA, prepared as described by Černá et al. (2002), was immobilized onto Covalink®NH microtiter plates (NUNC) using the method by Frost and Stern (1997) at a final concentration of 1µg/well bHA. The plates were incubated overnight at 4°C and washed three times in PBS, pH 7.2 containing 2 M NaCl and 50 mM $MgSO_4$. The plates with immobilized bHA were coated for 45 min with 1% BSA in PBS, then washed and equilibrated with assay buffer (0.1M acetate buffer, pH 5.0, 0.1 M NaCl, 0.1% Triton X-100) to adjust the pH for optimum sand fly salivary hyaluronidase activity (Černá et al., 2002). Four SGE samples for each colony were pipetted into the plates in triplicate at a final concentration of 0.5 salivary gland per well and incubated for 45 min at 37°C. To obtain a standard curve ranging from 0.5 to $7.8x10^3$ rTRU, hyaluronidase from bovine testes (Sigma), at a concentration of 0.01 TRU/µl, was diluted by two-fold serial dilution in 0.1M acetate buffer, pH 4.5, 0.1 M NaCl, 0.1% Triton X-100. Wells without bHA or enzyme were used as controls. The reaction was terminated by the addition of 200 µl/well of 6 M guanidine. After washing, avidin-peroxidase (Sigma, 2µg/ml) was added at a final concentration of 0.2 µg/ well and incubated for 30 min at room temperature. Color reaction was developed with o-phenylenediamine substrate in 0.1 M citrate-phosphate buffer, pH 5.5. Absorbance was measured at 492 nm using Infinite M 200 fluorometer (Schoeller Instruments). Raw data were evaluated by Measurement Parameters Editor Magellan 6 (Tecan) and the standard curve created using a 4-parameter logistic fit.

Apyrase activity analysis

Apyrase activity was determined using the Fiske and Subbarow method for measuring inorganic phosphate (Pi) released from ADP or ATP (Marinotti *et al*., 1996), with some modifications. Briefly, salivary glands were homogenized by one freeze-thaw cycle combined with a mechanical homogenization. Two µl of salivary gland homogenate (SGH) diluted 1:25 in assay buffer (50mM TRIS 150mM NaCl, pH 8.5 with 5mM $CaCl_2$ or 5mM $MgCl_2$) were mixed in wells with 78µl of assay buffer and 20µl of substrate to obtain a final concentration of 2mM ATP or ADP and 1/25 of gland pair per well. SGH samples were pipetted into the microtiter plate in series of six. Wells containing only assay buffer were used as negative controls. Plates were incubated for 15 min at 37°C. Then the enzymatic reaction was stopped by addition of 25µl of 1.25% ammonium molybdate in 1.25M sulfuric acid and 5µl of Fiske-Subbarow reducer (25mg/ml, F5428 Sigma) per well. The colorimetric reaction was read after 15 min by Tecan Infinite M 200 fluorometer (Schoeller Instruments) at 665 nm. The amount of Pi released from substrate was determined using potassium dihydrogen phosphate as a standard. The study of pH optimum was carried out within a range of pH 6.0 – 9.5. *Phlebotomus papatasi*, the species with previously described apyrase activity (Ribeiro *et al*., 1989), was used as a positive control. Amount of proteins within SGHs was determined using Bio-Rad *DC* Protein Assay with BSA as a standard according to the manufacturer instructions.

Western blotting

Salivary glands of both *P. orientalis* colonies were separated by SDS-PAGE on 10% gel under non-reducing conditions using Mini-Protean III apparatus (Biorad). Salivary proteins were transferred from gel to nitrocellulose membrane (NC) by Semi-Phor equipment (Hoefer Scientific Instruments) and cut into strips. The strips were then blocked with 5% low fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw) and subsequently incubated

with BALB/c mice sera (AZ – mice bitten 18 times; MW – mice bitten 17 times), diluted 1:100 in TBS-Tw, for 1 hour. After the washing with TBS-Tw, the strips were incubated for 1 hour with peroxidase-conjugated goat anti-mouse IgG (Serotec) diluted 1:1000 in TBS-Tw. The chromogenic reaction was developed using a substrate solution containing diaminobenzidine and H_2O_2.

Affinity blotting

Affinity bloting was performed using salivary glands from *P. orientalis* (MW) colony separated by SDS-PAGE as described above. After transfer, free binding sites on NC membrane were blocked with 5% bovine serum albumin in 20 mM TBS-Tw overnight at 4ºC. The strips were then incubated for 1.5 hour on the shaker at room temperature with biotinylated lectin from *Dolichos biflorus* (DBA, Vector), *Glycine max* (SBA, Vector), *Ulex europaeus* (UEA-I, Vector), *Tetragonolobus purpureas* (LTA, Sigma), *Canavalia ensiformis* (ConA, Sigma), and *Pisum sativum* (PSA, Vector). Lectins were diluted: 5 μg/ ml, 10 μg/ ml, 10 μg/ ml, 0.2 μg/ ml, 0.1 μg/ ml and 10 μg/ ml in TBS-Tw, respectively. To control the reaction specificity the aforementioned lectins were pre-incubated for 30 min with the appropriate saccharide inhibitors (Sigma) as follows: 0.25 M N-acetyl-D-galactosamine for DBA and SBA, 0.5 M L-fucose for UEA-I and LTA, 0.5 M methyl-α-D-mannopyranoside for ConA and PSA, and subsequently applied on the strips. After the washing with TBS-Tw, streptavidin-peroxidase (Sigma) was added to strips at a final concentration of 1 μg/ml and incubated for 1 h on the shaker at room temperature. The chromogenic reaction was developed as mentioned above.

Ethics statement

BALB/c mice 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). The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05, CZU 307/09) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic.

Results and Discussion

Sequencing of *P. orientalis* salivary gland cDNA libraries

Two cDNA libraries were constructed from salivary glands of *Phlebotomus orientalis* colonies originating from Addis Zemen (AZ) and Melka Werer (MW), Ethiopia. For each colony cDNA library, 940 clones were randomly selected and sequenced, which resulted in 835 and 749 high quality sequences from AZ and MW, respectively. Based on nucleotide homology, sequences were clustered into contigs, analyzed using the dCAS cDNA annotation software (Guo et al., 2009) and subsequently verified by manual annotation. From the AZ cDNA library, sequences were assembled into 263 contigs, where 185 of them were singletons (one sequence per contig). From the MW cDNA library, we obtained 242 contigs, including 171 singletons. In accordance with previously published cDNA libraries from sand fly salivary glands, the most abundant transcripts were those coding for putative salivary proteins (607 out of 835 in AZ; 567 out of 749 in MW). Of the nucleotide sequences encoding putative salivary proteins, 574 (AZ) and 506 (MW) salivary transcripts encoded a predicted signal peptide sequence. Those that didn’t posses sequences encoding a signal peptide were

298 truncated at the 5` end. Most of the contigs coding for putative salivary proteins were
299 comprised of more than one sequence (averaging 7.14 sequences per contig in AZ and 6.23 in
300 MW), whereas housekeeping proteins or proteins with unknown function were mostly
301 represented by singltons. All obtained ESTs were deposited in the NCBI dbEST database
302 under accession numbers NUMBERS for Addis Zemen and NUMBERS for Melka Werer
303 colony.
304
305 Members of 13 main protein families were found among the putative salivary proteins of the
306 two *P. orientalis* colonies: apyrase, yellow-related protein, antigen 5-related protein, odorant-
307 binding proteins (D7-related and PpSP15-like proteins), hyaluronidase, endonuclease,
308 phospholipase, pyrophosphatase, amylase, PpSP32-like protein, ParSP25-like protein, SP16-
309 like protein, and lufaxin (SP34-like protein). Detailed descriptions of each protein family are
310 listed in the following paragraphs. BLAST comparison of translated nucleotide sequences
311 with the non-redundant (NR) protein database showed high similarity with salivary proteins
312 of *P. perniciosus* and *P. tobbi* (both subgenus *Larroussius*). Sporadically, the best match was
313 found with salivary proteins of *P. arabicus* (subgenus *Adlerius*) or *P. argentipes* (subgenus
314 *Euphlebotomus*). Representative sequences of putative salivary proteins from both *P.*
315 *orientalis* colonies that were deposited into NCBI GenBank database are listed in Table 1 and
316 Table 2. Both tables include GenBank accession numbers, the predicted molecular weight,
317 isoelectric point, best match to the NR database, the sand fly species with the highest
318 homology, and presence in the proteome. Salivary proteins presented in the proteome were
319 identified by mass spectrometry and are shown in Figure 1.
320
321 The main objective of this work was broader comparison of two *P. orientalis* colonies
322 originating from non-endemic and endemic focus of visceral leishmaniasis. We investigated

the transcriptomes, proteomes, antigenic properties and level of glycosylation of the salivary proteins, as well as hyaluronidase and apyrase activity in the saliva of both colonies. We also evaluated the protein sequence homology with all other sand fly species from the subgenus *Larroussius* that have been analysed to date.

Yellow-related proteins

Yellow-related proteins are abundantly expressed in the sand fly salivary glands and have been detected in the saliva of all sand fly species tested, to date (Charlab et al., 1999, Valenzuela et al., 2001a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012, Kato et al., 2012, Rohousova et al., 2012). Yellow-related proteins were found in the cDNA library of the AZ (PorASP2/ KC170933; PorASP4/ KC170934) as well as the MW (PorMSP23/ KC170966; PorMSP24/ KC170967) *P. orientalis* colony and all of them were also present in the proteome (Figure 1). *P. orientalis* yellow-related proteins had similar predicted molecular mass (41.5-42.3 kDa) and wide range of pI (6.1-8.1) (Table 1, Table 2). All obtained sequences contained the whole major royal jelly protein (MRJP) domain, which is characteristic for the yellow-related proteins. The function of *Phlebotomus* yellow-related proteins has not been characterized but some advances have been made in describing the function of *Lutzomyia* yellow-related proteins. It was shown that recombinant yellow-related proteins from *Lutzomyia longipalpis* saliva (AAD32198, AAS05318) act as high affinity binders of prohemostatic and proinflammatory biogenic amins such as serotonin, catecholamines and histamine (Xu et al., 2011). The amino acid motif present in the ligand binding pocket of *L. longipalpis* was also discovered in the yellow-related proteins of *L. ayacuchensis* (BAM69111, BAM69185, BAM69109, BAM69110) suggesting similar anti-inflammatory function of these proteins in other *Lutzomyia* sand fly species (Kato et al.,

2012). Sand fly yellow-related proteins are homologous to the yellow protein of *Drosophila melanogaster* and to the MRJPs of honeybees. Similarly, sequences homologous to *D. melanogaster* yellow protein were also found in other bloodsucking insects; for example, the mosquito *Aedes aegypti* (Johnson & Christensen, 2001) and the tsetse fly *Glossina morsitans morsitans* (Alves-Silva et al., 2010).

Phylogenetic analysis shows that yellow-related proteins from *P. orientalis* saliva are divided into two clades (Figure 2). Both clades are represented by two yellow-related salivary proteins, one from each *P. orientalis* cDNA library (clade I - PorASP2, PorMSP23; clade II - PorASP4, PorMSP24). *Phlebotomus orientalis* sequences within the same clade revealed high degree of identity (99 and 100%, respectively), while comparison between clades showed 77% identity (Figure 3). Yellow-related proteins of other sand fly species from subgenus *Larrousius* were also splitted into two clades and these sequences are closely related to *P. orientalis* proteins (83-91% identity) (Figure 2, Figure 3).

Yellow-related proteins were shown to be highly immunogenic. These proteins were recognized by sera of repeatedly bitten hosts such as mice (Rohousova et al., 2005, Martin-Martin et al., 2012, Vlková et al. 2012), hamsters (Martin-Martin et al., 2012), dogs (Bahia et al., 2007, Hostomská et al 2008, Teixeira et al., 2010, Vlkova et al. 2011), foxes (Gomes et al., 2007, Teixeira et al., 2010) and humans (Gomes et al. 2002, Rohousova et al., 2005, Vinhas et al. 2007, Teixeira et al., 2010, Marzouki et al. 2011). Furthermore, recombinant yellow-related salivary proteins (AAD32198, AAS05318) were succesfully employed as the markers of sand fly exposure for individuals in endemic areas (Souza et al., 2010, Teixeira et al., 2010). Importantly, salivary yellow-related proteins seem to be promising candidates for anti-*Leishmania* vaccine. Inoculation of plasmids coding for *L. longipalpis* yellow-related

salivary proteins (AAD32198, AAS05318) into the skin elicited a strong delayed type hypersensitivity (DTH) reaction in various hosts (Gomes et al., 2008, Collin et al., 2009, Xu et al., 2011), which resulted in efficient killing of *L. infantum chagasi* parasites *in vitro* (Collin et al., 2009) and protection against *L. major* infection *in vivo* (Xu et al., 2011). According to the glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc), we found that PorASP4 and PorMSP24 are likely N-glycosylated and have three threonine sites for potential O-glycosylation. PorASP2 and PorMSP23 have four threonine for potential O-glycosylationand no N-glycosylation was predicted.

Apyrase

Sequences coding for apyrase were detected in the cDNA libraries of both the AZ (PorASP11/ KC170935; PorASP14/ KC170936; PorASP15/ KC170937) and the MW (PorMSP3/ KC170960; PorMSP4/ KC170961) *P. orientalis* colonies. All sequences had similar predicted molecular mass (33.2-35.6 kDa) and pI ranged from 8.8 to 10.0. For each of the *P. orientalis* apyrase sequences in the libraries the corresponding proteins were found in salivary gland proteomes (Figure 1). Apyrase is the principal anti-platelet aggregation enzyme which hydrolyses ATP and ADP to AMP and orthophosphate, thereby blocking the physiological signal of damaged cells and tissues. This enzyme has been found in the saliva of all sand fly species tested, to date (Charlab et al., 1999, Valenzuela et al., 2001a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Rohousova et al., 2012, Kato et al., 2012, Abdeladhim et al., 2012), but also in the saliva of other medically important haematophagous insects such as triatomine bugs (e.g. Santos et al., 2007, Assumpcao et al., 2008, Ribeiro et al., 2012), mosquitoes (e.g. Reno & Novak, 2005, Jariyapan et al., 2012), fleas (e.g. Andersen et al., 2007, Ribeiro et al., 2012), tsetse flies (e.g. Caljon et al., 2010), biting midges (e.g. Perez & Tabachnick, 1996), and

horseflies (e.g. Ma et al., 2009). Interestingly, apyrase has also been described in non-bloodsucking insects; for example, *Helicoverpa zea* (Wu et al., 2012) and *Drosophila melanogaster* (Fenckova et al., 2011), indicating that apyrase may have a broader functional potential than only the facilitation of blood acquisition.

Apyrases of the bloodfeeding insect are divided into three families: GTPase/CD-39, 5´-nucleotidase, and *Cimex* type (reviewed in Ribeiro et al., 2010). Apyrases from *P. orientalis* colonies as well as from other sand fly species are homologous to the bed bug apyrase, which defined the *Cimex* type family (Valenzuela et al., 1998). Phylogenic analysis showed that salivary apyrases from *P. orientalis* colonies are separated into two clades (Figure 4). Clade I includes two apyrases from the AZ colony and one from the MW colony (PorASP14, PorASP15, and PorMSP4); clade II contains two apyrases, one from each colony (PorASP11 and PorMSP3). Sequences of *P. orientalis* apyrase within the same clade revealed high degree of identity (95-99%), whereas the comparison between the clades showed an identity of 66% (Figure 5). Comparison with other sand fly species from the subgenus *Larroussius* showed that apyrases from *P. tobbi* (ADJ54077, ADJ54078) and *P. perniciosus* (ABB00906, ABB00907) saliva are closely related to *P. orientalis*, while apyrase from *P. ariasi* (AAX56357) saliva is more distinct (Figure 4, Figure 5).

Apyrase activity has been demonstrated in the saliva of *L. longipalpis* (Ribeiro et al., 1986, Charlab et al., 1999), *P. papatasi* (Ribeiro et al., 1989, Valenzuela et al., 2001b), *P. duboscqi* (Hamasaki et al., 2009), *P. perniciosus* (Ribeiro et al., 1989), *P. argentipes* (Ribeiro et al., 1989), and *P. colabaensis* (Ribeiro et al., 1989). In our experiments, apyrase activity was measured in the saliva of both AZ and MW *P. orientalis* colonies (Table 3). ATPase as well as ADPase activity, determined per the pair of salivary glands, was slightly higher from the

AZ colony, but recalculation of enzymatic activity per miligram of total proteins showed that apyrase activity in both colonies is comparable. Also, the ATPase/ ADPase ratio was equal in both colonies (Table 3). Comparison of *P. orientalis* and *P. perniciosus* apyrase activity (both subgenus *Larroussius*) showed that ATPase activity determined per pair of glands is comparable with MW colony, but ADPase activity is significantly lower (Ribeiro et al., 1989). Additionaly, in accordance with previous data (Ribeiro et al., 1986, Ribeiro et al., 1989, Charlab et al., 1999, Valenzuela et al., 2001b, Hamasaki et al., 2009), we showed that *P. orientalis* apyrase activity is dependent on presence of Ca2+ but not on Mg2+ ions.

Besides the antihemostatic effect of this enzyme, apyrase is also known as a powerful antigen. Specific antibodies from dogs bitten by *P. perniciosus* in the field, as well as under laboratory conditions, reacted strongly with two salivary apyrases (Vlkova et al., 2011). Apyrases from *P. perniciosus* and *P. papatasi* saliva were also recognized by sera of mice and hamsters immunized by homologous antigen (Martin-Martin et al., 2012, Vlkova et al., 2012). Furthermore, bacterially expressed recombinant *P. duboscqi* apyrase (ABI20147) was also recognized by specific antibodies from mice immunized with *P. duboscqi* saliva (Hamasaki et al., 2009), suggesting that antibody recognition is not solely targeted to the glycosylated parts of the antigen. On the other hand, inoculation of bacterially expressed recombinant *L. longipalpis* apyrase (AAD33513) into C57BL/6 mice did not elicit either antibody response or delayed type hypersensitivity reaction (Xu et al., 2011). These data indicates that the immunogenicity of the protein or saccharidic part of antigen may vary in different sand fly species. According to the glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc), *P. orientalis* apyrases PorASP14, PorASP15, and PorMSP4 are N-glycosylated, while no O-glycosylation sites were predicted.

Hyaluronidase

Hyaluronidase is an enzyme that degrades hyaluronic acid and other glycosaminoglycan constituents abundantly present in the vertebrate extracellular matrix. It is a well-known allergen occurring in the venom of bees, hornets, wasps, spiders, and snakes (reviewed in Stern & Jedrzejas, 2006, Muller, 2011), but hyaluronidase activity was also observed in the saliva of various bloodsucking Diptera (Ribeiro et al., 2000, Cerna et al., 2002, Volfova et al., 2008, Hostomska et al., 2009, Rohousova et al., 2012). Previously published data showed that hyaluronidase is able to promote the spreading of other components of bloodfeeding insect saliva within the skin, as well as enhance the success of potential parasite transmission (Volfova et al., 2008). Although positive enzymatic activity was detected in all sand fly species tested to date (Charlab et al., 1999, Ribeiro et al., 2000, Cerna et al., 2002, Volfova et al., 2008, Hostomska et al., 2009, Rohousova et al., 2012), transcripts coding for putative hyaluronidase were ascertained only in *L. longipalpis* (AAD32195), *P. arabicus* (ACS93505), *P. tobbi* (AEK98519) (Charlab et al., 1999, Valenzuela et al., 2004, Rohousova et al., 2012) and both *P. orientalis* colonies (PorASP112/ KC170958; PorMSP108/ KC170981) (Table 1, Table 2). The predicted molecular mass of AZ and MW hyaluronidase was 37.2 and 35.6 kDa, respectively, and the pI was 6.5 and 8.0, respectively. Hyaluronidase activity measured in the *P. orientalis* saliva was found to be the lowest among the other *Larroussius* species tested (Rohousova et al., 2012). While hyaluronidase activity expressed in the relative Turbidity Reducing Units (rRTU) reached approximately 0.48 rTRU/gland in *P. perniciosus* and 0.62 rTRU/gland in *P. tobbi* (Rohousova et al., 2012), enzymatic activity in *P. orientalis* saliva was 0.22 rTRU per gland.

Phlebotomus orientalis salivary hyaluronidase revealed high degree of homology (89-93%) with *P. tobbi* sequences, followed by *P. arabicus* enzyme (80-83%) and *L. longipalpis* hyaluronidase (56-58%). Comparison between AZ and MW colonies revealed identity

reaching 91 % and enzymatic activity per gland was similar in both strains: 0.22 ± 0.036 rTRU in AZ and 0.215 ± 0.045 rTRU in MW.

Moreover, glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc) showed that salivary hyaluronidase is the most glycosylated protein in both colonies, with twenty predicted N-glycosylation sites in the AZ and sixteen in the MW colony. In PorASP112, three O-glycosylated sites were predicted as well.

Other enzymes

None of the salivary enzymes listed in the following paragraphs were detected in the AZ or MW proteomes (Figure 1). It might be explained by the fact that extremely active enzymes do not need a huge amount of protein to be effective. Another enzyme that was identified among the transcripts from both *P. orientalis* cDNA libraries is a putative endonuclease (PorASP139/ KC170955; PorMSP101/ KC170979) (Table 1, Table 2). Both sequences contained the NUC Smart motif, which is typical for DNA/RNA non-specific endonucleases and phosphodiesterases. Predicted molecular mass of both AZ and MW endonucleases was 41.7 kDa and predicted pI was 9.3 and 9.4, respectively. Endonucleases function in sand fly saliva is still unclear, however, properties that facilitate blood acquisition are assumed. Endonucleases were detected in salivary gland cDNA libraries of some sand flies species tested (Valenzuela et al., 2004, Anderson et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Kato et al., 2012, Abdeladhim et al., 2012), but also in another bloodsucking Diptera (Ribeiro et al., 2004, Calvo & Ribeiro, 2006). Endonuclease of AZ and MW *P. orientalis* colony revealed 97 % identity and endonuclease from *P. perniciosus* (ABA43064) saliva was found to be the most relative sequence (92 % identity). Homology of *P. orientalis* enzymes with other sand fly endonucleases ranged from 44 – 80%. Endonuclease was also shown to have antigenic properties; sera of dogs from an endemic area of visceral leishmaniasis in Italy,

as well as dogs experimentally bitten by *P. perniciosus*, reacted with a 41 kDa salivary protein identified as the endonuclease (ABA43064) (Vlkova et al., 2011).

Transcripts coding for a putative phospholipase A2 (PLA2) were detected in both *P. orientalis* cDNA libraries (PorASP80/ KC170949; PorMSP129/ KC170982) (Table 1, Table 2). In both sequences, the whole PLA2 domain was present. The predicted molecular mass of PLA2 was 29.7 kDa and pI was 8.4 and 8.3 for AZ and MW, respectively. PLA2 was described as the main allergen in hymenopteran venom (reviewed in Muller, 2011), but the presence of this enzyme was also detected in the saliva of some sand fly species (Oliveira et al., 2006, Anderson et al., 2006, Hostomska et al., 2009). However, the exact function of PLA2 in sand flies remains to be elucidated. Sequences coding for PLA2 revealed a high degree of conservancy between the AZ and MW colonies and between species. The *P. orientalis* PLA2 of the AZ and MW colonies are almost identical (99%) and between *P. perniciosus* (ABA43062) and *P. ariasi* (AAX54852), species in the subgenus *Larroussius*, the identities are 99% and 94%, respectively. Moreover, comparing the PLA2 enzymes of *P. orientalis* and *P. arabicus* (ACS93491), subgenus *Adlerius*, showed 88 % identity.

A single 3´truncated transcript coding for a putative α-amylase was detected in the salivary gland cDNA library of Melka Werer *P. orientalis* (PorMSP27/ KC170968) (Table 1) but no homologous sequences were found in the Addis Zemen colony. Amylase is an enzyme which is likely not involved in bloodfeeding process, but participates in dietary starch digestion (reviewed in Jacobson et al., 2001). Transcripts coding for α-amylase were detected in the salivary gland cDNA libraries of *L. longipalpis* (AAD32192) (Charlab et al., 1999), *P. arabicus* (ACS93490) (Hostomska et al., 2009) and, recently, in *P. papatasi* (AAD32192) (Abdeladhim et al., 2012). Alpha-amylase activity was detected in the sand fly salivary glands (Charlab et al., 1999, Ribeiro et al., 2000, Jacobson & Schlein, 2001) and also in the gut of

phlebotomine larvae (Vale et al., 2012). The predicted molecular weight of the *P. orientalis* MW amylase was 33.4 kDa and the predicted pI was, 5.8 (Table 1). Amino acid sequence alignment of *P. orientalis* α-amylase shows 88% identity with the *P. arabicus* α-amylase (ACS93490) and 82% identity with *L. longipalpis* α-amylase (AAD32192).

A single sequence, truncated in the 3´region, coding for a putative salivary pyrophosphatase (PorASP262/ KC170959) was ascertained in the AZ cDNA library (Table 2). The predicted molecular mass was 32.9 kDa and the predicted pI was 7.2. Pyrophosphatase was detected also in MW colony, but these sequences did not contain signal peptides. Salivary pyrophosphatase was found also in saliva of other sand fly species from the genus *Phlebotomus* such as *P. duboscqi* (ABI20154) (Kato et al., 2006), *P. argentipes* (DQ136170) (Anderson et al., 2006), and *P. arabicus* (ACS93498) (Hostomska et al., 2009). Transcripts coding for pyrophosphatase did not reveal high degree of conservancy, as homology of the mentioned sequences ranged between 39 – 74 %.

Odorant-binding proteins

D7-related proteins belong to the odorant-binding protein family, which is composed of pheromone-binding proteins (PBP) and general-odorant-binding proteins (GOBP). D7 proteins are commonly present in the salivary glands of various bloodfeeding insects such as mosquitoes (James et al., 1991, Valenzuela et al., 2002a, Valenzuela et al., 2002b, Jariyapan et al., 2012), black flies (Andersen et al., 2009), biting midges (Campbell et al., 2005, Russell et al., 2009) and sand flies (Valenzuela et al., 2001a, Valenzuela et al., 2002a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012, Kato et al., 2012, Rohousova et al., 2012). In the *P. orientalis* cDNA libraries we found three contigs coding for D7-related proteins in the AZ colony (PorASP46/ KC170942, PorASP48/ KC170943, PorASP122/ KC170954) and four in the MW

549 colony (PorMSP28/ KC170969, PorMSP38/ KC170970, PorMSP43/ KC170971, PorMSP67/
550 KC170973) (Table1, Table 2). They all had a similar predicted molecular mass (26.7-27.3
551 kDa) and wide range of pI (6.4-9.2). Except for, All but two contigs, PorASP46 and
552 PorMSP43, represented the D7-related proteins that were detected in the proteome (Figure 1).
553 The function of sand fly salivary D7-related proteins remains unknown, although it might be
554 similar to mosquito D7 proteins; either as a binder of biogenic amines (Calvo et al., 2006,
555 Mans et al., 2007) or as an anticoagulant (Isawa et al., 2002, Alvarenga et al., 2010).
556
557 Phylogenetic analysis showed that *P. orientalis* D7-related salivary proteins are divided into
558 three clades (Figure 6). Clade I contains two 100% identical *P. orientalis* D7-related proteins,
559 one from each cDNA library (PorASP122, PorMSP67). Clade II contains only one *P.*
560 *orientalis* protein (PorMSP28) from the MW colony. Clade III includes two proteins from
561 each library, PorASP46 and PorASP48 from the AZ colony and PorMSP38 and PorMSP43
562 from the MW colony. The identity in this clade was 92 %. Sequences coding for salivary D7-
563 related proteins in *P. orientalis* species did not reveal high degree of conservancy as the
564 alignment of all D7-related proteins from both colonies reached only 31% identity (Figure 7).
565 Comparison with other sand fly species from subgenus *Larroussius* showed that *P. orientalis*
566 D7-related proteins are more related to *P. tobbi* and *P. perniciosus* than to *P. ariasi* (Figure 6,
567 Figure 7).
568
569 Based on the glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc), we
570 found that D7-related proteins have very limited glycosylation sites. Only PorASP122 and
571 PorMSP67 were predicted to have N-glycosylation and the other D7-related proteins are not
572 predicted to be glycosylated. Our data complies well with previously published results, where
573 most of the sand fly species studied so far contained in their salivary glands also mixture of

glycosylated and non-glycosylated D7-related proteins (Hostomska et al., 2009, Rohoušová et al. 2012).

D7-related proteins are antigenicand were recognized by specific antibodies from the sera of repeatedly bitten hosts, regardless of natural (Rohousova et al., 2005, Bahia et al., 2007, Marzouki et al., 2011, Vlkova et al., 2011) or experimental exposure (Hostomska et al., 2008, Vlkova et al., 2011, Martin-Martin et al., 2012, Vlkova et al., 2012). Recombinant *P. ariasi* D7-related protein (AAX55749) elicited the production of specific humoral response in immunized mice (Oliveira et al., 2006). Anti-*P. papatasi* saliva antibodies reacted with the 30 kDa recombinant *P. papatasi* D7-related protein (AAL11049) (Vlkova et al., 2012), but the same protein was not recognized by the human sera from an endemic area of cutaneous leishmaniasis in Tunisia (Marzouki et al., 2012). Moreover, recombinant 28 kDa D7-related protein from *P. papatasi* saliva (AAL11048) was not targeted by the specific antibodies of immunized mice (Vlkova et al., 2012). Thus, a broad use of D7-related salivary proteins as the reliable marker of sand fly exposure is not likely. Importantly, no significant cellular immunity was observed in various hosts after the inoculation of DNA plasmids coding for D7-related sand fly salivary proteins (Oliveira et al., 2006, Collin et al., 2009, Xu et al., 2011).

PpSP15-like proteins

Transcripts coding for PpSP15-like proteins represented the most abundant family in *P. orientalis* cDNA libraries. PpSP15-like proteins were detected in both the AZ (PorASP28/ KC170938; PorASP31/ KC170939; PorASP37/ KC170940; PorASP61/ KC170944; PorASP64/ KC170945) and MW colonies (PorMSP12/ KC170964; PorMSP74/ KC170974; PorMSP75/ KC170975; PorMSP90/ KC170977; PorMSP96/ KC170978) (Table 1, Table 2).

599 The predicted molecular mass ranged from 13.9 to 14.9 and the isoelectric point was slightly
600 basic (8.0-9.2). Three out of five PpSP15-like proteins in each library were found in the
601 proteomes (AZ: PorASP28, PorASP37, and PorASP61; MW: PorMSP12, PorMSP74, and
602 PorMSP96) (Figure 1).
603
604 Phylogenetic analysis showed that *P. orientalis* PpSP15-like proteins are divided into three
605 clades. Clade I contains two *P. orientalis* PpSP15-like proteins, one from each library
606 (PorASP37, PorMSP12). Clades II and III each contain four *P. orientalis* proteins, two from
607 each library (clade II: PorASP61, PorASP64, PorMSP74, PorMSP75; clade III: PorASP28,
608 PorASP31, PorMSP90, PorMSP96) (Figure 8). Alignment of known *Larroussius* and *P.*
609 *orientalis* PpSP15-like proteins revealed high degree of divergence (overall identity 24%)
610 among PpSP15-like proteins within the subgenous *Larroussius* but demonstrated that the MW
611 and AZ *P. orientalis* PpSP15-like proteins are nearly identical (Figure 9). Comparison of *P.*
612 *orientalis* and other *Larroussius* species PpSP15-like proteins within each clade showed
613 identity ranging from 61 to 96 %. Our results comply well with previous reports (Anderson et
614 al., 2006, Hostomska et al., 2009, Kato et al., 2012, Rohousova et al., 2012), where PpSP15-
615 like proteins of various sand fly species were described as extremely variable proteins, likely
616 occurring in multiple gene copies (Elnaiem et al., 2005).
617 PpSP15-like proteins belong to odorant-binding protein family but, so far, the exact function
618 of these proteins in sand flies remains unknown. However, SP15 protein from *P. papatasi*
619 saliva (AAL11047) was shown to elicit specific humoral and cellular immunity, which
620 resulted in the protection of immunized mice against *Leishmania major* infection (Valenzuela
621 et al., 2001a, Oliveira et al., 2008). Similarly, a DTH reaction was also observed in mice
622 immunized by the inoculation of a *P. ariasi* DNA plasmid coding for SP15-like salivary
623 protein (AAX56359) (Oliveira et al., 2006). On the other hand, DNA plasmids coding for *L.*

longipalpis SL1 protein (AAD32197) failed to promote the cellular immunity in experimental mice (Xu et al., 2011), hamsters (Gomes et al., 2008), and dogs (Collin et al., 2009). Glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc) revealed that *P. orientalis* PpSP15-like proteins are likely not glycosylated.

Antigen 5-related proteins

Antigen 5-related proteins (Ag5r) belong to the CAP family of proteins which is composed of **C**ysteine-rich secretory proteins, **A**ntigen 5, and **P**athogenesis-related 1 proteins. Proteins with the CAP domain are commonly present in a various organisms that include also prokaryotes or non-vertebrate eukaryotes (Yeats et al., 2003, Milne et al., 2003). Ag5r proteins were described from the venom of ants, wasps and other Hymenoptera (Lu et al., 1993, Hoffman, 1993, King & Spangfort, 2000), but were also found in salivary glands of various bloodsucking insects, including sand flies (Charlab et al., 1999, Valenzuela et al., 2001a, Valenzuela et al., 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Rohousova et al., 2012, Kato et al., 2012, Abdeladhim et al., 2012). The exact function of Ag5r in sand flies is still unknown, although biological properties of other proteins from the same family may give us some clue. The X-ray structure of NA-ASP-2 protein (pathogenesis –related 1 protein) from the human hookworm, *Necator americanus*, reveals structural and charge similarities to chemokines, suggesting that these proteins could potentially modulate the host immune response (Asojo et al., 2005).

Sequences coding for salivary Ag5r proteins were found in cDNA library from the AZ (PorASP74/ KC170947; PorASP76/ KC170948) and MW (PorMSP6/ KC170962; PorMSP8/ KC170963) *P. orientalis* colonies (Table 1, Table 2). The predicted molecular weight was 28.8 kDa and pI was slightly basic (8.9).All Ag5r contigs were also represented in the

proteomes (Figure 10). Phylogenetic analysis showed that Ag5r proteins from the saliva of sand fly species from the subgenus *Larroussius* are separated into two clades. The first clade contains only Ag5r protein from *P. ariasi* (AAX44092), whereas the second clade includes proteins of *P. tobbi* (ADJ54082, ADJ54083), *P. perniciosus* (ABA43055), and *P. orientalis* (Figure 10). *Phlebotomus orientalis* Ag5r proteins are divided into two groups. Both are represented by two salivary transcripts; one from each colony, respectively (PorASP74, PorMSP8 and PorASP76, PorMSP6). *Phlebotomus orientalis* Ag5r proteins revealed 100% identity within the same group and 99% identity between these two groups indicating that there is no difference in antigen 5 proteins between the AZ and MW colony. Sequences from *P. perniciosus* (92 % identity) and *P. tobbi* (88-93% identity) coding for Ag5r proteins were ascertained to be the closest relatives, while identity with *P. ariasi* protein reached 77% (Figure 11).

Antigenic properties of Ag5r proteins were demonstrated in various sand fly - host combinations. Salivary Ag5r proteins were recognized by sera of mice repeatedly bitten by *P. papatasi* (Vlkova et al., 2012) or *P. arabicus* (Hostomska et al., 2009), by sera of dogs bitten by *P. perniciosus* (Vlkova et al., 2011), as well as by sera of hamsters exposed to *P. tobbi* (Rohousova et al., 2012). On the other hand, inoculation of DNA plasmids coding for Ag5r protein from saliva of *P. ariasi* (AAX44092) or *L. longipalpis* (AAD32191) did not elicit a specific humoral response but did induced a cell-mediated immune response (Oliveira et al., 2006, Xu et al., 2011).. Glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc) showed that all *P. orientalis* Ag5r proteins are N- and O-glycosylated.

PpSP32-like proteins

The PpSP32-like protein family was described for the first time in the saliva of *P. papatasi* (Valenzuela et al., 2001a). These proteins occur solely in sand fly saliva and their exact function is unknown. PpSP32-like proteins were found in the transcriptomes of various sand flies (Valenzuela et al., 2001a, Oliveira et al., 2006, Anderson et al., 2006, Kato et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012, Kato et al., 2012, Rohousova et al., 2012) and sequences coding for these proteins were also found in both *P. orientalis* cDNA libraries (PorASP86/ KC170950; PorMSP15/ KC170964) (Table 1, Table 2). The predicted molecular mass was 25 kDa and the pI was very basic (10.1-10.2). *Phlebotomus orientalis* PpSP32-like proteins were not present in the proteomes (Figure 1). PpSP32-like proteins of AZ and MW colony revealed high degree of identity (98%); high identity was also obtained by comparing *P. orientalis* with other *Larroussius* sand fly species; 85-87% with *P. perniciosus* (ABA43053) and 81-83% with *P. tobbi* (ADJ54102). Glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc) showed a high degree of glycosylation of *P.orientalis* PpSP32-like proteins, which could be potentially responsible for their immunogenicity. Sera of mice experimentally bitten by *P. papatasi* recognized *P. papatasi* SP32 protein (AAL11050) (Vlkova et al., 2012) and human sera from endemic area of cutaneous leishmaniasis in Tunisia reacted strongly with recombinant PpSP32 (Marzouki et al., 2012). On the other hand, recombinant PpSP32-like protein from other sand fly species did not elicit either specific humoral or cellular response (AAX56358, AAS16906) (Oliveira et al., 2006, Xu et al., 2011).

ParSP25-like proteins

Transcripts coding for ParSP25-like proteins were identified in the cDNA library from the AZ (PorASP106/ KC170953) and MW (PorMSP65/ KC170972) *P. orientalis* colonies (Table 1, Table 2). The predicted molecular mass was 27.6 kDa and, due to the high proportion of

698 acidic residues present in the amino acid sequences, the pI was very acidic (4.7-4.8).
699 ParSP25-like proteins were detected in the saliva of sand flies from the subgenus *Larroussius*
700 (*P. ariasi*, *P. perniciosus*, *P. tobbi*), *Adlerius* (*P. arabicus*), and *Phlebotomus* (*P. papatasi*)
701 (Oliveira et al., 2006, Anderson et al., 2006, Hostomska et al., 2009, Rohousova et al., 2012,
702 Abdeladhim et al., 2012). ParSP25-like proteins have not yet been found in New World sand
703 fly species (Charlab et al., 1999, Valenzuela et al., 2004, Kato et al., 2012). The ParSP25-like
704 proteins of AZ and MW colonies are almost identical (98%). Homology of *P. orientalis*
705 proteins with other *Larroussius* species reached 85-86% for *P. tobbi* (ADJ54100), followed
706 by 73-74% for *P. perniciosus* (ABA43056) and 64% for *P. ariasi* (AAX55664). Although the
707 exact function of these proteins remains unknown, some ParSP25-like proteins were
708 demonstrated to be immunogenic. Sera from dogs, hamsters and mice bitten by *P. perniciosus*
709 reacted with salivary protein identified as the member of ParSP25-like family (Vlkova et al.,
710 2011, Martin-Martin et al., 2012). Similar to other sand fly species (Rohousova et al., 2012),
711 ParSP15-like proteins of *P. orientalis* are not predicted to be glycosylated.
712
713 **Lufaxin-like proteins**
714 A 32.4 kDa protein from *L. longipalpis* saliva belongs to a novel family of slow-tight factor
715 Xa inhibitors and displays antithrombotic and antiinflamatory activities and is named Lufaxin
716 (*Lutzomyia longipalpis* Factor Xa inhibitor) (Collin et al., 2012). Members of the Lufaxin
717 family were detected in saliva of various sand flies (Valenzuela et al., 2004, Anderson et al.,
718 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009, Abdeladhim et al., 2012,
719 Kato et al., 2012, Rohousova et al., 2012), but not in other bloodsucking insect. Sequences
720 coding for a Lufaxin-like protein, were detected in the cDNA library of MW *P. orientalis*
721 colony (PorMSP78/ KC170976) (Table 2). Transcripts similar to Lufaxin were also found in
722 AZ colony, but these sequences had low quality scores. The predicted molecular mass of MW

Lufaxin-like protein was 18.8 kDa and the pI was 8.4. *Phlebotomus orientalis* Lufaxin-like protein was not identified in either colony proteome. *Phlebotomus orientalis* Lufaxin-like protein was found to be highly homolougous with *P. perniciosus* (ABA43054) (88% identity) and *P. tobbi* (ADJ54104) (87% identity) Lufaxin-like proteins. According to the glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc), *P. orientalis* Lufaxin-like protein is N-glycosylated.

Lufaxin was previously shown to have antigenic properties. Sera of repeatedly bitten dogs recognized Lufaxin and the Lufaxin homologue from *P. perniciosus* (Teixeira et al., 2010, Vlkova et al., 2011). Recombinant Lufaxin (AAS05319) was also demonstrated to promote strong cellular immunity (Collin et al., 2009, Xu et al., 2011) and therefore was suggested as the promising candidate for vaccine against canine leishmaniasis (Collin et al., 2009).

Other putative salivary proteins

Several other putative salivary proteins were found in both cDNA libraries from *P. orientalis* saliva. Transcripts encoding a 16 kDa salivary protein, with a pI of 5.? and unknown function, were found in the AZ (PorASP150/ KC170956) and MW (PorMSP162/ KC170983) colonies (Table 1, Table 2). PorASP150 and PorMSP162 are closely related to 16 kDa salivary protein A (ACS93506) and protein B (ACS93507) from *P. arabicus* saliva. A high degree of homology was also found with salivary proteins from *P. argentipes* (ABA12153) and *P.sergenti* (ADJ54127). A related protein was recently identified in saliva of *P. papatasi* (ADJ54127). *Phlebotomus orientalis* 16 kDa proteins were not present in proteome and they are likely not glycosylated.

Three clusters, encodingsmall salivary proteins with unknown function, were identified in each *P. orientalis* cDNA library: 3.7 kDa protein (PorASP40/ KC170941; PorMSP169/ KC170984), 4.5 kDa protein (PorASP98/ KC170952; PorMSP104/ KC170980), and 5 kDa protein (PorASP68/ KC170946; PorMSP196/ KC170985) (Table 1, Table 2).

The proteins had small predicted molecular mass (3.9-5.6 kDa) and basic pI (9.2-11.0). None of these salivary proteins were detected in either proteome. *Phlebotomus orientalis* 3.7 kDa protein and 5 kDa protein were found to be closely related to the 3.7 kDa (ADJ54106) and 5 kDa (ADJ54105) *P. tobbi* proteins, respectively. Transcripts coding for a 4.5 kDa *P. orientalis* proteins were share predicted sequence homology with the 4.5 kDa protein of *P. tobbi* (ADJ54097), 7 kDa protein of *P. perniciosus* (ABA43060), and the 5.? kDa protein of *P. ariasi* (AAX55658). Although transcripts of small salivary proteins were also identified in other sand fly salivary gland cDNA libraries, there was no significant homology with *P. orientalis* small salivary proteins. Based on the glycosylation prediction servers (NetNGlyc, NetOGlyc, and NetCGlyc) we found that all *P. orientalis* small salivary proteins are likely O-glycosylated.

Antigens and glycoproteins

To identify the salivary antigens in both *P. orientalis* colonies and the degree of cross-reactivity between them, electrophoretically separated salivary proteins of each colony were incubated with sera from mice experimentally bitten by either the AZ or MW colony. By comparing the western blot analysis with the *P. orientalis* proteomes (Figure 1), we predict that the most intensive reactions detected the yellow-related proteins (AZ: PorASP2, PorASP4; MW: PorMSP23, PorMSP24), apyrases (AZ: PorASP11, PorASP14, PorASP15; MW: PorMSP3, PorMSP4), and antigen 5-related proteins (AZ: PorASP74, PorASP76; MW: PorMSP6, PorMSP8). All these proteins were recognized by all AZ and MW mice sera tested,

while D7-related proteins (AZ: PorASP48, PorASP122; MW: PorMSP28, PorMSP38, PorMSP67) and PpSP15-like proteins (AZ: PorASP28, PorASP37, PorASP61; MW: PorMSP12, PorMSP74, PorMSP96) were recognized only by some sera (Figure 12). Strong cross-reactivity was detected between AZ and MW *P. orientalis* colonies. The small differences in the intensity of reaction or the number of recognized protein bands was probably caused by the individual variability between mice. These data suggest that the salivary proteins in both colonies share similar antibody epitopes. Due to the identical predicted amino acid sequences, we chose only MW colony to study the level of glycosylation of *P. orientalis* saliva. Separated MW salivary proteins were incubated with biotinylated lectins (DBA, SBA, UEA-I, LTA, ConA, PSA) to detect mainly N- and O-glycosylation. To control the specificity of the reactions each lectin was preincubated with the appropriate saccharide inhibitor. The specific reaction was observed only with ConA, the other lectins did not bind specifically or they possess higher affinity for the glycoprotein, than for the saccharide inhibitor. We detected the specific binding of ConA to the protein bands corresponding to the 42 kDa yellow-related protein (PorMSP24), 36 kDa hyaluronidase (PorMSP108), 33 kDa salivary apyrase (PorMSP4), 29 kDa antigen 5-related salivary proteins (PorMSP6, PorMSP8), and 27 kDa D7-related salivary protein (PorMSP67), suggesting that these proteins are N-glycosylated (Figure 13). The strongest reaction was detected with salivary hyaluronidase and yellow-related protein, which indicates that these proteins are the most glycosylated. Our findings confirmed what was found using the NetNGlyc glycosylation prediction server.

Conclusions

Our study provides the first detailed description of the salivary proteins of *P. orientalis*, the important vector of visceral leishmaniasis in NorthEast Africa and SouthWest Asia.

Additionally, we make an important comparison between the salivary gland transcripts of two *P. orientalis* colonies derived from either an endemic focus of *Leishmania donovani* Addis Zemen and non-endemic area Melka Werer from Ethiopia to analyse whether the different epidemiology in these areas is caused by composition of sand fly saliva. Among the *P. orientalis* transcripts, we identified members of 13 main protein families. Most of the transcripts were represented equally in both colonies (Table 1, Table 2) and any absence of transcripts in one of the cDNA libraries was mainly caused by low sequence quality or by the low occurence of these transcripts in the number of randomly sequenced phage . Similarly, the proteomic analysis and apyrase and hyaluronidase assays showed that there is no difference between the sialomes of AZ and MW colonies. These findings were additionaly supported by the western blot analysis, which revealed high degree of cross-reactivity between these two colonies. Comparison of the *P. orientalis* sequences with other sand fly species from the subgenus *Larrousius* showed that *P. orientalis* is closely related to *P. tobbi* and *P. perniciosus*, while *P. ariasi* is likely the evolutionarily more distinct sand fly species.

Sand fly saliva is the critical factor affecting the outcome of *Leishmania* infection in vertebrate hosts. Therefore, the immunogenicity of individual salivary proteins is of intensive scientific interest. To date, the detailed investigation of salivary proteins in certain sand fly species has allowed the generation of individual recombinant salivary proteins that have been employed as reliable markers of *L. longipalpis* exposure (Teixeira et al., 2010, Souza et al., 2010) and were also shown to protect against cutaneous and visceral leishmaniases under laboratory condition (Morris et al., 2001, Valenzuela et al., 2001a, Gomes et al., 2008, Oliveira et al., 2008, da Silva et al., 2011, Tavares et al., 2011, Xu et al., 2011). Furthermore, the first reports of the cross-protectivity between two sand fly species were noticed (Tavares et al., 2011) and the protection was also demonstrated in the unnatural sand fly species –

Leishmania parasite combination (Xu et al., 2011). Thus, these data suggest that there si likely a way how to create the vaccine that could protect against leishmaniasis spread by more sand fly species. Moreover, deeper analysis of salivary proteins of other sand fly species may help to understand mechanism of blood sucking or to find biological activities of many of these novel sequences.

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Figure Legends

Figure 1. Proteomic analysis of salivary gland homogenates from *Phlebotomus orientalis*. *Phlebotomus orientalis* salivary proteins from Addis Zemen (AZ) and Melka Werer (MW) colony were identified using Mass Spectrometry. The name of sequences contained in each protein band and molecular weight in kDa (MW/ kDa) are indicated.

Figure 2. Phylogenetic analysis of the yellow-related family of sand fly salivary proteins. Phylogenetic analysis of yellow-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). The JTT model was used for this phylogenic analysis. Sequence names, GenBank accession numbers and branch values are indicated. Yellow-related salivary proteins from *Larroussius* sand fly species are divided into two distinct clades (Clade I, II).

Figure 3. Multiple sequence alignment of the sand fly yellow-related protein family. Multiple sequence alignment of yellow-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). Sequence names and the number of amino acids per line are indicated. Identical amino acid residues are highlighted black and similar residues grey.

Figure 4. Phylogenetic analysis of the apyrase family of sand fly salivary proteins. Phylogenetic analysis of salivary apyrases from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka

Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). The WAG model was used for this phylogenic analysis. Sequence names, GenBank accession numbers and branch values are indicated. Apyrases from *Larroussius* sand fly species are divided into two distinct clades (Clade I, II).

Figure 5. Multiple sequence alignment of the sand fly apyrase protein family. Multiple sequence alignment of salivary apyrases from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). Sequence names and the number of amino acids per line are indicated. Identical amino acid residues are highlighted black and similar residues grey.

Figure 6. Phylogenetic analysis of the D7-related family of sand fly salivary proteins. Phylogenetic analysis of D7-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). The WAG model was used for this phylogenic analysis. Sequence names, GenBank accession numbers and branch values are indicated. D7-related proteins from *Larroussius* sand fly species are divided into three distinct clades (Clade I - III).

Figure 7. Multiple sequence alignment of the sand fly D7-related protein family. Multiple sequence alignment of D7-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis*

(Lulo). Sequence names and the number of amino acids per line are indicated. Identical amino acid residues are highlighted black and similar residues grey.

Figure 8. Phylogenetic analysis of the PpSP15-like family of sand fly salivary proteins. Phylogenetic analysis of PpSP15-like salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). The JTT model was used for this phylogenic analysis. Sequence names, GenBank accession numbers and branch values are indicated. PpSP15-like proteins from *Larroussius* sand fly species are divided into three distinct clades (Clade I - III).

Figure 9. Multiple sequence alignment of the sand fly PpSP15-like protein family. Multiple sequence alignment of PpSP15-like salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). Sequence names and the number of amino acids per line are indicated. Identical amino acid residues are highlighted black and similar residues grey.

Figure 10. Phylogenetic analysis of the antigen 5-related family of sand fly salivary proteins. Phylogenetic analysis of antigen 5-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). The Dayhoff model was used for this phylogenic analysis. Sequence names, GenBank accession numbers and branch values are indicated. Antigen 5-related proteins from *Larroussius* sand fly species are divided into two distinct clades (Clade I, II).

Figure 11. Multiple sequence alignment of the sand fly antigen 5-related protein family. Multiple sequence alignment of antigen 5-related salivary proteins from *Phlebotomus ariasi* (Pari), *Phlebotomus perniciosus* (Pper), *Phlebotomus orientalis* Addis Zemen colony (PorA), *P. orientalis* Melka Werer colony (PorM), *Phlebotomus tobbi* (Ptob), and *Lutzomyia longipalpis* (Lulo). Sequence names and the number of amino acids per line are indicated. Identical amino acid residues are highlighted black and similar residues grey.

Figure 12. Humoral response to salivary gland antigens of Addis Zemen and Melka Werer *Phlebotomus orientalis* colony. Salivary proteins of Addis Zemen (AZ) and Melka Werer (MW) *P. orientalis* colony were separated under non-reducing conditions by SDS-PAGE electrophoresis. Western blot analysis was performed by two different sera of BALB/c mice experimentally bitten by AZ (lanes 1, 2, and 5, 6) and two sera of mice bitten byMW (lanes 3, 4, and 7, 8) colony. Serum from a naive mouse was used as the negative control (Neg). Molecular weight standard (STD), stained by amido black, labelled with the corresponding molecular weights (kDa).

Figure 13. *Phlebotomus orientalis* salivary gland glycoproteins. Salivary proteins of Melka Werer *P. orientalis* colony (SG) were separated under non-reducing conditions by SDS-PAGE electrophoresis and incubated with biotinylated lectin from *Dolichos biflorus* (DBA), *Glycine max* (SBA), *Ulex europaeus* (UEA-I), *Tetragonolobus purpureas* (LTA), *Canavalia ensiformis* (ConA), and *Pisum sativum* (PSA). Doublets were used to test reactivity of each lectin; the first line represents the reaction of lectin with SG, in the later one the lectins were pre-incubated with the appropriate saccharide inhibitors to prove the specificity of reaction. Molecular weight standard (STD), stained by amido black, labelled with the corresponding molecular weights (kDa).

1365 Table 1. Salivary gland trancripts of *Phlebotomus orientalis* – Addis Zemen colony

Cluster	Sequence name	Accession number	Proteome	MW	pI	Best match to NR protein database		
						Accession number	Species	E-value
PorASP2	42 kDa yellow-related salivary protein	KC170933	Y	41.54	6.09	ABA43049	*Phlebotomus perniciosus*	0.0
PorASP4	42.6 kDa yellow-related salivary protein	KC170934	Y	42.31	8.07	ADJ54080	*Phlebotomus tobbi*	0.0
PorASP11	35.5 kDa salivary apyrase	KC170935	Y	35.53	9.95	ABB00906	*Phlebotomus perniciosus*	0.0
PorASP14	35.2 kDa salivary apyrase	KC170936	Y	35.08	8.99	ADJ54077	*Phlebotomus tobbi*	0.0
PorASP15	35.2 kDa salivary apyrase	KC170937	Y	35.33	9.16	ADJ54077	*Phlebotomus tobbi*	0.0
PorASP28	14.6 kDa PpSP15-like salivary protein	KC170938	Y	14.53	8.88	ADJ54089	*Phlebotomus tobbi*	2e-75
PorASP31	14.4 kDa PpSP15-like salivary protein	KC170939		14.32	8.73	ADJ54088	*Phlebotomus tobbi*	6e-77
PorASP37	14.9 kDa PpSP15-like salivary protein	KC170940	Y	14.91	8.77	ADJ54084	*Phlebotomus tobbi*	3e-73
PorASP40	3.7 kDa-like salivary protein	KC170941		3.93	9.16	ADJ54106	*Phlebotomus tobbi*	2e-07
PorASP46	27 kDa D7-related salivary protein	KC170942		26.68	6.36	ABA43052	*Phlebotomus perniciosus*	4e-151
PorASP48	27.1 kDa D7-related salivary protein	KC170943	Y	26.93	8.26	ADJ54095	*Phlebotomus tobbi*	9e-162
PorASP61	13.8 kDa PpSP15-like salivary protein	KC170944	Y	13.88	9.07	ADJ54086	*Phlebotomus tobbi*	1e-68
PorASP64	14.7 kDa PpSP15-like salivary protein	KC170945		14.70	7.99	ADJ54085	*Phlebotomus tobbi*	8e-62
PorASP68	5.0 kDa-like salivary protein	KC170946		4.89	9.84	ADJ54105	*Phlebotomus tobbi*	5e-15
PorASP74	28.8 kDa antigen 5-related salivary protein	KC170947	Y	28.78	8.94	ADJ54083	*Phlebotomus tobbi*	3e-151
PorASP76	30 kDa antigen 5-related salivary protein	KC170948	Y	28.78	8.94	ABA43055	*Phlebotomus perniciosus*	1e-179
PorASP80	30 kDa salivary phospholipase A2	KC170949		29.66	8.44	ABA43062	*Phlebotomus perniciosus*	0.0
PorASP86	24.53 kDa PpSP32-like salivary protein	KC170950		24.97	10.14	ADJ54102	*Phlebotomus tobbi*	2e-125
PorASP98	4.5 kDa-like salivary protein	KC170952		5.63	10.51	ADJ54097	*Phlebotomus tobbi*	3e-18
PorASP106	38.8 kDa ParSP25-like salivary protein	KC170953		27.61	4.72	ADJ54098	*Phlebotomus tobbi*	1e-140
PorASP112	salivary hyaluronidase	KC170958		37.22	6.50	ACS93505	*Phlebotomus arabicus*	1e-178
PorASP122	27 kDa D7-related salivary protein SP10	KC170954	Y	26.76	9.20	ABA43058	*Phlebotomus perniciosus*	6e-155
PorASP139	41 kDa salivary endonuclease	KC170955		41.66	9.27	ABA43064	*Phlebotomus perniciosus*	0.0
PorASP150	16 kDa salivary protein A	KC170956		16.04	5.04	ACS93506	*Phlebotomus arabicus*	1e-42
PorASP262	47 kDa pyrophosphatase-like salivary protein SP132	KC170959		32.88	7.18	ABA12155	*Phlebotomus argentipes*	8e-163

Putatively secreted salivary proteins from AZ *Phlebotomus orientalis* colony with the number of cluster, GenBank accession number, presence in proteome, putative mature protein features (MW- molecular weight, pI- isoelectric point), and best match to NR protein database.

1416 Table 2. Salivary gland trancripts of *Phlebotomus orientalis* – Melka Werer colony
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Cluster	Sequence name	Accession number	Proteome	MW	pI	Best match to NR protein database		
						Accession number	Species	E-value
PorMSP3	35.5 kDa salivary apyrase	KC170960	Y	35.63	8.83	ABB00906	*Phlebotomus perniciosus*	0.0
PorMSP4	35.2 kDa salivary apyrase	KC170961	Y	33.22	8.89	ADJ54077	*Phlebotomus tobbi*	0.0
PorMSP6	30 kDa antigen 5-related salivary protein	KC170962	Y	28.78	8.94	ABA43055	*Phlebotomus perniciosus*	1e-179
PorMSP8	28.8 kDa antigen 5-related salivary protein	KC170963	Y	28.78	8.94	ADJ54083	*Phlebotomus tobbi*	3e-151
PorMSP12	14.9 kDa PpSP15-like salivary protein	KC170964	Y	14.9	8.77	ADJ54084	*Phlebotomus tobbi*	3e-73
PorMSP15	24.53 kDa PpSP32-like salivary protein	KC170965		25.02	10.24	ADJ54102	*Phlebotomus tobbi*	1e-127
PorMSP23	42 kDa yellow-related salivary protein	KC170966	Y	41.59	6.09	ABA43049	*Phlebotomus perniciosus*	0.0
PorMSP24	42.6 kDa yellow-related salivary protein	KC170967	Y	42.31	8.07	ADJ54080	*Phlebotomus tobbi*	0.0
PorMSP27	putative alpha-amylase	KC170968		33.4	5.75	ACS93490	*Phlebotomus arabicus*	4e-178
PorMSP28	27.0 kDa D7-related salivary protein	KC170969	Y	27.27	7.53	ADJ54096	*Phlebotomus tobbi*	1e-156
PorMSP38	27.1 kDa D7-related salivary protein	KC170970	Y	26.94	8.26	ADJ54095	*Phlebotomus tobbi*	3e-162
PorMSP43	27 kDa D7-related salivary protein SP04B	KC170971		26.7	6.71	ABA43052	*Phlebotomus perniciosus*	1e-151
PorMSP65	38.8 kDa ParSP25-like salivary protein	KC170972		27.56	4.78	ADJ54098	*Phlebotomus tobbi*	6e-140
PorMSP67	27 kDa D7-related salivary protein	KC170973	Y	26.76	9.2	ABA43058	*Phlebotomus perniciosus*	6e-155
PorMSP74	13.8 kDa PpSP15-like salivary protein	KC170974	Y	13.92	9.18	ADJ54086	*Phlebotomus tobbi*	4e-70
PorMSP75	14.7 kDa PpSP15-like salivary protein	KC170975		14.7	7.99	ADJ54085	*Phlebotomus tobbi*	8e-62
PorMSP78	33 kDa salivary lufaxin	KC170976		18.78	8.4	ABA43054	*Phlebotomus perniciosus*	4e-99
PorMSP90	14.4 kDa PpSP15-like salivary protein	KC170977		14.32	8.73	ADJ54088	*Phlebotomus tobbi*	6e-77
PorMSP96	14.6 kDa PpSP15-like salivary protein	KC170978	Y	14.5	8.88	ADJ54089	*Phlebotomus tobbi*	1e-74
PorMSP101	41 kDa salivary endonuclease	KC170979		41.7	9.44	ABA43064	*Phlebotomus perniciosus*	0.0
PorMSP104	4.5 kDa-like salivary protein	KC170980		5.63	10.97	ADJ54097	*Phlebotomus tobbi*	3e-18
PorMSP108	salivary hyaluronidase	KC170981		35.6	7.98	ACS93505	*Phlebotomus arabicus*	2e-163
PorMSP129	30 kDa salivary phospholipase A2	KC170982		29.72	8.31	ABA43062	*Phlebotomus perniciosus*	0.0
PorMSP162	16 kDa salivary protein A	KC170983		15.97	5.04	ACS93506	*Phlebotomus arabicus*	1e-41
PorMSP169	3.7 kDa-like salivary protein	KC170984		3.93	9.16	ADJ54106	*Phlebotomus tobbi*	2e-7
PorMSP196	5.0 kDa-like salivary protein	KC170985		4.97	10.18	ADJ54105	*Phlebotomus tobbi*	2e-14

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Putatively secreted salivary proteins from MW *Phlebotomus orientalis* colony with the number of cluster, GenBank accession number, presence in proteome, putative mature protein features (MW- molecular weight, pI- isoelectric point), and best match to NR protein database.

Table 3. Salivary apyrase in two *P. orientalis* colonies originated from Melka Werer (MW) and Addis Zemen (AZ) Ethiopia

		P. orientalis AZ	*P. orientalis* MW	*P. papatasi*
Total protein in µg/ gland pair		0.61 ± 0.05	0.52 ± 0.06	0.68 ± 0.07
Mean specific apyrase activity* at 37°C, pH 8.5:				
mUnits/ pair of glands**	ATPase	87.9 ± 2.9	74.3 ± 3.9	77.9 ± 5.3
	ADPase	99.3 ± 6.7	84.2 ± 6.7	89.80 ± 6.9
Units/ mg of total protein	ATPase	144	143	115
	ADPase	163	162	132
ATPase/ADPase ratio		0.88	0.88	0,87
pH optimum		8.5 - 9	8.5 - 9	nd
Activator cation		Ca^{2+}	Ca^{2+}	Ca^{2+}

*One unit of enzyme activity is defined as the amount of enzyme that releases one micromole of orthophsphate per minute from the nucleotide substrate at specified assay conditions.
**Individual specific activity was calculated per gland pair as *P. papatasi* is characterized by dissimilar size of salivary glands (Černá *et al.,* 2002).

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Figure 1. Proteomic analysis of salivary gland homogenates from *Phlebotomus orientalis*.

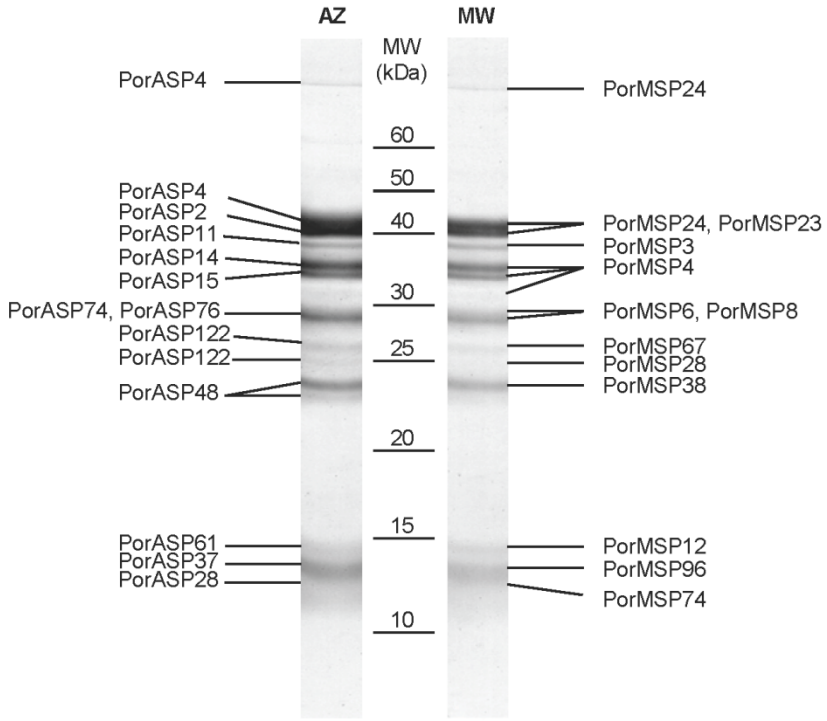


Figure 2. Phylogenetic analysis of the yellow-related family of sand fly salivary proteins.

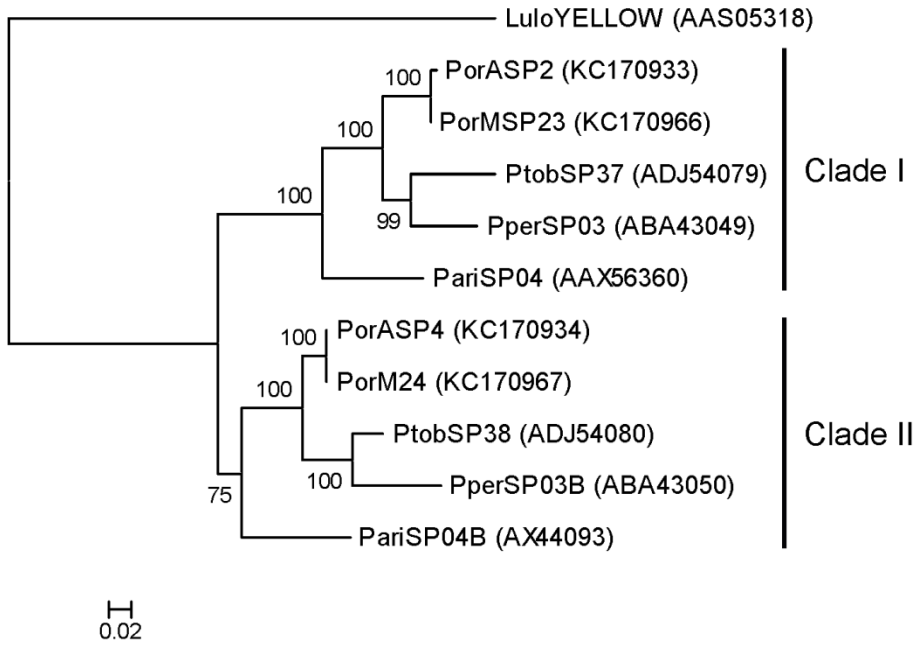


Figure 3. Multiple sequence alignment of the sand fly yellow-related protein family.

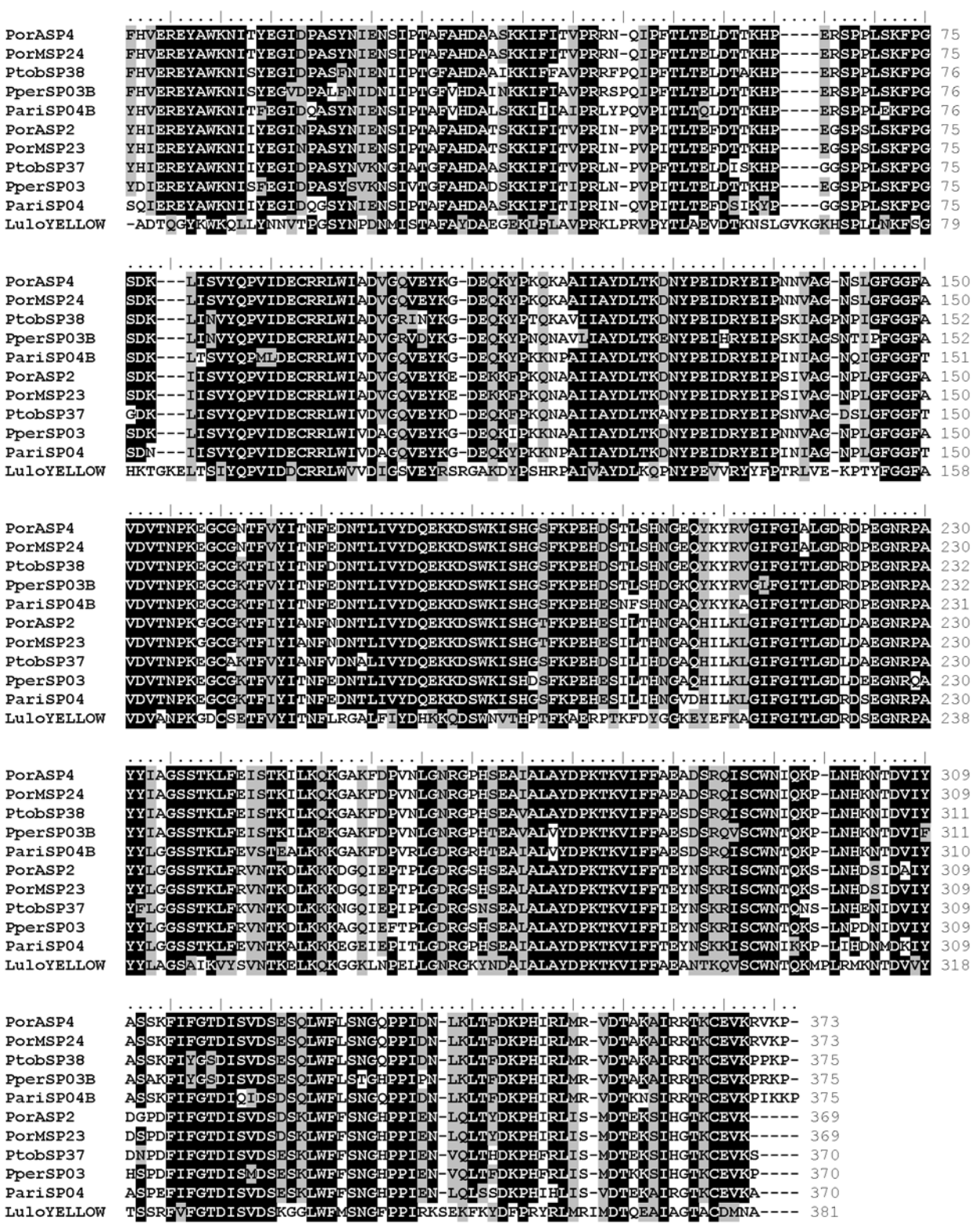


Figure 4. Phylogenetic analysis of the apyrase family of sand fly salivary proteins.

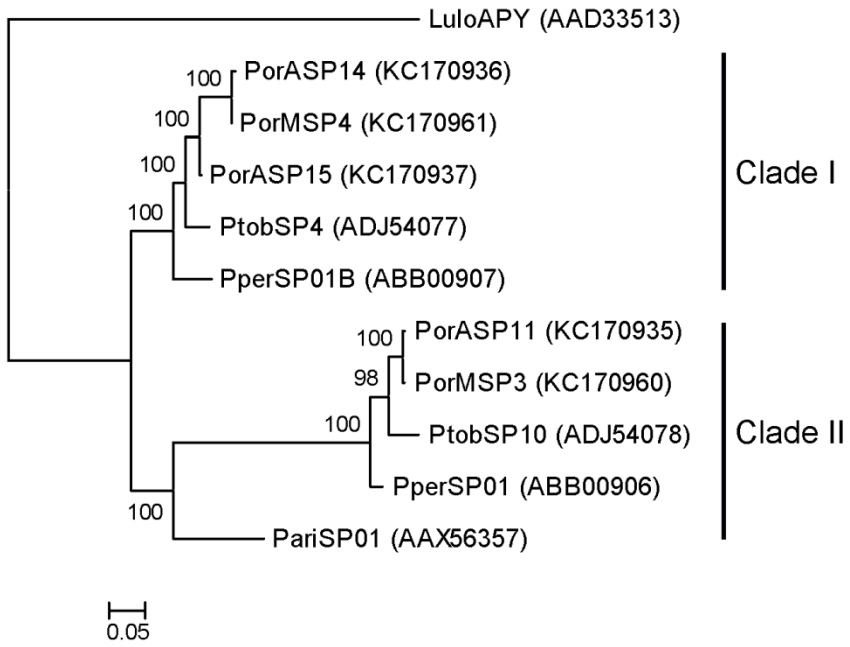


Figure 5. Multiple sequence alignment of the sand fly apyrase protein family.

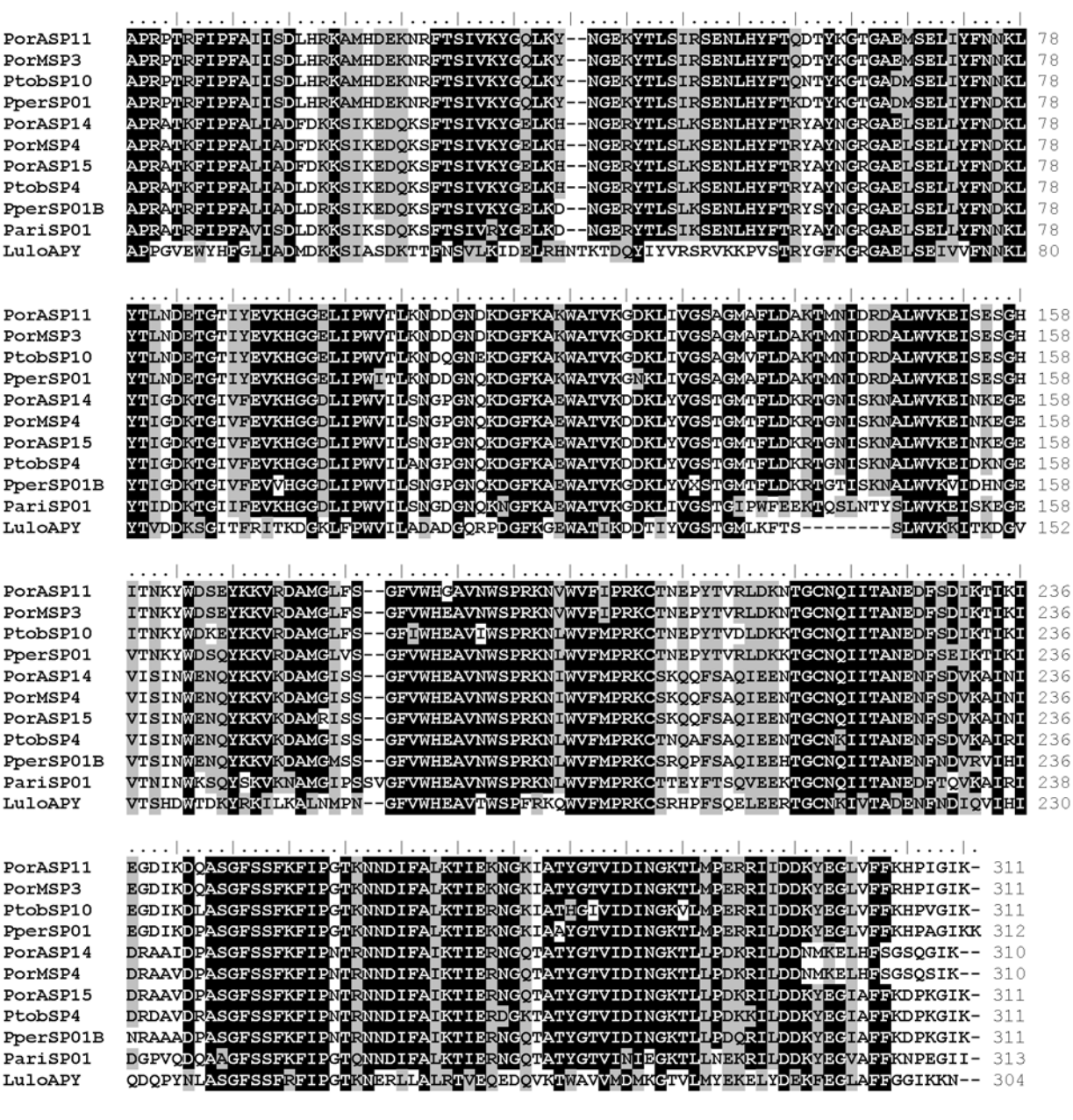


Figure 6. Phylogenetic analysis of the D7-related family of sand fly salivary proteins.

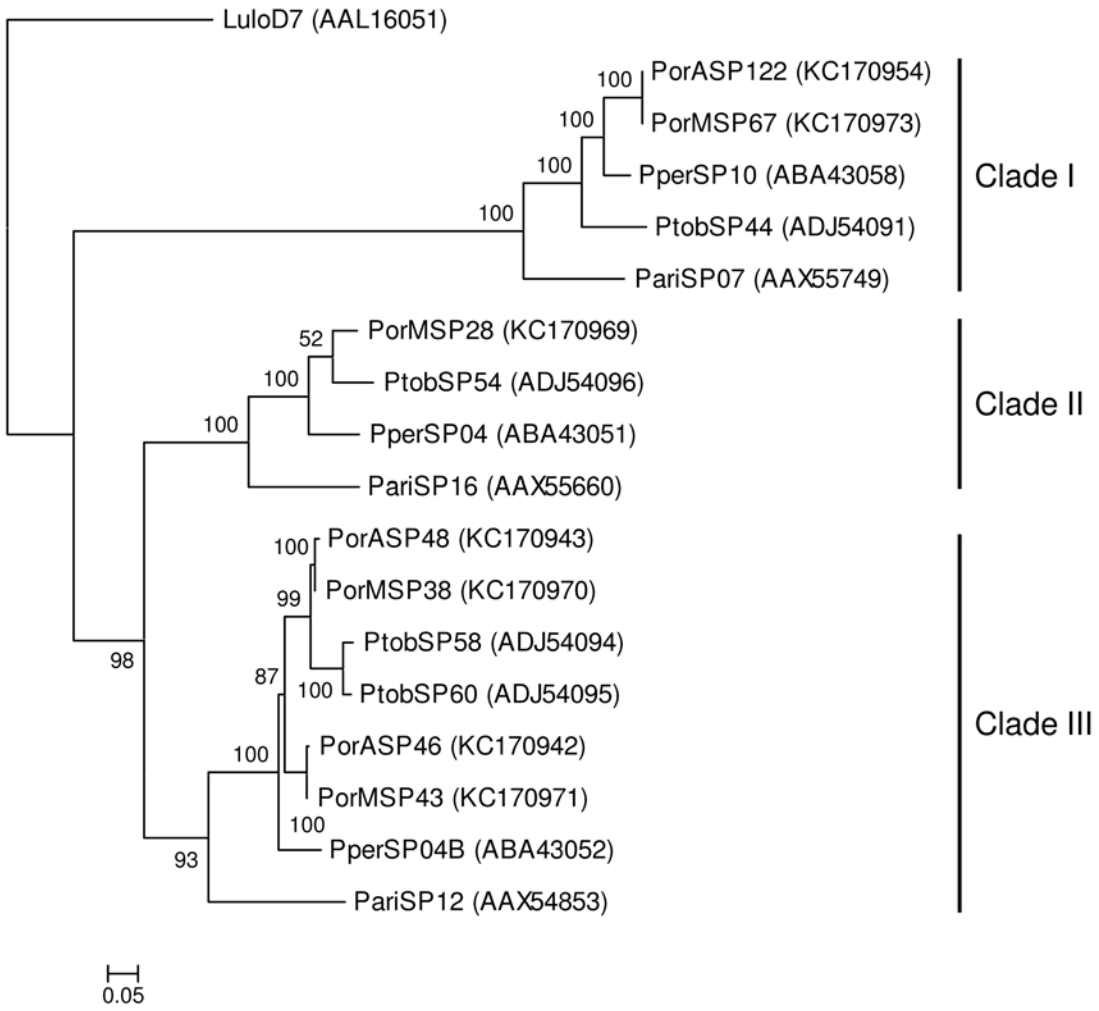


Figure 7. Multiple sequence alignment of the sand fly D7-related protein family.

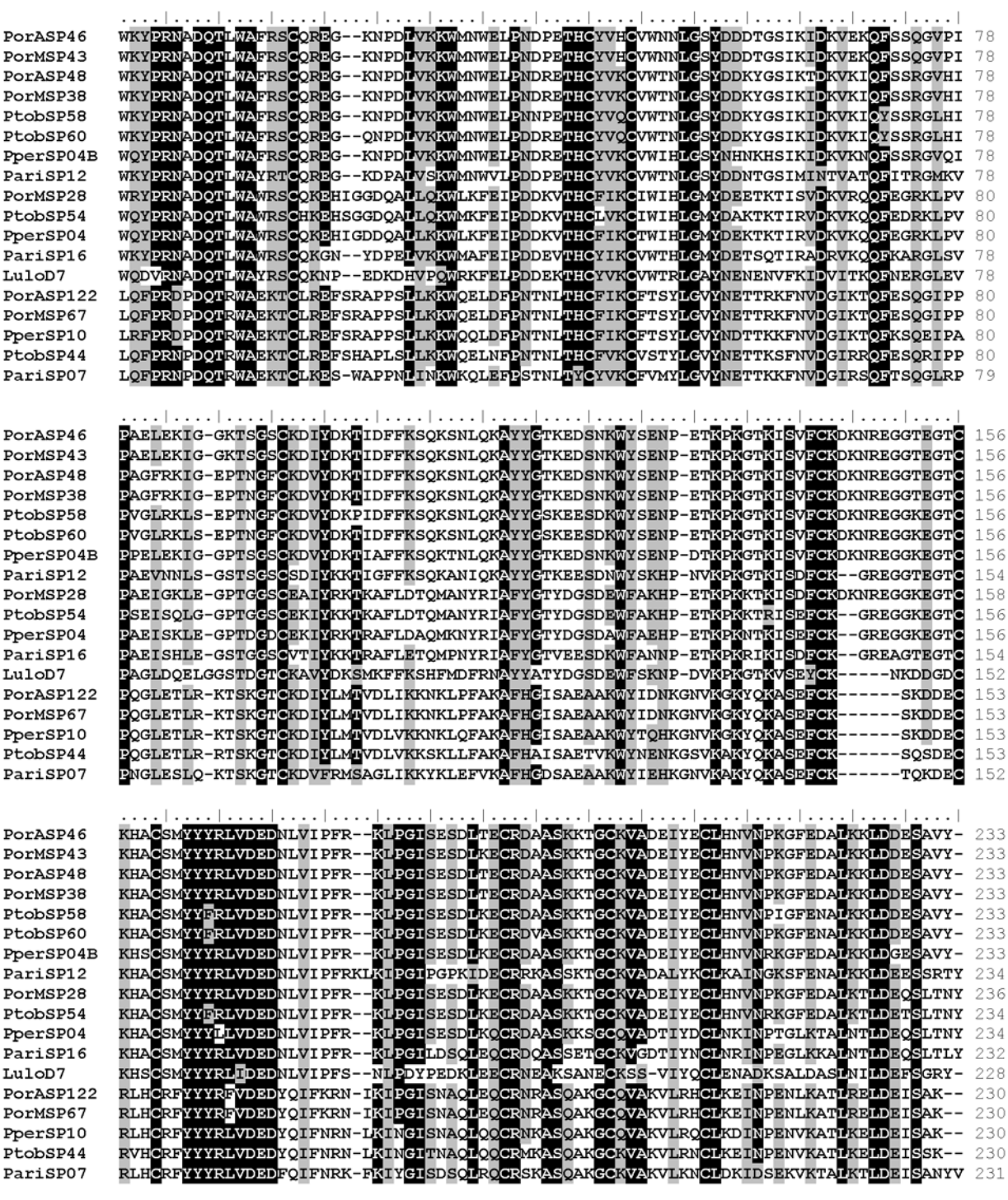


Figure 8. Phylogenetic analysis of the PpSP15-like family of sand fly salivary proteins.

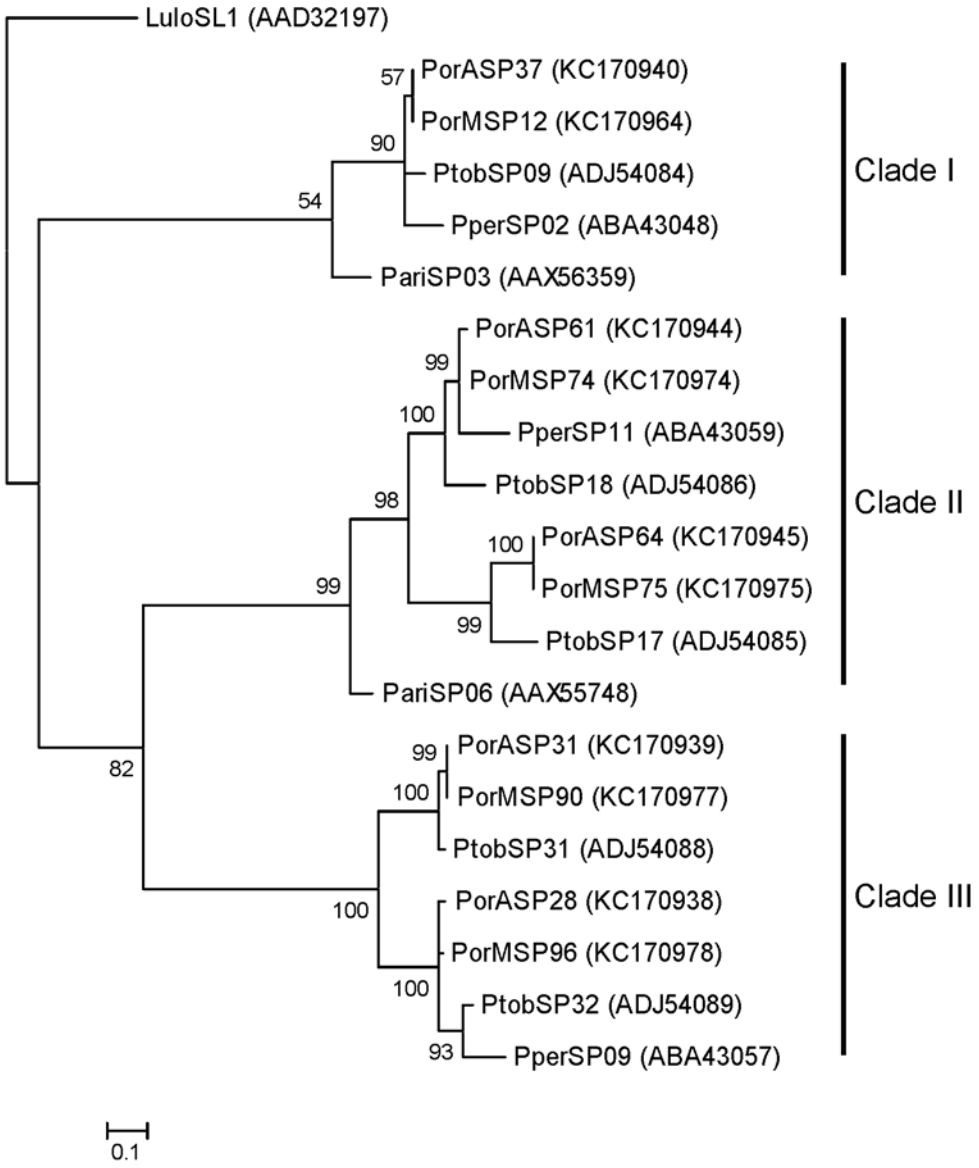


Figure 9. Multiple sequence alignment of the sand fly PpSP15-like protein family.

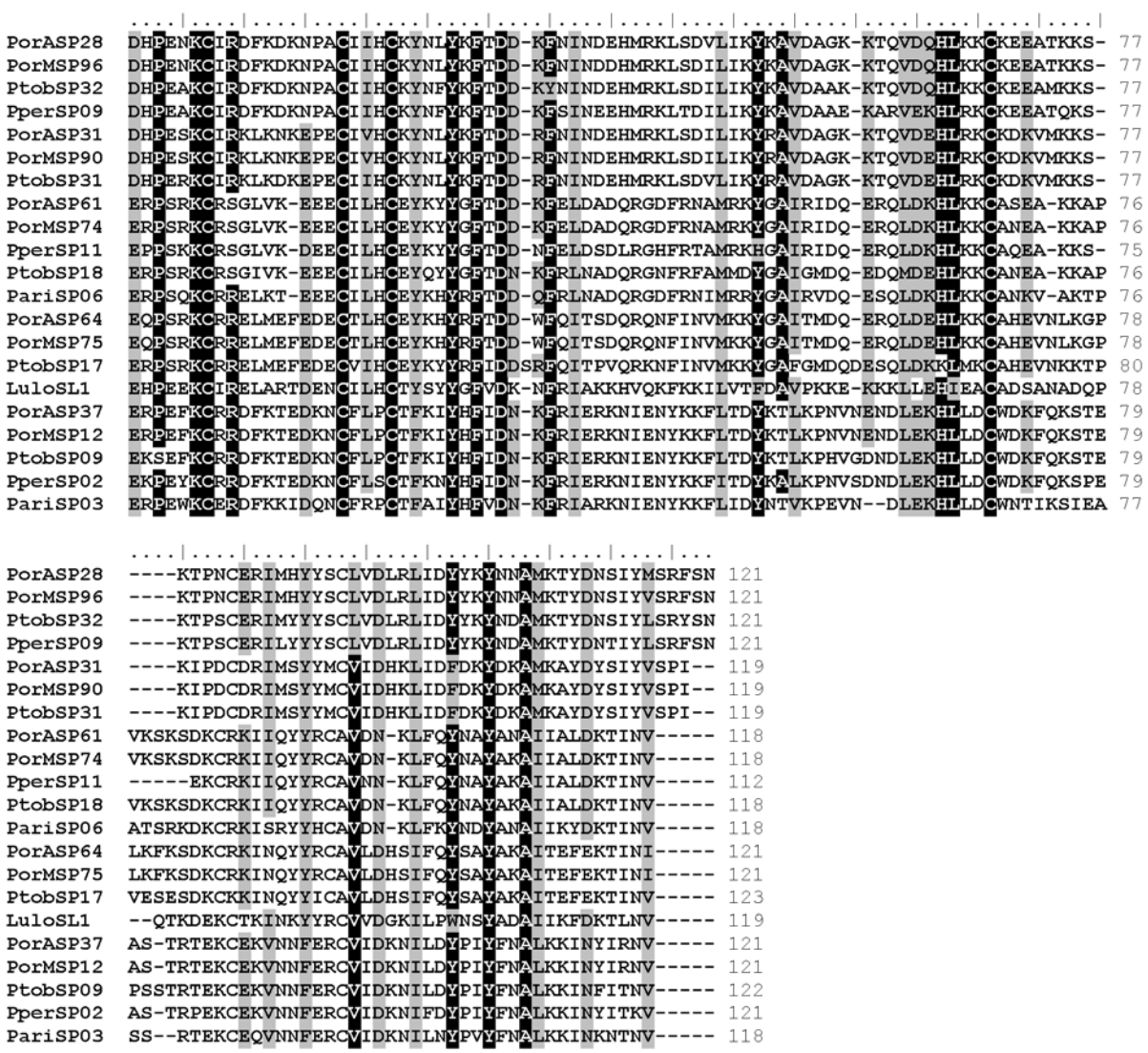


Figure 10. Phylogenetic analysis of the antigen 5-related family of sand fly salivary proteins.

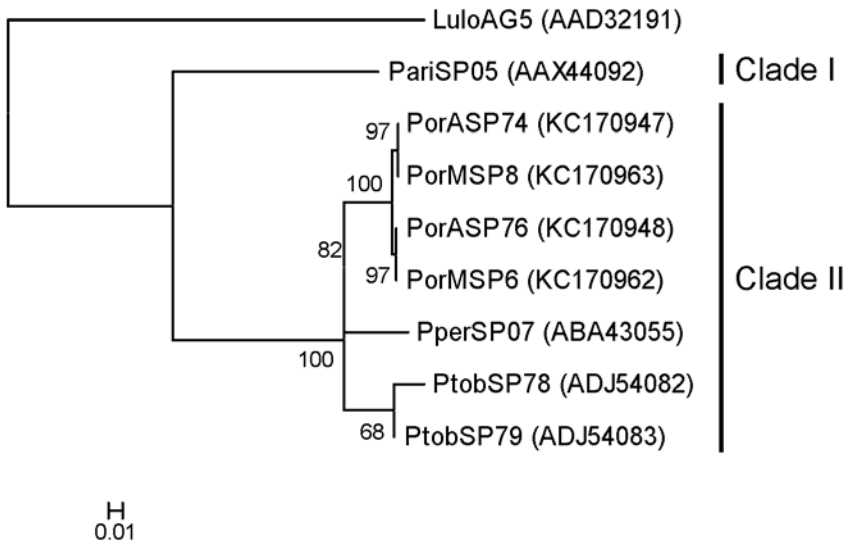


Figure 11. Multiple sequence alignment of the sand fly antigen 5-related protein family.

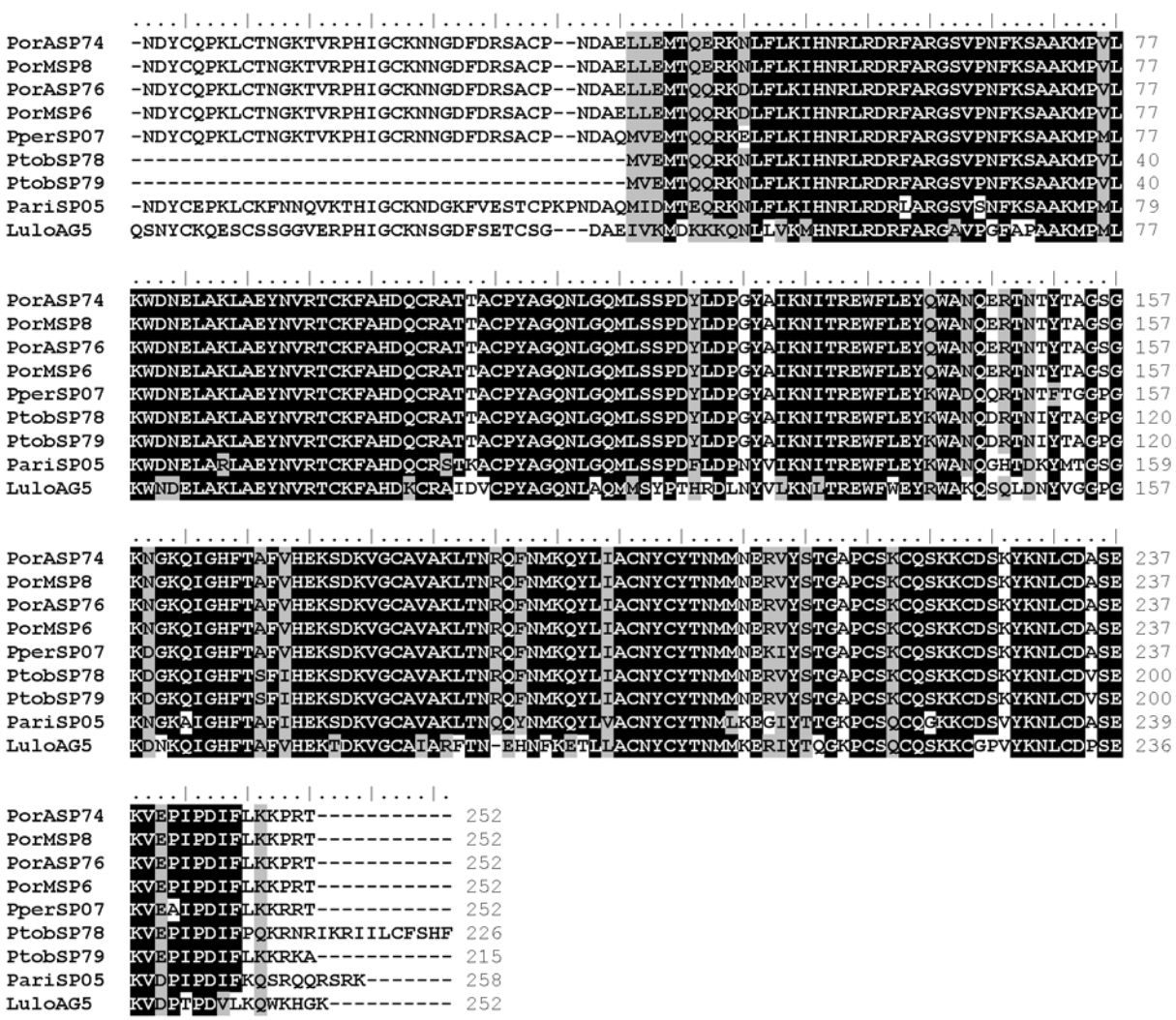


Figure 12. Humoral response to salivary gland antigens of Addis Zemen and Melka Werer *Phlebotomus orientalis* colony.

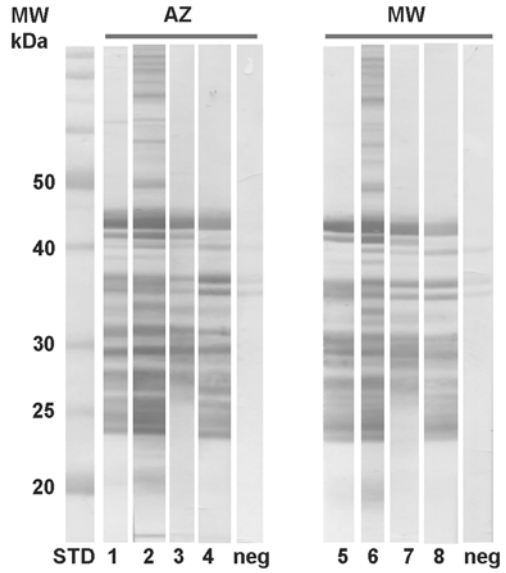
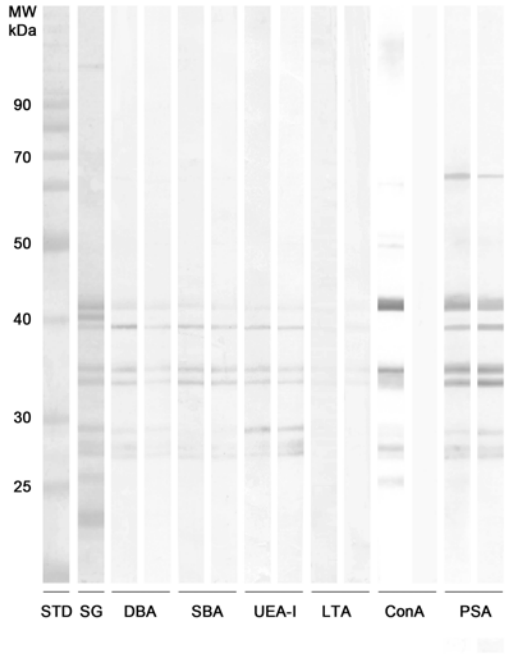


Figure 13. *Phlebotomus orientalis* salivary gland glycoproteins.



Results and Discussion

This thesis is comprised of the results from four projects that I participated on during my PhD study, with the common aim of all projects to deepen the knowledge about the immune aspects of *Leishmania* transmission. The results of the projects together with the integration into the general knowledge are briefly outlined in the following paragraphs.

First of all, we tried to mimic various immunization/ infection schedules commonly arising in the endemic areas with a focus on the feasibility of inducing the desenzitization in mice exposed to sand fly bites for prolonged period. For the first time, we demonstrated the limitations of the protective effect conferred by sand fly bites, firstly in the lenght of immunization before *Leishmania* infection, and secondly in the time interval between immunization and infection. In accordance with previously published data (Belkaid et al., 1998, Kamhawi et al., 2000), we showed that short-term exposure to sand fly bites elicits protective immunity against *Leishmania* infection. In our experiments, BALB/c mice bitten by *Phlebotomus duboscqi* twice in the week interval (short-term exposure) were protected against the subsequent *Le. major* infection; when compared to non-immunized controls, the size of *Leishmania* lesions was significantly reduces as well as the parasite burden within the mice tissues was diminished. Although *Le. major* established slight infection in short-term immunized group, the skin damage was mild or was not observed at all.

In contrast, different course of *Leishmania* infection was observed in mice exposed to *P. duboscqi* bites for fifteen consecutive weeks (long-term exposure). In these mice, the protective effect was completely abrogated; the parasite load as well as the destructiveness and size of *Leishmania* lesions was comparable to that in controls. Desenzitization elicited by the repeated exposure to blood-feeding insect was described in mosquitoes (Peng & Simons, 1998), flies (Benjamini et al., 1961), and triatomines (Sansom et al., 1992), nevertheless, here we demonstrated that similar phenomenon would be also feasible in sand flies. Thus, in the areas endemic for leishmaniasis the long-lasting history of uninfected sand fly bites may result in the non-protective immunity. We can assume that the Th1 DTH immune response to sand fly saliva, commonly accepted of being responsible for the protective effect (Valenzuela et al., 2001a, Oliveira et al., 2008), is suppressed and the immunity is likely polarized toward Th2 humoral profile. The underlying mechanism deserves further investigation; however, our hypothesis was supported by the fact that sera of long-term exposed mice contained a high levels of anti-*P. duboscqi* saliva antibodies suggesting that the severity of skin damage might

be connected with the potential deposition of exacerbative immune complexes within the mice skin. Except for the long-term immunized group and controls, similar immune pattern to *Le. major* infection was also detected in short-term immunized mice, infected with certain delay post immunization; thereby representing the other limitation of the protective effect. When applicated to the endemic areas; hosts bitten/immunized by uninfected sand flies at the end of sand fly season likely loose their protective immunity during the sand fly-free period, and thus become susceptible to *Leishmania* infection in the next season.

In the endemic area of cutaneous leishmaniasis, individuals with active *Leishmania* lesions possessed high levels of anti-sand fly saliva antibodies (Rohousova et al., 2005, de Moura et al., 2007, Marzouki et al., 2011) indicating that specific antibodies could be used as the marker of risk of *Leishmania* transmission. Similarly in our study, sera of mice in those groups, where the immunization schedule failed to protect against cutaneous leishmaniasis (except controls), revealed increased levels of anti-*P. duboscqi* antibodies. Moreover, these antibodies positively correlated with the parasite load in *Leishmania* lesions.

Although the laboratory experiments outline the factors that could affect the circulation of leishmaniasis in endemic regions, the immune response elicited by immunization under laboratory conditions and by the continuous exposure in leishmaniasis foci may differ. Indeed, the responsiveness of each host in the endemic area is influenced by many factors, such as genetic background, various enviromental factors or co-infections. The good example might be recent study of Oliveira et al. (2013), where they demonstrated that humans from an endemic focus of cutaneous leishmaniasis in Mali bitten by *P. duboscqi* produced several profiles of immunity, ranging from solely Th1 profile, through the combined Th1/ Th2 immunity to the Th2-polarized response (Oliveira et al., 2013).

In the other projects, we studied the specific immune response to sand fly saliva in dogs and rodents, the potential reservoir hosts of zoonotic visceral and cutaneous leishmaniasis, respectively. Furthermore, we tested whether the anti-saliva antibodies could be used as the marker of sand fly exposure and subsequently as a tool to evaluate the risk of *Leishmania* transmission. For our experiments, we choose dogs exposed to *P. perniciosus* bites under laboratory conditions as well as dogs bitten naturally in the endemic focus of *Le. infantum* in Italy. For the mouse study, we used BALB/c and C57BL/6 strains representing the widely accepted animal models sensitive and resistant to *Leishmania* infection, respectively (e.g. Belkaid et al., 1998, Kamhawi et al., 2000).

We demonstrated that repeated sand fly feeding elicits production of specific antibodies in dogs bitten by *P. perniciosus* as well as in mice exposed to *P. papatasi* bites. In

experimentally bitten dogs, we detected significantly increased levels of specific IgG, IgG1, and IgG2 that persisted in canine sera for more than six weeks after the last exposure. In accordance with previously published data (Hostomska et al., 2008, Collin et al., 2009), IgG2 was the predominant IgG subclass in immunized dogs and the levels of specific antibodies positively correlated with the intensity of *P. perniciosus* exposure. In our field trial, a significant increase of specific antibodies was detected in sera of *Leishmania*-positive and *Leishmania*-negative dogs as the sand fly season progressed. Importantly, when compared to the healthy dogs, *Leishmania*-positive animals revealed significantly lower levels of anti-*P. perniciosus* IgG2 and the IgG1/IgG2 ratio was significantly higher in these dogs.

There are two possible explanations for these findings. The first one that, similarly as in human studies from endemic foci of visceral leishmaniasis (Barral et al., 2000, Gomes et al., 2002, Aquino et al., 2010), the canine humoral response correlates with the protective cell-mediated immunity and thus animals with low levels of specific IgG2 are in the higher risk of being infected. The later one, that the *Leishmania* infection may decrease the production of IgG2 in bitten dogs. As IFN-γ was shown to positively correlate with the protective Th1 immunity (Collin et al., 2009) and, although not significantly, we detected higher levels of IFN-γ in *Leishmania*-negative dogs, thus we can speculate that the first hypothesis is more likely.

In experimentally bitten mice, exposure to *P. papatasi* bites led to a strong production of specific IgG and IgG1 in both mice strains, and additionally slightly increased the levels of specific IgG2b in BALB/c mice. These antibodies persisted in elevated levels in sera of BALB/c as well as C57BL/6 mice for more than five months after the last exposure. As the predominance of IgG1 subclass is the marker of Th2 profile of immune response in mice (Mosmann & Coffman, 1989), our results suggest that *P. papatasi* bites push the immune response toward the Th2 profile, regardless the mice strain. One of the possible explanations might be presence of adenosin in *P. papatasi* saliva, as this salivary compound was described to have powerful Th2-bias effect (Hasko et al., 1996, Hasko et al., 2000). However, saliva of *P. ariasi* or *L. longipalpis* also drives preferentially the production of specific IgG1 (Silva et al., 2005, Oliveira et al., 2006). These data indicates that saliva of more sand fly species polarizes the immune response to Th2 immunity. Furthermore, the presence of slightly elevated levels of specific IgG2b solely in BALB/c mice might be attributed to the different cytokine responsiveness to sand fly saliva in various mice strains, as switch to IgG2b subclass is initialized by production of TGF-β (Nimmerjahn & Ravetch, 2005).

Using the sera of dogs and mice bitten by sand flies, we looked into the most antigenic salivary proteins of *P. perniciosus* and *P. papatasi*, respectively. Specific antibodies in sera of experimentally bitten dogs recognized up to 11 salivary protein bands, where the most intensive reaction was achieved with the 42 kDa yellow-related protein (DQ150622) and 33 kDa apyrase (DQ192491). It should be pointed out that these two antigens represented also the most potent targets for specific antibodies in sera of dogs residing in endemic area, regardless of *Leishmania*-positivity. Later on, the same *P. perniciosus* salivary proteins were ascertained among the most antigenic spots, when the reactivity of mice and hamsters sera was tested using two-dimensional gel electrophoresis (Martin-Martin et al., 2012). In both strains of mice bitten by *P. papatasi*, the most intensive reaction was detected with the 30 kDa salivary band comprised of 30 kDa D7-related salivary protein (DQ205724) and 29 kDa antigen 5-related protein (AF335488). In parallel, *P. papatasi* 30 kDa salivary protein was also found to be the most antigenic for humans from endemic areas of cutaneous leishmaniasis (Rohousova et al., 2005, Marzouki et al., 2011).

In our experiments we tested the reactivity of four bacterially-expressed *P. papatasi* salivary proteins (two yellow-related proteins: PpSP44/AF335492, PpSP42/AF335491; two D7-related proteins: PpSP30/AF335489, PpSP28/AF335488) using sera of mice repeatedly bitten by *P. papatasi*. The most intensive reaction was detected with the PpSP30 protein, although, this protein was not recognized by all sera tested. None of the recombinant proteins was recognized by all mice sera, nevertheless, we can speculate that the combination of these salivary proteins could replace the salivary gland homogenate. This concept was successfully employed in experiments of Souza et al. (2010), where they showed that the combination of two recombinant *L. longipalpis* yellow-related proteins (AAS05318, AAD32198) is the full-fledged substitution of whole salivary glands (Souza et al., 2010). Our data proved that use of recombinant proteins instead of the sand fly salivary homogenate could sort out the limitations connected with maintainance of sand fly colonies and dissection of the salivary glands.

It was previously shown that *L. longipalpis* colonies originating from distinct areas of New World differ in the composition of saliva which may affect the pathology of leishmaniasis (Warburg et al., 1994). Therefore, we tried to find out whether the different epidemiology of visceral leishmaniasis observed in the Ethiopian non-endemic area Melka Werer and endemic focus of *Le. donovani* Addis Zemen is connected with differences in salivary proteins of the local *P. orientalis* colonies. Hence we constructed salivary gland

cDNA libraries of Melka Werer and Addis Zemen *P. orientalis* colonies and we analyzed their transcriptomes, proteomes and antigenicity of their salivary proteins.

In both colonies, we identified members of 13 main protein families: apyrase, yellow-related protein, antigen 5-related protein, odorant-binding proteins, hyaluronidase, endonuclease, phospholipase, pyrophosphatase, amylase, PpSP32-like protein, ParSP25-like protein, SP16-like protein, and lufaxin (SP34-like protein). We did not found any difference in transcriptoms, proteoms or in the enzymatic activity of salivary apyrase and hyaluronidase between *P. orientalis* colonies. Moreover, we showed that there is strong cross-reactivity in humoral response to Addis Zemen and Melka Werer saliva. When compared western blot analysis with *P. orientalis* proteoms, we can assume that in both colonies the most antigenic salivary proteins are yellow-related proteins (from Addis Zemen colony: PorASP2, PorASP4; from Melka Werer colony: PorMSP23, PorMSP24), apyrases (from Addis Zemen colony: PorASP11, PorASP14, PorASP15; from Melka Werer colony: PorMSP3, PorMSP4), and antigen 5-related proteins (from Addis Zemen colony: PorASP74, PorASP76; from Melka Werer colony: PorMSP6, PorMSP8).

Taken together, we demonstrated that the composition of salivary proteins as well as antigenic properties of both *P. orientalis* colonies are identical. Therefore, *P. orientalis* saliva is not the critical factor affecting the different epidemiology of the visceral leishmaniosis in Melka Werer and Addis Zemen. Thus, we can speculate that there are likely other factors being responsible for the endemicity of leishmaniasis in one area and non-endemicity in the other, for example differences in the presence of potential reservoir hosts or in the susceptibility of local human population.

In conclusion, our study proved that, in endemic areas, screening of the specific humoral response in hosts exposed to sand fly bites provide the valuable informations. Dogs and wild rodents represent the main reservoir hosts for zoonotic visceral and cutaneous leishmaniasis, respectively. However, sand flies also feed frequently on livestock and some studies suggest that the domestic animals could be employed in the *Leishmania donovani* life cycle as well (e.g. Gebre-Michael et al., 2010). Thus, screening of sera of domestic animals from endemic areas may help to indicate feeding preferences of vectors and evaluate the effectiveness of anti-vector campaigns. Moreover, in humans and dogs, the specific humoral response might serve as the marker of risk of *Leishmania* transmission. Our data also suggests that, for a broader use, whole salivary glands could be replaced by the combination of

recombinant salivary proteins, thereby solve the limitations stated by rearing of sand flies and dissections of their salivary glands.

Furthermore, we demonstrated that several salivary proteins are highly immunogenic for a broader scale of hosts. Deeper analysis of the individual salivary antigens could contribute to the research on anti-*Leishmania* vaccine. Many studies identified the potential candidates, proteins which successfully protected immunized hosts against cutaneous as well as visceral leishmaniasis; some even indicated the feasibility of cross-protectivity. However, the protective immunity elicited under laboratory conditions might fail in the endemic areas, where the outcome of *Leishmania* infection is affected by many enviromental factors and co-infections. Thus, the future studies should be focused on better understanding of the immune aspects that could influence *Leishmania* transmission and detailed knowledge about the immunity stimulated by individual salivary proteins.

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