CHARLES UNIVERSITY

FACULTY OF SCIENCE

UNIVERZITA KARLOVA

PŘÍRODOVĚDECKÁ FAKULTA

Ph.D. study program: Parasitology

Doktorský studijní program: Parazitologie

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Characterization and antigenic properties of salivary yellow-related proteins in phlebotomine sand flies

Charakterizace a antigenní vlastnosti yellow-related proteinů ze slinných žláz flebotomů

Ph.D. thesis / Disertační práce

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Prague / Praha, 2019

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Prague, April 16th, 2019
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I declare that Petra Cikrtová substantially contributed to the experimental work in four projects presented in her Ph.D. thesis and that she had a principal role in the writing of one publication and one manuscript presented.

Prohlašuji, že se Petra Cikrtová významně podílela na experimentální práci čtyř projektů shrnutých v této dizertační práci a je hlavní autorkou textu jedné uvedené publikace a jednoho rukopisu.

Prague, April 16th, 2019
Praha, 16. dubna 2019

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Prof. RNDr. Petr Volf, CSc.

ACKNOWLEDGMENTS / PODĚKOVÁNÍ

First and foremost, I would like to thank my supervisor Petr Volf for offering me a very interesting Ph.D. topic and for all his help, advices and support throughout my studies in his laboratory.

I would like to express my gratitude to Jesus G. Valenzuela for the opportunity to spend three months in his laboratory at National Institutes of Health in the United States and to the members of his lab for making it a great experience.

Thanks are due to my former and present colleagues from our lab. I wish to thank Táňa Spitzová, Terka Leštinová and Michal Šíma for a great time when working on the papers included in this study. I am grateful to Laura Willen, Nikola Polanská, Kristýna Hlavačková and Jana Glogarová for creating a friendly atmosphere in the lab and for further amusing me outside it. Special thanks are also due to my former colleague Blanka Ferencová and her baby Honzík, for lifting my spirits every time I see them.

Last but not least, I would like to thank my husband Michal and my parents for being patient and supportive through the whole period of my studies.

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LIST OF ABBREVIATIONS

CL	canine leishmaniasis
D.	*Drosophila*
DTH	delayed type hypersensitivity
E.	*Escherichia*
ELISA	enzyme-linked immunosorbent assay
HEK293	human embryonic kidney 293 cells
IFN-γ	interferon-γ
IgG	immunoglobulin G
IL-12	interleukin-12
L.	*Lutzomyia*
La.	*Larroussius*
Le.	*Leishmania*
MRJP	major royal jelly protein
MST	microscale thermophoresis
N.	*Nyssomyia*
NCBI	National Center for Biotechnology Information
P.	*Phlebotomus*
PDB	Protein Data Bank
S.	*Sergentomyia*
Sy.	*Synphlebotomus*
SGH	salivary gland homogenate
VL	visceral leishmaniasis
Th1	T-cell helper type 1
TNF-α	tumor necrosis factor-α
YRP	yellow-related protein

ABSTRACT

Yellow-related proteins (YRPs) form an abundant protein family, whose members were found in salivary gland transcriptomes of all sand fly species studied up to date. This protein family belongs to the group of MRJP/Yellow proteins occurring in insects and some other organisms. Though sharing similar folding as a six-bladed β-propeller comprising central tunnel, MRJP/Yellow proteins adopted different functions. The structure and biogenic amine-binding property described for the sand fly salivary YRPs was based on a single study conducted on *Lutzomyia longipalpis*.

In the present work, we have modelled the structures of 32 salivary YRPs belonging to 13 different sand fly species. We have shown the general structural similarity of these proteins along with both inter- and intra-specific differences in surface charge, tunnel parameters and in amino acids composition of the amine-binding motif. These modifications indicated divergence in function of individual YRPs, which was experimentally verified in the second project focused on identification of the amine-binding properties of YRPs in two important vectors of *Leishmania*; *Phlebotomus perniciosus* and *P. orientalis*. In each species, two YRPs differ in affinities for biogenic amines serotonin, histamine and catecholamines. However, in both sand fly species the highly abundant YRPs (rSP03B and rPorASP4, respectively) were demonstrated to bind with high-affinity serotonin. This suggested their potential ability to facilitate inhibition of the vasoconstriction and platelet aggregation at the fly feeding site.

Apart from their effect on haemostatic responses, the very same YRPs of *P. perniciosus* and *P. orientalis* were shown to elicit antibody response in bitten hosts, which can be used as a marker of sand fly exposure. In *P. perniciosus*, we have applied the recombinant YRP (rSP03B) to screen for canine exposure in a longitudinal field study in Italy. We have detected a high correlation between the canine anti-rSP03B antibody response and the response against the whole salivary glands. The kinetics of antibody response measured by both antigens revealed a comparable pattern related to the annual activity of *P. perniciosus*. Protein rSP03B was therefore shown as a valid tool to screen for canine exposure. Similarly, in *P. orientalis* a recombinant YRP (rPorASP4) was shown strongly antigenic for humans exposed to this sand fly in Sudan and Ethiopia. The antibody response against rPorASP4 correlated well with the response against the whole salivary glands. Combination of this protein with another antigen (antigen 5-related protein) significantly increased the performance of the test, and therefore represents the best tool to screen for human exposure to *P. orientalis* in Eastern Africa.

ABSTRAKT

Yellow-related proteiny (YRP) tvoří početnou rodinu proteinů, jejíž členové byli identifikováni v transkriptomech slinných žláz všech dosud studovaných druhů flebotomů. Tato rodina je zařazena do skupiny tzv. MRJP/Yellow proteinů, které se vyskytují u hmyzu i některých dalších organismů. Přestože tyto proteiny sdílejí podobnou terciární strukturu tvořenou šestiramenným beta-propelerem s centrálně umístěným tunelem, liší se ve svých funkcích. Struktura a amin-vázající vlastnosti YRP flebotomů byly popsány pouze na základě jediné studie u druhu *Lutzomyia longipalpis*.

V naší práci jsme namodelovali struktury celkem 32 YRP ze slinných žláz 13 druhů flebotomů. Zatímco celkový tvar a struktura všech proteinů byla obdobná, jednotlivé YRP se mezi druhy i v rámci jednoho druhu odlišovaly zejména v povrchovém náboji, vlastnostech tunelu a v aminokyselinovém složení vazebného místa pro biogenní aminy. Tyto modifikace ukazují na rozdílné funkce jednotlivých proteinů, jež byly experimentálně potvrzeny ve druhé části našeho projektu. Zaměřili jsme se na vazebné vlastnosti YRP druhů *Phlebotomus perniciosus* a *P. orientalis*, kteří jsou důležitými přenašeči prvoků rodu *Leishmania*. Síla vazby pro serotonin, histamin a katecholaminy se lišila jak mezi druhy, tak v rámci obou proteinů každého druhu. Přes tyto odlišnosti byla u jednoho YRP *P. perniciosus* (rSP03B) i *P. orientalis* (rPorASP4) prokázána schopnost vázat s vysokou afinitou serotonin.

Navíc jsme zjistily, že tytéž YRP obou studovaných druhů flebotomů vyvolávají u pobodaných hostitelů specifickou protilátkovou odpověď, která koreluje s mírou pobodání flebotomy. Rekombinantní YRP (rSP03B) *P. perniciosus* jsme využili ke stanovení míry expozice *P. perniciosus* u psů dlouhodobě sledovaných v endemické oblasti Itálie. Psí protilátková odpověď proti rSP03B silně korelovala s odpovědí naměřenou proti celým slinným žlázám. Kinetika protilátkové odpovědi stanovená pomocí obou antigenů vykazovala srovnatelnou křivku odpovídající sezónní aktivitě *P. perniciosus*. Protein rSP03B byl tedy prokázán jako platný nástroj pro stanovení expozice flebotomům u psů. Podobně u druhu *P. orientalis* byl rekombinantní YRP (rPorASP4) silně antigenní pro lidi vystavené pobodání tímto druhem flebotoma v Súdánu a Etiopii. Protilátková odpověď proti rPorASP4 korelovala s odpovědí na celé slinné žlázy. Kombinace proteinu rPorASP4 s dalším antigenním proteinem (antigen 5-related proteinem) dosáhla signifikantně vyšší korelace i dalších statistických veličin a je tak vhodným nástrojem pro měření míry expozice *P. orientalis* u lidí ve východní Africe.

INTRODUCTION

We have focused on structure, function and antigenic properties of salivary yellow-related proteins in two medically and veterinary important sand flies species, *Phlebotomus (Larroussius) perniciosus* and *P. (La.) orientalis*, which are both major Old-World vectors of *Leishmania donovani* complex (Kinetoplastida: Trypanosomatidae) causing visceral leishmaniases (VL) (reviewed in Dvorak et al., 2018).

The VL (also known as kala-azar) caused by *Le. donovani* is generally assumed to be mainly anthroponotic with the most endemic sites located in Indian subcontinent and in Eastern Africa (Elnaiem, 2011; Ready, 2014). Eastern Africa has a second largest numbers of human VL cases with high mortality rate and the incidence reaching 57,000 cases (Alvar et al., 2012). *Le. donovani* is focally endemic in Sudan, Ethiopia, South Sudan and Kenya, and it is transmitted by *P. orientalis*, *P. (Synphlebotomus) martini* and *P. (Sy.) celiae* (Dvorak et al., 2018; Elnaiem, 2011).

In this thesis, we have focused on two countries with highest reported incidence of VL in Eastern Africa; Sudan and Ethiopia (Alvar et al., 2012). In Sudan and northern Ethiopia, *P. orientalis* is an abundant sand fly species serving as a predominant vector of *Le. donovani* (Elnaiem, 2011; Gebresilassie et al., 2015a; Widaa et al., 2012). *P. orientalis* was shown to be exophilic and to feed preferentially on bovines, followed by other domestic and wild animals and humans (Gebre-Michael et al., 2010; Gebresilassie et al., 2015b, 2015c; Kirstein et al., 2018; Yared et al., 2017). The distribution of *P. orientalis* in Eastern Africa was associated with *Acacia sp.* and *Balanites sp.* trees which are growing on vertisols, whose cracks form the *P. orientalis* breeding sites (Kirstein et al., 2018).

Leishmania infantum, a second *Leishmania* species belonging to *L. donovani* complex, is the causative agent of potentially fatal canine leishmaniasis (CL) and human zoonotic leishmaniasis spread in regions of Europe, Africa, Asia and America. Domestic dogs serve as the main hosts and also the main reservoirs for both diseases, therefore the early detection of infected dogs is essential to impair transmission of *Le. infantum* to humans. The symptoms of systemic CL vary from subclinical to very severe, while both symptomatic and asymptomatic dogs were shown to contribute to the parasite transmission cycle (reviewed in Baneth et al., 2008; Maia & Campino, 2018; Solano-Gallego et al., 2011). In the Mediterranean basin, an annual incidence of human zoonotic leishmaniasis reaches 2000 cases in humans (Alvar et al., 2012), while the prevalence of CL was estimated to 2.5 million cases (Moreno & Alvar, 2002). *Le. infantum* is in this area transmitted by up to

14 sand fly species of subgenus *Larroussius*. In western and central Mediterranean region, the proven vectors are *P. perniciosus, P. (La.) ariasi*, *P. (La.) neglectus* and *P. (La.) perfiliewi* (Dvorak et al., 2018; Maroli et al., 1987).

In this thesis, we have focused on Italy, where *P. perniciosus* is the most widespread vector of *Le. infantum*. In comparison with France, Portugal and Spain, where *P. perniciosus* also serves as a vector, Italy has the highest median seroprevalence of CL (Franco et al., 2011; Maroli et al., 2013). *P. perniciosus* was shown as an opportunistic feeder, feeding both on humans, dogs and other domestic animals (Bongiorno et al., 2003; Rossi et al., 2008). It is present mainly at lower altitudes (100-300 m above the sea level) with temperate climate and high winter vegetation cover (Dvorak et al., 2018; Signorini et al., 2014).

To decrease the incidence of VL in both Mediterranean and Eastern Africa, vector control strategies adapted to different reservoir hosts and epidemiology of these diseases should be employed. To evaluate their efficiency, it is important to screen the hosts for exposure to vector sand fly species. The exposure to vectors could be determined by measuring hosts antibodies elicited against specific salivary antigens. To screen for these antibodies, either the salivary gland homogenate (SGH) or individual salivary proteins could be used (reviewed in Lestinova et al., 2017). This approach using SGH was already utilized to evaluate the effectiveness of insecticide-treated nets for humans exposed to *P. (Euphlebotomus) argentipes* in endemic localities of India and Nepal (Gidwani et al., 2011) and to *Anopheles sp.* mosquitoes in Angola (Brosseau et al., 2012; Drame et al., 2010b, 2010a). In this thesis we have focused on assessing the sand fly salivary YRPs as tools for screening canine exposure to *P. perniciosus* in Italy and for human exposure to *P. orientalis* in Sudan and Ethiopia.

In the following chapters, the sand fly salivary YRPs are classified as members of yellow family belonging to larger group of MRJP/Yellow proteins. The distribution across several insect orders, function and structure of this protein group is described. Sand flies' YRPs are subsequently reported as an abundant protein family found in salivary transcriptomes of all sand fly species studied up to date. Further on, YRPs are shown to influence host haemostatic and inflammatory processes and to interact with both humoral and cellular immune responses of the bitten hosts.

1. Yellow proteins

1.1. Yellow genes

The foremost "yellow" gene was studied in a fly *Drosophila melanogaster*. Its name corresponds to the abnormal light (yellow) phenotype of both cuticle of the adults and mouthparts of the larval instars, which was observed within the flies with yellow gene mutation (Brehme, 1941; Johnson et al., 2001). This classical yellow gene was as a visible genetic marker extensively studied during the last century (Han et al., 2002) and it was early presumed to be involved in the melanin pigmentation and in other possibly neural functions (Biessmann, 1985).

Although the mechanism how yellow gene influences melanisation process is not clear, it is known that the mutations at the yellow locus resulted in shifts in melanic pigmentation of the *Drosophila* wings, thorax and abdomen (reviewed in Ferguson et al., 2011). Genetic changes in *D. melanogaster* yellow gene affected also behaviour, as it was shown by decreased locomotor activity and a reduced level of male competitive mating success (Wilson et al., 1976). Other member of the *D. melanogaster* yellow gene family (yellow-g) was found essential for the proper eggshell formation (Claycomb et al., 2004), while others (yellow-f and yellow-f2) were demonstrated as dopachrome-conversion enzymes responsible for catalysing the conversion of dopachrome into 5,6-dihydroxy-indole in the insect melanisation pathway (Han et al., 2002). These enzymes shared an approximately 40 % similarity with a dopachrome-conversion enzyme characterized in a yellow fever mosquito *Aedes aegypti* (Fang et al., 2002; Johnson et al., 2001), which was shown to play role apart from cuticular tanning also in melanotic encapsulation reaction of parasites in the mosquitoes haemocoel (Huang et al., 2005; Li et al., 1994). The function of this enzyme and its similarity to some *Drosophila* yellow genes denote it as a member of yellow family (Xu et al., 2011).

A role of yellow family members in melanisation process was furthermore assumed to be conserved even across the insect orders. In a domestic silkmoth *Bombyx mori*, the homolog of one of *D. melanogaster* yellow genes (yellow-e) was shown to play a crucial role in pigmentation process by influencing the colour pattern of lepidopteran larvae (Ito et al., 2010). The role of lepidopteran yellow genes in melanisation pathway was further confirmed by their effect on the pigmentation, colour and pattern of the wings in *Heliconius erato* and *Vanessa cardui* (Ferguson et al., 2011; Zhang et al., 2017) and on the body pigmentation of the *Agrotis ipsilon* (Chen et al., 2018) butterflies. The orthologs of *Drosophila* yellow genes were also found necessary for melanin production or cuticle sclerotization in red flour beetle

Tribolium castaneum (Arakane et al., 2010), moreover one of the proteins was shown to have a vital waterproofing function (Noh et al., 2015). In an ant *Diacamma sp.*, the yellow gene was found responsible for the sexual colour dimorphism, through its higher expression in females leading to their darker phenotype in comparison to males (Miyazaki et al., 2014).

In mosquitoes, apart from abovementioned dopachrome-conversion enzyme, a transcript coding putative secreted yellow family protein was described also in salivary glands transcriptome of *Psorophora albipes* (Chagas et al., 2013). Members of yellow family were found also in other blood-sucking insect, such as in tsetse fly *Glossina morsitans morsitans*, where their ubiquitous tissue expression in salivary glands, fat body and midgut suggested potential role of these proteins in housekeeping (Alves-Silva et al., 2010). Putative yellow protein was found also in sialome of horse fly *Tabanus bromius* (Ribeiro et al., 2015) Uncharacterized yellow or yellow-like proteins were indicated also in human body louse *Pediculus humanus corporis* (NCBI accession number: XP_002427490.1), bed bug *Cimex lectularius* (XP_014260013.1), cat flea *Ctenocephalides felis* (XP_026464710.1) and in a stable fly *Stomoxys calcitrans* (XP_013111233.1). Members of yellow family called yellow-related proteins (YRPs) which are abundantly present throughout sialomes of Phlebotominae subfamily will be discussed in detail in a separate chapter.

Besides insects, yellow-like sequences have been identified in different species of bacteria and fungi. No yellow gene has been so far found in vertebrates, whereas the two yellow-like sequences described in the chordate known as a Florida lancelet (*Branchiostoma floridae*) were the most phylogenetically closest to them. One putative yellow-like gene was found in a protist *Naegleria fowleri* and one in a salmon louse belonging to crustaceans (Ferguson et al., 2011; Maleszka & Kucharski, 2000). Up to date, more putative yellow-like proteins emerged in different taxa indicating that it is a relatively extended group of proteins (InterPro database; Mitchell et al., 2018).

1.2. MRJP/Yellow protein family

All yellow, yellow-like and yellow-related proteins (YRPs) are characterized by the presence of a conserved region, the MRJP domain (Ferguson et al., 2011). This domain is named after the honeybees' **m**ajor **r**oyal **j**elly **p**roteins. MRJPs are secretory proteins forming a dominant protein component of larval jelly, which is a bee product synthesized and secreted by the cephalic glands of the nurse bees and fed to the larvae (Buttstedt et al., 2014; Schmitzova et al., 1998). MRJP family shares a common evolutionary origin with the yellow protein

of *D. melanogaster*, but the MRJPs in honeybees evolved a novel nutritional function (Albert et al., 1999). Apart from the larval jelly, MRJPs were further isolated also from honeybee brain, venom gland and other tissues (reviewed in Ferguson et al., 2011) implicating their role in other physiological functions including the eusocial behaviour (Drapeau et al., 2006). Besides from honeybees, MRJPs were described also in other Hymenoptera genera (Kupke et al., 2012; Liu et al., 2017; Werren et al., 2010).

In western honeybee *Apis mellifera*, both MRJPs and yellow genes are present (Buttstedt et al., 2014; Drapeau et al., 2006). Both protein groups were described to ensure diverse context-dependent physiological and developmental functions. As MRJP subfamily evolved from a yellow progenitor and due to their similarity, the MRJPs and yellow proteins were united into a protein family termed MRJP/Yellow (InterPro database code: IPR017996; Albert & Klaudiny, 2004; Buttstedt et al., 2014; Mitchell et al., 2018). All members of this family comprise the MRJP domain (Fig. 1, lower row). Sand fly YRPs were shown to contain the entire MRJP domain (Rohousova et al., 2012; Vlkova et al., 2014), whereas in some members of MRJP/Yellow family were found partial, fragmented or multiplied MRJP domains (Ferguson et al., 2011).

1.3. Structure of MRJP/Yellow proteins

Up to date, three crystal structure analyses were performed in the MRJP/Yellow protein family (Fig. 1). A crystal structure of putative hydrolase from cyanobacteria *Anabaena variabilis* is composed of two chains, both forming a six-bladed β-propeller and containing almost complete MRJP domain (Fig. 1a) (Protein Data Bank identifier: 2QE8; Joint Center for Structural Genomics). However, the detailed characteristics or roles of this protein were not further studied. The second available crystal structure was revealed for YRP LJM11 from sand fly *Lutzomyia (Lutzomyia) longipalpis* (Xu et al., 2011). Similar to the aforementioned protein, LJM11 fold as a six-bladed β-propeller and comprise the MRJP domain (Fig. 1b). Its function and properties will be further discussed in a separate chapter.

Recently, the crystal structure of the most abundant royal jelly protein MRJP1 from *Apis mellifera* was revealed (Tian et al., 2018). This protein plays a role in honeybee nutrition, caste differentiation, learning ability, social behaviour, and development. The native protein was shown to adopt the conformation of an oligomer which is composed of four lateral MRJP1 units, four honeybee specific peptides called apisimins located in the centre of the oligomer (Fig. 1c; lower row) and by eight 24-methylenecholesterol molecules serving as

cofactors. The MRJP1 share only a weak sequence homology with LJM11 (16.9 %), but a high structural similarity, as its units are folded into similar six-bladed β-propeller shape with a central channel (Fig. 1). In both proteins, each blade of the β-propeller consists of four-stranded β-sheets. The first strands from each blade are arranged in parallel to form the wall of the channel (Tian et al., 2018; Xu et al., 2011).

All three crystallized MRJP/Yellow proteins were shown to share similar position of the MRJP domain (Fig. 1; lower row) and of the core β-propeller, while their peripheral structures were found more variable (Fig. 1; upper row). MRJP1 differed from the other two proteins by the longer C-terminus (Fig. 1; upper row, blue colour) which interacts with adjoining MRJP1 unit forming the oligomer (Tian et al., 2018).

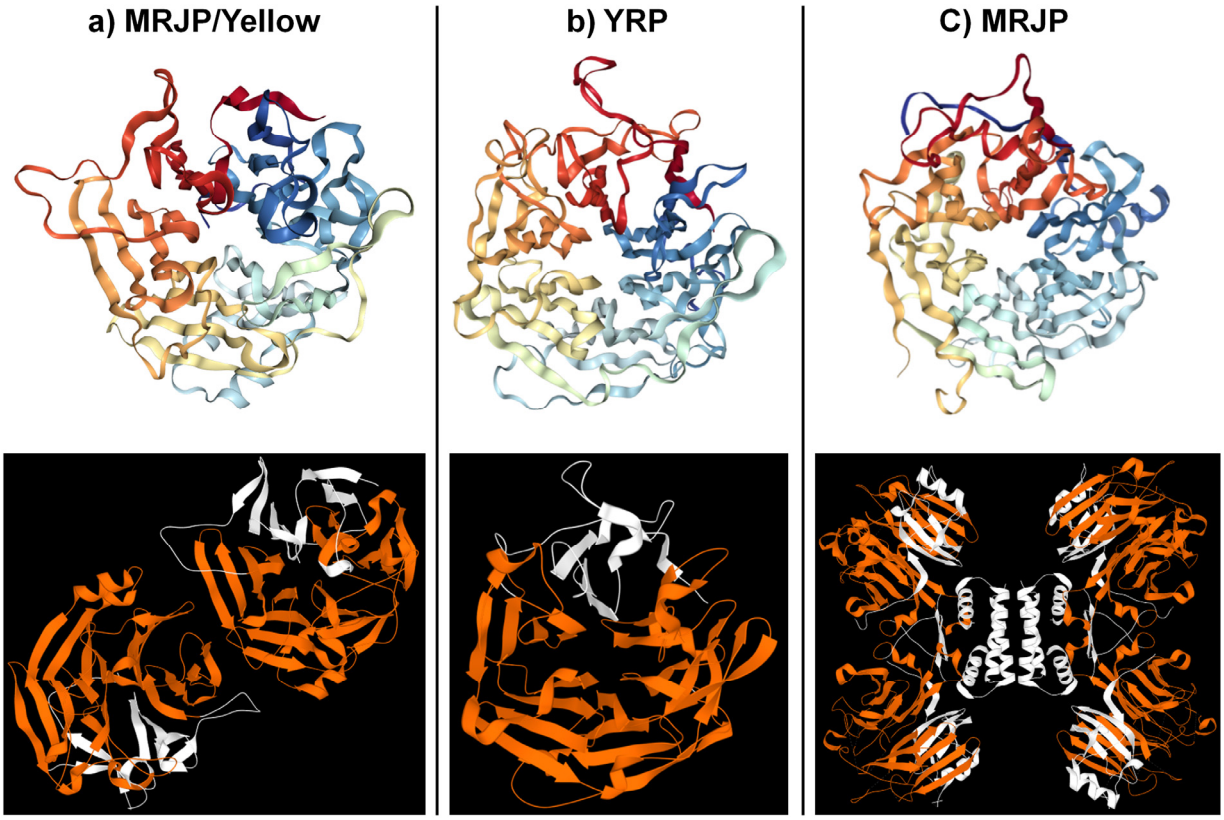

Fig 1. Structure of the members of MRJP/Yellow family. a) Uncharacterized MRJP/Yellow protein from *Anabaena variabilis* (PDB identifier: 2QE8); b) yellow-related protein (YRP) from *L. longipalpis* (LJM11; PDB identifier: 3Q6K); and c) MRJP protein from *Apis mellifera* (MRJP1; PDB identifier: 5YYL). Images were modified from the structures obtained from Protein Data Bank (Berman et al., 2000). Upper row represents aligned models of monomer units coloured according to atom index numbers (red-yellow-blue colour scheme) using NGL Viewer (Rose et al., 2018); lower row visualize the location of MRJP domains (orange) on the whole protein structure using LiteMol viewer (Sehnal et al., 2017).

Based on the shared structure of the aforementioned proteins and on the InterPro database (Mitchell et al., 2018), MRJP/Yellow family of proteins is classified as a member of homologous superfamily of six-bladed β-propellers, TolB-like (InterPro database code: IPR011042). The name of this superfamily is derived from the β-propeller forming C-terminal domain of the periplasmic TolB protein playing role in a cellular integrity of Gram-negative bacteria (Abergel et al., 1999). Into this protein superfamily belongs enzymes and proteins of different functions sharing the six-bladed β-propeller domain consisting of six four-stranded β-sheet motifs. The structure usually resembles the barrel shape with a central cavity comprising the ligand-binding site located at its top side (Chufan et al., 2009; Oubrie et al., 1999; Shin et al., 2001; Xu et al., 2011). The β-propeller fold adopts a highly symmetrical and rigid structure, with the β-sheets radially arranged around the central tunnel. The structure is stabilized by the hydrophobic interactions between the sheets and the circle is usually closed by the interactions between the first and last blades of the propeller (Fulop & Jones, 1999).

2. Yellow-related proteins

2.1. Yellow-related proteins across the phlebotomine sand flies

The yellow related proteins (YRPs) are a conserved group of proteins, whose members were so far detected in saliva of all sand fly species studied up to date (Table 1; Abdeladhim et al., 2012, 2016; Anderson et al., 2006; Coutinho-Abreu & Valenzuela, 2018; Hostomska et al., 2009; Kato et al., 2006, 2013; de Moura et al., 2013; Oliveira et al., 2006; Rohousova et al., 2012; Valenzuela et al., 2001, 2004; Vlkova et al., 2014). In three species (*P. papatasi*, *P. duboscqi* and *P. orientalis*); two cDNA libraries comparing the sand flies originating from geographically distant localities were constructed. The salivary YRPs of these species showed a high degree of conservancy independent on the sand flies' origin (Abdeladhim et al., 2012; Kato et al., 2006; Vlkova et al., 2014). In all sand fly salivary cDNA libraries studied up to date, YRPs belong to the most abundant protein families. The numbers of identified YRPs per species vary from one in *P. (Adlerius) arabicus*, *P. (La.) kandelakii*, *P. argentipes,* and in *L.* (*Nyssomyia) intermedia* to five in *P. (Paraphlebotomus) sergenti* (Table 1). The differences in numbers of YRPs are assumed to be caused by duplications in discrete clades formed by sand fly YRPs (Fig. 2). In average per species, one or more duplication events were computed within the YRPs of New-World sand flies and in *Phlebotomus* plus *Paraphlebotomus* subgenera, while in *Larroussius* together with *Adlerius* and *Euphlebotomus* less than one duplication event was described (Coutinho-Abreu & Valenzuela, 2018).

Table 1. A list of identified sand flies salivary YRPs.

Subgenus	Species	Name	MW	pI	AN (NCBI)	cDNA library
Phlebotomus	*P. papatasi*	PPTSP42	42.3	9.1	JQ988885	Abdeladhim et al., 2012
		PPTSP44	**43.6**	**8.4**	**JQ988886**	**Abdeladhim et al., 2012**
		PpSP42	*44.4*	*NA*	*AF335491*	*Valenzuela et al., 2001*
		PpSP44	***43.6***	***NA***	***AF335492***	***Valenzuela et al., 2001***
	P. duboscqi	**PduM10**	**43.4**	**7.7**	**DQ826519**	Kato et al., 2006
		PduM35	42.6	9.3	DQ826522	Kato et al., 2006
		PduK04	***43.4***	***7.7***	***DQ835365***	***Kato et al., 2006***
		PduK06	*42.5*	*9.3*	*DQ835367*	*Kato et al., 2006*
Paraphlebotomus	*P. sergenti*	PsSP18	42.2	9.0	HM569361	Rohousova et al., 2012
		PsSP19	42.5	8.9	HM560865	Rohousova et al., 2012
		PsSP20	42.4	9.8	HM560866	Rohousova et al., 2012
		PsSP22	42.3	5.7	HM560867	Rohousova et al., 2012
		PsSP26	**43.9**	**8.1**	**HM569362**	**Rohousova et al., 2012**
Larroussius	*P. perniciosus*	PpeSP03	41.8	6.0	DQ150621	Anderson et al., 2006
		PpeSP03B	**42.7**	**8.6**	**DQ150622**	**Anderson et al., 2006**
	P. orientalis	PorASP2	41.5	6.1	KC170933	Vlkova et al., 2014
		PorASP4	**42.3**	**8.1**	**KC170934**	**Vlkova et al., 2014**
		PorMSP23	*41.6*	*6.1*	*KC170966*	*Vlkova et al., 2014*
		PorMSP24	***42.3***	***8.1***	***KC170967***	***Vlkova et al., 2014***
	P. tobbi	PtSP37	41.5	6.0	HM140618	Rohousova et al., 2012
		PtSP38	**42.6**	**8.5**	**HM140619**	**Rohousova et al., 2012**
	P. ariasi	ParSP04	41.7	5.8	AY845196	Oliveira et al., 2006
		ParSP04b	**42.9**	**8.3**	**AY850692**	**Oliveira et al., 2006**
	P. kandelakii	PkanSP04	NA	NA	NA	Coutinho-Abreu & Valenzuela, 2018
Adlerius	*P. arabicus*	**PabSP26**	**42.9**	**8.4**	**FJ410293**	**Hostomska 2009**
Euphlebotomus	*P. argentipes*	PagSP04	43.2	8.8	DQ136151	Anderson et al., 2006
Lutzomyia	*L. longipalpis*	LJM11	43.2	9.3	AY445935	Valenzuela et al., 2004
		LJM17	**45.2**	**5.7**	**AF132518**	**Charlab et al., 1999; Valenzuela et al., 2004**
		LJM111	43.0	4.9	ABB00904	Valenzuela et al., 2004
Helcocyrtomyia	*L. ayacuchensis*	**LayS118**	**43.8**	**8.0**	**AK416844**	**Kato et al., 2013**
		LayS22	43.4	9.4	AK416768	Kato et al., 2013
		LayS23	43.5	9.4	AK416769	Kato et al., 2013
		LayS24	43.5	9.4	AK416770	Kato et al., 2013
Bichromomyia	*L. olmeca*	LolYLWa	42.5	5.7	KX011388	Abdeladhim et al., 2016
		LolYLWb	43.0	9.4	KX011389	Abdeladhim et al., 2016
		LolYLWc	**44.5**	**9.2**	**KX011390**	**Abdeladhim et al., 2016**
Nyssomyia	*L. intermedia*	**Linb-21**	**44.0**	**8.4**	**KA660057**	**de Moura et al., 2013**

For each YRP, the molecular weight (MW; kDa), isoelectric point (pI), NCBI accession number (AN) and the reference (cDNA library) are indicated. The proteins belonging to the same species, but differing in geographical origin are written in italics. The higher molecular YRPs belonging to "44 kDa" subgroup are written in bold. The colour code corresponds with the colours used in the Fig. 2 bellow. NA denoted not available.

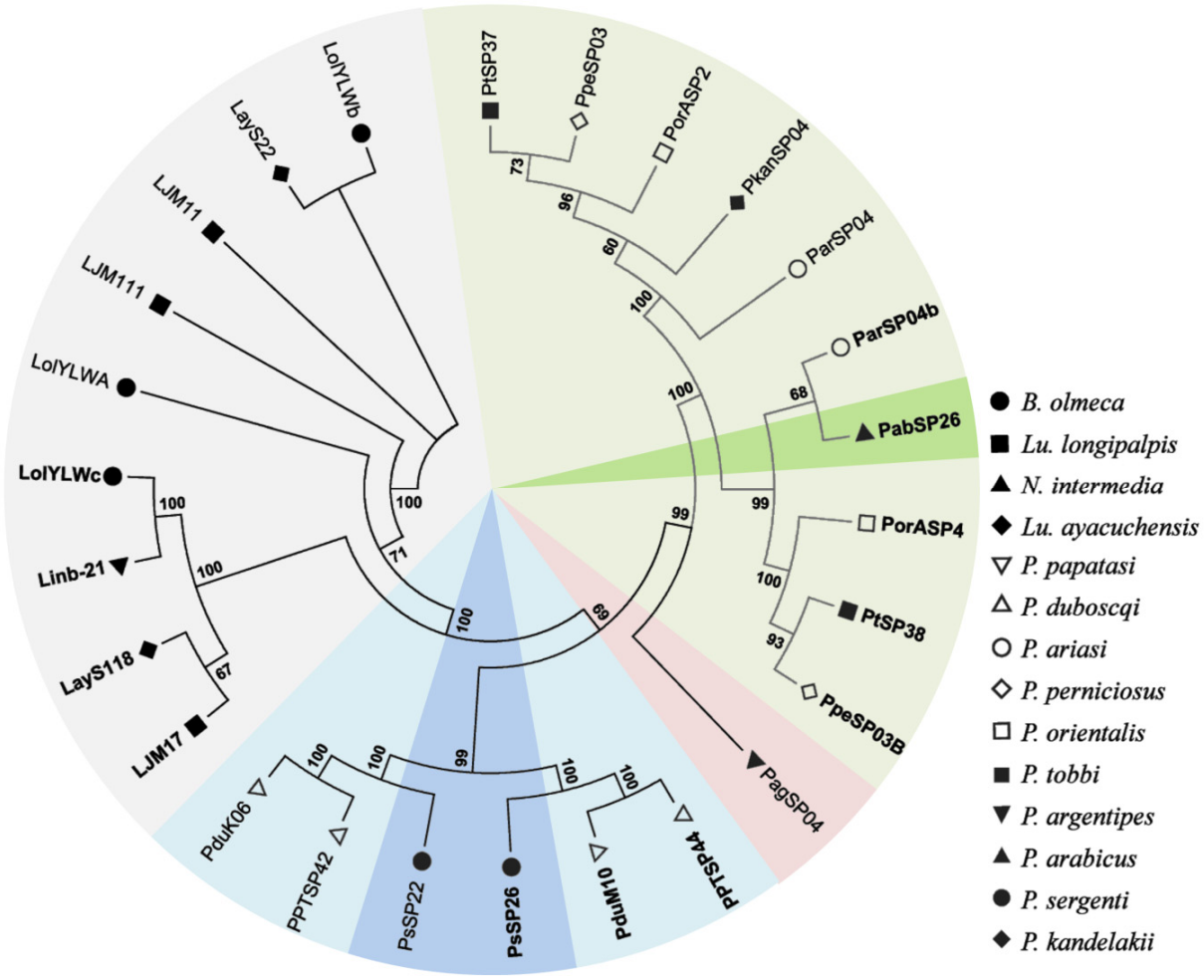

Fig. 2. Molecular phylogenetic analysis of selected sand flies YRPs. Sand fly species are indicated by the different symbols described in the legend on the right. Colour code of the tree represents individual subgenera: green colour marks the *Larroussius* (light green) and *Adlerius* (bright green) subgenera; red colour indicates the *Euphlebotomus* subgenus; blue colour indicates proteins of the *Phlebotomus* (light blue) and *Paraphlebotomus* (bright blue) subgenera; and grey colour unites the proteins of the New-World sand flies. Proteins belonging to the 44 kDa orthologs are written in bold. The phylogenetic tree was modified from Coutinho-Abreu & Valenzuela, 2018.

Within all sand flies, YRPs adopt similar predicted molecular mass (41.5-45.2 kDa) and a wide range of isoelectric points (4.75-9.8). In most of the sand fly species, the YRPs were shown to split into two subgroups of "42 kDa" (lower molecular weight orthologs: 41.5-43.5 kDa) and "44 kDa" (higher molecular weight orthologs: 42.3-45.2 kDa) proteins (Abdeladhim et al., 2016). The differences between both subgroups of proteins are the most pronounced in *Larroussius* subgenus, where the proteins belonging to "42 kDa" or "44 kDa" group share similar molecular weight and acidic (5.8-6.1) or basic (8.1-8.5) isoelectric points, respectively (Table 1).

The phylogeny based on YRPs is in accordance with the sand fly species phylogeny inferred from the small subunit nuclear ribosomal DNA (Aransay et al., 2000). The genus *Phlebotomus* is divided into two main clades, one clade including the YRPs of *Phlebotomus* and *Paraphlebotomus* subgenera while the other comprising the sequences of *Larroussius*, *Euphlebotomus*, and *Adlerius* subgenera (Fig. 2). Phylogenetic analyses revealed a clear separation between the orthologs belonging into groups of "42 kDa" and "44 kDa" YRPs in both New-World and Old-World sand fly species (Table 1; Fig. 2) (Abdeladhim et al., 2016; Coutinho-Abreu & Valenzuela, 2018; Vlkova et al., 2014). The YRPs from both New-World and Old-World sand flies were shown to cluster more frequently in the same branches with their paralogs from other species, than with same species orthologs. The cause of this separation might be multiple gene conversions identified in paralogs of Old-World sand flies "42 kDa" and "44 kDa" YRPs-encoding genes (Abdeladhim et al., 2016).

2.2. Structure and function of yellow-related proteins

Structure and function of sand fly yellow-related proteins remained for long time elusive. The potential function of YRPs was hypothesized based on the sequence similarities with yellow protein described in *D. melanogaster*. The involvement of this protein in cuticle pigmentation which is associated with catecholamine metabolism early led to the hypothesis that YRPs might catabolise vasoconstrictor catecholamines (Bahia et al., 2007; Charlab et al., 1999). Into account was also taken the possibility that, similar to yellow-like proteins in mosquitoes, the YRPs may act as dopachrome-converting enzymes. However, no dopachrome-convertase activity was detected in salivary glands of any sand fly species tested (Alves-Silva et al., 2010; Hostomska et al., 2009; Ribeiro et al., 2010).

In 2011, the amine-binding properties of all three *L. longipalpis* YRPs were revealed (Xu et al., 2011). The binding potential of these YRPs was tested with serotonin, histamine and catecholamines. These amines are involved in host haemostatic processes including platelet aggregation and vasoconstriction which form together with blood clotting and anti-inflammatory response a barrier to efficient blood feeding. Degranulation of mast cells lead to the release of mainly histamine and serotonin both inducing itch and swelling and also triggering the pain response to the insect bite, which can prevent feeding of the vector (Julius & Basbaum, 2001; Ribeiro et al., 2010). These vasoactive substances act within a minute or two after their release, but they are shortly metabolized or washed out from the tissue (Ribeiro & Francischetti, 2003). Serotonin and catecholamine epinephrine are released by activated

platelets whereas together they potentiate their aggregation (Francischetti, 2010) and also blood vessel constriction, both participating in haemostasis at the bite site (Ribeiro, 1995). Additionally, other catecholamine norepinephrine also acts as a vasoconstrictor through stimulating adrenergic receptors in the vasculature (Calvo et al., 2009).

To wipe out the biogenic amines, blood-feeding arthropods employ different groups of salivary proteins. The anopheline peroxidases destroy serotonin and norepinephrine through oxidative activities (Ribeiro & Nussenzveig, 1993; Ribeiro & Valenzuela, 1999). Other proteins dispose of biogenic amines by binding them. These proteins belong, apart from sand fly YRPs, either into lipocalin protein family including amine-binding proteins of kissing bugs (Andersen & Montfort, 2002; Andersen et al., 2003; Xu et al., 2013) and both soft ticks (Mans et al., 2008; Neelakanta et al., 2018) and hard ticks (Paesen et al., 1999; Sangamnatdej et al., 2002); or to D7 protein family comprising amine-binding proteins of mosquitoes (Calvo et al., 2006, 2009; Jablonka et al., 2019; Mans et al., 2007). Lipocalins were never found in sand fly sialomes, while D7-related proteins were identified in all sand fly species (Lestinova et al., 2017). However, sand flies D7-related proteins were proposed not to have a biogenic amine-binding function due to the lack of amino acids conservancy with the amine-binding site of mosquito kratagonists (Mans et al., 2007). The absence of amine-binding pocket was recently validated by a crystal structure analysis of *P. (Phlebotomus) papatasi* and *P. (P.) duboscqi* D7-related proteins. Even though these proteins did not bind biogenic amines, they were shown to inhibit platelet activation through binding cysteinyl leukotrienes and thromboxane A_2 by their N-terminal domain (Jablonka et al., 2019).

In some of the amine-binding proteins the crystal structures were revealed, showing both similarities and differences with the published structure of *L. longipalpis* YRP. As mentioned above, *L. longipalpis* YRP (LJM11) occupy six-bladed β-propeller fold which comprise amine-binding pocket located in the cavity of the propeller (Xu et al., 2011). The highly conserved structure of amine-binding lipocalins is formed by eight β-stranded antiparallel β-sheets closed into a barrel structure and by two α-helixes located outside the barrel fold (reviewed in Flower et al., 2000), while the C-terminal amine-binding domain of D7 proteins is formed by 7-8 α-helixes but no β-sheets (Calvo et al., 2009). All amine-binding proteins in blood-feeding arthropods are characterized by a similar ligand-binding pocket and their structure is stabilized by conserved disulfide bonds (Calvo et al., 2009; Flower et al., 2000; Mans et al., 2007). For instance, the structure of the protein LJM11 is stabilized by two disulfide bonds (Xu et al., 2011) formed by four cysteine residues conserved in all sand flies YRPs (Coutinho-Abreu & Valenzuela, 2018).

In *L. longipalpis*, recombinant yellow-related proteins LJM11 and LJM17 were shown to tightly bind serotonin, while the third YRP LJM111 bound with highest affinity epinephrine and norepinephrine (for recombinant YRPs see Table 2). Histamine was proposed to be a target ligand only for LJM17, while dopamine was bound with quite high activity by all YRPs, even though it is an unlikely target for sand flies salivary proteins owing to its negligible role in haemostasis or inflammation. The amine-binding site was identified and characterized on the LJM11 crystals soaked with serotonin. Only one amine-binding site which is located at the top part of the protein cavity and is able to bind a single molecule was discovered. Its amine-binding motif is composed by 11 closest amino acids, from which three may interact with the ligand through hydrogen bonds (Xu et al., 2011).

The amino acids responsible for binding to serotonin have been shown to be highly conserved in YRPs of different sand fly species, suggesting similar amine-binding function in other members of this family of proteins (Abdeladhim et al., 2012, 2016; Kato et al., 2013; de Moura et al., 2013; Vlkova et al., 2014). On contrary, this amine-binding motif was not found in any proteins outside *Lutzomyia* and *Phlebotomus* genera suggesting absence of amine-binding function in other members of MRJP/yellow protein family (Tian et al., 2018; Xu et al., 2011). Different binding affinities of *L. longipalpis* YRPs indicated the occurrence of functional divergence in this protein family. The similarity of dopachrome and biogenic amines structure suggested a possibility that the progenitor of salivary YRPs might have been a another member of MRJP/yellow family, a dopachrome-conversion enzyme (Xu et al., 2011).

Additionally, several other functions or potential abilities were described in some YRPs. Recombinant protein LJM111 (Table 2) from *L. longipalpis* was characterized as a potent anti-inflammatory molecule acting through inhibiting the production of pro-inflammatory molecules and by affecting the dendritic cells maturation in the experimental arthritis model. Further on, LJM111 was able to reduce pain sensitivity in mice (Grespan et al., 2012). A different, not specified activity was speculated for LJM17, the second YRP of *L. longipalpis*, which was reported to possess a DNA binding domain, occurring usually in proteins acting as transcription factors (Bahia et al., 2007). Of interest is also the special ability of LJM11, the third YRP of *L. longipalpis*. This recombinant protein (Table 2) was proposed to trigger a human autoimmune disease through molecular mimicry based on sharing some conformational epitopes with human desmoglein 1, thus inducing the cross-reactive auto-antibodies in susceptible individuals (Qian et al., 2012). In *P. duboscqi*, a protein identified as salivary YRP corresponding to PduM35 (Table 1) was

characterized as a lectin with potential function in midgut development of *Leishmania*. The lectin was present in sand fly salivary glands and in the lumen of both thoracic and abdominal parts of midgut. It was suggested that at least part of the lectin activity might be due to swallowing the saliva by the fly (Volf et al., 2002).

2.3. Antigenicity of yellow-related proteins

Humans and animals repeatedly exposed to sand fly bites develop antibodies to certain salivary proteins. The possibility to measure this antibody response prompted research groups to utilize it as a marker of exposure to different sand fly species. At first, the whole SGH was utilized as an antigen to detect the antibody response in the host exposed to sand flies.

The anti-saliva antibodies were shown to be species-specific and to correlate with the intensity of sand fly exposure (reviewed in Lestinova et al., 2017). The intensity of the specific human, mice or canine anti-saliva antibody response significantly decrease in few weeks to months post exposure, while it can still persist in low levels to the following sand fly season (Clements et al., 2010; Hostomska et al., 2008; Vlkova et al., 2012). The next exposure then significantly increase the anti-sand fly antibody titres indicating the development of antibody memory response and making anti-saliva antibodies a suitable tool to measure even the recent exposure to sand flies (Clements et al., 2010; Velez et al., 2018; Vlkova et al., 2012).

To undertake studies aimed at the use of anti-saliva antibodies as a marker of either sand fly exposure, of risk for *Leishmania* transmission or of cell-mediated immune response to sand flies, or as a tool to determine putative reservoir hosts, a large numbers of dissected salivary glands are needed (reviewed in Lestinova et al., 2017). To overcome this demand, the specific proteins responsible for the antigenic properties of sand fly saliva were further characterized and used in recombinant form as antigens to assess the host anti-sand fly antibody response (recombinant YRPs are summarized in Table 2). In addition, only the synthetic amino acid sequence coding the antigenic parts of the proteins (B-cell epitopes) could be employed (Sima et al., 2019).

The most antigenic salivary proteins across the sand fly species belong to the yellow-related, D7-related, apyrase, antigen 5-related and PpSP15-like protein families (reviewed in Lestinova et al., 2017). Salivary YRPs were shown antigenic for different hosts in both Old-World and New-World sand flies species; details of their antigenic properties are for each species discussed bellow.

Table 2: A list of sand flies recombinant YRPs.

Species	Recombinant protein	Properties	Expression system	Reference
P. papatasi	**PPTSP44**	vaccine candidate: human	mammalian	Tlili et al., 2018
	PpSP42	antigen: mice	bacterial	Vlkova et al., 2012
	PpSP44	antigen: mice	bacterial	Vlkova et al., 2012
P. orientalis	**rPorSP24** (≈PorASP4)	antigen: dog, goat, sheep	bacterial	Sima et al., 2016
P. perniciosus	**rSP03B** (=PpeSP03B)	antigen: dog and mice antigen: dog	bacterial bacterial	Drahota et al., 2014 Kostalova et al., 2017; Martín-Martín et al., 2014; Velez et al., 2018; Willen et al., 2018
L. longipalpis	**LJM11**	anti-haemostatic	bacterial	Xu et al., 2011
		structure	bacterial	Xu et al., 2011
		antigen: dog, human	mammalian	Teixeira et al., 2010
		antigen: chicken	mammalian	Soares et al., 2013
		vaccine candidate: mice	mammalian	Cunha et al., 2018; Gomes et al., 2012; Xu et al., 2011
		autoimmunity trigger	mammalian	Qian et al., 2012
	LJM17	anti-haemostatic	bacterial	Xu et al., 2011
		antigen: dog, human, fox	mammalian	Teixeira et al., 2010
		antigen: chicken	mammalian	Soares et al., 2013
		vaccine candidate: dog	mammalian	Abbehusen et al., 2018
	LJM11+LJM17	antigen: human	mammalian	Souza et al., 2010
	LJM111	anti-haemostatic	bacterial	Xu et al., 2011
		antigen: human	mammalian	Teixeira et al., 2010
		anti-inflammatory	mammalian	Grespan et al., 2012
L. intermedia	**Linb-21**	antigen: human	mammalian	Carvalho et al., 2017

For each YRP, the species, name, tested properties (antigen, vaccine candidate, structure or function), expression system and references are denoted.

In some species, the antigenicity of YRPs has been studied only to a limited extent. In *P. (La.) tobbi*, both YRPs were shown antigenic for rabbits repeatedly exposed to this sand fly species (Rohousova et al., 2012), but they were not tested with any other hosts. In *P. ariasi*, the mice immunised with plasmid coding its YRP ParSP04b produced antibodies recognizable by *P. ariasi* SGH (Oliveira et al., 2006). The antibody response against SGH of *L.* (*N.) intermedia* in naturally exposed humans was similar to the response measured using its recombinant YRP (Linb-21), however the correlation achieved low predictive values, discarding it as a suitable marker of human exposure to this sand fly species (Carvalho et al., 2017).

In *P. papatasi*, both salivary YRPs were found reactive by sera from experimentally bitten mice, whereas in recombinant form, none of the two YRPs reacted with sera of all bitten animals; therefore a combination of more recombinant antigens was suggested (Vlkova et al., 2012). In humans, salivary YRPs were lightly detected by only two thirds of serum samples of SGH-positive individuals from Brazil and by some of the volunteers from Egypt and Jordan (Geraci et al., 2014; Marzouki et al., 2011). Therefore, in this sand fly species the YRPs were not pointed out as suitable antigens to measure the host exposure to sand flies. The valid antigen for replacement of SGH in epidemiological studies aimed at human exposure to *P. papatasi* was shown to be a recombinant salivary protein belonging to a different, SP32-like protein family (Marzouki et al., 2015).

Both YRPs of *P. orientalis* were shown antigenic for experimentally bitten mice, while only PorSP24 (=KC170934; Table 1) was recognized by dogs naturally exposed to *P. orientalis* in Ethiopia (Sima et al., 2016; Vlkova et al., 2014). The recombinant rPorSP24 expressed in bacterial cells (Table 2) was highlighted as the most promising antigen for evaluating the sand fly exposure of dogs, sheep and goat from endemic area in Ethiopia. For these hosts, the antibody response against rPorSP24 correlated well with the response against *P. orientalis* SGH (Sima et al., 2016). *P. orientalis* is the first sand fly species where to measure host antibody response a novel type of antigen was tested. In addition to the recombinant proteins, which might be laborious to express and purify, a short peptides coding for B-cell epitopes predicted on the surface of antigenic salivary proteins, were synthesised. These peptides were adapted for routine ELISA (enzyme-linked immunosorbent assay) method and tested with the same sera samples as the above-mentioned recombinant YRP. One of the two peptides (OR24 P2) derived from the YRP PorSP24 was found suitable for antibody screening of naturally exposed dogs and goats. The correlation coefficients and other statistical values measured for OR24 P2 corresponded well with the ones achieved for the recombinant protein, validating this short peptide as a novel tool to measure exposure to *P. orientalis* (Sima et al., 2019).

In *P. perniciosus*, salivary YRPs were shown antigenic for dogs (Risueno et al., 2018), mice, hares and rabbits (Martin-Martin et al., 2014, 2015). Specifically, the YRP PpeSP03B was shown as a major antigen for dogs experimentally and naturally exposed to *P. perniciosus* (Kostalova et al., 2017; Vlkova et al., 2011), while the second YRP PpeSP03 was faintly recognized only by sera of experimentally bitten dogs (Vlkova et al., 2011). On contrary, PpeSP03 was recognized with a higher intensity than PpeSP03B by sera of exposed hamsters and both proteins were detected with similar strength by sera from experimentally

bitten mice (Martin-Martin et al., 2012). Both YRPs were further expressed in bacterial system (Table 2), however protein rSP03 (≈PpeSP03) was tested only in one study where, for dogs naturally exposed to *P. perniciosus*, it was considered to be a non-immunogenic antigen (Kostalova et al., 2017).

Antigenic properties of the other recombinant YRP, rSP03B (≈PpeSP03B) were thoroughly studied in several studies. The antigenicity of rSP03B was revealed in immunoblots and ELISA experiments with experimentally bitten mice and dogs (Drahota et al., 2014) and further described in dogs, hares and rabbits naturally exposed to *P. perniciosus* in an endemic area of Spain (Martin-Martin et al., 2014). The anti-rSP03B response of all tested animals significantly correlated with the antibody response against SGH. A robust correlation was also found in a large-scale study aimed at naturally exposed dogs sampled in two distant regions of Italy, and in Portugal. Further on, the rSP03B was shown to share similar antigenic epitopes with the native antigen PpeSP03B (Kostalova et al., 2017). In a longitudinal study carried out in 12 locations in Spain, the canine ani-rSP03B antibody responses followed the dynamics of anti-SGH antibodies and corresponded with the expected annual activity of *P. perniciosus* (Velez et al., 2018). These studies all demonstrated the applicability of rSP03B-based ELISA test to screen for canine exposure to *P. perniciosus* bites in various locations of its distribution.

To enable a more practical, immediate application of this approach in the field conditions, a new tool was recently proposed. A simple and fast immunochromatographic test based on the rSP03B protein was validated as a replacement for the standard ELISA. This so-called sero-strip is composed by both anti-dog IgG and control colloidal gold conjugates impregnated on an absorbent pad, sample deposition line, rSP03B protein in test line and a control line with antibodies that bind to control conjugate. The principle of the test is based on the migration of the sample and the conjugate through the strip while capturing the anti-rSP03B antibodies from the sample at the test line. The agreement between SGH-ELISA, rSP03B-ELISA and rSP03B-sero-strip was evaluated with sera of dogs experimentally exposed to *P. perniciosus*. The rSP03B-sero-strip showed a high sensitivity and specificity and an almost perfect agreement with SGH-ELISA, indicating its great potential as a tool to rapidly screen anti-*P. perniciosus* antibodies in dogs from endemic areas (Willen et al., 2018).

The salivary YRPs of *L. longipalpis* were recognized by sera of exposed dogs (Hostomska et al., 2008), foxes and humans (Teixeira et al., 2010). The most abundant YRP of *L. longipalpis*, the LJM17 was specifically recognized by canine (Bahia et al., 2007), chicken (Soares et al., 2013) and human sera (Gomes et al., 2002). The second YRP, protein

LJM11 was recognized by sera of chicken (Soares et al., 2013), while the salivary protein LJM111 was never identified as an antigen. Nevertheless, all *L. longipalpis* YRPs might have wider spectrum of antigenic properties, as salivary proteins of similar molecular weight (43-45 kDa) were previously identified as antigens in humans (Gomes et al., 2002; Vinhas et al., 2007), mice (Silva et al., 2005), foxes and dogs (Gomes et al., 2007).

Well studied is the immune response induced by immunization with plasmids coding *L. longipalpis* YRPs. Plasmid coding LJM17 was shown to induce strong humoral response in dogs (Collin et al., 2009), which is in accordance with antigenicity of the native salivary protein (Bahia et al., 2007). In mice and hamsters, anti-saliva IgG antibodies were detected after immunization with plasmids coding both LJM11 and LJM17, but not with LJM111 (Gomes et al., 2008; Xu et al., 2011). All three *L. longipalpis* YRPs were further produced in mammalian expression system and their antigenic properties were tested with sera from humans, dogs and foxes from endemic localities in Brazil (Teixeira et al., 2010). The least antigenic protein LJM111 was recognised only by human sera, while LJM11 was antigenic also for dogs and LJM17 reacted with sera from all three different hosts. Antigenic properties of recombinant LJM11 and LJM17 were further evaluated by ELISA with human (Souza et al., 2010) and chicken (Soares et al., 2013) sera samples. Both proteins were able to distinguish majority of SGH-positive samples, whereas for chicken sera the anti-SGH antibody response correlated only with response against recombinant LJM11 (Soares et al., 2013), while for human sera the combined antigens yielded the best results. The ability of the combination of LJM11+LJM17 to substitute the SGH as an antigen was further confirmed in a large-scale study with more than 1,000 human serum samples (Souza et al., 2010), making this combination a valid replacement for SGH in studies aimed at screening anti-*L. longipalpis* antibodies in humans.

The host-specific antigenicity of different YRPs from one sand fly species was shown for both *P. perniciosus* and *L. longipalpis.* This specificity may be described by different intensity of reaction (Martin-Martin et al., 2012) or by different recognition pattern for each host (Teixeira et al., 2010). Differences in antigenicity might be caused by different electrostatic potential at the surface of individual YRPs, as it was proposed for positively charged antigenic protein LJM11 and negatively charged low-immunogenic LJM111 (Xu et al., 2011).

A dog is the main reservoir host of *Leishmania sp.* transmitted by both *P. perniciosus* and *L. longipalpis* (Belo et al., 2013; Moreno & Alvar, 2002), which emphasise the necessity of biomarkers facilitating screening canine exposure to these sand flies. This highlights the

utilization of YRPs reactive for dogs: *P. perniciosus* recombinant rSP03B (Kostalova et al., 2017; Martin-Martin et al., 2014; Velez et al., 2018; Willen et al., 2018) and LJM17 + LJM11 from *L. longipalpis* (Souza et al., 2010; Teixeira et al., 2010). Together with *P. orientalis* PorSP24, the recombinant YRPs of three epidemiologically important sand fly species were shown as valid tools to measure the anti-sand fly antibody response and therefore to screen for vector exposure in bitten hosts. Moreover, recently have emerged new tools employing YRPs through novel technique such as rSP03B-serostrip (Willen et al., 2018), or by using a different type of antigen like OR24 P2 B-epitope (Sima et al., 2019). The studies highlighting the YRPs as the best biomarkers of vector exposure in several sand fly species emphasised the idea, that YRPs of different species are recognized and presented in a analogous way by the host immune system (Coutinho-Abreu & Valenzuela, 2018).

2.4. Yellow-related proteins as anti-leishmaniasis vaccine components

The bites of uninfected sand flies or specific salivary proteins were shown to protect hosts repeatedly exposed to sand flies against *Leishmania* infection. This protection was mediated mainly by hosts delayed-type hypersensitivity (DTH) reaction and enhanced interferon-γ (IFN-γ) and/or interleukin-12 (IL-12) production at the sand fly feeding site. Sand fly salivary proteins are therefore targeted as the feasible compounds of a vaccine against leishmaniases (reviewed in Lestinova et al., 2017).

Vaccine LbSapSal aimed against canine VL in Brazil combined the whole salivary extract of *L. longipalpis*, *Le. braziliensis* crude antigens and the saponin as an adjuvant (Aguiar-Soares et al., 2014; Giunchetti et al., 2007; Resende et al., 2016). Dogs were vaccinated by three doses of this vaccine and afterwards challenged by the intradermal inoculation of *Le. infantum* and the salivary gland extract of *L. longipalpis* (Aguiar-Soares et al., 2014; Resende et al., 2016). Immunization with LbSapSal was able to induce enhanced amounts of tumour necrosis factor-α (TNF-α), IL-12 and IFN-γ cytokines. The establishment of a prevailing pro-inflammatory immune response was further supported by the increased levels of nitric oxide production facilitating a reduction in numbers of parasites in spleen (78.9 %) and therefore indicated the LbSapSal vaccine as a potential tool to control the *Le. infantum* infection (Resende et al., 2016). The *L. longipalpis* salivary proteins associated with the resistance pattern against *Leishmania* infection were determined as an apyrase and an YRP (Giunchetti et al., 2007). In the present study, we further focus only on the YRPs and their potential to serve as components of anti-leishmaniases vaccine.

To identify the DTH-inducing salivary proteins the method termed reverse antigen screening was employed. Animals previously exposed to sand flies/SGH are injected with plasmids encoding salivary proteins and after 24-48 hours scored for DTH response (Oliveira et al., 2006). In *L. longipalpis*, the potential of all three YRPs to induce DTH response was tested in dogs, hamsters and mice. In dogs, the strongest DTH response was elicited by DNA plasmid coding YRP named LJM17, while the second YRP (LJM11) induced weaker response and the third YRP (LJM111) did not react at all. Dogs immunized with LJM17 showed, upon challenge with *Le. infantum* infected sand flies, a polarized protective T-cell helper type 1 (Th1) immune response characterized by a significant induction of IFN-γ and IL-12 (Collin et al., 2009). Novel vaccination strategy for the canine model therefore comprised LJM17 and consisted of a single dose of DNA plasmid encoding LJM17 followed by two doses of recombinant *Canarypoxvirus* expressing LJM17 and later on by challenge with *Le. infantum* promastigotes and *L. longipalpis* SGH. During the 10 month follow-up period, the LJM17-based vaccine was shown to induce an immune profile consistent with the protective immunity against CL (Abbehusen et al., 2018). Of *L. longipalpis* DNA plasmids coding YRPs, both LJM11 and LJM17, but not LJM111 induced the DTH response in hamster model, while none of the proteins showed a long term protection against *Le. infantum* (Gomes et al., 2008). On the contrary, in mice vaccinated with *L. longipalpis* YRPs, LJM11 elicited statistically significant DTH response, while LJM17 raised only an anti-saliva-specific IgG antibody response, and LJM111 did not induce either humoral or cellular response (Xu et al., 2011).

Recombinant protein LJM11 was recognized by mice immune response generated against the SGH. The immunity to LJM11 was described as Th1-biased and systemic, thus conferring a protection against the intradermally inoculated *Le. major* (Xu et al., 2011). Similarly, in another study focused on mice model, intradermal immunization with recombinant LJM11 induced long-lasting Th1 immunity resulting in ulcer-free protection against *Le. major* delivered by vector bites, thus mimicking in a better way the natural conditions (Gomes et al., 2012). Utilizing the same parasite-host model, different immunization strategy using an attenuated strain of *Listeria monocytogenes* as an expression system for LJM11 was implemented. The delivery of LJM11 by bacteria was shown as a promising vaccination strategy against *Le. major*, as it induced IFN-γ and TNF-α based long-term protection against ulcer formation after a natural challenge with infected sand flies (Abi Abdallah et al., 2014). The protective potential of recombinant LJM11 was tested also for *Le. braziliensis* infection in mice. In LJM11 immunized mice challenged with *Le. braziliensis*

and *L. longipalpis* salivary gland sonicate a significant reduction of parasite numbers was observed. On the contrary, when challenged with salivary glands sonicate of a sympatric species *L. intermedia*; no reduction in parasite load was detected. Protein LJM11 was therefore shown as a species-specific protein incapable to confer cross-protection against *Le. braziliensis* (Cunha et al., 2018).

YRPs were suggested as potential vaccine candidates also in *P. papatasi*, an important vector of cutaneous leishmaniases in Old-World. In mice, DNA plasmids coding both *P. papatasi* YRPs elicited DTH response; however immunization with YRP PpSP44 produced distinct immune profile resulting in susceptibility to *Le. major* infection and disease enhancement (Oliveira et al., 2008). On contrary, in humans, both PpSP44 and the second YRP PpSP42 were postulated as potential vaccine candidates. Peripheral blood mononuclear cells from donors naturally immune to *P. papatasi* were transfected with the DNA plasmids coding these proteins, while measuring the cell proliferation and cytokine production. Both YRPs, with a special emphasis to PPTSP44 (≈PpSP44; Table 1), recalled a Th1-polarized immune response in immune cells from volunteers previously exposed to *P. papatasi* saliva (Tlili et al., 2018).

Discrepancies between the polarization of cellular immune response to different YRPs in both *L. longipalpis* and *P. papatasi* show that proteins immunogenic for rodents might not be immunogenic for human or dogs and vice versa. This finding is in accordance with abovementioned host-specificity described for humoral immune responses to YRPs.

OBJECTIVES

In this Ph.D. thesis, we have focused on deepening our knowledge of the structure, binding properties and anti-haemostatic function of sand fly yellow-related proteins. In both species studied, *P. perniciosus* and *P. orientalis*, YRPs were found as suitable antigens to screen host exposure to sand flies. We have validated the utilization of *P. perniciosus* YRP as a tool to measure anti-saliva response in long term study monitoring the canine exposure to this sand fly in Italy, and also extended the use of *P. orientalis* YRP from screening sera of domestic animals to evaluation of human exposure to *P. orientalis* bites in Eastern Africa.

The main objectives of this thesis were to:

1. model the structures of YRPs across sand fly species, compare the sequences and structural variability of their putative ligand-binding site and their phylogenetic relationships.

2. experimentally validate amine-binding potential in genus *Phlebotomus* by measuring and further comparing the amine-binding interactions of recombinant YRPs from *P. perniciosus* and *P. orientalis*.

3. evaluate the utilization of *P. perniciosus* recombinant YRP as a substitution for SGH to screen canine anti-*P. perniciosus* antibodies in a longitudinal study sampling dogs over two sand fly seasons in southern Italy.

4. assess the antigenicity of recombinant *P. orientalis* YRPs for a human host and to determine their potential to replace saliva in epidemiological studies screening exposure to *P. orientalis* bites in humans from endemic areas of Eastern Africa.

PUBLICATIONS

1. Sima, M., Novotny, M., Pravda, L., **Sumova, P.**, Rohousova, I., & Volf, P. (2016). The diversity of yellow-related proteins in sand flies (Diptera: Psychodidae). PLoS One *11*, e0166191.

2. **Sumova, P.**, Sima, M., Kalouskova, B., Polanska, N., Vanek, O., Oliveira, F., Valenzuela, J.G., & Volf, P. (manuscript). Amine-binding properties of salivary yellow-related proteins in phlebotomine sand flies.

3. Kostalova, T., Lestinova, T., **Sumova, P.**, Vlkova, M., Rohousova, I., Berriatua, E., Oliva, G., Fiorentino, E., Scalone, A., Gramiccia, M., Gradoni, L., & Volf, P. (2015). Canine antibodies against salivary recombinant proteins of *Phlebotomus perniciosus*: A longitudinal study in an endemic focus of canine leishmaniasis. PLoS Negl. Trop. Dis. *9*, e0003855.

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The diversity of yellow-related proteins in sand flies (Diptera: Psychodidae)

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PLOS ONE

RESEARCH ARTICLE

The Diversity of Yellow-Related Proteins in Sand Flies (Diptera: Psychodidae)

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Citation: Sima M, Novotny M, Pravda L, Sumova P, Rohousova I, Volf P (2016) The Diversity of Yellow-Related Proteins in Sand Flies (Diptera: Psychodidae). PLoS ONE 11(11): e0166191. doi:10.1371/journal.pone.0166191

Editor: Yara M. Traub-Csekö, Instituto Oswaldo Cruz, BRAZIL

Received: August 9, 2016

Accepted: October 24, 2016

Published: November 3, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Charles University (GAUK project no. 120516/B-BIO, http://www.cuni.cz/UKEN-1.html, PS; UNCE 204013, http://www.cuni.cz/UKEN-1.html, MS). LP was supported by Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601) (http://www.msmt.cz/?lang=2, LP) Czech Science Foundation (Project Number: P506 13-05292S, https://gacr.cz/en/, to MS, PS, IK, PV). The funders had no role in study

Abstract

Yellow-related proteins (YRPs) present in sand fly saliva act as affinity binders of bioamines, and help the fly to complete a bloodmeal by scavenging the physiological signals of damaged cells. They are also the main antigens in sand fly saliva and their recombinant form is used as a marker of host exposure to sand flies. Moreover, several salivary proteins and plasmids coding these proteins induce strong immune response in hosts bitten by sand flies and are being used to design protecting vaccines against *Leishmania* parasites. In this study, thirty two 3D models of different yellow-related proteins from thirteen sand fly species of two genera were constructed based on the known protein structure from *Lutzomyia longipalpis*. We also studied evolutionary relationships among species based on protein sequences as well as sequence and structural variability of their ligand-binding site. All of these 33 sand fly YRPs shared a similar structure, including a unique tunnel that connects the ligand-binding site with the solvent by two independent paths. However, intraspecific modifications found among these proteins affects the charges of the entrances to the tunnel, the length of the tunnel and its hydrophobicity. We suggest that these structural and sequential differences influence the ligand-binding abilities of these proteins and provide sand flies with a greater number of YRP paralogs with more nuanced answers to bioamines. All these characteristics allow us to better evaluate these proteins with respect to their potential use as part of anti-*Leishmania* vaccines or as an antigen to measure host exposure to sand flies.

Introduction

During intake of a bloodmeal, sand flies (Diptera: Phlebotominae) and other bloodsucking insects inject saliva into the host skin. This saliva contains a mixture of various proteins, which play a major role in preventing host haemostatic and inflammatory responses of different pathways, e.g. platelet activation, coagulation, inflammation, mast cell function, and vasoconstriction (reviewed in [1]). In sand flies, the vectors of *Leishmania* protozoan parasites, these salivary proteins have been studied for decades due to their biological activities and possible

design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

use in anti-*Leishmania* vaccines (reviewed in [2]). In repeatedly bitten hosts, several salivary proteins elicit a strong antibody response, which can be utilized for the detection of exposure to sand flies in epidemiological studies [3–7].

Transcripts of yellow-related proteins (YRPs) have been present in the salivary cDNA libraries of all sand fly species tested to date [8–21]. Usually, they are found in more than one homolog, which may occur in N-glycosylated, O-glycosylated, C-glycosylated, or non-glycosylated forms [16, 20].

YRPs are known as (1) kratagonists that remove small molecule mediators of haemostasis by high affinity ligand-binding proteins, and as (2) antigens that elicit a host immune response, both antibody and cell-mediated. All sequences of sand fly YRPs contain the entire insect-specific MRJP (major royal jelly protein) domain, which defines this protein family across several insect orders/families including *Drosophila melanogaster* [22], honeybees [23], mosquitoes [24], and tse-tse flies [25]. In *Lutzomyia longipalpis*, Xu et al. [26] demonstrated that YRPs are high affinity binders of pro-haemostatic and pro-inflammatory biogenic amines, such as serotonin, histamine and catecholamines. Blocking of these small molecules by YRPs results in vasodilatation, platelet deactivation, and a decrease in vascular permeability [27, 28]. The YRP of *Phlebotomus duboscqi* was shown to have lectin-like activity and is swallowed into the midgut, together with saliva [29].

Sand fly salivary recombinant YRPs are the most promising antigens for measuring exposure in naturally bitten hosts, and have been the subject of large epidemiological studies [30–34]. Besides eliciting an antibody response, YRPs as well as plasmids coding these proteins of *Lutzomyia longipalpis* induce a strong delayed type hypersensitivity (DTH) reaction, which leads to protection against *Leishmania major* in vivo [26, 35] and against *L. infantum* in vitro [36]. This suggests a possible use of these proteins in an anti-*Leishmania* vaccine.

In 2011, the crystal structure of *L. longipalpis* YRP LJM11 (GenBank ACCN: AAS05318) was published as 3Q6K (Protein Data Bank ID) with a description of the ligand-binding pocket [26]. Based on this structure and available amino acid sequences obtained from GenBank, we constructed 3D models of all YRPs identified so far in sand fly sialomes. We predicted their phylogenetic relationships, glycosylation sites, surface electrostatic potentials, compared their sequences and characterized the ligand-binding tunnel. Our results show differences among individual proteins within one species as well as differences among various species. Our results may lead to a better understanding of the biological function of YRPs.

Methods

Phylogenetic analysis

Amino acid sequences of YRPs were identified in public databases at NCBI using BLAST [37] based on similarity with *Lutzomyia longipalpis* LJM11 (Protein Data Bank: 3Q6K, for the purpose of this study called 3Q6K_Llon1), the best explored protein from this group, [26]. All these analyses were performed for the sequences without a signal peptide, which was identified using SignalP 4.0 [38]. Sequences were consequently aligned using ClustalX (version 2.0) [39]. The best substitution matrix for creating a phylogenetic tree of sand fly salivary YRPs was determined in ProtTest software 2.0 [40]. TREEPUZZLE 5.2 [41] was used to create a maximum likelihood phylogenetic tree from the protein alignment using the WAG model [42] and quartet puzzling with 10000 puzzling steps. The resulting tree for all 31 proteins from 11 sand fly species in two genera was visualized in MEGA 4 [43] and rooted by the related protein from *Drosophila melanogaster* (ACCN: NP650247). Clustal Omega [44] was used with default settings to calculate a Percent Identity Matrix among all sand fly salivary YRPs.

Prediction of glycosylation

Putative N-, O-, and C- glycosylation sites for all 31 protein sequences were determined using NetNGlyc 1.0, NetOGlyc 4.0 [45], and NetCGlyc 1.0 [46] servers with default settings.

3D models construction

All proteins were modeled using 3Q6K, the only available structure of sand fly salivary YRP in Protein Data Bank (PDB, [47]), from where its PDB file and fasta sequence were downloaded. The alignment of template and target sequence was done in Clustal Omega [44] for all proteins. Scripts for Python version 2.7 (Python Software Foundation) were prepared in a txt file and ran in MODELLER [48]. Five models were calculated for each protein, with the best one chosen based on the lowest energy levels of molpdf and the DOPE score. All models were displayed and analyzed in PyMOL (The PyMOL Molecular Graphics System, Version 1.5 Schrödinger, LLC.). Electrostatic surface potentials were calculated using the APBS Tools2 plugin [49] in PyMOL.

Tunnel analysis

Tunnels in the protein structures were detected and characterized using the channel analysis tool MOLE 2.0 [50]. All proteins were superimposed using PyMOL in order to use the same settings, and therefore obtain comparable tunnels. In brief, MOLE 2.0 calculates a Delaunay triangulation/Voronoi diagram of the atomic centers. Next, tunnels are identified between every tuple of user defined end points within a protein structure using Dijkstra's algorithm. The resulting tunnels are defined by their centerline and are uniformly divided into layers. Each layer is defined by the residues lining it. A new layer starts whenever there is a change in residues lining it along its length. Additionally, MOLE 2.0 inferrs basic physicochemical properties for each tunnel as well as layers. These were calculated from a unique set of lining residues averaging tabulated values for hydrophobicity [51]. WebLogo 2.8.2. [52] and Clustal Omega [44] were used to visualize differences among protein tunnels.

Results

Identification of sand fly salivary YRPs

Thirty two sand fly salivary YRPs were identified based on their similarity with PDB ID: 3Q6K from *L. longipalpis* [26], the only known 3D structure of YRPs of phlebotominae sand flies. These YRPs were detected in all 13 sand fly species with a published salivary gland transcriptome: 9 *Phlebotomus* species from 5 subgenera (*Phlebotomus*, *Paraphlebotomus*, *Larroussius*, *Adlerius*, and *Euphlebotomus*), and 4 *Lutzomyia* species from 3 subgenera (*Lutzomyia*, *Nyssomyia*, and *Helcocyrtomyia*). In summary, there are currently 33 different YRPs described in the Phlebotominae subfamily (Table 1). There are large differences in the number of these proteins found in various species, ranging from 1 in *P. arabicus* and *P. argentipes* to 5 in *P. sergenti*.

Phylogenetic analysis

The phylogenetic analysis was performed using the maximum likelihood phylogenetic tree of all 33 YRPs from 13 sand fly species. A higher variability in *Lutzomyia* YRPs compared to *Phlebotomus* proteins was found: two clusters from seven *Lutzomyia* YRPs were detected but four other *Lutzomyia* proteins created own branches. Twenty two *Phlebotomus* proteins clustered together in one branch with high bootstrap support. A closer phylogenetic relationship was discovered between the subgenera *Phlebotomus* (*P. papatasi* and *P. duboscqi*) and *Paraphlebotomus* (*P. sergenti*), as well as between *Larrousius* (*P. tobbi*, *P. orientalis*, *P. perniciosus*, and *P.*

Table 1. Identified sand fly salivary YRPs.

ACCN or name	Sand fly species	Subgenus	Published by	Identifier
BAM69109	*L. ayacuchensis*	*Helcocyrtomyia*	[19]	Laya1
BAM69110	*L. ayacuchensis*	*Helcocyrtomyia*	[19]	Laya2
BAM69111	*L. ayacuchensis*	*Helcocyrtomyia*	[19]	Laya3
BAM69185	*L. ayacuchensis*	*Helcocyrtomyia*	[19]	Laya4
AFP99235	*L. intermedia*	*Nyssomyia*	[17]	Lint
AAS05318, LJM11, 3Q6K	*L. longipalpis*	*Lutzomyia*	[10, 25]	3Q6K_Llon1
AAD32198, LJM17	*L. longipalpis*	*Lutzomyia*	[8]	Llon2
ABB00904, LJM111	*L. longipalpis*	*Lutzomyia*	[10]	Llon3
ANW11467	*L. olmeca*	*Nyssomyia*	[21]	Lolm1
ANW11468	*L. olmeca*	*Nyssomyia*	[21]	Lolm2
ANW11469	*L. olmeca*	*Nyssomyia*	[21]	Lolm3
ACS93501	*P. arabicus*	*Adlerius*	[14]	Para
ABA12136	*P. argentipes*	*Euphlebotomus*	[12]	Parg
AAX44093	*P. ariasi*	*Larroussius*	[11]	Pari1
AAX56360	*P. ariasi*	*Larroussius*	[11]	Pari2
ABI15938	*P. duboscqi*	*Phlebotomus*	[13]	Pdub1
ABI15941	*P. duboscqi*	*Phlebotomus*	[13]	Pdub2
ABI20172	*P. duboscqi*	*Phlebotomus*	[13]	Pdub3
AGT96460	*P. orientalis*	*Larroussius*	[20]	Pori1
AGT96461	*P. orientalis*	*Larroussius*	[20]	Pori2
AAL11051	*P. papatasi*	*Phlebotomus*	[9]	Ppap1
AAL11052	*P. papatasi*	*Phlebotomus*	[9]	Ppap2
AGE83094	*P. papatasi*	*Phlebotomus*	[15]	Ppap3
AGE83095	*P. papatasi*	*Phlebotomus*	[15]	Ppap4
ABA43049	*P. perniciosus*	*Larroussius*	[12]	Pper1
ABA43050	*P. perniciosus*	*Larroussius*	[12]	Pper2
ADJ54114	*P. sergenti*	*Paraphlebotomus*	[16]	Pser1
ADJ54115	*P. sergenti*	*Paraphlebotomus*	[16]	Pser2
ADJ54116	*P. sergenti*	*Paraphlebotomus*	[16]	Pser3
ADJ54122	*P. sergenti*	*Paraphlebotomus*	[16]	Pser4
ADJ54123	*P. sergenti*	*Paraphlebotomus*	[16]	Pser5
ADJ54079	*P. tobbi*	*Larroussius*	[16]	Ptob1
ADJ54080	*P. tobbi*	*Larroussius*	[16]	Ptob2

Sequence GenBank accession numbers (or names), sand fly species (ordered alphabetically), references, and identifier names used in this study are provided for each protein.

doi:10.1371/journal.pone.0166191.t001

ariasi) and *Adlerius* (*P. arabicus*) species. *Euphlebotomus* (*P. argentipes*) protein created a single branch, which is closer to species from subgenera *Larroussius* and *Adlerius* (Fig 1).

Based on the identity matrix from Clustal Omega, the similarity among all 33 YRPs (528 combinations) varied from 44 to 99% (S1 Table).

Glycosylation

For all 33 proteins, potential N-, O- and C-glycosylation sites were predicted. In 11 proteins, only N-glycosylation sites were predicted. In four YRPs of *P. sergenti* (Pser1-Pser4), only O-glycosylation sites were identified, and C-glycosylation sites were predicted for only two

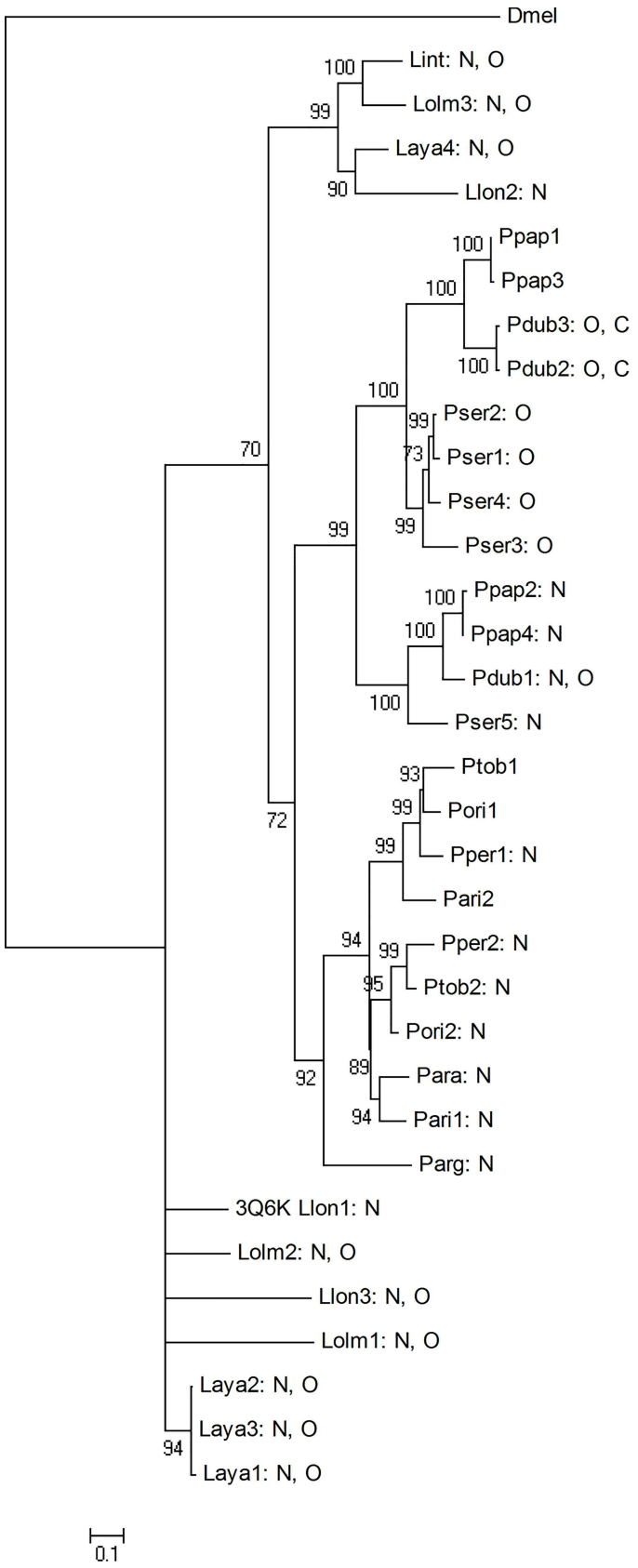
Dmel
100
Lint: N, O
99
Lolm3: N, O
Laya4: N, O
90
Llon2: N
100
Ppap1
100
Ppap3
Pdub3: O, C
100
Pdub2: O, C
100
99
Pser2: O
70
73
Pser1: O
Pser4: O
99
99
Pser3: O
100
Ppap2: N
100
Ppap4: N
Pdub1: N, O
100
Pser5: N
93
Ptob1
99
Pori1
72
99
Pper1: N
Pari2
94
99
Pper2: N
95
Ptob2: N
Pori2: N
89
92
Para: N
94
Pari1: N
Parg: N
3Q6K Llon1: N
Lolm2: N, O
Llon3: N, O
Lolm1: N, O
Laya2: N, O
Laya3: N, O
94
Laya1: N, O
0.1

Fig 1. Phylogenetic tree of 33 sand fly salivary YRPs with putative glycosylation sites marked. The maximum likelihood phylogenetic tree was created in TREEPUZZLE with WAG model using quartet puzzling with 10000 puzzling steps. For rooting, the related protein (ACCN: NP650247) from *Drosophila melanogaster* (Dmel) was used. Bootstraps with support for branching are shown. The letters N, O, and C indicate putative N-, O-, and C-glycosylation sites, respectively. Protein codes refer to Table 1.

doi:10.1371/journal.pone.0166191.g001

proteins from *P. duboscqi* (Table 2). In eleven proteins, more than one type of putative glycosylation was found.

The predicted glycosylation sites for the majority of YRPs (77% and 53% of putative N-glycans and O-glycans, respectively) share the same positions (Table 2). Importantly, proteins clustering together in the phylogenetic tree (Fig 1) had similar predicted glycosylation patterns.

Table 2. Putative glycosylation sites.

Identifier	N-glycosylation	O-glycosylation	C-glycosylation
Laya1	Asn 164, Asn 196	Ser 208	-
Laya2	Asn 164, Asn 196	Ser 113, Ser 208	-
Laya3	Asn 164, Asn 196	Ser 208	-
Laya4	Asn 11, Asn 264	Ser 262	-
Lint	Asn 11	Thr 262, Thr 263	-
3Q6K_Llon1	Asn 195	-	-
Llon2	Asn 11	-	-
Llon3	Asn 123	Ser 300	-
Lolm1	Asn 122	Thr 262	-
Lolm2	Asn 194	Thr 261	-
Lolm3	Asn 11	Thr 197	-
Para	Asn 11	-	-
Parg	Asn 11, Asn 18, Asn 307	-	-
Pari1	Asn 11, Asn 201	-	-
Pari2	-	-	-
Pdub1	Asn 11, Asn 65	Thr 367	-
Pdub2	-	Ser 208	Trp 338
Pdub3	-	Ser 208	Trp 338
Pori1	-	-	-
Pori2	Asn 11	-	-
Ppap1	-	-	-
Ppap2	Asn 11, Asn 65	-	-
Ppap3	-	-	-
Ppap4	Asn 11, Asn 65	Ser 264	-
Pper1	Asn 11	-	-
Pper2	Asn 11	-	-
Pser1	-	Ser 81, Ser 208, Thr 263	-
Pser2	-	Ser 208, Thr 263	-
Pser3	-	Ser 208, Thr 263	-
Pser4	-	Ser 208	-
Pser5	Asn 11, Asn 65, Asn 250	-	-
Ptob1	-	-	-
Ptob2	Asn 11	-	-

N-, O- and C-glycosylation sites were predicted for each protein using glycosylation servers. Numbers and three-letter abbreviations indicate the positions of predicted glycosylation and the amino acid where the glycosylation occurs (Asn–asparagine, Thr–threonine, Ser–serine, and Trp–tryptophan).—shows cases where no glycosylation sites were identified. Protein codes refer to Table 1.

doi:10.1371/journal.pone.0166191.t002

3D model analysis

The amino acid sequence alignment of 33 sand fly salivary YRPs showed high conservancy in several regions, with 57 invariant sites identified among all sequences, representing approximately 15% of the total sequence. Five out of eleven amino acids creating the ligand-binding pocket known from Xu et al. [26] were identical across all proteins. The most variable position in the ligand-binding site was the one annotated as Phe 344 in the crystal structure of YRP 3Q6K—this position is occupied by six different amino acids (His, Gln, Tyr, Phe, Met, or Lys) in our studied set of proteins. Two most common amino acids on this position are histidine and glutamine (Fig 2).

Models for 32 different proteins were created based on the template sequence 3Q6K from *L. longipalpis* (S1 Fig). All of them folded as a six-bladed beta-propeller, similarly as in 3Q6K [26]. In 3Q6K, 11 ligand-binding amino acids were identified [26]. Three of them (in the case of 3Q6K –Thr 327 with 2 bonds, Asn 342 with 2 bonds, and Phe 344 with 1 bond) play a major role in binding abilities, because of the hydrogen bonds between the protein amino acids and

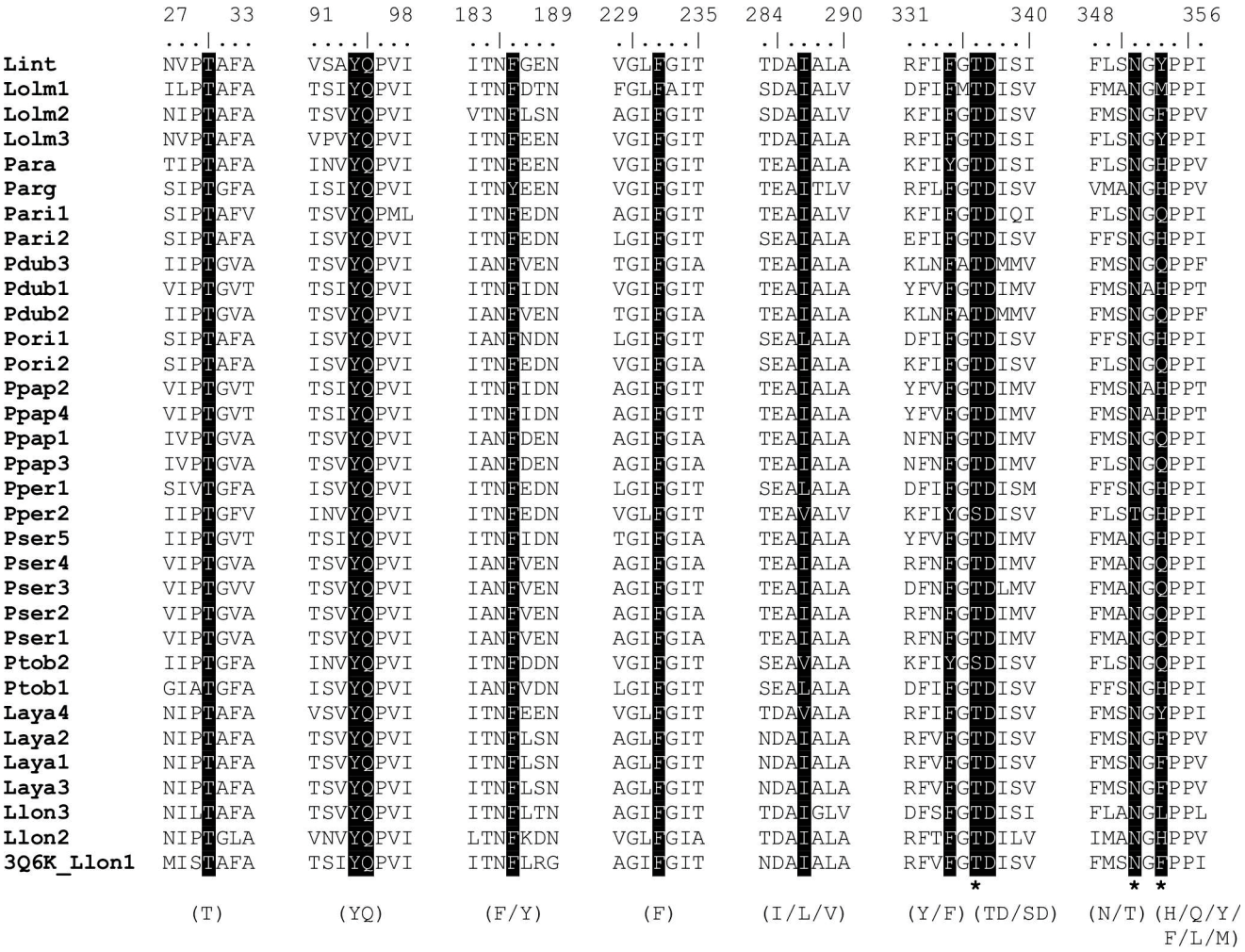

Fig 2. Amino acid alignment within the YRP ligand-binding sites. The alignment was created in ClustalX, and shows high conservancy in binding sites (letters in brackets below the sequences, slashes indicate amino acid substitutions in appropriate positions). Ligand binding sites are highlighted in black, three bold stars below the sequences represent the main binding sites where hydrogen bonds between amino acids and the ligand are predicted. Protein codes refer to Table 1.

doi:10.1371/journal.pone.0166191.g002

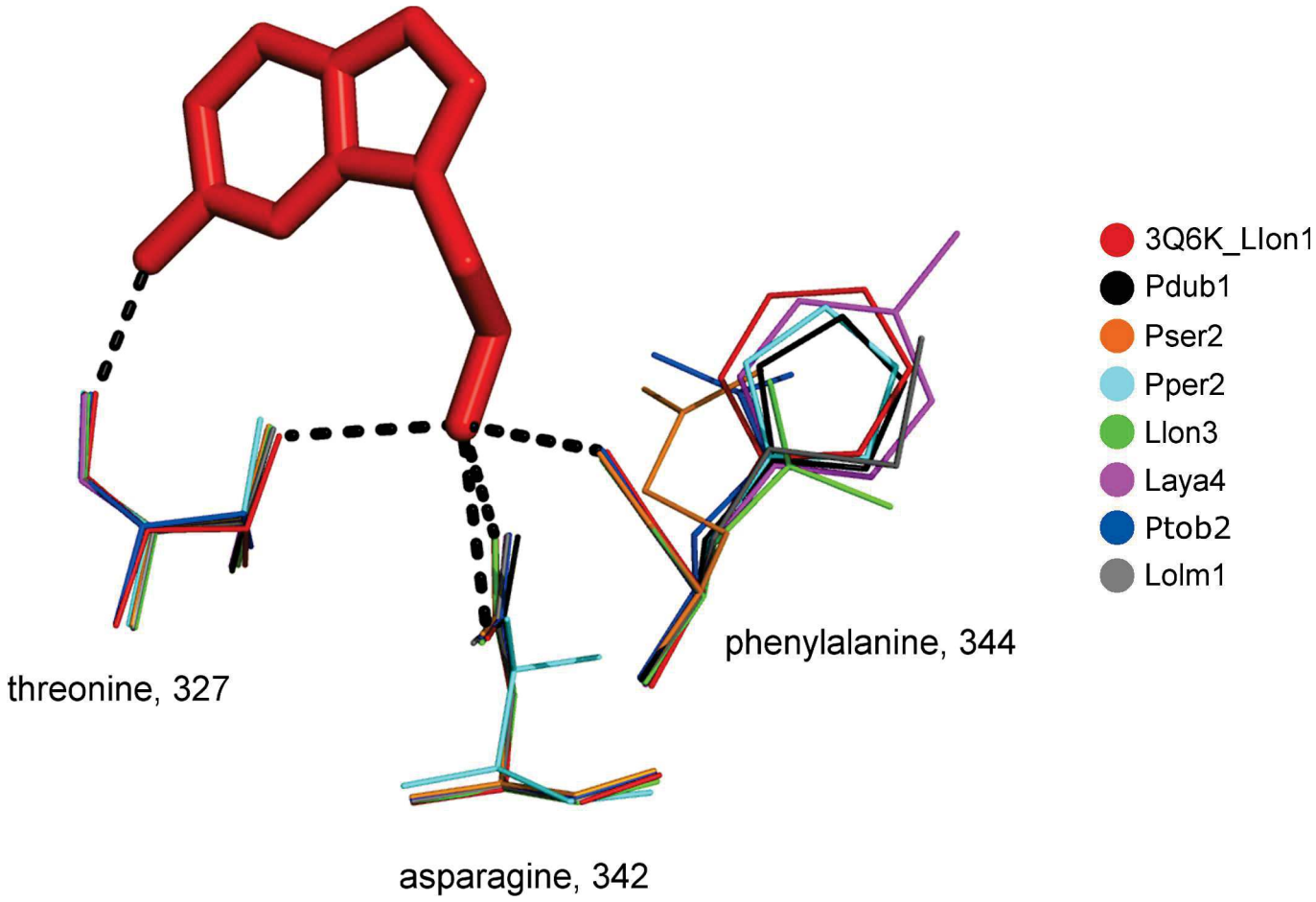

Fig 3. Hydrogen-binding between serotonin and ligand-binding amino acids in sand fly salivary YRPs. The figure shows the variability in hydrogen bonds between serotonin and 3Q6K (provided by Thr 327, Asn 342, and Phe 344) described in Xu et al. [26] and other YRP models created during this study and visualized in PyMOL. The red bold molecule symbolizes the ligand serotonin and the black dashed line show the hydrogen bonds between amino acids and serotonin in 3Q6K. Other colors indicate one representative member of one protein group. A group was defined as having at least one unique mutation from the template structure in one of the three described amino acids binding the ligand. From each group, the protein with the most abundant amino acid rotamer was chosen as being representative for this visualization. Protein codes refer to Table 1.

doi:10.1371/journal.pone.0166191.g003

the ligand. The other 8 amino acids bind serotonin by Van der Waals or hydrophobic interactions. These three amino acids were visualized in the 3D alignment of all 33 proteins. Nine different amino acids were found at three positions that create hydrogen bonds with serotonin from 3Q6K (Fig 3).

As well as in 3Q6K_Llon1, in Laya1-3 and Lolm1, the amino acids creating hydrogen bonds with serotonin are Thr 327, Asn 342, and Phe 344 (using the numbering for 3Q6K). The largest group includes 11 proteins (Llon2, Para, Parg, Pari, Pdub1, Pori2, Ppap2, Ppap4, Pper1, Pser5, and Ptob1). In comparison with the group containing 3Q6K_Llon1, there is a substitution of histidine instead of phenylalanine in the last binding position. A substitution in the same position (glutamine instead of phenylalanine) also occurs in the second biggest group, which includes 10 proteins (Pari1, Pdub2, Pdub3, Pori2, Ppap1, Ppap3, Pser1, Pser2, Pser3, and Pser4). The last group with more than one protein (Laya4, Lint, and Lolm3) has substitution in the last binding position, as well. In this case, there is tyrosine instead of phenylalanine. Pper2 has substitutions in all three positions (serine, threonine, and histidine). Because of these

substitutions (mainly due to the presence of Thr instead of Asn) these proteins are likely to have different binding ability to biamines from 3Q6K_Llon1. In the last binding positions of Llon3 and Lolm1 there are leucine and methionine, respectively, instead of phenylalanine. Ptob2 contains a substitution in the first binding position, with threonine replaced by serine. The distribution of YRPs in these groups partially corresponds with branches in the phylogenetic tree (S2 Fig).

Tunnel in YRPs

In Xu et al. [26], YRP 3Q6K from *L. longipalpis* was described as a hollow barrel with two possible entrances. Here, based on the models created using other sand fly YRPs and MOLE analysis, we found a very similar hollow barrel in all proteins we studied (Table 3). It is therefore likely that all studied proteins retain the ability to bind a ligand. The differences in a length and minimal radius of the identified tunnels have limited relevance due to the fact that they are calculated on theoretical models. On contrary, differences in hydrophobicity should be less influenced by homology modeling process and could therefore indicate (where present) changes in binding affinities among paralogs within species.

The ligand-binding site for serotonin in 3Q6K is located approximately one third of the way along this tunnel (Fig 4). The comparison based on the APBS tools in PyMol of all models and the 3Q6K structure revealed that the surface electrostatic potential of the entrance closer to the binding site varies from negative to positive (S2 Fig), and the size of this entrance is smaller than the opposite entrance. In contrast, the entrance more distant from the binding site is larger and neutrally or negatively charged in all these proteins (Table 3). The distribution of the surface potentials in most of these proteins corresponds with the phylogenetic relationships (Fig 1) and predicted glycosylation sites (Table 2).

The sequence conservation of the amino acids lining the tunnels was explored using WebLogo server (Fig 5). The WebLogo was based on the multiple sequence alignment (S3 Fig) of all amino acids lining the accessible tunnels towards the ligand-binding site, as described by MOLE. The analysis showed that there are not any absolutely conserved amino acids, but both entrances to the tunnel are dominated by negatively-charged residues; however, there are proteins (e.g. Pdub1, Pari2, Ppar1, Ppap4, and Pser5) that are positively charged at least at one of the entrances. The middle section of the tunnel lining is occupied mostly by hydrophobic and small polar amino acids.

Discussion

Here we focused on the bioinformatic characterization of sand fly salivary YRPs. These proteins have high affinity binding properties for pro-haemostatic and pro-inflammatory biogenic amines [26], but they are also highly antigenic for hosts bitten by sand flies [30–34] and can be used for anti-*Leishmania* vaccine development [53]. We searched published transcriptomes and recombinant proteins and found 33 different proteins from this family among 13 species from the two genera–Old World *Phlebotomus* and New World *Lutzomyia*. We analyzed their phylogenetic relationships, predicted glycosylation sites, constructed 3D models, identified tunnels passing through these structures, described the ligand-binding site, and compared their surface electrostatic potentials. The number of detected YRPs in these 13 sand fly species varied from one in *P. arabicus* [14], *P. argentipes* [12], and *L. intermedia* [17] to five in *P. sergenti* [16]. Such interspecies variability might be partially attributed to the sensitivity of sequencing methods; however, this is not likely the case of YRPs that were analyzed in the same laboratory using the same method. For example, Anderson et al. [12] reported a single YRP in *P. argentipes* but two in *P. perniciosus*, while Rohousova et al. [16] reported two in *P. tobbi* but five in *P.*

Table 3. A comparison of tunnels in sand fly salivary YRPs.

Identificator	Length	Min. Radius	Hydrophobicity	Ligand-side	Opposite-side
Laya1	38.320	1.769	-0.13	Positive	Negative
Laya2	38.707	1.813	-0.22	Positive	Negative
Laya3	41.045	0.962	-0.17	Positive	Negative
Laya4	41.295	1.785	-0.25	Neutral (negative)	Neutral (negative)
Lint	39.194	1.539	-0.32	Negative	Negative
3Q6K_Llon1	41.245	1.892	-0.23	Positive	Neutral (negative)
Llon2	39.722	1.361	-0.07	Negative	Negative
Llon3	43.634	1.831	-0.29	Negative	Negative
Lolm1	38.595	2.055	-0.21	Negative (neutral)	Neutral
Lolm2	41.696	1.997	-0.45	Positive (neutral)	Neutral (negative)
Lolm3	43.098	1.416	-0.86	Neutral (negative)	Negative (neutral)
Para	45.294	1.229	-0.53	Negative	Negative
Parg	40.506	1.262	-0.20	Negative	Negative
Pari1	45.068	1.673	-0.02	Negative	Negative
Pari2	41.717	1.880	-0.29	Negative	Negative
Pdub1	37.292	1.744	-0.14	Negative	Neutral (negative)
Pdub2	39.912	1.433	0.03	Neutral (positive)	Neutral (negative)
Pdub3	35.967	2.021	-0.34	Negative	Neutral (positive, negative)
Pori1	42.203	1.677	-0.22	Negative	Negative
Pori2	32.368	1.824	-0.18	Negative	Neutral (negative)
Ppap1	38.163	1.834	-0.23	Negative	Neutral (positive)
Ppap2	40.508	1.780	-0.05	Neutral (negative)	Negative (neutral)
Ppap3	40.849	2.053	-0.08	Negative	Neutral (positive)
Ppap4	46.137	1.518	-0.27	Neutral	Neutral (negative, positive)
Pper1	41.652	1.419	-0.18	Negative	Negative
Pper2	41.944	1.948	-0.25	Neutral (negative)	Neutral (negative)
Pser1	50.023	2.047	-0.27	Negative	Neutral
Pser2	37.843	1.755	-0.05	Negative	Neutral
Pser3	38.013	1.757	0.02	Neutral	Negative
Pser4	31.949	1.812	0.01	Negative	Negative
Pser5	40.761	1.853	-0.01	Negative	Neutral
Ptob1	33.603	1.740	-0.31	Negative	Negative
Ptob2	34.118	1.822	-0.22	Neutral (positive)	Neutral (negative)

The length and minimum radius (in Ångströms) and hydrophobicity are shown for the tunnels of each protein. All values were calculated using MOLE. Surface electrostatic potentials around protein entry sides were calculated in PyMOL using the APBS tool. "Ligand-side" refers to the side with the shorter distance from the beginning of the tunnel to the ligand-binding site, while "Opposite-side" is the other side of the tunnel. The most common electrostatic surface potentials of both entrances to the tunnel are shown. Charges in brackets indicate that the charge around the entrance is not uniform, and small parts of the entrance are charged differently from the majority of the entrance. Protein codes refer to Table 1.

doi:10.1371/journal.pone.0166191.t003

sergenti. It is also possible that occurence of various numbers of YRPs is caused by differential gene expression. Genes with lower expression might be recorded less frequently as it was shown for other sand fly protein families [54].

Our phylogenetic analysis suggested a higher variability in *Lutzomyia* than in *Phlebotomus* YRPs. Sequence identity among YRPs ranged between 44 and 99 percent, with a majority of proteins having sequence identity around 50 percent. Further analysis divided these proteins into several branches, with closer relationships within sand flies from the same subgenera

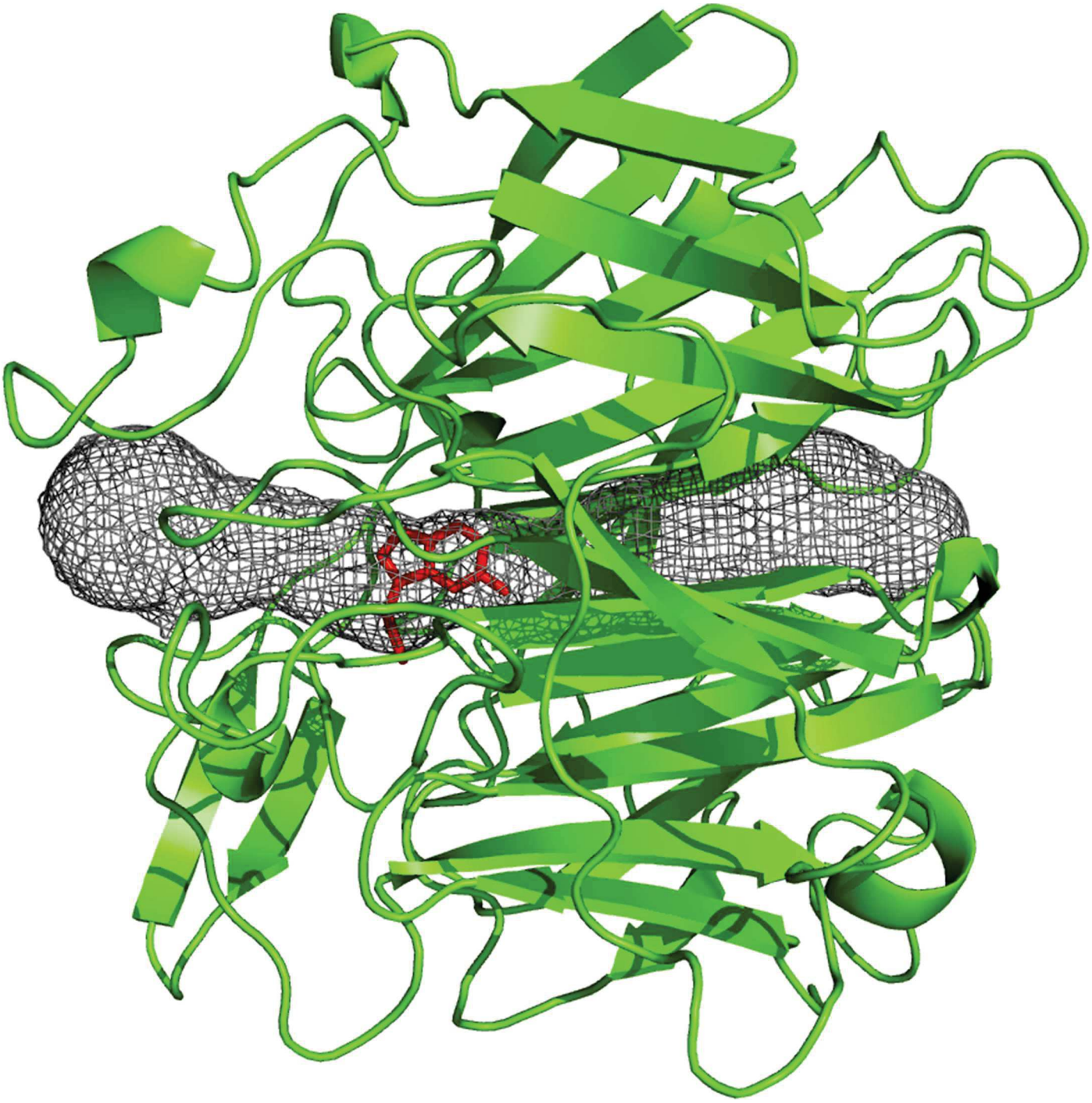

Fig 4. Visualization of the tunnel in 3Q6K_Llon1. The structure was visualized in PyMOL using the MOLE script for calculating the tunnel. The 3Q6K_Llon1 protein is drawn in green, the tunnel through this protein structure is in black mesh and the red-stick molecule represents serotonin.

doi:10.1371/journal.pone.0166191.g004

(*Phlebotomus*, *Paraphlebotomus*, and *Larrousius*). This confirmed previous analyses based on amino acid sequences [16, 20] and also corresponds with an analysis based on small subunit nuclear ribosomal DNA [55] showing two main clades–*Phlebotomus* with *Paraphlebotomus*

Fig 5. Sequence conservation of the amino acids lining the ligand-binding tunnel. The WebLogo server was used to visualize the sequence conservation of the amino acids lining the ligand-binding tunnel in 31 YRPs. The amino acids are colored according to their physical-chemical properties. LS and OS below the x-axis indicate the side closer to the ligand-binding site and the opposite side of the tunnel, respectively.

doi:10.1371/journal.pone.0166191.g005

subgenera clustering together and leaving other subgenera (e.g. *Euphlebotomus*, *Larroussius*, *and Adlerius*) in the second clade.

The biogenic function of proteins may be affected by the presence or absence of posttranslational modifications [56, 57]. For example, the antibody response against some glycoproteins can be directed strictly to the glycan part [58]. In accordance with our previous studies [16, 20, 54, 59], we showed both inter- as well as intra-species variability in the glycosylation of sand fly YRPs, which corresponded well with the phylogenetic analysis. Moreover, the same positions of putative glycosylation sites among YRPs mirrored the presumed effects of glycosylation on protein folding and stability, suggesting a highly conserved tertiary structure. It would be interesting to evaluate similarities in the antigenic potential of the proteins belonging to the same clusters.

The only protein with an experimentally solved 3D structure, LJM11 (3Q6K) from *Lutzomyia longipalpis*, has a unique six-bladed beta propeller fold that has only been identified in this protein. It is characterized by a long ligand-binding tunnel with two entrances–one on each side of the protein. Our 3D models of other YRPs showed similar structures as 3Q6K, and the tunnel is present in all studied proteins. Its length or minimum width vary between and within species but it remains uninterrupted in all modeled proteins, suggesting it is very important feature of these proteins. LJM11 is known to bind positively charged biogenic amines [26], and our structural analysis of YRPs from other sand fly species showed that in all tested sequences the ligand-binding tunnels were negatively charged and could therefore bind positively charged ligands of bioamine size. Nevertheless, we observed that the proteins and more specifically the paralogs within one species differ in the charge of tunnel entrances. Our data indicate that paralogs within one species will bind similar ligands with different affinities, and the ligands may travel different paths to the binding site according to the charge of the entrances. YRPs also differed within and among species in the length of their tunnels, the minimum radius of the tunnel and its hydrophobicity. We expect that all these parameters affect the nature and the affinity of the preferred ligand, which results in a greater binding versatility of the host bioamines.

In the 3Q6K YRP structure, the serotonin ligand is stabilized by hydrogen bonds from three amino acids in the main chain and side chain and by a number of additional hydrophobic interactions [26]. Both our sequence and structure analyses showed the diversity of this crucial site among the 33 proteins studied here. The largest diversity was observed on the position denoted in 3Q6K as Phe 344 (see Fig 3). The amino acid at this position participates in ligand-binding through a hydrogen bond from the main chain. Substitutions at this position should

not play a major role in ligand-binding, yet it is the only amino acid of the ligand-binding-site that is different among three *L. longipalis* yellow-related proteins that have been shown to have different affinities for serotonin and other tested bioamines [26]. Our models of the other YRPs suggest that at least in the case of the Phe344Gln mutation, the glutamine side chain could provide a further hydrogen bond to the ligand (serotonin). Interestingly, our studied YRPs can be divided into several groups according to their ligand-binding composition, and some groups correlate with the branches identified in phylogenetic distribution as well as with the predicted glycosylation patterns. For example, Ppap2, Ppap4, Pdub1, and Pser5 (proteins from the largest group with binding amino acids Thr-Asn-His) or Pser1-4 (proteins from the second largest group with binding amino acids Thr-Asn-Glu) are located in two branches in the phylogenetic tree and share the same N-glycosylation and O-glycosylation pattern, respectively (see S2 Fig).

The lining of the tunnel is not conserved, but the entrances to the tunnel are composed mostly of negatively charged residues and the inner part of the tunnel is lined by hydrophobic and small polar amino acids (threonines). There are however several proteins (Pdub1, Ppap4, Pser5 etc.) that have positively charged residues forming one of the entrances (typically the one closer to ligand-binding site within the tunnel)–this might allow interaction with different ligands.

In summary, we found interesting diversity among these important proteins. All 33 sand fly salivary YRPs studied here share similar folding and contain a ligand-binding tunnel. Nevertheless, modifications found among these proteins influence the charge of entrances to the tunnel, the length of the tunnel and/or its hydrophobicity. We suggest that these modifications provide sand fly species with multiple paralogs of YRPs with more nuanced answers to host bioamines. Further experiments are needed to validate these models, but we assume, that our results should allow a better understanding of the biological role of these important proteins with respect to their potential use in anti-*Leishmania* vaccines or as host exposure markers to sand flies.

Supporting Information

S1 Fig. Modeled 3D structures of sand fly yellow-related proteins. Zipped PDB files of 32 sand fly yellow-related proteins calculated in MODELLER based on 3Q6K as the template structure. Protein codes refers to Table 1.
(ZIP)

S2 Fig. Visual summary of the diversity of yellow-related proteins in sand flies. The maximum likelihood phylogenetic tree was created in TREEPUZZLE with WAG model using quartet puzzling with 10000 puzzling steps. For rooting, the related protein (ACCN: NP650247) from *Drosophila melanogaster* (Dmel) was used. Bootstraps with support for branching are shown. Protein codes refer to Table 1. Symbols preceding the protein codes indicate the most common surface electrostatic potential of the entrance to the protein tunnel closer to the ligand-binding site (LS) as shown in Table 3 (◊ positive, ○ neutral, and □ negative). Colors in symbols indicate the affiliation to protein groups with the same ligand-binding amino acids as shown in Fig 3. The letters N, O, and C indicate putative N-, O-, and C-glycosylation sites, respectively, based on Table 2.
(PDF)

S3 Fig. Alignment of amino acids creating tunnels in yellow-related proteins. For each protein, amino acids creating tunnel were determined using MOLE. Clustal Omega was used to visualize differences of these protein tunnels. In sequences, small letters indicate main chain

interaction and capital letters represent side chain interactions. Above the sequences, LS and OS describe the side closer to the ligand binding site and the opposite side of the tunnel, respectively. Protein codes refer to Table 1.
(PDF)

S1 Table. Percent Identity Matrix of sand fly yellow-related proteins. Clustal Omega was used to calculate this sequence identity matrix among all identified yellow-related proteins. The similarity between the proteins is shown in percents. Different colors represent 10 percent similarity intervals. Protein codes refer to Table 1.
(PDF)

Acknowledgments

We appreciate Helena Kulikova and Lenka Zitkova for excellent administrative assistance and Simon Kerdik for the help with graphical images.

Author Contributions

Conceptualization: MN.

Formal analysis: MN.

Investigation: MS.

Methodology: MS.

Software: LP.

Supervision: IR PV.

Visualization: MS MN.

Writing – original draft: MS MN LP PS.

Writing – review & editing: MN PV IR.

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Amine-binding properties of salivary yellow-related proteins in phlebotomine sand flies

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Amine-binding properties of salivary yellow-related proteins in phlebotomine sand flies

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Abbreviations: YRP, yellow-related protein; MST, microscale thermophoresis; ITC, isothermal titration calorimetry; HEK293S, human embryonic kidney 293S; Kd, dissociation constant

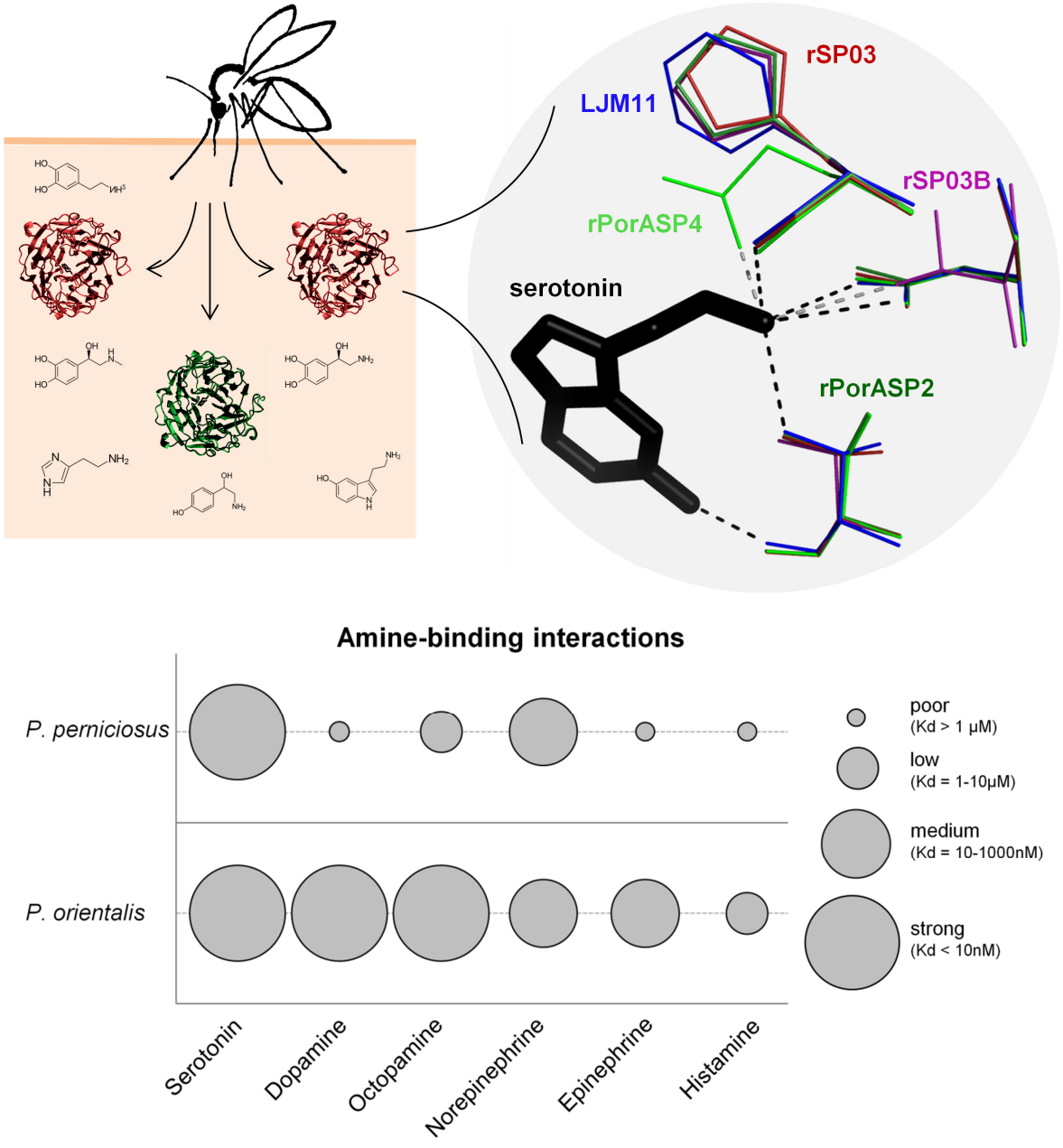
Abstract

The amine-binding properties of sand fly salivary yellow-related proteins (YRPs) were described only in *Lutzomyia longipalpis* sand flies. Here, we experimentally confirmed the kratagonists function of YRPs in the genus *Phlebotomus*. We utilized microscale thermophoresis technique to determine the amine-binding properties of YRPs in saliva of *Phlebotomus perniciosus* and *P. orientalis*, the Old-World vectors of visceral leishmaniases causative agents. Expressed and purified YRPs from three different sand fly species were tested for their interactions with various biogenic amines, including serotonin, histamine and catecholamines. Using the *L. longipalpis* YRP LJM11 as a control, we have demonstrated the comparability of the microscale thermophoresis method with conventional isothermal titration calorimetry described previously. By homology *in* silico modeling, we demonstrated the surface charge and both amino-acids and hydrogen bonds of the amine-binding motifs to influence the binding affinities between closely related YRPs. All YRPs tested bound at least two biogenic amines, while the affinities differ both among and within species. Low affinity was observed for histamine. The salivary recombinant proteins rSP03B (*P. perniciosus*) and rPorASP4 (*P. orientalis*) showed high-affinity binding of serotonin, suggesting their capability to facilitate inhibition of the blood vessel contraction and platelet aggregation.

Keywords

yellow-related protein, *Phlebotomus perniciosus*, *Phlebotomus orientalis*, salivary protein, microscale thermophoresis, biogenic amine

41 **Graphical abstract**



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Highlights:

- MST was proven as a valid method to measure amine-binding properties of sand fly yellow-related proteins (YRPs).
- The amine-binding function was experimentally verified for YRPs of genus *Phlebotomus*.
- All YRPs bound at least two biogenic amines, while the affinities differ both among and within species.
- During feeding, YRPs potentially facilitate counteracting platelet aggregation and vasoconstriction via high affinity binding of serotonin.

1. Introduction

Phlebotomus perniciosus and *P. orientalis* are closely related sand fly species (Diptera: Phlebotominae) belonging to the subgenus *Larroussius*. *P. perniciosus* is distributed through the western and central parts of Mediterranean region and it serves as an important vector of *Leishmania infantum,* a causative agent of visceral leishmaniases in Southern Europe and Northern Africa, while *P. orientalis* is a proven vector of *Leishmania donovani* in Sudan, Ethiopia and Kenya (Dvorak et al., 2018). The number of human cases of visceral leishmaniasis were estimated to annually reach up to 20000 and 56700, in Mediterranean and East African regions, respectively (Alvar et al., 2012).

To facilitate successful blood feeding, sand fly injects into the host skin saliva containing a vast variety of pharmacologically active compounds that interact with the host haemostatic processes (Lestinova et al., 2017). To ease the spread of these biomolecules, sand fly saliva contains hyaluronidase, which enzymatic activity facilitates the enlargement of the feeding site by degrading the extracellular matrix (Volfova et al., 2008; Volfova and Volf, 2018). To stop the blood coagulation, sand fly employ anticoagulants which affects the components of coagulation cascade (Chagas et al., 2014; Collin et al., 2012) or inhibits

68 the activators of coagulation (Alvarenga et al., 2013). Some sand fly saliva components such
69 as maxadilan (Lerner and Shoemaker, 1992) and adenosine (Ribeiro et al., 1999) act directly
70 as vasodilatators, while other proteins, such as salivary apyrase prevent the ATP-induced
71 aggregation of platelets as reviewed in (Lestinova et al., 2017).

72 Host haemostatic responses to insect bites are triggered also by biogenic amines such
73 as serotonin, histamine or catecholamines. Serotonin is released by platelets and initiates
74 vasoconstriction resulting in limitation of the flow of blood to the insects mouthparts (Ribeiro,
75 1995). Histamine is secreted upon the tissue damage from basophiles and mast cells granules
76 and influence the hydrostatic pressure of capillaries and their permeability for plasma
77 containing immune cells and other factors (Paesen et al., 1999; Ribeiro and Francischetti,
78 2003). Both serotonin and histamine have also roles in inducing itch and pain response to
79 the insect bite (Julius and Basbaum, 2001; Yosipovitch et al., 2018). Other biogenic amines
80 playing roles in host haemostatic responses belong to the catecholamine family.
81 Norepinephrine stimulates vasoconstriction via adrenergic receptors in the vasculature (Calvo
82 et al., 2009; Xanthos et al., 2008). When released by the local nerves in response to bleeding,
83 epinephrine also initiates constriction of blood vessels and it potentiates platelet aggregation
84 (Andersen et al., 2003).

85 Bloodsucking insects prevent haemostatic responses through binding biogenic amines
86 in the pocket of hollow barrel structure forming the amine-binding proteins belonging into
87 three different protein families (reviewed in Lestinova et al., 2017). Lipocalins serve this
88 function in ticks and triatomine bugs (Andersen et al., 2003; Mans and Ribeiro, 2008;
89 Sangamnatdej et al., 2002; Xu et al., 2013) while D7-related proteins are known kratagonists
90 in mosquitoes (Calvo et al., 2006; Jablonka et al., 2019; Mans et al., 2007). The amine-
91 binding potential of sand fly salivary yellow-related proteins YRPs was firstly hypothesized
92 by Charlab et al. (1999). In 2011, the crystal structure and antihaemostatic properties of YRPs

of the New-World sand fly *Lutzomyia longipalpis* were revealed. All three *L. longipalpis* YRPs were shown to bind with different affinities serotonin, histamine and catecholamines - epinephrine, norepinephrine, dopamine and octopamine (Xu et al., 2011). Although predicted, the binding of biogenic amines was not yet experimentally demonstrated for YRPs of sand flies from the genus *Phlebotomus*.

To measure the amine-binding interactions of YRPs, several methods can be employed. So far, the isothermal titration calorimetry (ITC) was the method of choice for the characterization of salivary amine-binding proteins of blood-sucking arthropods (Andersen et al., 2003; Calvo et al., 2009, 2006; Jablonka et al., 2019; Ma et al., 2012; Xu et al., 2013, 2011). In sand flies, this method was also utilized to characterize the mechanism of coagulation pathway inhibition in anticoagulants (Alvarenga et al., 2013; Collin et al., 2012). Microscale thermophoresis was demonstrated as a method comparable with ITC, requiring less sample volume and time (Scheuermann et al., 2016; Seidel et al., 2013; Wienken et al., 2010) but has never been used to characterize binding properties of proteins from blood sucking arthropods.

Here, we have expressed recombinant YRPs from two closely related *Phlebotomus* species, *P. orientalis* (rPorASP4 and rPorASP2) and *P. perniciosus* (rSP03 and rSP03B), and experimentally tested their ability to bind host biogenic amines through MST. To validate the accuracy of our chosen method, we have also expressed and measured binding affinities of *L. longipalpis* YRP LJM11, which ability to bind amines was previously determined using ITC (Xu et al., 2011). We have utilized the 3D models of YRPs to evaluate the effect of the amino acid composition of the amine-binding site and of the surface electrostatic potential on the differences in YRPs binding affinities to different biogenic amines.

2. Methods

2.1. Expression of recombinant yellow-related proteins

For binding experiments five salivary YRPs were expressed in human cell line (Table 1). For *P. perniciosus* YRPs, the gene construct was prepared by isolating the total RNA from one day old *P. perniciosus* females by the High Pure RNA Tissue Kit (Roche), after which it was transcribed using anchored-oligo $(dT)_{18}$ primers into the cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's protocol. The cDNA fragments were amplified by PCR and subcloned into the pTW5sec expression plasmid, a derivative of pTT5 (Blaha et al., 2015; Durocher et al., 2002). Proteins expressed using this plasmid contain additional ITG- and -GTHHHHHHHHG sequences at their N- and C-termini, respectively.

Table 1. Recombinant salivary yellow-related proteins.

Name	Species	MW (kDa)	ε ($M^{-1}cm^{-1}$)	GenBank ACCN	Salivary protein codes
rSP03	*P. perniciosus*	43.20	51590	ABA43049	PpeSP03[1]; Pper1[2]
rSP03B	*P. perniciosus*	44.26	51590	ABA43050	PpeSP03B[1]; Pper2[2]
rPorASP2	*P. orientalis*	42.96	53080	AGT96427	Pori1[2], rPorASP2[3]
rPorASP4	*P. orientalis*	43.74	53080	AGT96428	Pori2[2], rPorASP4[3], PorSP24[4]
LJM11	*L. longipalpis*	44.67	65000	AAS05318	3Q6K Llon1[2]

List of recombinant YRPs based on the salivary proteins of *P. perniciosus*, *P. orientalis* and *L. longipalpis*. Designation, species, molecular weight (MW), extinction coefficient (ε), GenBank accession numbers and the codes of parallel salivary proteins are indicated. [1] retrieved from Anderson et al., 2006; [2] Sima et al., 2016b; [3] Sumova et al., 2018; [4] Sima et al., 2016a.

The rSP03B protein was transiently expressed in human embryonic kidney 293S (HEK293S) GnTI$^-$ cell line (ATCC CRL-3022), as previously described in Bláha et al., 2015. Briefly, suspension adapted cells were grown in EX-CELL293 medium supplemented with 4 mM L-glutamine (Sigma) in square-shaped glass bottles at 37 °C and 5% CO_2 in a humidified incubator and shaken at 135 rpm. For transient transfection, the cell culture was transferred into EX-CELL293 medium at 20×10^6 cells/ml cell density. The expression plasmid (diluted in PBS; 1 µg of DNA per 1×10^6 cells) and 25 kDa linear polyethylenimine (in a 1:4 w/w ratio to total amount of DNA) were added directly into the high-density cell culture. After 4 hours of incubation, the culture was diluted with EX-CELL293 medium to 2×10^6 cells/ml.

Due to low protein yields, expression cassette of rSP03 was sub-cloned into vector permitting generation of stably transfected HEK293S GnTI$^-$ cell line using piggyBac system (Li et al., 2013). After selection, pools of stably transfected cells were expanded, and protein expression was induced by doxycycline (1 mg/ml) when cell density reached 3×10^6 cells/ml.

Culture medium was harvested five to seven days post-transfection (rSP03B) or induction (rSP03) by centrifugation (10,000 × g, 30 min) and filtered thereafter (0.22 µm Steritop filter; Millipore, USA). Before purification, the harvested medium was diluted with an equal volume of buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM NaN_3, pH 7.5). Histidine-tagged proteins were then purified by IMAC chromatography using HiTrap Talon Crude columns (GE Healthcare) by isocratic (rSP03B) or gradient elution (rSP03). Affinity chromatography was followed by size exclusion chromatography using Superdex 200 Increase 10/300 GL column (GE Healthcare).

Recombinant yellow-related proteins derived from *P. orientalis* and *L. longipalpis* were produced and purified as described elsewhere (Gomes et al., 2012; Sumova et al., 2018). Briefly, the synthetic DNA fragments (GeneArt Strings, ThermoFisher Scientific) coding

recombinant proteins including histidine tag at the C-terminus were cloned into VR2001-TOPO vector (Oliveira et al., 2006). Plasmids were sent to Leidos, NCI, Protein Expression Laboratory (Frederick, MD) for transient transfection and expression. Transfected FreeStyle HEK293 C18 (ATCC CRL-10852) cell cultures were harvested after 72 h. Recombinant proteins were purified in one step in a HPLC system (Bio-Rad) using the HiTrap Chelating HP columns (GE Healthcare) by gradient elution with imidazole.

2.2. Quality check of recombinant proteins

All recombinant YRPs were purified and subsequently stored in phosphate-buffered saline (PBS; pH 7.5). Protein concentrations were measured using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific) at 280 nm and calculated using the theoretical molar extinction coefficients and molecular weights of the proteins (Table 1).

Oligomeric state of all recombinant YRPs was analyzed in analytical ultracentrifuge ProteomeLab XL-I equipped with an An-50 Ti rotor (Beckman Coulter, USA) using the sedimentation velocity experiment. Samples of proteins in PBS buffer were spun at 48,000 rpm at 20 °C, and 100 scans with 0.003 cm spatial resolution were recorded at 280 nm in 5-min steps using absorbance optics. Buffer density and protein partial specific volumes were estimated in SEDNTERP (www.jphilo.mailway.com). Data were analyzed with Sedfit (Schuck, 2000) using a c(s) continuous size distribution model, figure illustrating AUC data was prepared in GUSSI (Brautigam, 2015).

To check the quality of expressed proteins, 1 μg of each eligible protein fractions acquired from chromatography was electrophoretically separated on 12% polyacrylamide gel under non-reducing conditions using a Mini-protean apparatus (Bio-Rad). One gel with separated proteins was silver stained. Separated protein bands from parallel gel were transferred onto a nitrocellulose membrane using the iBLOT system (Invitrogen) and blocked

in 5% non-fat milk diluted in a Tris-buffered saline with 0.05% Tween 20 (TBS-Tw) overnight at 4 °C. Subsequently, the membrane was incubated for 1 hour with a monoclonal anti-polyhistidine-peroxidase antibody (Sigma Aldrich) diluted 1:1,000 in TBS-Tw. After the washing step with TBS-Tw, the chromogenic reaction was developed using a substrate solution containing diaminobenzidine and H_2O_2. Identity and high purity of the proteins was further verified by mass spectrometry.

2.3. Mass spectrometry

Mass spectrometry was used to confirm the high purity of expressed proteins, to estimate the proportion of *P. orientalis* and *P. perniciosus* YRPs in the total amount of salivary glands proteins and to determine the ratio of the two YRPs in both species. The analyses were performed in OMICS Proteomics laboratory Biocev, Czech Republic. For the analysis, salivary glands were dissected from 5 to 7 day old sand fly females into aliquots of 20 glands per 20 µl of 100 mM triethylammonium bicarbonate buffer with 2% sodium deoxycholate and boiled at 95 °C for 5 min. Protein samples (20 µg of each YRP per sample) were mixed with 4 volumes of cold ice acetone and kept for 30 min at -20 °C, then centrifuged for 15 min at 16,000 × g at 4 °C. Supernatants were discarded and pellets were resuspended in the same buffer as for salivary glands.

Subsequently, cysteines were reduced by 5 mM tris(2-carboxyethyl)phosphine (TCEP; 60 min at 60 °C), blocked with 10 mM of methylmethanethiosulfonate and incubated 10 min at RT. Samples were digested with trypsin at 37 °C overnight, after which they were acidified with trifluoroacetic acid up to a final concentration of 1%. Finally, sodium deoxycholate was removed by extraction to ethyl acetate (Masuda et al., 2008) and the peptides were desalted on a Michrom C18 column.

The Nano Reversed phase column (EASY-Spray PepMap C18 column, 50 cm × 75 µm ID, 2 µm particles, 100 Å pore size) was used for the LC/MS analysis. The mobile phase A was composed of 0.1% formic acid, while the mobile phase B was composed of acetonitrile and 0.1% formic acid. Samples were loaded onto the peptide trap column (Acclaim PepMap300 C18, 5 µm, 300 Å pore size, 300 µm × 5 mm) at a flow rate of 15 µl/min. The loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. Peptides were eluted with gradient of B from 4% to 35% during 60 min at a flow rate of 300 nl/min. The eluted peptides were converted to gas phase ions using electrospray ionization and subsequently analyzed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo). Survey scans of peptide precursors from 350 to 1,400 m/z were performed at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.5 Th with the quadrupole, higher energy collisional dissociation fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS 2 ion count target was set to 10^4 and the maximum injection time was 35 ms. Only the precursors with a charge state of 2-6 were sampled for MS 2. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles (Hebert et al., 2014).

All data were analyzed and quantified with the MaxQuant software (version 1.5.3.8) (Cox et al., 2014). The false discovery rate was set to 1% and we specified a minimum length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against the database obtained from the available sand fly transcriptomes (for samples from salivary glands), or from the Human database (downloaded from Uniprot on September 2017, containing 20,142 entries). Enzyme specificity was set as C-terminal to arginine and lysine, also allowing cleavage at proline bonds and a maximum of two missed cleavages.

Dithiomethylation of cysteine was selected as the fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. Finally, data analysis was performed using Perseus 1.5.2.4 software (Tyanova et al., 2016). Proteins purities/ratios were calculated as percentages of intensities of particular proteins from summed intensities of all identified proteins.

2.4. Microscale thermophoresis

Microscale thermophoresis (MST) was used to measure the binding between recombinant YRPs and their potential ligands, the biogenic amines. This approach is based on the measurement of the ligand binding induced change in directed movement of molecules along a temperature gradient. The change in movement is caused by differences in size, charge, or solvation energy of the studied protein itself versus in complex with ligand. This change is measured by monitoring the fluorescence of label attached to the protein (Baaske et al., 2010).

The highly pure recombinant YRPs were fluorescently labeled by Monolith Protein Labeling Kit RED-NHS (Nanotemper) according to manufacturer instructions. Fluorescent YRPs were then diluted to 6 nM concentration (corresponding to 260 ng/ml) in the MST buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 10 mM $MgCl_2$; 0.05% Tween-20) and centrifuged for 10 min at 15,000 × g at 4 °C to get rid of protein aggregates. The biogenic amines serotonin, histamine, dopamine, octopamine, norepinephrine and epinephrine (Sigma) were dissolved in MST buffer. For each tested recombinant YRP, a titration series with constant concentration of fluorescently labeled YRP and equal amount of two-fold dilution series of a single unlabeled ligand was prepared in the MST buffer. Binding experiments were performed on a Monolith NT.115PicoRed (Nanotemper). Samples were loaded into the Monolith NT.115 Premium Capillaries (Nanotemper) and ran with 40% MST power and 10-20% LED power based on the fluorescence signal of each protein. The Kd (dissociation

constant) model binding curves expressing the dependence of normalized fluorescence on the ligand concentration were fitted to the average of three independent repetition of each experiment. The Kd values, confidence intervals, amplitudes and the signal to noise levels were calculated using the NanoTemper analytical software package. The amplitude of the binding interaction expressed the difference in normalized fluorescence values between the bound and unbound state (signal). The noise level was defined as the average standard deviation of all data points compared to the Kd model curve. The signal-to-noise ratio was used to assess the quality of the binding outcome, with a ratio higher than 5 being desirable and ratio higher than 12 reflecting an excellent assay according to manufacturer's manual. For the purposes of this study we have defined the strength of the binding interaction as high when the measured Kd was lower than 10 nM, medium when Kd was in range of 10-1,000 nM, low for Kd in range of 1-10 μM and poor for Kd higher than 10 μM.

2.5. 3D models

The YRPs were modeled as described in Sima et al (2016b). Models were based on the crystal structure of LJM11, the only available sand fly YRP in Protein Data Bank (3Q6K; Xu et al., 2011). Models and their amine-binding site were displayed and analyzed in PyMOL (The PyMOL Molecular Graphics System, Version 1.5, Schrödinger, LLC.). Electrostatic surface potentials were calculated using the APBS Tools2 plug-in (Baker et al., 2001) in PyMOL.

3. Results and Discussion

Yellow-related proteins (YRPs) are characterized by the presence of major royal jelly protein domain (Schmitzova et al., 1998) and their name is derived from the role of "yellow" protein in cuticle pigmentation in *Drosophila* (Geyer et al., 1986). They are abundant in saliva of all sand fly species studied up-to-date (Abdeladhim et al., 2016, 2012; Anderson et al., 2006;

Coutinho-Abreu and Valenzuela, 2018; de Moura et al., 2013; Hostomska et al., 2009; Kato et al., 2013, 2006; Oliveira et al., 2006; Rohousova et al., 2012; Valenzuela et al., 2004; Vlkova et al., 2014). In each sand fly species, 1-5 YRPs of distinct sequences with molecular weight of 41-45 kDa were described. In sand fly saliva, YRPs are predicted to act as high affinity binders of host's pro-haemostatic and pro-inflammatory biogenic amines, such as serotonin, histamine and catecholamines but previously this binding was experimentally verified only for YRPs from New-World sand fly species *L. longipalpis* (Xu et al., 2011). Here, we have tested the amine-binding capability of YRPs in the two species of the Old-World genus *Phlebotomus* and validate microscale thermophoresis (MST) as an accurate method to determine the binding affinities of biogenic amines to YRPs.

3.1. YRPs in salivary glands

Among proteins determined by mass spectrometry in the *P. perniciosus* and *P. orientalis* salivary glands, YRPs constitute a high proportion; 40 % and 35.4 %, respectively. These portions correspond to approximately 160 ng and 100 ng of YRPs per a pair of salivary glands of *P. perniciosus* and *P. orientalis*, respectively (Sumova et al., 2018; Velez et al., 2018). The two YRPs in each species tested were present in ratio 2.67 for rSP03B/rSP03 and 2.52 for rPorASP4/rPorASP2 making the closely related proteins rSP03B and rPorASP4 considerably more abundant than the other two YRPs (https://data.mendeley.com/datasets/ryz2djytsz/draft?a=e7e8b4d1-bc85-4ce8-856d-f8e4e5002280). The potential of both rSP03B and rPorASP4 to sequester biogenic amines is therefore enhanced by the higher quantities of these proteins salivated into the host skin.

To antagonize host haemostasis through sequestering the biogenic amines at the biting site, the amine-binding proteins should presumably achieve the concentrations corresponding to 0.2-2 μM, which in mosquitoes correspond to 0.03-0.3 μg of amine-binding D7 proteins

(Calvo et al., 2006). In mosquitoes, both the blood meal (2.4-3.3 µl) and the salivary proteins amount per a pair of glands (1.4-4 µg) are in average 5 times larger than in studied sand flies (0.6 µl and 0.3-0.4 µg, respectively) (Jeffery, 1956; Nascimento et al., 2000; Pruzinova et al., 2015). Therefore, after recalculation according to YRPs molecular weight, we can consider 17-174 ng of YRPs necessary for physiological relevance. As both mosquitoes and sand fly discharge during blood feeding approximately half of the salivary proteins content (Marinotti et al., 1990; Ribeiro et al., 1989), we can consider the measured amount of YRPs sufficient to mop local biogenic amines. YRPs are therefore present in salivary glands in physiologically significant quantities.

3.2. Recombinant yellow-related proteins

All YRPs with a histidine-tag were expressed in a HEK293 cell line and purified by chromatography; the representative results for *P. perniciosus* and *P. orientalis* YRPs are shown in Fig. 1a. All recombinant proteins were analyzed in an analytical ultracentrifuge by performing sedimentation velocity experiment. The resulting size distributions of sedimenting species are shown in Fig. 1b. Apart from rSP03, all other proteins have standard $s_{20,w}$ sedimentation coefficient of 3.5-3.6 S, corresponding well to anticipated 43-45 kDa monomeric proteins. Protein rSP03 sedimented slower with $s_{20,w}$ of 3.2-S, suggesting slightly different shape of the particle when compared to other measured YRPs. The purity of proteins was verified by SDS-PAGE and western blot analysis with an anti-histidine-tag antibody. Both the silver stained gel and the western blot assay showed only one major band of expected molecular weight for all YRP tested (Fig. 1c). The purity of all YRPs was verified by high-resolution mass spectrometry and reached 91 % in average. The major contaminants were identified as keratins, whose presence in samples resulted from protein handling and do not interfere with YRPs performance.

The amino acid similarities between studied recombinant YRPs are summarized in Table 2. The highest similarity was found between YRPs originating from different *Phlebotomus* species, reaching 86% amino acid sequence identity for rSP03 and rPorASP2, and 85% for rSP03B and rPorASP4. High degree of identity found between rSP03/rPorASP2 and also between rSP03B/rPorASP4 is in accordance with their position on phylogenetic tree, where they form the same clusters together with YRPs from related *Larroussius* species *P. tobbi* (Abdeladhim et al., 2016; Coutinho-Abreu and Valenzuela, 2018; Sima et al., 2016b). As expected for a species from different genera, *L. longipalpis* LJM11 share only 51-55.6 % identity with *Phlebotomus* YRPs.

Table 2: Recombinant YRPs amino acid sequence identity

	LJM11	**rSP03**	**rPorASP2**	**rPorASP4**	**rSP03B**
LJM11	100.00	51.06	53.32	55.56	51.45
rSP03	51.06	100.00	85.98	75.65	68.49
rPorASP2	53.32	85.98	100.00	76.96	68.25
rPorASP4	55.56	75.65	76.96	100.00	84.82
rSP03B	51.45	68.49	68.25	84.82	100.00

Percent identity matrix of recombinant YRPs created in Clustal Omega (Sievers et al., 2011). Shading indicates the resemblance of proteins to each other.

3.3. Ligand binding analysis using microscale thermophoresis

Microscale thermophoresis was used to determine whether the expressed recombinant YRPs bind different biogenic amines. Binding curves modeled for each protein with binding amines are shown in Fig. 2. Dissociation constants (Kd) of the interaction of each YRP with all ligands derived from these data are summarized in Table 3. The calculated amplitudes and signal to noise ratios were high enough to confirm the significances of the binding curves for all measured binding interactions.

Table 3: Amine-binding properties of recombinant YRPs

YRP	Ligand	Kd (nM)	Kd CI	Amplitude	Signal to Noise
rSP03	Serotonin	20,558	±7,062	5.1	10.0
	Dopamine	>20,000	NA	NA	NA
	Norepinephrine	534	±131	9.3	13.1
	Epinephrine	18,418	±1,920	9.8	33.6
	Octopamine	5,935	±1,160	9.0	17.1
	Histamine	13,138	±4,431	3.3	9.6
rSP03B	Serotonin	9.6	±6.2	16.1	5.4
	Dopamine	NB	±NA	NA	NA
	Norepinephrine	NB	±NA	NA	NA
	Epinephrine	NB	±NA	NA	NA
	Octopamine	NB	±NA	NA	NA
	Histamine	23,708	±4,602	10.9	16.5
rPorASP2	Serotonin	37.5	±10	6.1	13.0
	Dopamine	76.4	±20.8	8.6	12.0
	Norepinephrine	NB	±NA	NA	NA
	Epinephrine	NB	±NA	NA	NA
	Octopamine	4.2	±1.2	7.4	13.2
	Histamine	75,107	±20,800	8.3	16.2
rPorASP4	Serotonin	1.1	±0.3	13.3	13.2
	Dopamine	3.2	±0.7	11.9	15.7
	Norepinephrine	74.8	±8.4	15.8	28.9
	Epinephrine	745	±79.2	15.2	30.4
	Octopamine	122	±16.2	17.5	24.3
	Histamine	5,753	±378	17.6	48.7
LJM11	Serotonin	4.5	±1.1	14.1	15.5
	Dopamine	12.4	±4.0	17.0	10.5
	Norepinephrine	182	±24.6	20.4	23.8
	Epinephrine	4,212	±465	23.4	29.3
	Octopamine	396	±61.9	17.8	20.6
	Histamine	178,801	±23,426	26.6	24.6

Dissociation constants (Kd; nM), Kd confidence intervals (CI), amplitudes and the signal to noise ratios for rSP03, rSP03B, rPorASP2, rPorASP4 and LJM11. NB – no measurable binding interaction, NA – not applicable.

Protein rSP03 acted as a medium affinity binder of norepinephrine, low affinity binder of octopamine and a poor affinity binder of histamine, epinephrine and serotonin. The binding curve for dopamine was affected by the ligand induced change in fluorescence which precluded plotting it into graph. We have estimated the Kd for dopamine to be higher than 20 μM thus making it a poor affinity ligand. The second *P. perniciosus* YRP, protein rSP03B, had affinity for two ligands only. It served as a high affinity binder of serotonin and it also interacted poorly with histamine. Protein rPorASP2 bound with high affinity octopamine, with medium affinity serotonin and dopamine and it poorly bound histamine. rPorASP2 had no measurable affinity for catecholamines epinephrine and norepinephrine. Protein rPorASP4 interacted with different strength with all ligands. It displayed a high affinity for serotonin and dopamine, medium affinity to norepinephrine, octopamine and epinephrine, and it interacted with low affinity with histamine. The binding interactions of LJM11 were similar to those measured for rPorASP4; the only variation was detected in the lower affinities of LJM11 for epinephrine and histamine. The binding affinities of LJM11 measured by MST correspond well with the ones measured previously by the ITC technique (Xu et al., 2011). We have therefore shown that for this type of study the results obtained either by ITC or MST are comparable.

Although the binding of biogenic amines to YRPs varied both among and within sand fly species, we can draw the following conclusions: In both *P. orientalis* and *P. perniciosus*, at least one YRP was shown to bind with high affinity serotonin (Kd < 10 nM) and with medium affinity norepinephrine (Kd = 10-1,000 nM), suggesting that both sand flies effectively sequester these compounds during feeding. This YRPs capability might inhibit the blood vessels contraction and impede platelet activity by increasing the agonist threshold concentration for the platelet aggregation (Andersen et al., 2003; Calvo et al., 2006). This effect can be further emphasized by the high abundance of both high affinity binders of

384 serotonin (rSP03B and rPorASP4) in sand fly saliva. On the contrary, all tested proteins
385 bound histamine with affinity lower than 1 μM which was indicated as insufficient to prevent
386 interaction of histamine with its physiological receptors (Mans et al., 2008; Xu et al., 2011).
387 The binding affinities for epinephrine, dopamine and octopamine varied among the individual
388 YRPs. When comparing the two *Phlebotomus* species, the binding affinities were
389 in *P. perniciosus* much lower for all these potential ligands. Despite the tight binding
390 of dopamine and octopamine to *P. orientalis* YRPs, these ligands are improbable
391 physiological targets for the salivary YRPs, as they do not play a major role in haemostasis or
392 inflammation (Xu et al., 2011). Therefore, the inter-specific difference in binding of these
393 catecholamines is not supposed to have an application in physiological conditions.
394
395 **3.4. Amine-binding site**
396 The YRPs amine-binding site was formerly determined from LJM11 crystals with added
397 serotonin (Xu et al., 2011). The site was shown to be composed by 11 amino acids from
398 which eight bound biogenic amines through Van der Waals forces and hydrophobic
399 interactions. Five of these amino acids are conserved in all YRPs modeled up to date. Three
400 extra amino acids (Thr327, Asn342 and Phe344) of the LJM11 binding motif were able to
401 bind serotonin not only by above mentioned non-covalent intermolecular interactions, but also
402 by hydrogen bonds (Fig. 3), which are predicted to play major role in the binding event (Sima
403 et al., 2016b; Xu et al., 2011).
404 We have modeled the major amino acids together with their hydrogen bond
405 interactions in the amine-binding site of all YRPs tested (Fig. 3). In *P. orientalis* rPorASP4,
406 the binding motif differed only in one amino acid (position 344; Gln instead of Phe) from
407 *L. longipalpis* LJM11, though the amino acid sequence identity of these two proteins is only
408 55.6 %. This non-conservative substitution should not alter the potential hydrogen bond of the

carbonyl oxygen; on the contrary it could facilitate the link of additional hydrogen bond to the glutamine side chain and therefore tightening the interaction with serotonin (Sima et al., 2016b). This assumption was emphasized by the measured high affinity interaction of rPorASP4 with serotonin, which achieved the lowest Kd of all proteins tested.

The highly related proteins rSP03 and rPorASP2 (86 % identity) shared the same amine-binding motif differing from LJM11 in one non-conservative substitution at position 344 (His instead of Phe), which should not affect the distance of putative hydrogen bond; and one conservative substitution within the other amino acids of the binding motif.

Protein rSP03B varied in all 3 amino acids playing major role in the ligand binding in addition to conservative substitutions in the other two amino acids of the amine-binding motif. Conservative substitution at the position 327 (Ser instead of Thr) and non-conservative substitution at the position 344 (His instead of Phe) probably did not affect the putative hydrogen bonds to serotonin. On the contrary, the conservative substitution at the position 342 (Thr instead of Asn) probably resulted in the switch of two potential hydrogen bonds to only one. Previously described site-directed mutagenesis of an asparagine to alanine at the position 342 resulted for LJM11 in elimination of both potential hydrogen bonds. This mutation led to the complete loss of binding to norepinephrine and epinephrine and to the large reduction of the affinity for serotonin and dopamine (histamine and octopamine were excluded from the analysis) (Xu et al., 2011). This finding is in accordance with the measured absence of binding of all catecholamines by rSP03B. The fact that SP03B was shown to bind serotonin with high affinity can be explained by the protein retaining one hydrogen bond at the side chain of threonine which is longer and therefore closer to the serotonin than in the case of alanine (Fig. 3.). The expected decrease of affinity to catecholamines could have been further enhanced by the substitutions in other amino acids possibly influencing the interaction of rSP03B with the secondary and phenolic hydroxyls, which were previously shown to have

434 an important role in the binding interactions of mosquitoes amine-binding proteins (Calvo et
435 al., 2009; Mans et al., 2007).

436 The majority of the measured substitutions in the amine-binding motif had presumably
437 no effect on the putative hydrogen bonds holding the ligand in the binding pocket. We can
438 therefore hypothesize that there are other factors which should be considered when analyzing
439 the differences in the YRPs affinities for different ligands. For instance, the low affinity
440 binding of histamine by all YRPs might be due to the absence of hydrogen bond formation
441 towards hydroxyl group, which are not present in the structure of this ligand. Even though the
442 ligand-binding sites of insect amine-binding proteins were by crystallography (Calvo et al.,
443 2009; Mans et al., 2007), site-directed mutagenesis (Mans et al., 2007; Xu et al., 2011) or by
444 ligand saturation experiments (Andersen et al., 2003) repeatedly shown to be analogous for
445 different biogenic amines, it is possible that the ligands may in each protein accommodate
446 a slightly different position disabling, reducing or conversely increasing their binding affinity
447 (Calvo et al., 2009; Xu et al., 2013).

448

449 **3.5. 3D models and electrostatic potential of YRPs**

450 *L. longipalpis* YRPs share a similar six-bladed β-propeller fold with all sand fly YRPs studied
451 up to date, which were all shown by homology modeling to comprise a ligand-binding site
452 within their barrel structure (Sima et al., 2016b). The electrostatic potential of the
453 *P. perniciosus*, *P. orientalis* and *L. longipalpis* YRPs surface was compared on their 3D
454 models (Fig. 4). The models of both entrance sides of the six-bladed β-propeller structure
455 showed that the cavity of the channel has in all cases negative charge, which enables the
456 binding of positively charged bioamines in all YRPs studied. This is in accordance with the
457 negatively charged amine-binding site of structurally similar D7 proteins in mosquitoes
458 (Calvo et al., 2009).

Closely related proteins rSP03 and rPorASP2 were shown to share the negative electrostatic potential at both channel openings. Other proteins shared predominantly neutral charge at the entrance of the side further from the ligand-binding site. The charge on the side closer to the ligand was found more variable, with the proteins LJM11, rPorASP4 and rSP03 displaying positive, negative and predominantly neutral electrostatic potential, respectively. Proteins rPorASP4 and LJM11, which were shown to have similar binding affinities, had opposite charges at the channel entrance closer to the binding site, but shared the same charges on the other side of the channel (Fig. 4). We can therefore hypothesize that the ligands preferably enter the barrel structure from the side further from the binding pocket. This assumption is in agreement with already described larger size of this entrance in all YRPs, which could facilitate the entry of the ligand (Sima et al., 2016b). If this hypothesis is valid, the surface charge will probably have less pronounced effect on binding interactions than expected.

The observed variance in the binding affinities even of closely related YRPs was shown also in the three *L. longipalpis* YRPs indicating the occurrence of functional divergence in the family (Xu et al., 2011). Divergence in YRPs function might be caused also by other factors apart from the variations in the amine-binding motif and charge. This can be illustrated on proteins rPorASP2 and rSP03, which share the same amine-binding motif and electrostatic potential but differed in binding affinities for all ligands. Interestingly, rSP03 protein showed slightly lower value of sedimentation coefficient than expected according to its molecular weight and also lower than all other analyzed YRPs (Fig. 1b), pointing to more elongated shape or slightly different folding of this protein compared to other tested YRPs, which might be the cause of different binding properties of rSP03. Distinct interactions of rSP03 and rPorASP2 might be also influenced by the absence of N-glycosylation in rPorASP2 (Sima et al., 2016b; Vlkova et al., 2014) presumably affecting the protein

folding and subsequently its structure and therefore also binding properties (Katoh and Tiemeyer, 2013). The particular sand fly YRPs were also found versatile in other parameters including the length, minimum radius and hydrophobicity of their channels, which could also affect the binding affinities even of closely related proteins (Sima et al., 2016b).

In conclusion, we have validated MST as a useful tool to study the amine-binding interactions of the sand fly YRPs. We have shown that although the 3D structure of all YRPs is highly conserved, differences in the amino acids of the ligand-binding motif, conformation of the potential ligands and other factors might affect the binding affinities even of closely related proteins. Nevertheless, both *P. orientalis* and *P. perniciosus* express at least one YRP which bound serotonin with high affinity, while none of the proteins was shown to bind histamine with significant affinity. We can therefore propose that both *P. perniciosus* and *P. orientalis* YRPs potentially contribute to counteracting of the platelet aggregation and vasoconstriction.

Acknowledgement

We are grateful to Shannon Townsend and Morgan Karetnick for the help with protein expression in mammalian system. Our thanks are due to Vera Volfova for providing us with the sand fly image. Acknowledgment to Karel Harant and Pavel Talacko from Laboratory of Mass Spectrometry, Biocev, Charles University, Faculty of Science, where proteomic and mass spectrometric analysis had been done. We appreciate Helena Kulikova and Lenka Krejcirikova for excellent technical and administrative support.

Funding

This work was supported by the Charles University (GAUK project no. 120516/B-BIO, PS), Czech Science Foundation (Project Number: P506 17-10308S, MS, PS, NP, PV), the Ministry of Education, Youth and Sports of the Czech Republic (LTC17065 in frame of the COST Action CA15126), Centre for research of pathogenicity and virulence of parasites (CZ.02.1.01/0.0/0.0/16_019/ 0000759; PV) and by the project „BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University“ (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases (FO, JGV). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure captions

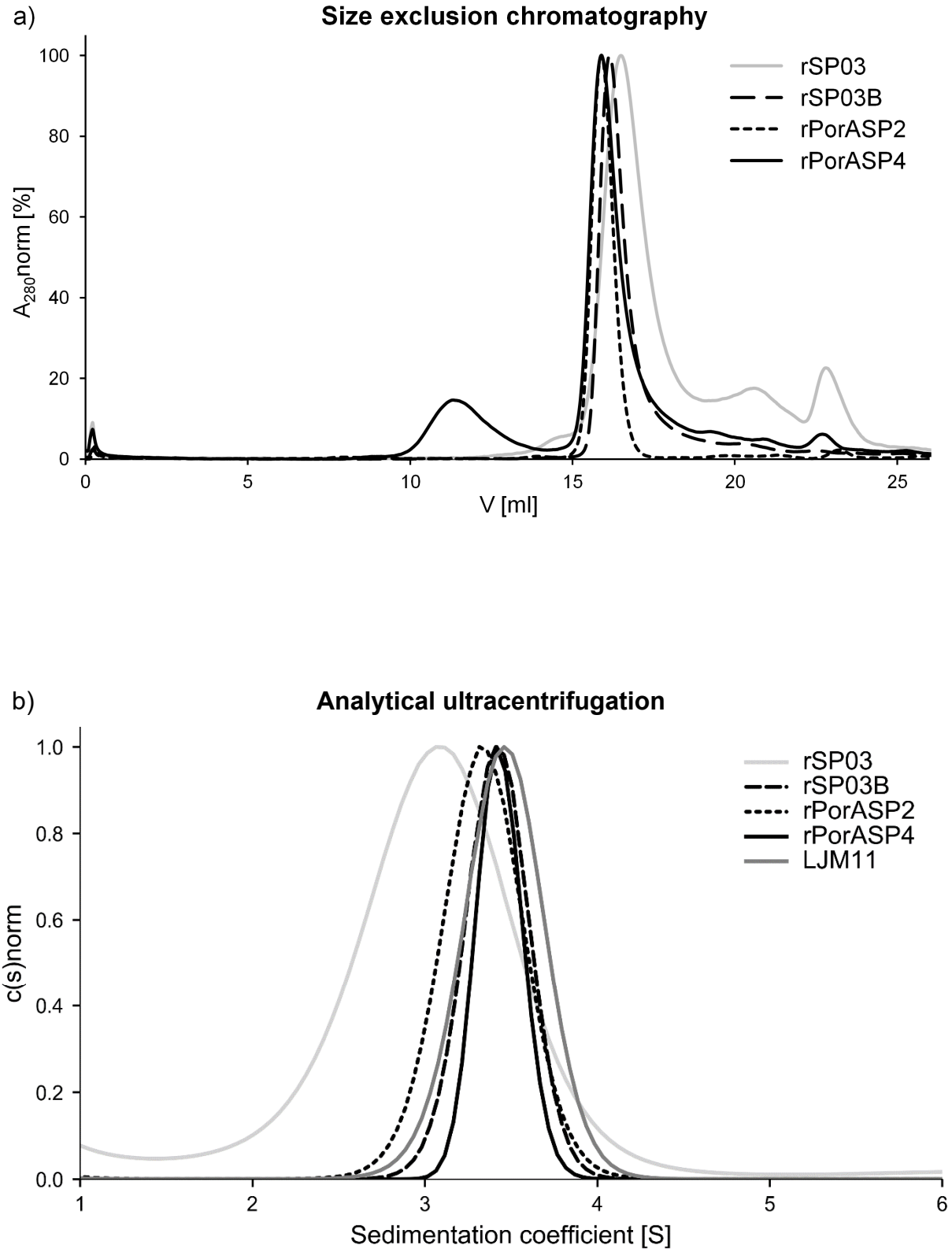
Fig. 1. Purification and quality control of recombinant proteins. a) Representative results of size exclusion chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare); peaks for each protein are shown with distinct type of line (see legend on the right). b) Recombinant YRPs were analyzed in analytical ultracentrifuge by sedimentation velocity experiment and an overlay of normalized size distributions for individual proteins is shown. c) Recombinant YRPs were run by SDS-PAGE under non-reducing conditions. Silver-stained gel is shown on the left. Western blot analysis was performed with anti-polyHistidine-peroxidase antibody. Molecular weights (kDa) of standard (STD; BenchMark Protein Ladder, ThermoFisher Scientific) are indicated.

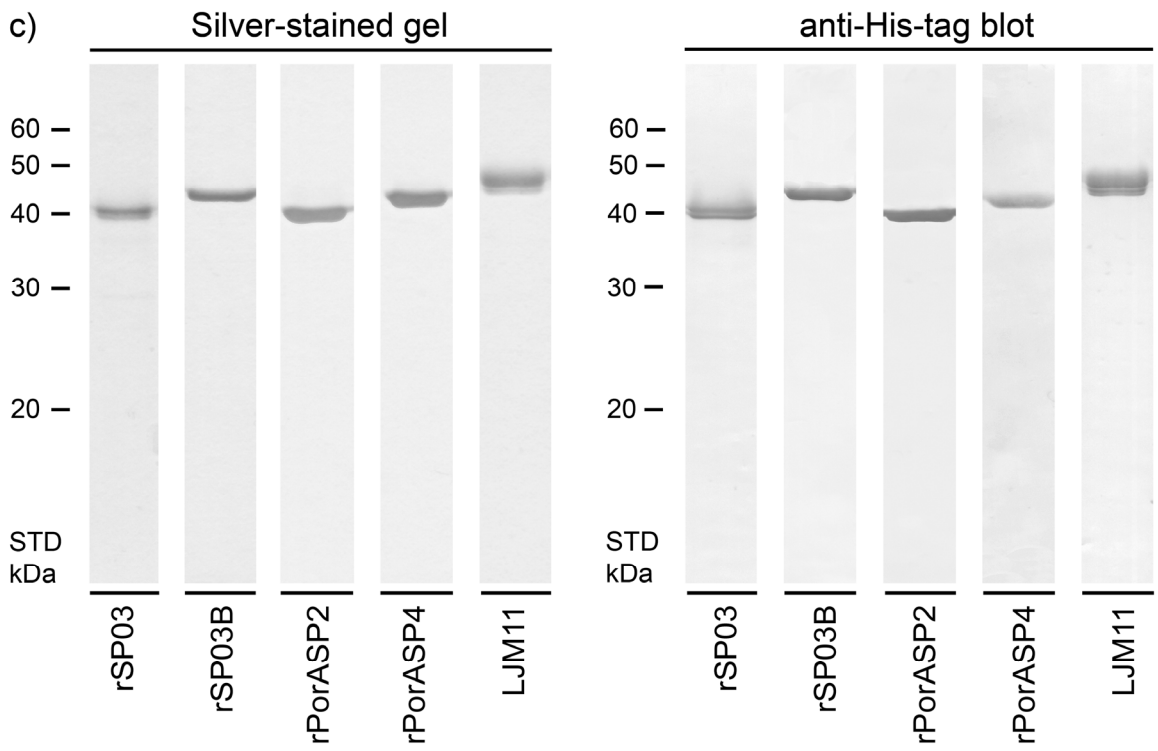
Fig. 2: MST showing binding of biogenic amines to each YRP. Graphs show the MST data fitted with Kd model binding curves for each YRP and all the biogenic amines it bound. For the amines for which no binding was detected, no graphical visualization is shown. Each curve and data point represents an average of three independent experiments. Curves for each amine are shown with distinct type of line and data points (see legend in frame).

Fig. 3. Amine-binding pocket of YRPs. The model depicts the relevant amino acids of the serotonin binding site for both *P. perniciosus* (rSP03 - red, rSP03B - violet) and *P. orientalis* (rPorASP4 - light green, rPorASP2 - dark green) YRPs and *L. longipalpis* protein LJM11 (blue). Numbering of amino acids was adapted from Xu et al., 2011. Dashed lines represent putative hydrogen bonds which bind serotonin (black) inside the pocket, the numbers mark the actual donor-acceptor distances in Ångströms, in grey are marked the bonds formed by the amino acid substitutions. Asterisks mark the substitutions in amino acids participating in hydrogen bond formation.

Fig. 4: Electrostatic potential at YRPs channel entrances. Models depict each recombinant YRP from side closer (ligand side) and further (other side) to the ligand-binding site inside the channel. Color and its brightness correspond to the scale of negative (red) to positive (blue) charge.

Fig. 1. Purification and quality control of recombinant proteins.




c)
Silver-stained gel
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rPorASP4
LJM11

Fig. 2: MST showing binding of biogenic amines to each YRP.

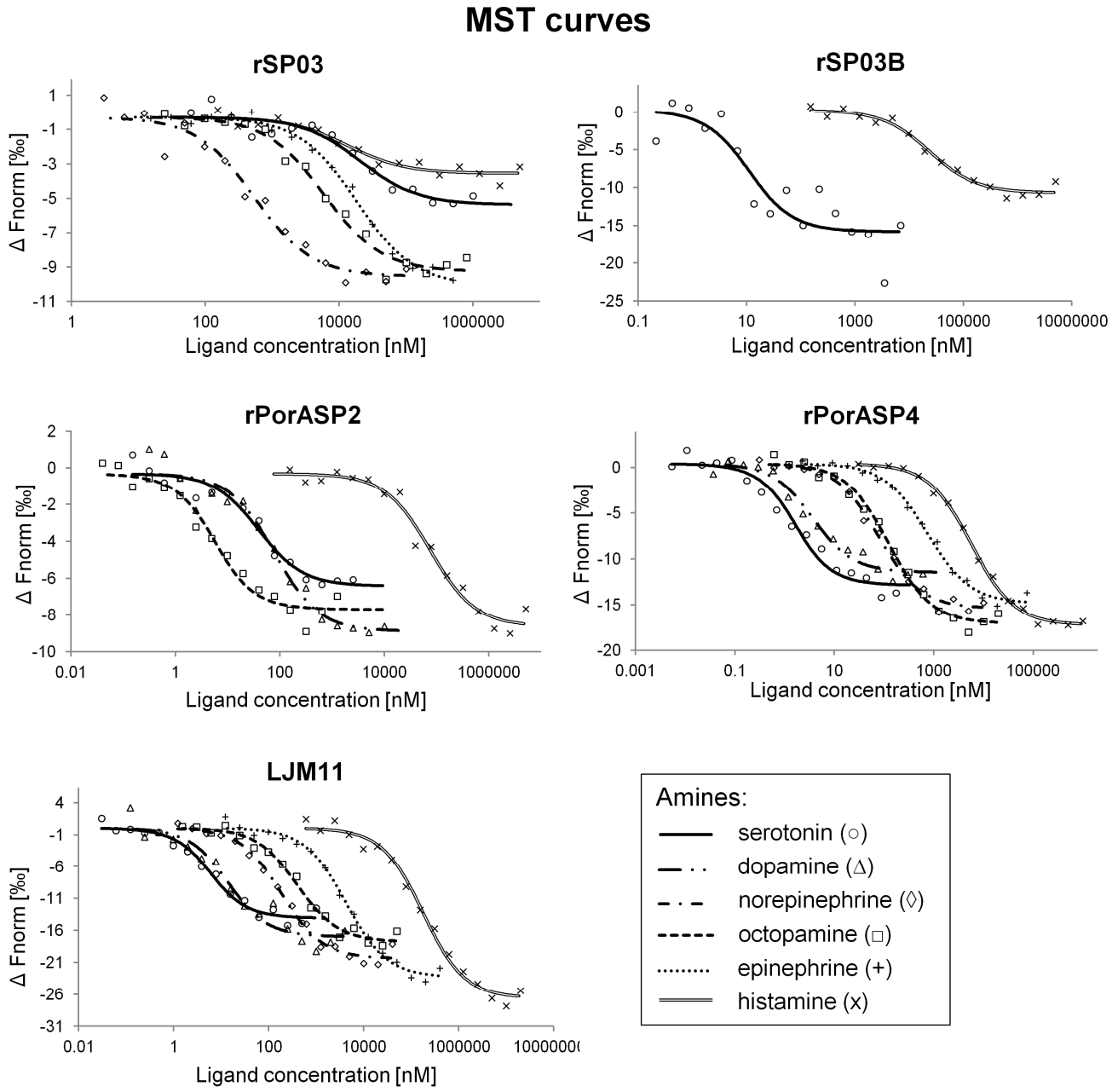


Fig. 3. Amine-binding pocket of YRPs.

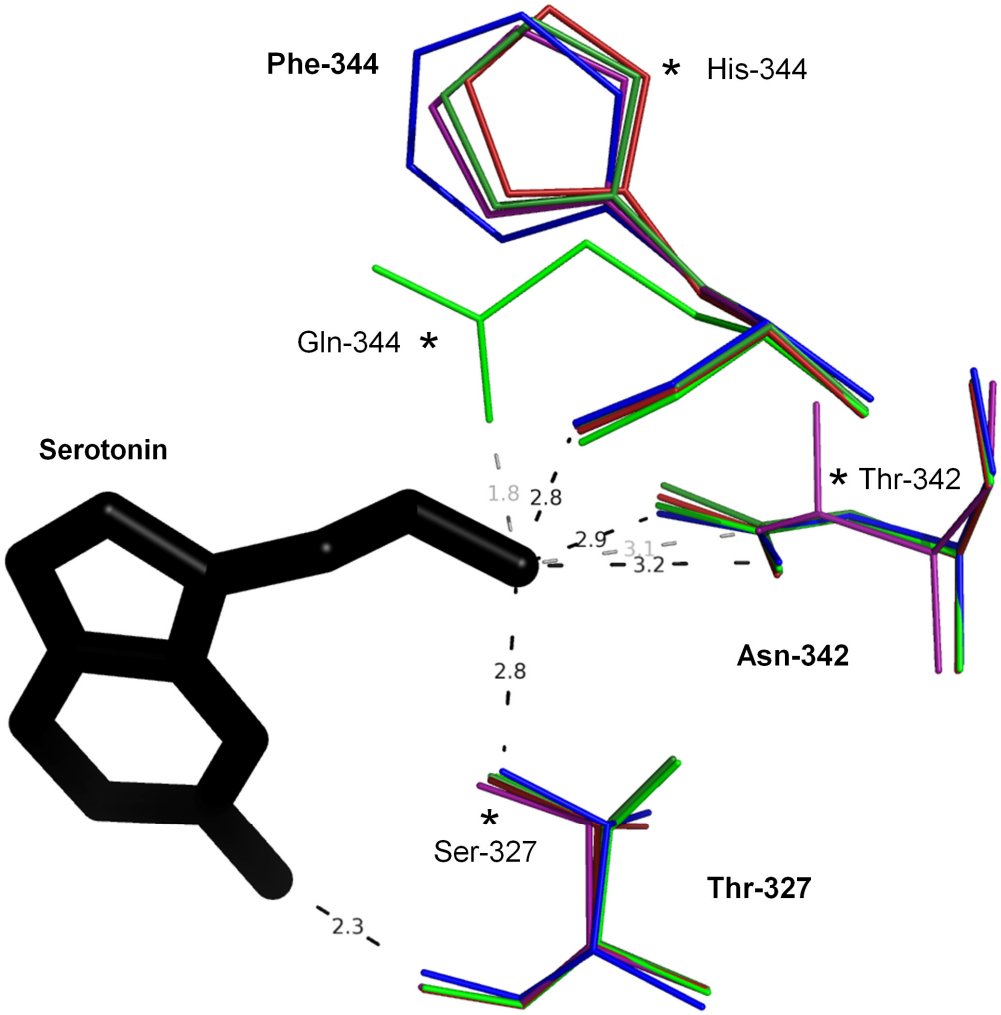
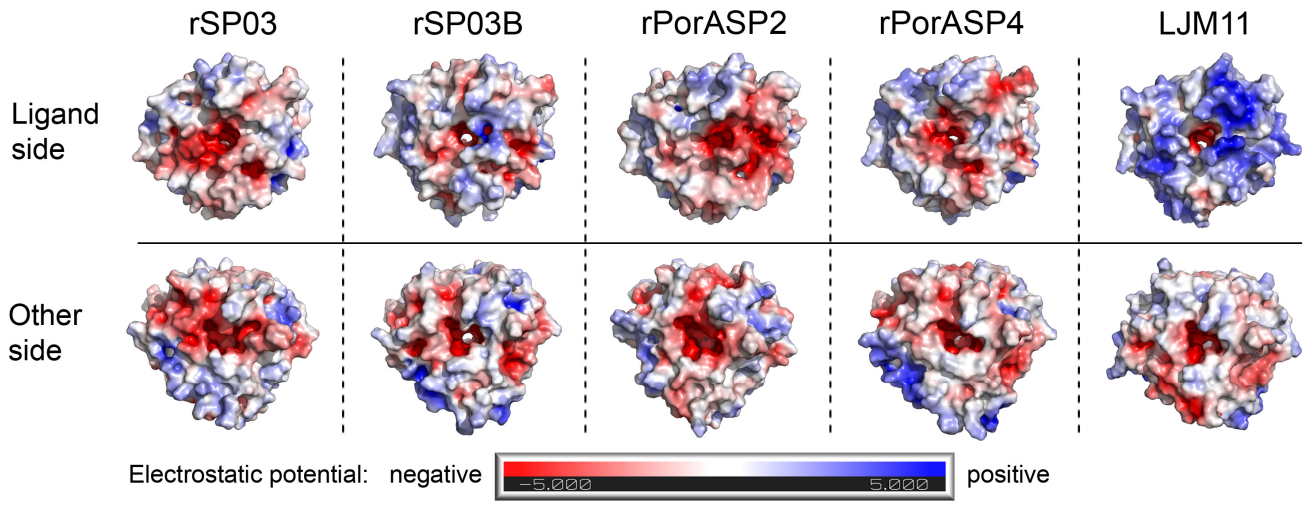


Fig. 4: Electrostatic potential at YRPs channel entrances.



Canine antibodies against salivary recombinant proteins of *Phlebotomus perniciosus*: A longitudinal study in an endemic focus of canine leishmaniasis

Kostalova, T., Lestinova, T., **Sumova, P.**, Vlkova, M., Rohousova, I., Berriatua, E., Oliva, G., Fiorentino, E., Scalone, A., Gramiccia, M., Gradoni, L., & Volf, P. (2015). PLoS Negl. Trop. Dis. *9*, e0003855.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Canine Antibodies against Salivary Recombinant Proteins of *Phlebotomus perniciosus*: A Longitudinal Study in an Endemic Focus of Canine Leishmaniasis

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OPEN ACCESS

Citation: Kostalova T, Lestinova T, Sumova P, Vlkova M, Rohousova I, Berriatua E, et al. (2015) Canine Antibodies against Salivary Recombinant Proteins of *Phlebotomus perniciosus*: A Longitudinal Study in an Endemic Focus of Canine Leishmaniasis. PLoS Negl Trop Dis 9(6): e0003855. doi:10.1371/journal.pntd.0003855

Editor: Alain Debrabant, US Food and Drug Administration, UNITED STATES

Received: February 6, 2015

Accepted: May 28, 2015

Published: June 25, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was partially funded by Charles University (GAUK – 1642314/2014), by the EU grant FP7-261504 EDENext, and the paper is catalogued by the EDENext Steering Committee as EDENext317 (http://www.edenext.eu) and by the EurNegVec COST Action TD1303. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Phlebotomine sand flies are vectors of *Leishmania* parasites. During blood feeding, sand flies deposit into the host skin immunogenic salivary proteins which elicit specific antibody responses. These anti-saliva antibodies enable an estimate of the host exposure to sand flies and, in leishmaniasis endemic areas, also the risk for *Leishmania* infections. However, the use of whole salivary gland homogenates as antigen has several limitations, and therefore, recombinant salivary proteins have been tested to replace them in antibody detection assays. In this study, we have used for the first time sand fly salivary recombinant proteins in a longitudinal field study on dogs.

Methodology/Principal Findings

Sera from dogs naturally exposed to *P. perniciosus* bites over two consecutive transmission seasons in a site endemic for canine leishmaniasis (CanL) were tested at different time points by ELISA for the antibodies recognizing whole saliva, single salivary 43 kDa yellow-related recombinant protein (rSP03B), and a combination of two salivary recombinant proteins, 43 kDa yellow-related protein and 35.5 kDa apyrase (rSP01). Dogs were also tested for *Leishmania infantum* positivity by serology, culture, and PCR and the infection status was evaluated prospectively. We found a significant association between active CanL infection and the amount of anti-*P. perniciosus* saliva antibodies. Importantly, we detected a high correlation between IgG antibodies recognizing rSP03B protein and the whole salivary antigen. The kinetics of antibody response showed for both a whole saliva and rSP03B a similar pattern that was clearly related to the seasonal abundance of *P. perniciosus*.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

These results suggest that *P. perniciosus* rSP03B protein is a valid alternative to whole saliva and could be used in large-scale serological studies. This novel method could be a practical and economically-sound tool to detect the host exposure to sand fly bites in CanL endemic areas.

Author Summary

Canine leishmaniasis (CanL) is a widespread zoonosis caused by protozoan parasite *Leishmania infantum*. CanL is endemic in more than 70 countries, including regions of south-western Europe, where the main vector is *Phlebotomus perniciosus*. Saliva inoculated into the host during blood feeding provoke species-specific antibody response. They could be used as a serological marker of exposure to sand flies and, in leishmaniasis endemic areas, also as an indication of *Leishmania* infection risk. However, in case of large-scale serological studies, it is difficult to obtain appropriate amounts of crude salivary antigen from laboratory-reared sand flies. Therefore, we studied if specific salivary recombinant proteins could be suitable to replace whole saliva as an antigen for serological assays. Antibodies recognizing recombinant salivary protein in naturally bitten dogs were highly correlated with antibody response against whole saliva. Seasonal exposure of dogs to sand flies led to antibody response fluctuations related to the period of activity and abundance of vectors. In a context of high CanL endemicity, we found a significant association between progressive CanL infections and antibodies against sand fly saliva. Thus, salivary recombinant proteins could be used as suitable tool for investigations host exposure to sand flies in endemic settings.

Introduction

Canine leishmaniasis (CanL), caused by protozoan parasite *Leishmania infantum*, is a systemic and potentially fatal disease [reviewed in [1, 2]]. It may affect any organ or body fluid [reviewed in [1]] and can manifest variable clinical signs [reviewed in [2, 3]]. However, the majority of infected dogs do not develop any clinical signs. Importantly, *L. infantum* is also a causative agent of human visceral leishmaniasis and both symptomatic and asymptomatic dogs have a crucial role in the epidemiology of this disease, serving as reservoirs [reviewed in [2]]. CanL is endemic in many regions of southern Europe and Latin America, however, climate changes and socioeconomic factors, particularly increased travelling of dogs between endemic and non-endemic areas, led to changes in the distribution of CanL in both continents [reviewed in [1, 2, 4]].

Diagnosis of CanL should be based on an integrated approach considering signalment, history, clinical findings, and results of basic laboratory analyses that detect the parasite or evaluate the immune response in the host [reviewed in [3]]. The commonly used diagnostic methods include direct detection of the parasite by culture or polymerase chain reaction (PCR) and indirect techniques for detection of antibodies against *Leishmania*, such as immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [5–8]. However, there is still lack of practical methods for detection of the risk of *Leishmania* transmission according to the exposure of dogs to sand fly vectors.

Results from studies on human and canine antibody response against sand fly saliva revealed high immunogenicity of sand fly salivary proteins as well as high specificity of anti-

saliva IgG [9–12]. Studies based on dynamics of IgG response in dogs experimentally exposed to the main *L. infantum* vectors, *Lutzomyia longipalpis* and *Phlebotomus perniciosus* [12, 13], suggest that monitoring canine antibody response to sand fly saliva could be a useful epidemiological tool in CanL foci. However, the use of whole sand fly saliva in such studies is limited by time-consuming salivary gland dissection and complicated by potential cross-reactivity with saliva from sand fly species with no role in *Leishmania* transmission [11] or from other blood sucking insects [14]. These problems could be overcome by using specific immunogenic sand fly salivary recombinant proteins [reviewed in [15]]. The use of recombinant proteins was already shown for *P. papatasi* [16, 17], *Lu. longipalpis* [18, 19] and *P. perniciosus* [20, 21]. Although these studies confirmed the advantages of salivary recombinant proteins, most of them were tested on small sets of samples and never in association with naturally transmitted *Leishmania* infections.

Previous studies on different hosts (dogs, foxes, and humans) bitten by *P. perniciosus* or *Lu. longipalpis* revealed high antigenicity of salivary yellow-related proteins and apyrases [12, 13, 19]. Apyrases are enzymes with potent anti-hemostatic activity hydrolyzing the platelet activator ADP [22]. The role of yellow-related proteins is less clear [23] but most probably they act as a histamin-binding molecules [24]. Recent work using a low number of sera from dogs experimentally exposed to *P. perniciosus* showed that recombinant forms of these proteins could be used as potential candidates for markers of canine exposure to sand flies [20]. Therefore, herein we used the recombinant 43 kDa yellow-related protein (rSP03B) from *P. perniciosus* and its combination with recombinant *P. perniciosus* 35.5 kDa apyrase (rSP01) to study the specific antibody response in a large number of dogs naturally exposed to *P. perniciosus* over two years in a focus endemic for *L. infantum*.

Methods

Ethical statement

The technical protocol for the investigation of natural canine *Leishmania* infections performed in the frame of experimental drug trials was approved by the Veterinary Board of the Italian Ministry of Health (authorization no.4051/P) following the European Directive 86/609/EEC, adopted by the Italian Government with the Law 116/1992.

Experimental design and background

A longitudinal study including two sand fly seasons was performed on 56 Beagle dogs housed in the same open-air kennel sited in a rural municipality of the Naples province (Campania region, southern Italy) where both human visceral leishmaniasis and CanL are highly endemic. Here, *P. perniciosus* is the only *Leishmania* vector, whose activity period ranges from the end of May through late October [25]. *Leishmania infantum* infection rates in dissected sand flies were found to range from 2.8% to 6.2% in places not far from the kennel [26]. In cohorts of naïve dogs previously exposed in the same kennel, the annual incidence of CanL infection and clinical disease was reported to average about 40% and 20%, respectively [8]. The dogs included in our study were part of experimental trials of pharmacological products against CanL. The animals were born in a breeding facility sited in a non-endemic area of northern Europe, and were confirmed to be CanL-free at the time they were moved to the study site when they were about 6 months old. Once housed in the open-air kennel, the use of topical or environmental insecticides was avoided to allow natural exposure of dogs to sand fly bites in the warm season.

Antibodies against sand fly salivary proteins were analyzed retrospectively in dog sera taken for routine clinical examination and periodical CanL serology, and that were stored frozen. Available samples included sera obtained shortly before the dogs were transferred to the

endemic area in July (first year), and they served as pre-immune sera (n = 56). Responses to salivary antigens were then analyzed on selected samples collected at four-time points in the first year [August (n = 53), September (n = 54), October (n = 34), December (n = 54)] and at five occasions during the second year [January (n = 56), March (n = 56), July (n = 56), August (n = 56), September (n = 54)]. As per established protocols [5], follow-up analyses for the detection and classification of natural CanL infections were performed at the beginning of the study in the first year in July (n = 56), twice in the second year [March (n = 56) and July (n = 56)] and once at the beginning of the third year [March (n = 53)].

Analysis of *Leishmania* infection

Dogs were examined by serology, culture, and PCR. Detection of anti-*Leishmania* IgG antibodies was performed by an in-house IFAT assay using *L*. *infantum* promastigotes as antigen and following the protocol recommended by the Office International des Epizooties [27]. The cut-off dilution for *Leishmania* exposure was set at 1:40 (i.e. the upper part of the so-called "IFAT grey zone") [3]. Bone-marrow aspirate material was examined by nested-PCR assay as previously described [28]. Lymph-node aspirate material was cultured from each popliteal node in Evans' Modified Tobie's medium and cultures were periodically examined for promastigotes growth during one month.

At each assessment, the dogs were classified as follows with regards to the infection status: i) "*Leishmania* negative"if found negative by all assays, ii)"*Leishmania* exposed"if tested positive by IFAT at low titers and negative by other tests, iii) having a"subpatent *Leishmania* infection" if bone-marrow PCR tested positive, IFAT was either negative or positive at low titers, and lymph node culture was negative, iv) having an"active *Leishmania* infection" if both bone-marrow PCR and lymph node culture were tested positive. In this infection stage, IFAT can be found negative or positive at low titer initially, but converts shortly to very elevated titers. Once established, active infections do not regress spontaneously towards negative or subpatent conditions and dogs invariably progress to clinical disease [8].

Sand flies and salivary proteins

A colony of *P*. *perniciosus* was reared under standard conditions as described in [29]. Salivary glands, dissected from 4–6 day old female sand flies, were pooled in 20 mM Tris buffer with 150 mM NaCl and stored at -20°C. Recombinant salivary proteins from *P*. *perniciosus*, 35.5 kDa apyrase (rSP01, Genbank accn. DQ192490) and 43 kDa yellow-related protein (rSP03B, Genbank accn. DQ150622) were obtained from Apronex s.r.o. (Prague) as mentioned in [20]. The concentrations of these proteins were quantified by the Lowry method (Bio-Rad) following the manufacturer´s protocol.

Detection of anti- *P*. *perniciosus* IgG

Anti-*P*. *perniciosus* IgG were measured by enzyme-linked immunosorbent assay (ELISA) as described in [12] with minor modifications. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (0.2 salivary gland per well) or with rSP03B (5μg/ml) or with rSP03B+rSP01 (5μg/ml of each protein) in 20 mM carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were incubated with blocking solution, 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine sera were diluted 1:200 for SGH and 1:100 for recombinant proteins in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. Absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). Each serum was tested in duplicate and the experiment was repeated twice.

Statistical analysis

Antibodies against sand fly saliva are reported as optical densities (OD) with subtracted blanks and multiplied by 100 for easier readability. In case of linear mixed models, ODs were log transformed (OD value+1).

Statistical analyses were carried out using R software (http://cran.r-project.org/). Repeatability defined as the degree of agreement between repeated OD measures on the same samples was estimated within and between plates by calculating the concordance correlation coefficient (CCC) that ranges from 0 (no concordance) to 1 (perfect concordance) [30].

Antibodies against sand fly saliva and *Leishmania* infection status frequency distributions were analysed over time. Proportions and medians between time points were compared using McNemar's chi-square test for paired data and Wilcoxon signed rank sum test, respectively. Correlations were analysed using Spearman rank correlation test [31].

The "nlme" package [32] was then used to develop multilevel linear regression models to investigate the relationship between IgG anti-saliva antibodies as continuous dependent variable, and sampling months and *L. infantum* infection status (as described above) included as categorical predictor variables, taking into account the correlation between repeated measures of the same dogs over time. Two hierarchical levels were considered in the analysis with repeated measures within individual dogs as the level-1 units and individual dogs as the level-2 units. The random variation at the dog level was examined at both the intercept and at the slopes and significance was assessed testing the -2 log likelihood ratios between the model with and the model without the random effect [33]. The correlation between level 1 units was considered as having a compound-symmetry (CS) or autoregressive with a lag of 1 (AR-1) structure or as unstructured (UN) and Akaikes's Information Criterion (AIC) was used to compare the goodness of fit of models with different correlation structures, selecting those with the smallest values of these statistics [33, 34]. Parameter estimates for fixed effects were estimated using restricted maximum likelihood estimation (REML) and significance was assessed using conditional t-tests and F-tests, and alpha was taken at the 5% ($p<0.05$) level for a two-tailed test.

Results

Dynamics of *L. infantum* infection status

The percentage of dogs classified as negative, *Leishmania* exposed, subpatently and actively infected is presented in Fig 1.

At the beginning of the study (July in the first year) all dogs were negative by all tests, thereafter, the proportion of *L. infantum* infected dogs increased throughout the trial ($p<0.05$) (Fig 1). In the following sampling, in March, 32% of dogs were classified as *Leishmania* exposed, while subpatent and active infections were found in 2% and 4% of dogs, respectively. The percentage of dogs in each category was similar 3 months later in July (Fig 1). In contrast, by the end of the study in March of the third year, the percentage of subpatently and actively infected dogs sharply increased to 17% and 13%, respectively. By this time, 36% of dogs were classified as *Leishmania* exposed and 34% as negative ($p<0.05$) (Fig 1).

Distribution and dynamics of antibody response against salivary proteins using SGH, rSP03B, and rSP03B+rSP01 as antigens

The overall median values of ELISA ODx100 using SGH, rSP03B, and rSP03B+rSP01 antigens were 10 (range: 2–194), 24 (1–234) and 37 (11–189), respectively ($p<0.05$). However, the median increased significantly with time, following a similar pattern for all three antigens

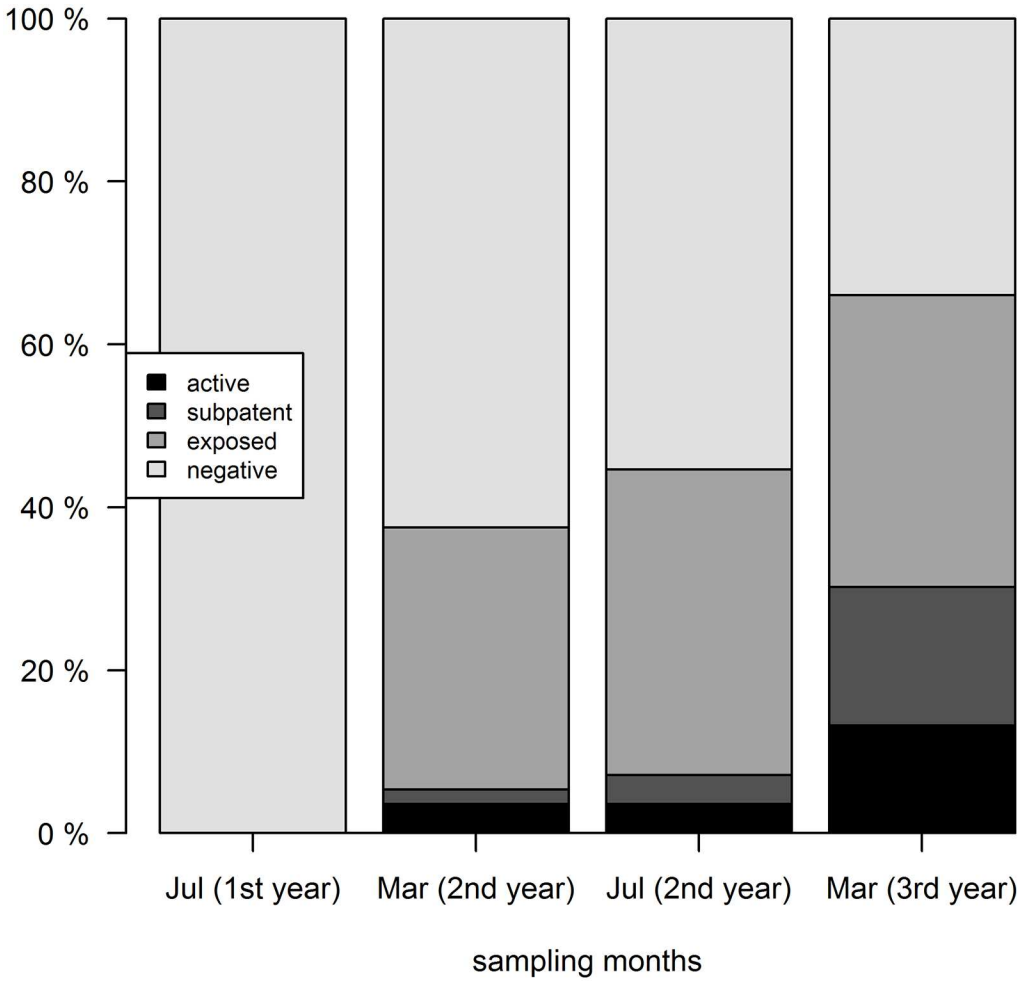

Fig 1. Dynamics of *L. infantum* infection statuses in dogs from a CanL endemic site in southern Italy. Dogs were screened for *L. infantum* infection on four occasions, 56 dogs in July first year, 56 dogs in March the second year, 56 dogs in July the second year and 53 dogs in March the third year.

doi:10.1371/journal.pntd.0003855.g001

tested, most significantly for SGH and rSP03B (Fig 2). For example, the median ODx100 for SGH was 5 at the baseline in July of the first year, increased significantly to 11 through October, decreased thereafter to 8 in January, increased again up to a peak of 24 detected in July of the second year, and remained similar until September (Table 1). The sharp OD increase observed in summer of the second year for SGH and rSP03B was less pronounced for rSP03B+rSP01 (Table 1). Moreover, correlation of antibody response between SGH and rSP03B was stronger (r = 0.77) than between rSP03B+rSP01 and SGH (r = 0.65) (Fig 3). In addition, we detected high correlation between antibodies recognizing rSP03B and antibodies recognizing combination of rSP03B+rSP01 (r = 0.75) (Fig 3).

Within plate repeatability was high for each antigen and the CCC varied from 0.94 (95% CI: 0.93–0.95) to 0.99 (95% CI: 0.98–0.99), depending on the antigen. However, the CCC between plate was moderately high for whole SGH (0.77, 95% CI: 0.73–0.8) and rSP03B (0.88, 95% CI: 0.87–0.9) and low for the combination of rSP03B+ rSP01 (0.53, 95% CI: 0.47–0.59).

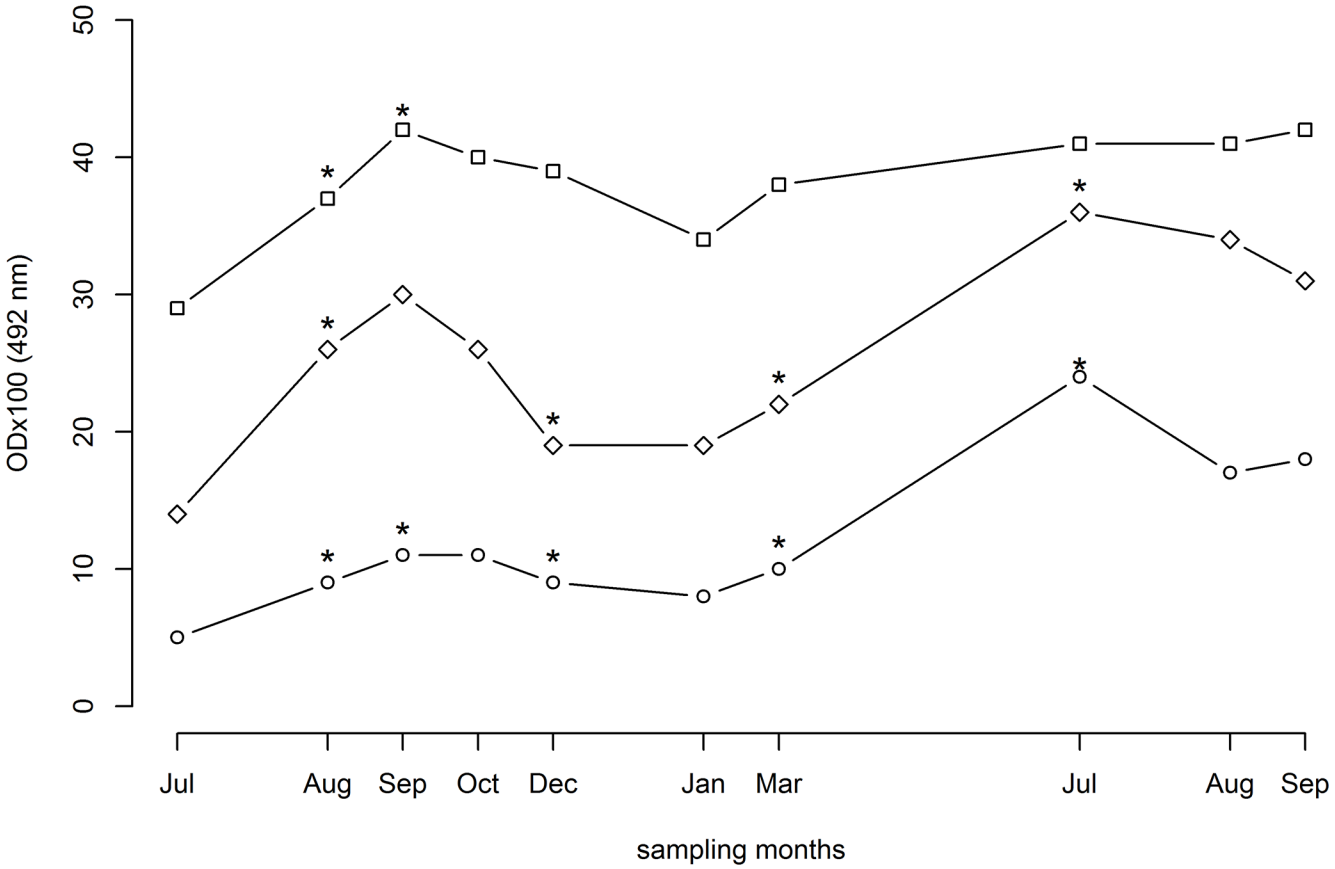

Fig 2. Dynamics of IgG antibody response against sand fly salivary proteins in dogs naturally exposed to *P. perniciosus* over two years in endemic foci. Canine sera were tested by ELISA for the antibodies recognizing SGH (open circle), rSP03B protein (open diamond) and combination of rSP03B+rSP01 proteins (open square). Data are presented as median values for each sampling month. Asterisk represents significant change in the median compared to previous sampling. ODx100 = optical density multiplied by 100.

doi:10.1371/journal.pntd.0003855.g002

Table 1. Median and range antibody OD values (multiplied by 100) for each used antigen according sampling month.

Study year	Sampling month		SGH	rSP03B	rSP03B+rSP01
		n	Median(range)	Median(range)	Median(range)
First	July	56	5 (3–12)	14 (7–36)	29 (19–49)
	August	53	9 (4–42)*	26 (14–75) *	37 (16–71) *
	September	54	11 (4–56) *	30 (11–165)	42 (19–116)*
	October	34	11 (3–59)	26 (11–93)	40 (17–85)
	December	54	9 (4–50) *	19 (10–81) *	39 (21–100)
Second	January	56	8 (2–49)	19 (1–69)	34 (19–93)
	March	56	10 (4–62)*	22 (9–89)*	38 (11–117)
	July	56	24 (6–161) *	36 (9–223) *	41 (17–177)
	August	56	17 (5–129)	34 (11–154)	41 (18–157)
	September	54	18 (6–194)	31 (11–234)	42 (18–189)
	Total		10 (2–194)	24 (1–234)	37 (11–189)

n = number of dogs

* significant change in the median compared to previous sampling

doi:10.1371/journal.pntd.0003855.t001

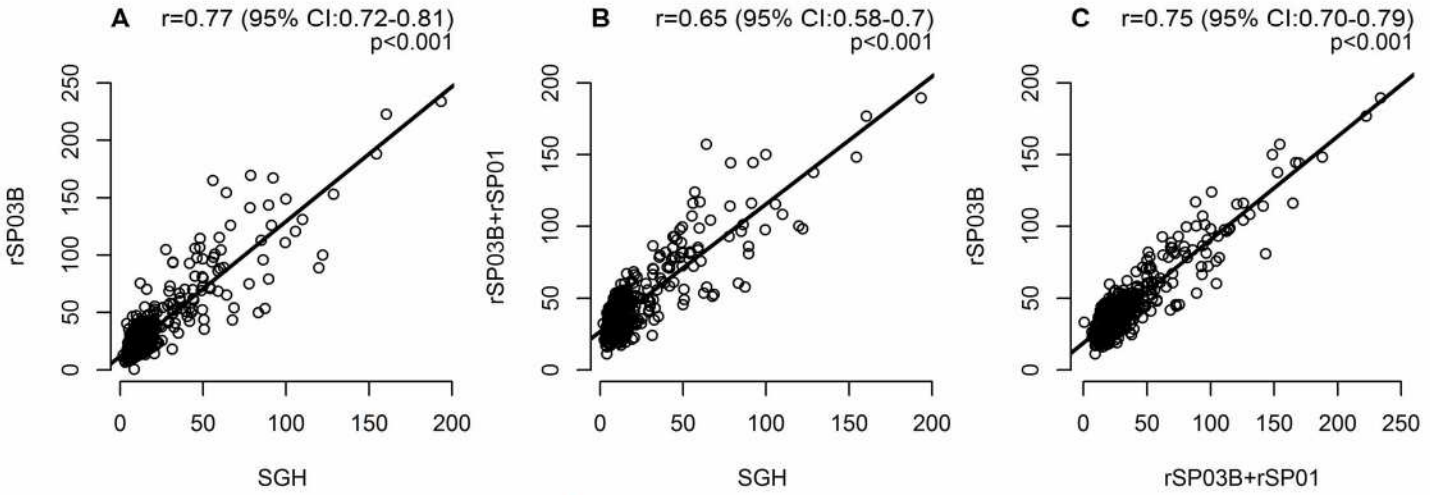

Fig 3. Correlations between IgG antibodies recognizing SGH and recombinant salivary proteins in dogs naturally exposed to sand flies. Correlation between SGH and rSP03B protein (A), between SGH and combination of rSP03B+rSP01 proteins (B) and between rSP03B protein and combination of rSP03B+rSP01proteins (C) was performed using Spearman rank correlation. OD values were multiplied by 100. r = correlation index, CI = confidence interval.

doi:10.1371/journal.pntd.0003855.g003

Multivariable relationship between salivary antibodies, season, and *L. infantum* infection

Multilevel models confirmed the strong association between antibodies against SGH and recombinant proteins and sampling month, and between SGH and active *L. infantum* infection.

Tables 2 and 3 present parameters estimates for models including only sampling date (model a) and both sampling date and *L. infantum* infection status (model b) for SGH and rSP03B, respectively (for combination of rSP03B+rSP01 data are showed in S1 Table). Estimates for sampling months alone reflect a significant increase in log OD values by September in comparison to July of the first year, when the study started, then they started to decrease in October, and raised again in March and especially in July of the second year, with the highest log OD estimate detected in the following month of September ($p<0.05$) (Tables 2 and 3, model a). Including dog as a random effect significantly improved the model and the variance estimate indicated that for SGH it was 33% [(10.06/(10.06+20.35))*100] of the variation in log OD dog related. For rSP03B it was 38% [(17.33/(17.33+28.67))*100]. This can be appreciated in Fig 4A and 4B representing the sampling month-specific ODs for the 56 study dogs. Finally, model b shows the sharp increase in log OD between March and July of the second year (Tables 2 and 3) and highlights the strong association between the amount of antibodies against whole saliva and dogs with active *L. infantum* infection ($p<0.05$) (Table 2). The association between active infection and anti-rSP03B antibodies (Table 3) and combination of rSP03B+rSP01did not reach statistical significance. The model also shows that the greatest variation in log ODs between dogs was observed in July of the second year (Tables 2 and 3). There was no evidence of correlation between repeated measures in any of the above models.

Discussion

This paper describes for the first time the dynamics and diagnostic potential of antibodies recognizing *P. perniciosus* salivary recombinant proteins in dogs following natural exposure to the sand flies over two years.

Table 2. Estimates of the multilevel linear regression model of the relationship between log transformed SGH OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		4.33	0.74	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	1.56	0.87	0.073
	September (first year)	2.85	0.86	0.001
	October (first year)	2.49	0.99	0.012
	December (first year)	1.60	0.86	0.064
	January (second year)	1.55	0.85	0.069
	March (second year)	2.36	0.85	0.006
	July (second year)	9.21	0.85	<0.001
	August (second year)	7.84	0.85	<0.001
	September (second year)	9.62	0.86	<0.001
Random effects	**Variance**			
Dog	10.06			
Residual	20.35			
b) sampling month and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		4.33	0.11	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	1.71	0.56	0.003
	July (second year)	8.46	1.2	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.39	0.90	0.13
	Subpatent	1.28	2.96	0.67
	Active	5.11	2.38	0.03
Random effects	**Variance**			
Dog	0.11			
March (second year)	10.42			
July (second year)	71.52			
Residual	0.51			

SE = standard error

doi:10.1371/journal.pntd.0003855.t002

The study area is considered traditionally endemic for *L. infantum* [35]. The rate of dogs with active infection was increasing over the two-year period, and this pattern was in accordance with previous studies carried out in the area [5, 8]. Our results indicate that there is a significant positive association between anti-*P. perniciosus* saliva antibodies and active *L. infantum* infection. This finding could reflect that these dogs had a greater sand fly challenge compared to other dogs and therefore developed a stronger humoral response against salivary proteins, thus had bigger chance to become infected. Alternatively, because active *L. infantum* infection in dogs is characterized by a mixed Th1/Th2 response associated with marked antibody production [reviewed in [36]], it is possible that our *Leishmania* infected dogs were more sensitive to develop a humoral antibody response against sand fly salivary gland proteins. So far, there is only one report describing an association between CanL and positivity for anti-sand fly saliva antibodies. The study, performed in a *L. infantum* focus in Apulia region of

Table 3. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		8.18	0.91	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	4.15	1.03	<0.001
	September (first year)	5.87	1.02	<0.001
	October (first year)	3.61	1.18	0.002
	December (first year)	2.14	1.02	0.037
	January (second year)	1.53	1.01	0.131
	March (second year)	2.88	1.01	0.005
	July (second year)	10.81	1.01	<0.001
	August (second year)	8.50	1.01	<0.001
	September (second year)	9.67	1.02	<0.001
Random effects	**Variance**			
Dog	17.33			
Residual	28.67			
b) sampling date and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		8.18	0.32	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	2.26	0.74	0.003
	July (second year)	10.13	1.49	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.47	1.11	0.188
	Subpatent	-1.11	3.59	0.758
	Active	4.68	3.14	0.139
Random effects	**Variance**			
Dog	2.39			
March (second year)	15.34			
July (second year)	104.54			
Residual	3.31			

SE = standard error

doi:10.1371/journal.pntd.0003855.t003

Italy, showed that *Leishmania* positive and negative dogs did not differ in IgG and IgG1 production against the whole *P. perniciosus* salivary lysates but the *Leishmania* positive ones had significantly decreased levels of IgG2 antibodies [12]. However, we did not find any association between antibodies against rSP03B protein and active CanL infection. Although, the use of rSP03B protein as a marker of exposure to sand flies is promising, use of recombinant proteins as risk markers for infection need more investigation.

In the present study *P. perniciosus* salivary recombinant proteins, rSP03B (yellow- related protein) and its combination with rSP01 (apyrase) were used. For rSP03B, the repeatability of ELISA test was even higher than for SGH, proving this recombinant protein to be better antigen for large scale studies. The achieved high correlation score between SGH and rSP03B was in agreement with results from previous studies [20, 21]. However, the combination of two recombinant proteins (rSP03B and rSP01) did not show better performance than a single

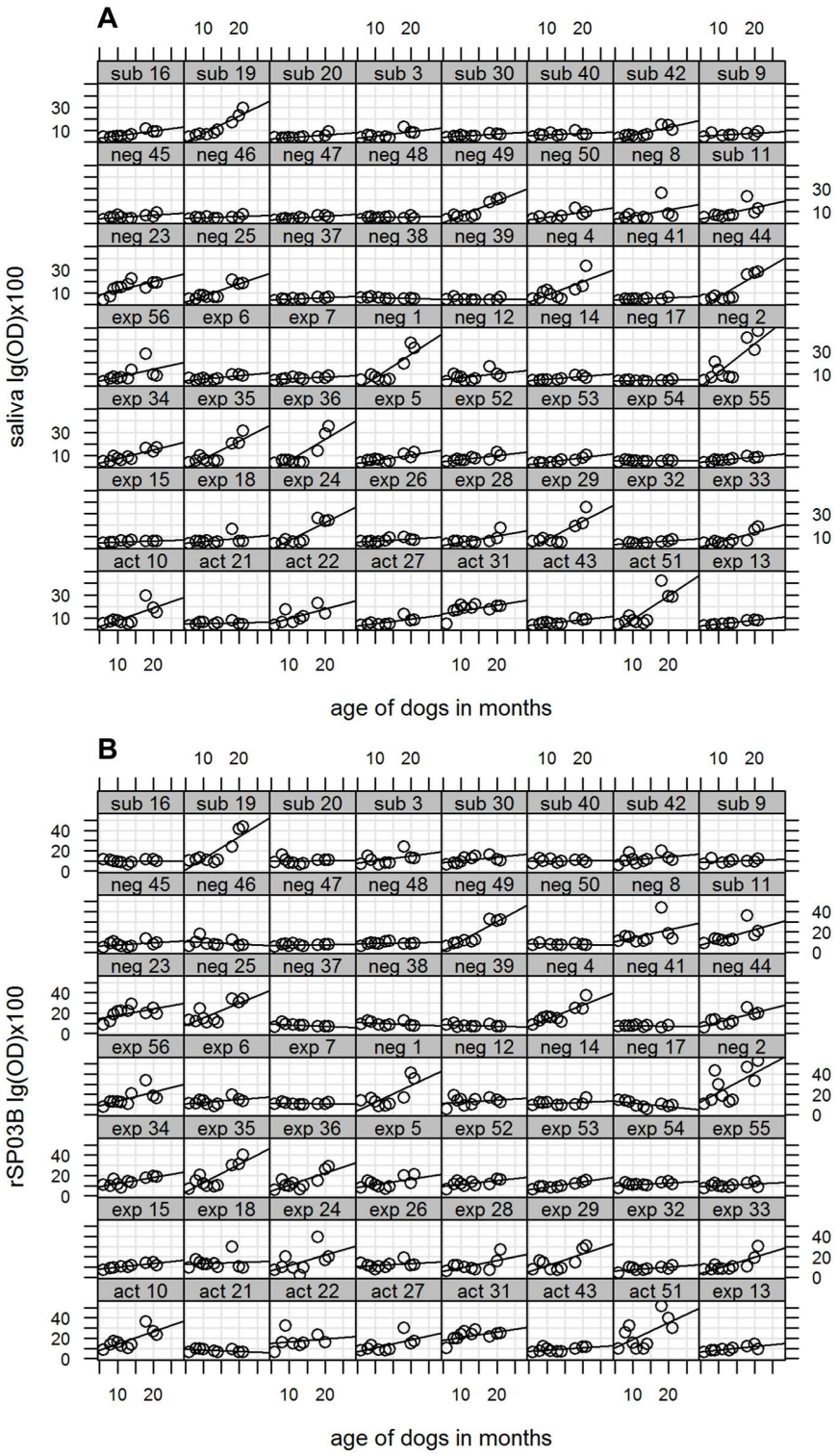

Fig 4. Dynamics of IgG antibodies recognizing SGH (A) and rSP03B protein (B) in individual dogs from CanL endemic locality. *Leishmania* infection status was assigned for 53 dogs according their infection status at the end of the study in March third year and for 3 dogs according their infection status in July second year (dog number 7, 8, 49). OD values were log transformed and multiplied by 100. OD = optical density, neg = negative, exp = exposed, sub = subpatent, act = active.

doi:10.1371/journal.pntd.0003855.g004

SP03B. Repeated experiments suggest that this could be due to instability of rSP01 protein and its higher susceptibility to repeated thawing and freezing. Previously, better results for recombinant proteins combination were obtained for detection of anti-*Lu. longipalpis* antibodies when two recombinant yellow-related proteins were combined together [18].

Kinetics of anti-SGH and anti-rSP03B IgG antibodies developed with similar pattern and were clearly seasonal: rising during summer months when sand fly density is the highest and decreasing during winter months when sand flies are not active. This positive correlation between antibodies against salivary proteins and seasonal abundance of blood feeding insects has been previously reported in mosquitoes [37, 38]. The smaller increase of antibodies against *P. perniciosus* salivary proteins during the first transmission season was probably due to the fact that dogs were exposed to the vector for the first time and were moved to an endemic locality in the middle of the transmission season (July). In central and southern peninsular Italy, the sand fly season usually lasts from late May to late October, with two density peaks [25, 39]. The significant increase of IgG antibodies for SGH and rSP03B protein in March compared to January in the second year (Fig 2) remains unexplained, since it cannot be attributed to such an early activity of sand flies. Even in the southernmost region of Italy (Sicily) the earliest collection of *P. perniciosus* was recently reported to be in April [40]. The marked increase in antibody levels in the second transmission season is much probably caused by re-exposure of dogs to sand flies following antigenic priming in the previous season. Similar antibody responses to sand fly saliva were observed in mice re-exposed to *P. papatasi* [17] and in humans re-exposed to *P. argentipes* [11]. Moreover, the vector population density could vary between years due to the different climatic conditions [39] and therefore, the dogs could have been exposed to higher sand fly challenge during the second transmission season. However, as shown by the multilevel models, there was significant variation in the amount of anti-saliva antibodies between dogs, particularly during July of the second year, the reason of which remains unclear. It could be associated with innate differences in their antibody responses to sand fly salivary antigens or in attractiveness to sand fly bites, or with other unknown factors that need to be investigated. Interestingly, it was recently reported that Beagle dogs may exhibit markedly different attractiveness to *P. perniciosus* under experimental exposure [41].

In conclusion, the dynamics of antibody response against *P. perniciosus* salivary proteins clearly showed seasonal changes due to the expected sand fly abundance. Our study confirmed that recombinant yellow-related protein rSP03B of *P. perniciosus* is a valid alternative to whole sand fly saliva as marker of sand fly exposure. Serology tests based on this recombinant protein could be a practical and economically-sound tool for investigations dog exposure to sand flies in endemic settings of zoonotic visceral leishmaniasis.

Supporting Information

S1 Table. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B+rSP01 OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).
(DOCX)

Acknowledgments

We would like to thank Helena Kulikova, Lenka Zitkova and Kristina Simkova for great technical and administrative support.

Author Contributions

Conceived and designed the experiments: PV IR LG MG. Performed the experiments: GO AS LG MG EF TK TL PS MV. Analyzed the data: TK EB. Contributed reagents/materials/analysis tools: PV LG MG. Wrote the paper: TK PV LG MG EB.

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Sumova, P., Sima, M., Spitzova, T., Osman, M.E., Guimaraes-Costa, A.B., Oliveira, F., Elnaiem, D.-E.A., Hailu, A., Warburg, A., Valenzuela, J.G., & Volf, P. (2018). PLoS Negl. Trop. Dis. *12*, e0006981.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Human antibody reaction against recombinant salivary proteins of *Phlebotomus orientalis* in Eastern Africa

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OPEN ACCESS

Citation: Sumova P, Sima M, Spitzova T, Osman ME, Guimaraes-Costa AB, Oliveira F, et al. (2018) Human antibody reaction against recombinant salivary proteins of *Phlebotomus orientalis* in Eastern Africa. PLoS Negl Trop Dis 12(12): e0006981. https://doi.org/10.1371/journal.pntd.0006981

Editor: Walderez O. Dutra, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, BRAZIL

Received: August 28, 2018

Accepted: November 7, 2018

Published: December 4, 2018

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: The research was supported by Bill and Melinda Gates Foundation Global Health Program (OPPGH5336; https://www.gatesfoundation.org/), by Charles University Research Centre (UNCE 204072; MS, TS; https://www.cuni.cz/UKEN-246.html), by Centre for research of pathogenicity and

Abstract

Background

Phlebotomus orientalis is a vector of *Leishmania donovani*, the causative agent of life threatening visceral leishmaniasis spread in Eastern Africa. During blood-feeding, sand fly females salivate into the skin of the host. Sand fly saliva contains a large variety of proteins, some of which elicit specific antibody responses in the bitten hosts. To evaluate the exposure to sand fly bites in human populations from disease endemic areas, we tested the antibody reactions of volunteers' sera against recombinant *P. orientalis* salivary antigens.

Methodology/Principal findings

Recombinant proteins derived from sequence data on *P. orientalis* secreted salivary proteins, were produced using either bacterial (five proteins) or mammalian (four proteins) expression systems and tested as antigens applicable for detection of anti-*P. orientalis* IgG in human sera. Using these recombinant proteins, human sera from Sudan and Ethiopia, countries endemic for visceral leishmaniasis, were screened by ELISA and immunoblotting to identify the potential markers of exposure to *P. orientalis* bites. Two recombinant proteins; mAG5 and mYEL1, were identified as the most promising antigens showing high correlation coefficients as well as good specificity in comparison to the whole sand fly salivary gland homogenate. Combination of both proteins led to a further increase of correlation coefficients as well as both positive and negative predictive values of *P. orientalis* exposure.

Conclusions/Significance

This is the first report of screening human sera for anti-*P. orientalis* antibodies using recombinant salivary proteins. The recombinant salivary proteins mYEL1 and mAG5 proved to be

virulence of parasites (CZ.02.1.01/0.0/0.0/16_019/0000759; TS, PV; https://opvvv.msmt.cz/), by Czech Science Foundation (P506 17-10308S; PS, MS, PV; https://gacr.cz/en/), by KalaCORE program of DFiD and WHO, Khartoum office (MO, DE; http://www.kalacore.org/) and by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases (JGV; https://irp.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

valid antigens for screening human sera from both Sudan and Ethiopia for exposure to *P. orientalis* bites. The utilization of equal amounts of these two proteins significantly increased the capability to detect anti-*P. orientalis* antibody responses.

Author summary

Hosts repeatedly bitten by phlebotomine sand flies develop species-specific antibody responses against certain sand fly salivary antigens. Salivary gland homogenate (SGH) is frequently used to evaluate the levels of this antibody response in host. However, SGH is less suitable for large-scale studies, since obtaining sufficient numbers of salivary glands is labor intensive and requires expertise in dissection. To replace SGH as antigen to screen for exposure to sand fly bites, specific recombinant salivary antigens were utilized. Our study assessed the human antibody reactions against recombinant salivary proteins of *Phlebotomus orientalis*. This sand fly species is a vector of *Leishmania donovani*, the causative agent of severe visceral leishmaniasis in Eastern Africa. To identify valid markers of exposure to *P. orientalis* in humans, we screened for anti-*P. orientalis* antibody responses in serum samples from individuals residing in Sudan and Ethiopia. We tested nine recombinant salivary antigens and found a combination of yellow-related protein (mYEL1) and antigen 5-related protein (mAG5) the best marker of exposure, accurately correlating with the levels of exposure to *P. orientalis* bites as determined using SGH. Thus the combination mYEL1+ mAG5 can comprise a useful epidemiological tool to determine levels of exposure to *P. orientalis* in populations living in endemic areas of Eastern Africa, which could help in monitoring the distribution of *P. orientalis* and therefore assessing suitable anti-vector campaigns.

Introduction

Phlebotomine sand flies (Diptera: Phlebotominae) are blood sucking insects that transmit parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). *Phlebotomus orientalis* is an important vector of *Leishmania donovani* in Sudan, Ethiopia and Kenya [1]. In these countries, *Le. donovani* is a causative agent of life-threatening visceral leishmaniasis (VL), with an estimated incidence reaching 39 thousand people annually [2]. The optimal strategy for controlling VL depends on understanding the epidemiology and transmission of *Le. donovani* by its vectors. Visceral leishmaniasis in Eastern Africa was believed to be anthroponotic, but recent findings of *Leishmania* DNA and seroprevalence against *Leishmania* antigen in domestic animals indicates the possibility of anthropozoonotic transmission [3–5]. Moreover, *P. orientalis* feeds on humans as well as on domestic and wild animals [3,6–8].

During blood-feeding, female sand flies salivates into the host skin. Saliva contains a cocktail of pharmacologically active proteins that facilitate successful blood feeding on host [9]. Some salivary proteins elicit antibody response, which may be species-specific [10,11] and can, therefore, serve as a marker of exposure to sand flies in bitten hosts [12]. In humans, the predominant subtypes of antibody responses to sand fly saliva differ between vector species [9]. For this reason, in most studies that determined the level of antibody responses to sand fly bites, authors measured basic IgG levels in sera of bitten people. The exposure of human populations to sand fly bites was evaluated through measuring specific antibody responses against salivary gland homogenate (SGH) of several sand fly species including *L. longipalpis* in Brazil

[13,14], *P. sergenti* and *P. papatasi* in Turkey [15] and Israel [16], *P. papatasi* in Tunisia [17,18], *P. arabicus* in Israel [16], and *P. orientalis* in Ethiopia [8]. The aforementioned human antibody response against salivary proteins was also utilized to validate efficacy of vector control programs aimed against *P. argentipes* and *P. papatasi* in India [19,20].

These studies required laborious and time consuming dissections of large quantities of salivary glands. To make large-scale studies more feasible, the most antigenic salivary proteins need to be identified and expressed in recombinant form. Recombinant proteins have already proven their utility in large-scale studies screening anti-phlebotomine antibodies in human populations. In Brazil, recombinant salivary proteins LJM17 and LJM11 [21], and rLinB-13 [22] are able to detect anti-*L. longipalpis* and anti-*L. intermedia* antibodies, respectively. Similarly, a recombinant protein (rPpSP32) was used to screen for exposure to *P. papatasi* bites in Saudi Arabia and Tunis [23,24]. All four proteins were produced in a mammalian expression system and were demonstrated to be valid substitution for SGH in serological tests [21–23].

The current study is the first one aimed at validation of antigenic properties of *P. orientalis* salivary proteins produced in different expression systems, and evaluating their potential to serve as a substitution for SGH in serological surveys screening exposure to *P. orientalis* bites in humans from endemic areas of Eastern Africa.

Methods

Ethical statement

Written informed consent was obtained from all the adult volunteers who participated in the study and for inclusion of young children written consent was obtained from their parents or guardians. For Ethiopian samples, the study protocols were reviewed and approved by the ethical review committee at the Medical Faculty, Addis Ababa University and the National Research Ethics Review Committee (NRERC) at the Ethiopian Ministry of Science and Technology. For Sudanese samples, the study protocols were approved by the National Ethical Committee of the federal Ministry of Health, Sudan. Each volunteer was treated in accordance with international bio-ethical rules and laws for human sampling. Blood was sampled by certified trained Health officers or nurses.

Serum samples

Fifty human serum samples from Sudan and 235 samples from Ethiopia were used for specific anti-*P. orientalis* antibody detection. Eighteen serum samples were used as negative controls: i) nine samples were collected in USA from volunteers with known travel history. and ii) nine human serum samples were collected from residents of Khartoum (Sudan), where *Phlebotomus orientalis* is not present. All sera were obtained from volunteers which did not travel to locations were *P. orientalis* is abundant. Sera were stored at -80˚C and aliquoted before use.

Sand flies and salivary gland homogenate

A laboratory colony of *P. orientalis* originating from Ethiopia (established in 2008) [25] was reared under standard conditions [26]. Salivary glands of *P. orientalis* were dissected from 4–6 day old female sand flies into 20mM Tris buffer with 150mM NaCl (20 glands per 20 μl of buffer) and stored at -80˚C. Before use, salivary glands were disrupted by three freeze-thawing cycles in liquid nitrogen into salivary gland homogenate (SGH).

Recombinant proteins

Nine recombinant proteins based on six different *P. orientalis* salivary antigens were prepared in bacterial (two proteins), mammalian (one protein), or both (three proteins) expression systems (Table 1) and used as antigens for detection of anti-*P. orientalis* IgG in human sera. Bacterially expressed proteins were selected based on their antigenicity in ELISA using domestic animal sera from Ethiopia [11]. The most antigenic proteins recognized by antisera from domestic animals (YEL1 and PAR25) were expressed also in mammalian system. The second yellow-related protein (mYEL2) was expressed in mammalian system due to its sequence similarity to mYEL1 (78%). Mammalian AG5 protein was prepared based on the reactivity of the native protein from SGH on immunoblot with human sera from Sudan and Ethiopia.

The bacterial forms of the recombinants with His-tag were expressed in *Escherichia coli* BL21 (DE3) gold cells (Agilent) and purified using Ni-NTA column (Bio-Rad) as described in [11]. Mammalian counterparts of these proteins were produced and purified as described elsewhere [27]. Briefly, the synthetic DNA fragments (GeneArt Strings, ThermoFisher Scientific) coding recombinant proteins including His-tag at the C terminal end were cloned into VR2001-TOPO vector [28] and transformed by heat shock in TOP-10 cells (ThermoFisher Scientific). Plasmids were isolated and sent to Leidos, NCI, Protein Expression Laboratory (Frederick, MD) for transfection and expression. Transfected FreeStyle human embryonic kidney 293-F cell cultures (HEK293) were harvested after 72 h. Supernatant was concentrated from 2 liter to 400 ml using a Stirred Ultrafiltration Cell unit (Millipore) with ultrafiltration membrane (Millipore). Recombinant proteins were purified by a HPLC system (Bio-Rad) using a HiTrap Chelating HP columns (GE Healthcare Biosciences) charged with nickel by a gradient of imidazole. Protein concentration was measured using a NanoDrop ND-1000 (ThermoFisher Scientific) spectrophotometer at 280 nm and calculated using the extinction coefficient of the protein. Identity and high purity of the proteins was verified by mass spectrometry.

Study design

To determine the best candidates among recombinant proteins, experiments were conducted in several subsequent steps. In the first one, human serum samples from individuals living in areas where *P. orientalis* was prevalent were tested by ELISA with *P. orientalis* SGH as antigen to detect the levels of specific IgG antibodies in these sera. The most antigenic *P. orientalis*

Table 1. Recombinant *P. orientalis* salivary proteins.

Name	Protein family	Expression system	GenBank ACCN
bAPY	Apyrase	*E. coli*	AGT96431
bYEL1	Yellow-related	*E. coli*	AGT96428
bPAR25	ParSP25-like	*E. coli*	AGT96466
bD7	D7-related	*E. coli*	AGT96467
bAG5	Antigen 5-related	*E. coli*	AGT96441
mYEL1	Yellow-related	HEK293	AGT96428
mYEL2	Yellow-related	HEK293	AGT96427
mPAR25	ParSP25-like	HEK293	AGT96466
mAG5	Antigen 5-related	HEK293	AGT96441

List of nine recombinant proteins based on salivary antigens of *P. orientalis*. Designation, protein families, expression systems and GenBank accession numbers are indicated.

https://doi.org/10.1371/journal.pntd.0006981.t001

salivary proteins were defined by immunoblot. To determine their antigenicity, all nine recombinant proteins were tested by ELISA using five different serum samples (three anti-*P. orientalis* SGH positive and two anti-*P. orientalis* SGH negative (2^{nd} step). The promising recombinants were used in ELISA with twenty human sera displaying varying anti-*P. orientalis* SGH reactivity and five non-exposed human sera controls (3^{rd} step). Several combinations of antigen-sera dilutions were tested to determine the optical density (OD) values matching in a best way with anti-SGH. In the final series of experiments (4^{th} step), only the most promising recombinant proteins were tested on immunoblot with five human sera, and by ELISA with all available samples. The dilutions of antigens and sera in each set of ELISA experiments are indicated in S1 Table.

ELISA

ELISA plates (ThermoFisher Scientific) were coated with SGH (28 ng of proteins per well; corresponding to 0.2 gland per well) or recombinant proteins (exact amounts specified in S1 Table) diluted in 20mM carbonate-bicarbonate buffer (pH 9.5) at 4˚C overnight. After washing with phosphate-buffered saline with 0.05% Tween 20 (PBS-Tw), plates were blocked with 6% non-fat dried milk (Bio-Rad) diluted in PBS-Tween and incubated for one hour at 37˚C. After another washing step, plates were incubated with sera diluted in 2% non-fat dried milk for 1.5 hour at 37˚C (sera dilutions specified in S1 Table). Plates were washed and incubated for 45 minutes at 37˚C with peroxidase-conjugated Anti-Human IgG antibody (Sigma-Aldrich) diluted 1:1000 in PBS-Tween. The chromogenic reaction was developed for six minutes in McIlwain phosphate-citrate buffer (pH 5.5) with OPD (orthophenylendiamine, Sigma-Aldrich) and hydrogen peroxide in dark. The reaction was stopped by adding 10% sulfuric acid and measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). In each step, 100 μl of solution was used and each sample was tested in replicates.

Immunoblot

The immunogenicity of the *P. orientalis* salivary proteins was tested by the immunoblot technique. Salivary gland homogenate (4.2 μg of total salivary proteins per lane, equivalent to 30 glands) and recombinant proteins mAG5, mYEL1, and mAG5 + mYEL1 (3 μg of each protein per lane) were electrophoretically separated on a 12% polyacrylamide gel under non-reducing conditions using a Mini-protean apparatus (Bio-Rad). Separated protein bands were transferred onto a nitrocellulose membrane using the iBLOT system (Invitrogen) and then cut into strips. Strips were either stained with amidoblack (Merck; 0.1% solution in 25% isopropanol and 10% acetic acid) or blocked in 5% non-fat milk diluted in Tris-buffered saline with 0.05% Tween 20 (TBS-Tw) overnight at 4˚C and subsequently incubated for 1 hour with human sera diluted 1:50 in TBS-Tw. After the washing step with TBS-Tw, the strips were incubated with peroxidase-conjugated Anti-Human IgG Antibody (Sigma-Aldrich) diluted 1:500 in TBS-Tw. The chromogenic reaction was developed using a substrate solution containing diaminobenzidine and H_2O_2.

Statistical analysis

The OD values of anti-SGH antibodies were used as the gold standard to validate the recombinant proteins in ELISA tests by calculating the respective positive and negative predictive values (PPV and NPV, respectively), sensitivity, and specificity. The cut-off value was calculated as the average OD of negative controls plus three times the standard deviation of negative controls. All serum samples were used in duplicates, averages of measured ODs were calculated and the blank value (average OD from wells without antigen, antibodies, and conjugate) was

subtracted from each sample. Statistical analyses were carried out using R software (http://cran.r-project.org/). The non-parametric Spearman rank correlation test was used to assess correlations between total anti-SGH and anti-recombinant proteins IgG levels. To compare medians between locations and antigens, the non-parametric Kruskal-Wallis test and as a post-hoc test Wilcoxon rank sum test with Holm correction were used. A P-value of < 0.05 was considered to indicate statistical significance. The results were graphically presented using „ggplot2"package in R software [29].

Results

Human sera of individuals from endemic areas recognize *P. orientalis* SGH

Human sera (303 samples) were tested in duplicates with SGH of *P. orientalis* (S1 Fig). The ELISA cut-off value (0.238) was calculated from 18 negative controls. The positivity or negativity of all samples was defined based on the cut-off value. In total, 52% of the sera samples from Sudan and 34.5% samples from Ethiopia were determined as anti-*P. orientalis* positive. Median OD values for Sudan and Ethiopia were 0.25 (range: 0.06–1.6) and 0.19 (range: 0.04–1.4), respectively. When compared to the median value of negative controls (0.1, range: 0.04–0.19) they differed significantly ($p < 0.0001$). The difference in anti-*P. orientalis* antibody levels between Sudan and Ethiopia was marginally significant ($p = 0.08$).

Immunoblot with SGH and human sera

Antigenic *P. orientalis* salivary proteins were identified by immunoblot (Fig 1). In total, 10 highly reactive human serum samples from Sudan and Ethiopia and two negative samples from USA were chosen according to their optical densities in anti-SGH ELISA. Anti-*P. orientalis* positive serum samples recognized up to 10 protein bands with different intensities. The most prominent bands of approximately 40 kDa and 28 kDa corresponded to the molecular weight of the yellow-related protein (sYEL1) and antigen 5-related protein (sAG5), respectively. These two proteins were recognized by the majority of sera samples.

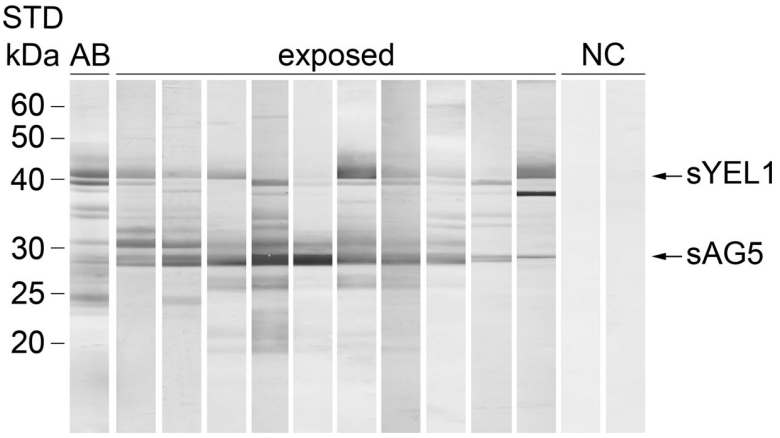

Fig 1. Immunoblot profile of *P. orientalis* salivary antigens. *P. orientalis* salivary proteins were electrophoretically separated under non-reducing conditions. Western blot analysis was performed with ten anti-SGH ELISA positive human sera (exposed) and two negative sera (NC). AB stands for salivary gland protein profile stained by amidoblack; sYEL1 and sAG5 stands for salivary yellow-related protein 1 and salivary antigen 5-related protein, respectively. Molecular weights (kDa) of standard (STD; BenchMark Protein Ladder, ThermoFisher Scientific) are indicated.

https://doi.org/10.1371/journal.pntd.0006981.g001

Assessment of antigenic properties of recombinant proteins

In total, six different *P. orientalis* salivary proteins were expressed in recombinant form. Five proteins (bAPY, bYEL1, bPAR25, bD7, bAG5) were expressed in a bacterial system; three of their counterparts (mYEL1, mPAR25, mAG5) as well as a second *P. orientalis* yellow-related protein mYEL2, were expressed in a mammalian system. In the preliminary experiments using ELISA, antigenic properties of all proteins were tested with five human serum samples. Three different dilutions of antigens and two dilutions of sera (S1 Table: 2nd step) were used to assess the optimal concentration for more extensive experiments. For recombinant proteins expressed in the mammalian system, lower concentrations were used owing to higher purity of these proteins. Results are summarized graphically in S2 Fig.

Based on these initial results, five proteins were excluded from further experiments, the reason was either no difference in OD of anti-SGH positive/negative sera (bAG5, bYEL1, bD7) or false positivity in negative sera (bAPY, mYEL2). For four promising proteins which were subsequently used for further experiments (bPAR25, mPAR25, mYEL1 and mAG5), the lowest eligible concentrations and sera dilutions were selected (S1 Table: 3rd step).

Evaluation of promising recombinant antigens using ELISA

Four promising antigens (bPAR25, mPAR25, mYEL1 and mAG5) were tested by ELISA with 20 Sudanese human serum samples covering the range of anti-SGH positivity/negativity and with five negative control samples from USA and Sudan. Apart from mYEL1, all proteins were tested in single concentration and sera dilution. For mYEL1, two different sera dilutions were tested to justify usage of lower dilution (1:100). Results are summarized in S2 Table. Recombinant ParSP25-related protein expressed in both bacterial and mammalian system was excluded from more extensive experiments due to high cut-off values, low correlation coefficient and big difference in serum positivity in comparison to SGH. Antibody response against mYEL1 correlated well with antibody reaction against SGH in both sera dilution, but the cut off and median values were slightly higher in dilution 1:50, which led to subsequent use of dilution 1:100. Together with mYEL1, antibody reaction against mAG5 also corresponded to anti-SGH response and both proteins showed low cut off values. These two antigens were therefore further analyzed as promising candidates for assessing exposure of human populations to *P. orientalis* bites.

Immunoblot with recombinant salivary proteins mYEL1 and mAG5

The relative intensity of antibody reactions against mYEL1, mAG5 and their combination is shown on an immunoblot (Fig 2). In total, four serum samples positive against both SGH and recombinant proteins in ELISA and one negative control serum were used. All anti-SGH positive samples reacted with both proteins and their combination. Western blot analysis showed that some serum samples recognize mYEL1 with more intensity than mAG5 or vice versa, reinforcing the use of combined antigens to enhance statistical variables of ELISA results.

ELISA with mYEL1 and mAG5

IgG antibody response against SGH was compared by ELISA with response against mYEL1 and mAG5 (each one on its own or in combination). All Sudanese and Ethiopian samples were tested as well as negative control human sera.

For both recombinant proteins and their combination, the cut-off values were comparable with SGH (Table 2). The percentage of positive samples from both localities was highest for SGH, followed by mAG5, combination of mAG5 + mYEL1, and mYEL1 (Table 2). Statistical

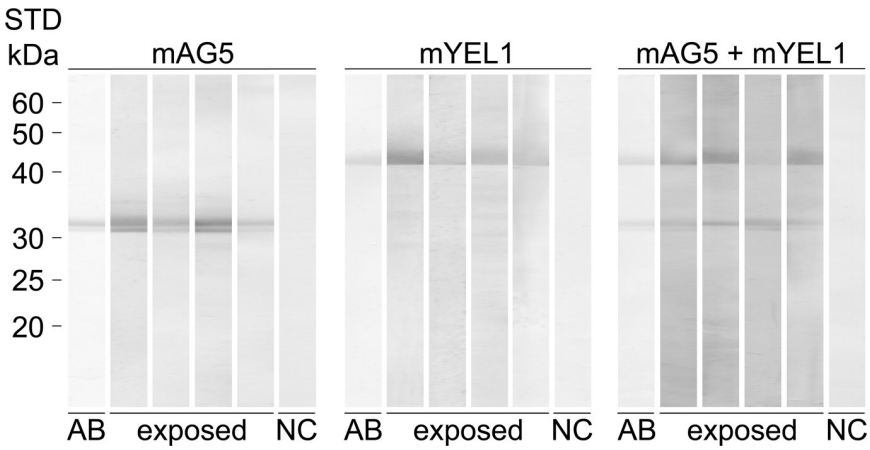

Fig 2. Antibody reaction against mYEL1, mAG5 and their combination on immunoblot. Recombinant mYEL1, mAG5, or both in equal amounts were run by SDS-PAGE under non-reducing conditions. Western blot analysis was performed for four human sera samples anti-recombinant proteins positive in ELISA (exposed) and one negative control serum (NC). AB stands for recombinant protein strip stained by amidoblack. Molecular weights (kDa) of standard (STD; BenchMark Protein Ladder, ThermoFisher Scientific) are indicated.

https://doi.org/10.1371/journal.pntd.0006981.g002

variables comparing the antibody reactions against all antigens (PPV, NPV, specificity, and sensitivity) achieved quite high values in case of PPV, NPV, and specificity (PPV ranged from 0.67 to 1, NPV ranged from 0.63 to 0.82, specificity ranged from 0.83 to 1), moderate values were achieved for sensitivity (range from 0.41 to 0.81) (Table 2). The highest values of all above mentioned statistical variables were achieved for the combination of mAG5 and mYEL1.

Correlation coefficients between anti-SGH antibody reaction and anti-mAG5 antibodies were slightly higher than coefficients between anti-SGH and anti-mYEL1 antibodies, with ranges from 0.68 to 0.84 and from 0.64 to 0.7, respectively. The highest correlation coefficients were achieved when both antigens were combined (range from 0.76 to 0.82) (Fig 3).

Medians of antibody responses to mYEL1 and combination of mAG5 + mYEL1 correspond well to the ones achieved for SGH; while medians calculated for mAG5 were significantly

Table 2. Comparison of antibody reaction of human sera from Sudan, Ethiopia and both localities together against SGH, mAG5, mYEL1, and combination of both proteins.

	SGH			mAG5			mYEL1			mAG5 + mYEL1		
	S	E	S + E	S	E	S + E	S	E	S + E	S	E	S + E
Cut-off	0.238			0.145			0.335			0.284		
Median	0.25	0.19	0.19	0.10	0.11	0.11	**0.24**	**0.16**	**0.17**	**0.24**	0.15	0.16
Q_1 (25%)	0.11	0.13	0.13	0.06	0.06	0.06	**0.11**	**0.10**	**0.10**	0.12	**0.10**	**0.10**
Q_3 (75%)	0.58	0.29	0.32	0.22	0.17	0.17	0.36	**0.28**	**0.30**	**0.47**	0.27	**0.30**
Positive (%)	52.0	34.5	37.5	36.0	**31.1**	**31.9**	36.0	17.9	21.1	**44.0**	22.6	26.3
PPV	not applicable			**1.00**	0.67	0.74	0.78	0.79	0.78	0.96	**0.83**	**0.87**
NPV				0.75	**0.80**	0.79	0.63	0.75	0.73	**0.82**	0.78	**0.80**
specificity				**1.00**	0.84	0.87	0.83	**0.94**	**0.98**	0.96	**0.94**	0.94
sensitivity				0.69	**0.61**	**0.63**	0.54	0.41	0.44	**0.81**	0.54	0.61

Cut-off values, medians, Q_1 (25^{th} percentile), Q_3 (75^{th} percentile), positivity, positive and negative predictive values (PPV and NPV respectively), specificity, and sensitivity (true and false positive and negative values are available in S3 Table) for SGH, mAG5, mYEL1, and mAG5 + mYEL1 are indicated in this table. S, E and S + E represents Sudan, Ethiopia and both localities together, respectively. In total, 50 Sudanese, 235 Ethiopian, and 18 negative control human serum samples were used. The highest values or the best resemblance with SGH for each locality are written in bold.

https://doi.org/10.1371/journal.pntd.0006981.t002

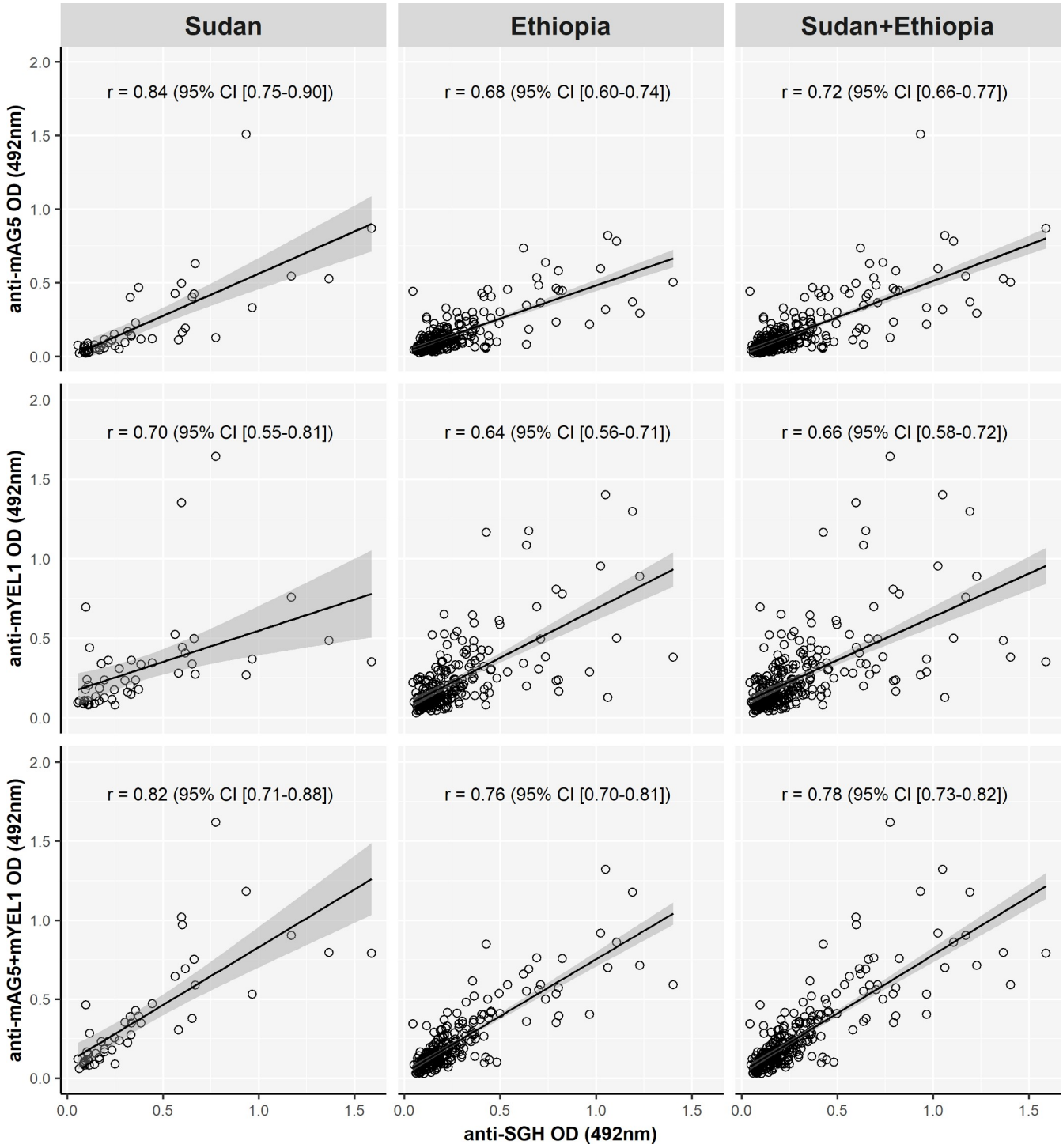

Fig 3. Correlations between IgG antibodies recognizing SGH and mAG5, mYEL1, or mAG5 + mYEL1 in human from Sudan and Ethiopia. Correlation between anti-SGH and anti-mAG5 (first row), between anti-SGH and anti-mYEL1 (second row) and between anti-SGH and combination of mAG5 and mYEL1 (third row) with Sudanese, Ethiopian, and serum samples from both localities together were performed by Spearman-Rank analysis. P value achieved in all analysis was below 0.0001. Correlation coefficients (r) and confidence intervals (CI) are indicated.

https://doi.org/10.1371/journal.pntd.0006981.g003

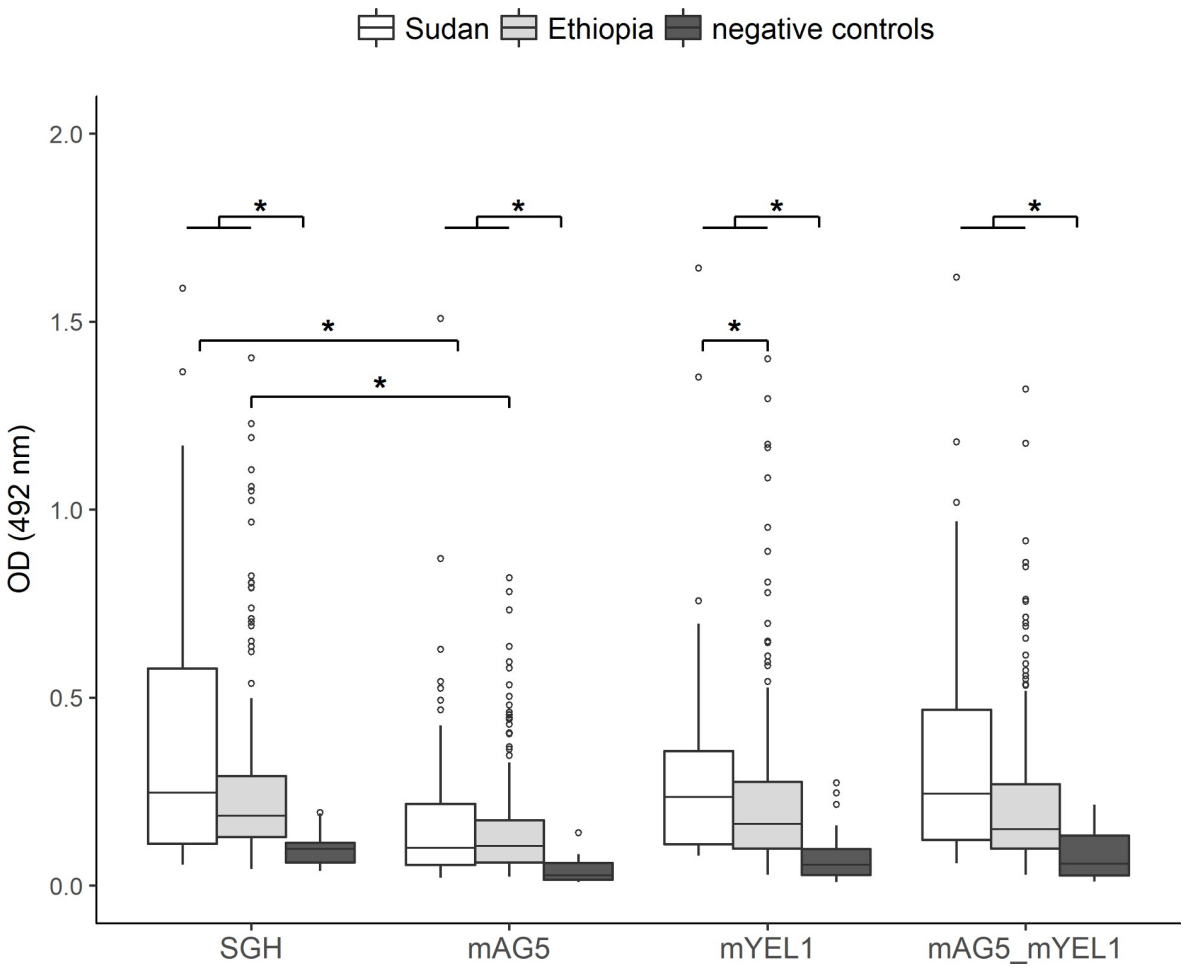

Fig 4. Antibody response against mAG5, mYEL1, their combination, and SGH. Plotted optical densities obtained from ELISA with serum samples from Sudan (50), Ethiopia (235), and 18 negative control samples. Results are shown in box plot graphs where boxes display the 25th-75th percentiles of OD values and median value; vertical lines represent minimum to maximum values and dots the outliers. Asterisks notes statistical significance of $p < 0.05$.

https://doi.org/10.1371/journal.pntd.0006981.g004

lower ($p < 0.004$) (Fig 4). There was no statistical difference in levels of antibody response in Sudan versus in Ethiopia against SGH, mAG5 and mYEL1, even though medians calculated for SGH and mYEL1 were distinctly lower in Ethiopia than in Sudan (Fig 4). Significantly lower levels of IgG reaction in Ethiopia in comparison to Sudan were observed for combination of mAG5 and mYEL1 only ($p = 0.02$) (Fig 4).

Discussion

Nine recombinant antigens (five expressed in *E. coli* and four expressed in human embryonic kidney cells) were tested as potential markers of anti-*P. orientalis* antibody response using human serum samples from Sudan and Ethiopia. Proteins, all belonging to the most abundant sand fly salivary protein families [30], were selected based on the immunoblot reactions with human sera and their known antigenicity for dogs or other domestic animals from Ethiopia, and for mice experimentally bitten by *P. orientalis* [11]. Yellow-related protein mYEL1, antigen 5-related protein mAG5 and especially their combination were shown to be promising candidates for replacing SGH in specific anti-*P. orientalis* salivary antigen IgG detection.

Phlebotomus orientalis is an only species from subgenus *Larroussious* which inhabits areas of interest. Other sand fly species present in both Sudan and Ethiopia include *P. papatasi* and *Sergentomyia schwetzi* which may bite humans but do not transmit visceral leishmaniasis [31]. Antibodies against salivary antigens of these two species do not cross-react with *P. orientalis* SGH or recombinant salivary proteins (bYEL1 and bAG5) confirming that they are species-specific [11]. We found 52% of the sera samples from Sudan and 34.5% samples from Ethiopia as anti-*P. orientalis* positive. The exposure and percentage of positive individuals was comparable or even higher than in other studies [21, 23]. The level of exposure to sand flies can be affected by many variables, including the closeness of the village to the vertisols (breeding sites of *P. orientalis*), the presence of domestic animals, the level of urbanization, the outdoor activities of the participants, etc. The lower positivity of participants from Ethiopia might be due to low numbers of *P. orientalis* in some of the villages where the samples were collected [8].

Proteins AG5, YEL1, and SP25 were expressed in both mammalian and bacterial systems. Although bPAR25 showed similar antigenic properties to mPAR25, both proteins were excluded from further analysis due to high cut-off values, low correlation coefficients with SGH, and big difference in samples positivity in comparison to SGH. In contrast with AG5 and YEL1 expressed in mammalian systems, the same antigens expressed in bacterial cells were not found antigenic for human. Different antigenic properties and low resemblance to anti-SGH antibody reaction in proteins expressed in bacterial system could be due to some *E. coli* impurities remaining in protein preparations even after purification process. These bacterial contaminants can lead to nonspecific serological reaction with anti-*E. coli* antibodies, which are present even in sera of healthy individuals [32]. As *E. coli* is highly prevalent in many African countries [33], anti-*E. coli* antibodies can be expected in a considerable part of the population. Since both antigen 5-related and yellow-related protein are in a native state glycosylated [30], other possible issue could be absence of glycosylation in bacterially expressed proteins, which could affect not only protein folding [34], but could also change their antigenic potential [35].

Antigen 5-related proteins were previously recognized as potent SGH antigens by sera of mice [30,36], dogs [37], rabbits [38], and hamsters [39] bitten by several Old World sand fly species. Recombinant antigen 5-related proteins were tested as potential markers of exposure in three sand fly species only: in *P. perniciosus*, antigen 5-related protein (expressed in *E. coli*) was not recognized by either sera from dogs or mice bitten by this sand fly [40] and in *P. orientalis*, bAG5 was specifically recognized by sera from experimentally bitten mice, but not by those from naturally exposed dogs, goats or sheep from Ethiopia [11]. In *L. intermedia*, recombinant antigen 5-related protein rLinB-13 expressed in mammalian system was specifically recognized by human sera from endemic areas of cutaneous leishmaniasis in Brazil [22]. Our study is therefore the first one describing recombinant antigen 5-related protein as a potent tool to screen exposure to Old World sand fly species in human. Recombinant mAG5 was specifically recognized in immunoblot and its ability to substitute SGH was verified in ELISA. The antibody reaction against mAG5 corresponds to response against SGH with a high correlation coefficient, similar to the one obtained for rLinB-13 in Brazil [22]. The mAG5 achieved high values of specificity, NPV, PPV, and moderate sensitivity. However, the median of antibody responses against mAG5 was significantly lower than the median of responses against SGH in both Sudan and Ethiopia. This finding suggests that the mAG5 cannot capture all antibodies aimed on SGH, thus reinforcing the use of mAG5 in combination with an additional antigen.

The antigenicity of yellow-related proteins was well recognized in various host and sand fly species in both Old and New World [11,15,37,38,41–46]. In *P. papatasi*, two yellow-related proteins, expressed in *E. coli*, were specifically recognized by sera of some experimentally

bitten mice, but were not tested in any large scale survey [36]. In *L. longipalpis*, recombinant yellow-related proteins were produced in mammalian expression system. Proteins LJM11 and LJM17 were identified as suitable antigens to screen exposure in bitten people [21], while LJM11 only was shown also as a marker of exposure in chickens [46]. On contrary, human antibody response against recombinant yellow-related protein from related species *L. intermedia* did not correlate with response against SGH [22]. Recombinant yellow-related protein rSP03B (expressed in bacterial system) derived from saliva of *P. perniciosus*, a sand fly species closely related to *P. orientalis* was at first described as a potential candidate for measuring exposure in dogs and mice experimentally bitten by *P. perniciosus* [40]. Consequently, rSP03B was shown as a suitable tool to screen exposure in hares, rabbits and dogs naturally exposed in Spain, where anti-rSP03B antibody reaction strongly correlated with response against SGH [47]. A strong correlation between anti-rSP03B and anti-SGH antibody response was also achieved in dogs naturally exposed to *P. perniciosus* in Italy and Portugal [48,49]. Sero-strip based on rSP03B was able to rapidly screen dogs living in endemic areas for the presence of *P. perniciosus*, which made it the first feasible immunochromatographic test in the field of vector exposure [50]. It is worth mentioning, that protein rSP03B shares 86% homology to recombinant mYEL1 protein tested in our study. Recombinant bYEL1 from *P. orientalis* which was found non-antigenic to human subjects in our study, was shown as a potent tool to screen exposure to *P. orientalis* bites in dogs, goats, and sheep from Ethiopia [11]. The statistical variables describing anti-bYEL1 antibody response of sheep was similar to the ones obtained in our study for humans and mYEL1. The mYEL1 achieved high specificity and PPV; moreover median values measured for mYEL1 did not statistically differ to the ones measured for SGH. On the other hand, correlation coefficients, NPV, and sensitivity were in both Sudan and Ethiopia distinctly lower than for the other potent candidate, mAG5, thus further reinforcing combining both antigens.

Combining recombinant proteins mAG5 and mYEL1 in an ELISA test led to a distinct increase of correlation coefficients, PPV, and NPV. Correlation coefficient reached r = 0.8 in both Sudan and Ethiopia, a number comparable to studies carried out on dogs [11,47–49], but more promising than the coefficients previously achieved for human host [21,23]. Furthermore, the combination of mAG5 + mYEL1 showed non-significant difference from standard SGH ELISA. Similarly as it was for SGH and mYEL1, differences in medians of OD values between Sudan and Ethiopia were observed also for antigen combination. However this difference was significant only in case of combination mAG5 + mYEL1. This strengthens the conception of using this combination as a replacement for the SGH ELISA in further epidemiological studies. The utilization of combined antigens was further supported by western blot analysis showing that some serum samples recognize with more intensity mYEL1 and with less intensity mAG5 and vice versa. Two dominant antigens used together can therefore resemble in a better way the whole salivary glands, as was previously shown also for two recombinant yellow-related proteins from *L. longipalpis* [21]. On the contrary, in dogs naturally exposed to *P. perniciosus*, combining recombinant yellow-related protein with apyrase did not increase the performance of the yellow-related protein itself, even so it still attained quite high correlation with antibody response against SGH [48].

In conclusion, this study identified the combination of two major *P. orientalis* salivary antigens, yellow-related protein mYEL1 and antigen 5-related protein mAG5, as a valid tool to screen sand fly exposure in human from both Sudan and Ethiopia. This combination of stable recombinant proteins is able to substitute *P. orientalis* SGH and thereby to simplify studies aimed on detecting antibodies in bitten people in endemic areas of visceral leishmaniasis. On the basis of these results, protocols incorporating these recombinant proteins will be used to determine impact of large scale spraying of insecticides on lowering exposure to the bites of *P.*

orientalis in Sudan. The trial will involve thousands volunteers from a hyperendemic region in Gedarif state, where above samples were obtained.

Supporting information

S1 Fig. Anti-*P. orientalis* IgG response against SGH in serum samples from Sudan and Ethiopia. Plotted optical densities obtained from ELISA with 50 Sudanese, 235 Ethiopian, and 18 negative control (NC) serum samples. Each circle represents one serum sample; dashed line represents cut-off value, black lines represents medians, asterisk denotes statistical significance ($p < 0.0001$).
(TIFF)

S2 Fig. Antigens and sera dilution titrations. Summarized optical densities (ODs) of antibody reactions of three anti-*P. orientalis* SGH positive (empty circle) and two negative (full circle) human sera in two dilutions (indicated as S) with nine recombinant proteins in three concentrations (indicated as Ag; μg/well).
(TIF)

S1 Table. Dilutions of antigens and sera for ELISA. Table of antigens and sera dilutions divided into four groups according to the order of experiments as mentioned above. For SGH, the concentration of 0.028 μg/well and the 1:100 dilution of sera was same in all sets of experiments. bRP and mRP stands for recombinant proteins expressed in bacterial and mammalian system, respectively.
(DOCX)

S2 Table. Comparison of antibody reaction of SGH and recombinant proteins. Cut-off values, medians, positivity, and correlation coefficients between antibody response against bPAR25, mPAR25, mYEL1 in two serum dilutions (A stands for 1:50, B stands for 1:100), and antibody response against SGH are indicated in this table. In total, 25 serum samples were used for this experiment. NA stands for not applicable.
(DOCX)

S3 Table. True and false positive and negative values. True positive, true negative, false positive and false negative values describing antibody reaction of all available human serum samples against SGH and recombinant proteins mAG5, mYEL1 and mAG5 + mYEL1 are indicated in this table. S, E and S+E represents Sudan, Ethiopia and both localities together, respectively. NA stands for not applicable.
(DOCX)

Acknowledgments

We are grateful to Oscar Kirstein for participating in field sample collection and would like to express special thanks to Dr Orin Courtenay for his valuable help during the field work. Thanks are due to Prof Omran F Osman, University of Khartoum, for coordinating and supervising the work in Sudan. Valuable efforts of Mr Osman Dakeen, KalaCORE Research Assistant, in obtaining the Sudanese samples are highly appreciated. Thanks are due to Dr. Atia Altaiabi and staff at WHO office in Khartoum for valuable help. We are grateful to Shannon Townsend and Morgan Karetnick for help with protein expression in mammalian system. We appreciate Jana Glogarova and Helena Kulikova for excellent technical and administrative support.

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SUMMARY AND CONCLUSIONS

This Ph.D. thesis constitutes of the results of four projects connected by the main subject, the sand fly salivary yellow-related proteins. The results emphasized the distinct role of this abundant group of salivary proteins in blood-feeding and its potential to serve as a marker of exposure to sand flies across different vectors and hosts species. We have studied the structure and amine-binding function of salivary YRPs and evaluated their antigenic properties in two important vectors of VL causative agents.

In order to validate the biogenic amine-binding properties of the sand fly salivary YRPs, we have first modelled their structure. Models of all 32 YRPs from 13 sand fly species were constructed based on the published structure of *L. longipalpis* YRP LJM11. Protein LJM11 is the first sand fly salivary protein in which the crystal structure was described (Xu et al., 2011). All 33 YRPs were shown to share similar folding as a six-bladed β-propeller, which forms a hollow barrel with two entrances on the opposite sides of the protein that are connected by the ligand-binding tunnel with varying length, width and hydrophobicity. On the visualized models, the potential ligand-binding site was located closer to the smaller entrance. The surface electrostatic potential of the entrance closer to the binding site varied in different YRPs from negative to positive, while the charge of the larger entrance was found more uniform by being neutral to negative in all YRPs. The ligand-binding tunnel was in all proteins charged negative, enabling the binding of positively charged bioamines similarly to amine-binding proteins in mosquitoes (Calvo et al., 2009).

The amino acids alignment of all 33 YRPs shown 44-99 % similarity, sharing 15 % invariant sites including five identical amino acids forming the biogenic amines binding motif established for LJM11 (Xu et al., 2011). On contrary, other seven amino acids of the binding site, including the three major amino acids (Thr-327, Asn-342 and Phe-344) participating in the hydrogen bonds with amine serotonin in LJM11, were shown to vary between species. The presence and type of substitutions in these amino acids differed both between and within species, though the most of the proteins varied from LJM11 only in the last major amino acid. The only YRP which differed from the LJM11 in all hydrogen bonds-associated amino acids was protein PpeSP03B from *P. perniciosus* (labelled as Pper2). The resemblance of YRPs in the hydrogen bonds-associated amino acids was found partially in accordance with their position on the phylogenetic tree, which was created using the maximum likelihood method. The detected relations between YRPs which were visualized on the phylogenetic tree showed

a greater divergence of YRPs in *Lutzomyia* than in *Phlebotomus* genera. The observed relations corresponded well with a recently published phylogenetic tree which additionally comprised YRP from *P. kandelakii* (Fig. 2; Coutinho-Abreu & Valenzuela, 2018).

For most of the YRPs, the positions on the phylogenetic tree were in agreement with their predicted glycosylation sites. In accordance with other studies, the majority of proteins were predicted to be glycosylated and were shown to vary in their glycosylation pattern both between and within species (Rohousova et al., 2012; Vlkova et al., 2014). As the N- or O-glycans were predicted mainly at the same amino acid positions, the glycosylation was assumed to enhance protein folding and stability of the tertiary structure together with a possible effect on the function of YRPs (Katoh & Tiemeyer, 2013; Reddy et al., 2000). Both inter- and intra-specific differences observed in glycosylation pattern, amine-binding motif, electrostatic potential, length, width and hydrophobicity of the tunnel suggested versatility of YRPs in binding affinities for different amines. These differences were even more pronounced in paralogs within one sand fly species, supporting the suggested functional divergence of YRPs enabling nuanced reactions to host bioamines (Xu et al., 2011).

The results obtained from the bioinformatics analyses of the sand fly YRPs revealed overall similarity in their 3D structures along with both inter- and intra-specific modifications which may affect function even of closely related proteins. Therefore, we have decided to confirm the presumed amine-binding function of selected YRPs by an experimental approach. In *L. longipalpis*, all three YRPs were shown as high affinity binders of host pro-haemostatic and pro-inflammatory amines including serotonin, catecholamines or histamine. The affinities for each ligand were measured by isothermal titration calorimetry and were shown to vary between the individual YRPs (Xu et al., 2011). The binding of biogenic amines was not yet demonstrated for genus *Phlebotomus*, therefore we have focused on revealing the amine-binding affinities of YRPs in two important vectors of VL causative agents from this genus*;* *P. perniciosus* and *P. orientalis*. Both species belong to the subgenus *Larroussius* and each comprises two YRPs. It is of interest, that greater amino acids identity of the four YRPs was found not inside but across these two species, which is in accordance with the phylogenetic tree constructed in the first project and with published findings dividing the YRPs to higher ("44 kDa") and lower ("42 kDa") molecular weight subgroups (Abdeladhim et al., 2016; Coutinho-Abreu & Valenzuela, 2018). The higher molecular proteins of both *P. orientalis* (PorASP4) and *P. perniciosus* (PpeSP03B) were found considerably more abundant in the salivary glands of both species then the other two proteins (PorASP2 and PpeSP03).

We have expressed the *P. orientalis* and *P. perniciosus* YRPs in mammalian cell line of human embryonic kidney 293 (HEK293) cells; our choice of mammalian system for expression was supported by the abovementioned putative glycosylation of YRPs (besides PorASP2). To determine their amine-binding affinities, we have employed for the first time the microscale thermophoresis technique (MST), which express the affinity of the interaction by the dissociation constant (Kd). To confirm validity of the results from MST we have re-measured the amine-binding properties of LJM11 previously ascertain by conventional isothermal titration calorimetry (Xu et al., 2011) and yield the comparable values of Kd. All *P. orientalis* and *P. perniciosus* recombinant YRPs bound at least two biogenic amines, while the affinities for individual ligands vary both intra- and inter-species. On contrary to *L. longipalpis* LJM17 (Xu et al., 2011), *Phlebotomus* YRPs were all shown as low affinity binders of histamine (Kd > 1000), therefore they were assumed to be unable to prevent its interaction with physiological receptors at the feeding site. Similarly to both LJM11 and LJM17 (Xu et al., 2011), recombinant YRPs rSP03B (*P. perniciosus*) and rPorASP4 (*P. orientalis*) bound with high affinity serotonin (Kd < 10), which might enable them to facilitate inhibition of platelet aggregation and vasoconstriction. This proposed function could be further emphasized by high abundance of these proteins in sand fly saliva and by measured medium affinity to other pro-haemostatic ligand norepinephrine in both species.

To elucidate the differences in binding affinities between the YRPs, we have look further on the 3D models of the selected proteins. The most pronounced difference in amine-binding motif was shown for the abovementioned protein rSP03B (≈Pper2), where the changes in amino acids participating in hydrogen bond formation led to the loss of one potential hydrogen bond, which might be the reason for the absence of catecholamine-binding property of this protein. The binding affinities of protein rPorASP4 were shown highly similar to *L. longipalpis* LJM11, even though they differed in the surface charge of the cavity entrance closer to the amine-binding site, which led us to hypothesize that the ligands enter the barrel-like structure from the more uniform larger opening (mentioned above). Proteins rSP03 (*P. perniciosus*) and rPorASP2 (*P. orientalis*) shared the same amine-binding motif and also similar surface charge at the tunnel entrances, while they differed in the binding affinities for the ligands. The distinct binding profile of these two YRPs might be due to assumed slightly different folding or more elongated shape of rSP03 in comparison to other YRPs. Binding event might be affected also by additional parameters of tunnel inside the barrel structure or by distinct glycosylation patterns (Katoh & Tiemeyer, 2013).

To summarize these two parts of our project, we have demonstrated that while retaining similar structure, even the closely related YRPs diverge in their function. However, when salivated into the feeding site, we can assume that both *P. perniciosus* and *P. orientalis* YRPs potentially facilitate counteracting platelets aggregation and blood vessels constriction.

Our work elucidated amine-binding properties of YRPs of sand flies belonging to genus *Phlebotomus.* As YRPs of *Lutzomyia longipalpis* were previously described (Xu et al 2011) it might be very interesting to validate the structure and function of YRPs in the third sand fly genus, *Sergentomyia*. We may focus on *S. schwetzi*, a species distributed in sub-Saharan Africa, which we have in laboratory colony. Although not being a competent vector of *Le. donovani*, *S. schwetzi* is an important African species with man-biting tendencies which in some places co-occur or even outnumber *P. orientalis* (Sadlova et al., 2013).

Apart from the revealed anti-haemostatic properties, sand flies YRPs also trigger the host immune system, especially the humoral part of it. When salivated into the feeding site, YRPs were shown to elicit specific host antibody response, which can be measured and subsequently used to serve as a marker of exposure to sand flies (reviewed in Lestinova et al., 2017). To make large scale serological studies more feasible and decrease the potential inter-specific cross-reactivity (Clements et al., 2010; Volf & Rohousova, 2001) the YRPs expressed in recombinant form were utilized. In two following projects we have focused on evaluating the potential of recombinant YRPs of *P. perniciosus* and *P. orientalis* to serve as tools to detect canine and human exposure, respectively.

In *P. perniciosus*, we have focused on dogs, the main hosts and also the reservoir hosts of *Le. infantum* in Mediterranean region (Dvorak et al., 2018). A longitudinal study across two sand fly seasons was performed on 56 naive dogs which were relocated to southern Italy. In this location, endemic for both CL and human zoonotic leishmaniases, *P. perniciosus* is the only vector species (Gramiccia et al., 2013; Maroli et al., 2001). The dogs were sampled before the transport (pre-immune sera), in the first transmission season (three times), during the winter (three times) and in the second sand fly season (three times). One more sampling was performed in March of the third year to evaluate dogs follow-up *Le. infantum* status.

We have described for the first time the dynamics of IgG antibodies recognizing both *P. perniciosus* SGH and recombinant salivary proteins in dogs naturally exposed to sand flies for the period of the two following years. To measure the canine anti-*P. perniciosus* antibody response we used (apart from the SGH) recombinant YRP rSP03B and its combination with apyrase (rSP01; NCBI accession number: DQ192490). Both proteins were expressed in

bacterial cells (*Escherichia coli*; Bl21) and found antigenic in previous small-scale studies (Drahota et al., 2014; Martin-Martin et al., 2014).

The second *P. perniciosus* YRP rSP03 was not used, as it was found non-antigenic for dogs naturally exposed to *P. perniciosus* (Kostalova et al., 2017; Vlkova et al., 2011). The anti-*P. perniciosus* SGH antibody response of all canine serum samples throughout our study correlated with high correlation coefficient (r=0.77) with the IgG response against rSP03B, while the combination of YRP with apyrase did not increase the performance of rSP03B alone. Moreover, protein rSP03B was shown as a better antigen than SGH for the large scale studies, as the repeatability of the ELISA test using rSP03B achieved higher value.

When taken month by month, both anti-SGH and anti-rSP03B IgG antibody responses developed with similar pattern, corresponding to the *P. perniciosus* seasonal abundance. This positive correlation between anti-vector antibodies and its annual activity was previously shown for mosquitoes (Fontaine et al., 2011) and recently also for *P. perniciosus* in dogs screened in Spain (Velez et al., 2018) and for dogs exposed to *L. longipalpis* (Quinnell et al., 2018). In the present study, naive dogs were introduced into the endemic locality in the middle of the sand fly season (Maroli et al., 2001), which is in accordance with a smaller increase in the antibody levels during the late summer of the first year. In winter, both anti-SGH and anti-rSP03B antibodies significantly decreased. The re-exposure of dogs to sand flies in the second summer season led to a pronounced increase of the antibody levels when compared to the first season. This corresponds with the previously indicated antigenic priming during the first rounds of exposure and expected long term persistence of memory immune cells (Clements et al., 2010; Vlkova et al., 2012).

We have found a significant positive association between the active *Le. infantum* infection status and canine anti-*P. perniciosus* IgG antibodies when using SGH as an antigen, while no association was found for recombinant YRP rSP03B, indicating that the rSP03B protein cannot be utilized as a risk marker for infection. The use of anti-saliva antibodies to estimate the risk of *Le. infantum* infection in dogs is controversial, as the detected association in published studies varied from positive to negative or none (Kostalova et al., 2017; Quinnell et al., 2018; Velez et al., 2018; Vlkova et al., 2011).

In summary, this large scale longitudinal study confirmed the recombinant protein rSP03B as a valid alternative to *P. perniciosus* SGH for screening sand fly exposure of dogs from area endemic for *Le. infantum.* The applicability of rSP03B was further emphasized by the most recent studies (Velez et al., 2018; Willen et al., 2018).

In *P. orientalis*, we have focused on humans living in *Le. donovani* endemic countries in Eastern Africa. Previously, the recombinant YRP corresponding to rPorASP4 was identified as a best tool to screen antibodies in dogs, goats and sheep naturally exposed to *P. orientalis* (Sima et al., 2016). To identify valid markers of exposure to *P. orientalis* in humans, we have screened for *anti-P. orientalis* IgG antibody responses in serum samples from individuals living in Sudan and Ethiopia using either SGH or individual recombinant salivary proteins as antigens. With the use of *P. orientalis* SGH, 52 % and 34.5 % of human serum samples were found positive in Sudan and Ethiopia, respectively. These numbers are comparable to other studies conducted on humans living in endemic areas of other *Leishmania sp*., using as antigen SGH of local vector species (Carvalho et al., 2017; Kammoun-Rebai et al., 2017; Marzouki et al., 2015; Souza et al., 2010). The lower exposure to sand flies in Ethiopia was probably caused by low abundance of *P. orientalis* in most villages sampled (Kirstein et al., 2018).

We have expressed and tested six different *P. orientalis* salivary antigens selected according to their strong reaction with canine sera (Sima et al., 2016). The most promising antigens including YRP rPorASP4 (labelled in this study as YEL1) were expressed in both bacterial (*E. coli*, Bl21) and mammalian system (HEK293), the other proteins including the second YRP rPorASP2 (labelled in this study as mYEL2) were expressed in one system only. YRP rPorASP4, together with the other promising protein from antigen 5-related protein family (AG5, NCBI accession number: AGT96441) were found antigenic for human host only when expressed in mammalian system (labelled as mYEL1 and mAG5). The reason for low antigenicity of bacterially expressed proteins might be the absence of glycosylation, which was shown to affect proteins antigenic properties (Lisowska, 2002). As bacterially expressed rPorASP4 (labelled as bYEL1) was previously shown antigenic for domestic animals (labelled as rPorSP24; Sima et al., 2016), we can presume that its inability to distinguish between anti-*P. orientalis* SGH positive and negative human sera might be also due to some bacterial contaminants persisting in protein preparation, which interact with anti-*E. coli* antibodies anticipated in African population (Paudyal et al., 2017; Yip et al., 2007).

The human IgG antibody response against rPorASP4 showed in both Sudan and Ethiopia high correlation coefficients (r=0.64-0.70) as well as good specificity and positive predictive values in comparison to the antibody response against the whole *P. orientalis* SGH. It is of interest that protein rPorASP4 share 85 % homology with above discussed *P. perniciosus* antigen rSP03B. On contrary to abovementioned study conducted on *P. perniciosus*, combining equal amounts of rPorASP4 YRP with the second antigenic

P. orientalis salivary protein mAG5 further strengthen the correlation coefficients (r=0.76-0.82) as well as both positive and negative predictive values of *P. orientalis* exposure in humans. The statistical values achieved for combination of rPorASP4 + mAG5 were even more promising than the ones previously achieved for human host using combinations of two salivary proteins of *L. longipalpis* (Souza et al., 2010) or single recombinant protein of *P. papatasi* (Marzouki et al., 2015), while they were comparable to above mentioned study and also to other studies carried out on dogs (Kostalova et al., 2017; Martin-Martin et al., 2014). The combination of two recombinant salivary proteins was therefore proposed as a valid tool for screening human sera from Eastern Africa for exposure to *P. orientalis* bites.

On contrary to rPorASP4, the second YRP rPorASP2 was not found specific enough to be a valid antigen for screening human exposure, even though it was also expressed in mammalian system. This finding is in accordance with low/no antigenicity of its above mentioned *P. perniciosus* ortholog rSP03. We can hypothesize that the low antigenicity of these YRPs might be together with other factors caused by their lower abundance in sand fly saliva, when compared to highly antigenic YRPs rPorASP4 and rSP03B (mentioned above).

In the future work it might be interesting to focus on developing a sero-strip incorporating the combination of *P. orientalis* recombinant proteins rPorASP4 + mAG5 which would thereby extend the use of this novel tool to people living in *Le. donovani* endemic areas in Eastern Africa. The sero-strip could be used to determine the impact of large-scale spraying of insecticides on decreasing the exposure to *P. orientalis* in a hyper-endemic area for *Le. donovani* in Sudan.

In conclusion, salivary YRPs from related species *P. perniciosus* and *P. orientalis* were shown to diverge both in function and in antigenic properties. However, the YRPs of these two species were shown to share similar structure and proposed to facilitate sand fly blood-feeding by potentiating inhibition of platelet aggregation and vasoconstriction. In both *P. orientalis* and *P. perniciosus*, the highly abundant higher molecular weight proteins (rPorASP4 and rSP03B) were found as valid tools to screen host exposure to sand flies, while the other two proteins (rPorASP2 and rSP03) had low/no antigenicity. This led us to hypothesize that in subgenus *Larroussius* the members of “44 kDa” subgroup might have the antigenic properties in common. It would be interesting to validate this hypothesis for other members of *Larroussius* subgenus, such as *P. tobbi*, which also comprise two YRPs.

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