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Comparison and characterization of salivary proteins from *Sergentomyia* and *Phlebotomus* sand flies

Vlastnosti slinných proteinů flebotomů rodu *Sergentomyia* a *Phlebotomus*

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Ph.D. thesis/Disertační práce

Supervisor/Školitel: Prof. RNDr. Petr Volf, CSc.

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AUTHOR'S DECLARATION / PROHLÁŠENÍ AUTORKY

I declare that I elaborated this Ph.D. thesis on my own. The results presented within this thesis were accomplished by myself or in the collaboration with the co-authors of the presented papers. I also proclaim that the literary sources were cited properly and neither this work nor the particular data have been used as a final work towards any other university degree.

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Prague, July 13th, 2020

V Praze, 13. července 2020

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I declare that Nikola Polanská 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 presented.

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

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ABSTRACT

Sand flies (Diptera, Phlebotominae) are small biting insects and vectors of *Leishmania* spp. which cause medically and veterinary important disease – leishmaniasis. During the piercing of the host skin, sand fly females inject saliva to facilitate the blood feeding. The sand fly saliva is composed of many bioactive molecules which were shown to possess anti-inflammatory and anti-haemostatic functions. The saliva affects host's immunity in the bite site and consequently enhances the survival and development of transmitted pathogens.

Most of the studies focus on salivary proteins and enzymes of sand flies belonging to *Phlebotomus* and *Lutzomyia* genera, while salivary proteins from sand flies of the third genus *Sergentomyia* were neglected so far. In this thesis we focused on comparison of salivary proteins from two *Phlebotomus* species, namely *Phlebotomus perniciosus* and *Phlebotomus orientalis*, and *Sergentomyia schwetzi*. These sand fly species differ not only by the ecology and geographical distribution but also by host preferences. Both *Phlebotomus* species prefer large or medium-size mammals as the bloodmeal source, particularly rabbits, hares and dogs for *P. perniciosus* and cattle, goats, sheep and humans for *P. orientalis*. Contrarily, *Sergentomyia* sand flies are known for preferred feeding on reptiles but mammal biting behaviour was also reported.

In the first part of this thesis we characterised main salivary proteins from *S. schwetzi*. Moreover, to reveal the adaptation on different hosts, we compared salivary proteins from two *S. schwetzi* lineages adapted to feeding either on geckos or on mice. To do so we used various studying approaches, particularly transcriptome sequencing, RNA-seq, proteome analysis by mass spectrometry and enzyme assays. We identified *S. schwetzi* homologues of all main sand fly salivary protein families. Interestingly, we detected secreted salivary ribonuclease, the enzyme previously found only in mosquitoes. The lineage comparison revealed higher hyaluronidase activity and higher expression of PpSP15-like protein in saliva of the lineage adapted to mice while the comparison of the proteome showed six proteins more abundant in saliva of lineage adapted to geckos.

The second part of the thesis was focused on salivary yellow-related proteins (YRPs), which are known to bind host's biogenic amines. We expressed *P. perniciosus*, *P. orientalis* and *S. schwetzi* YRPs in mammalian expression system and compared their amine-binding properties by microscale thermophoresis method and for *P. perniciosus* and *P. orientalis* also by *in silico* 3D structure modelling. Our analysis showed that each YRP

homologue has different binding abilities. Importantly, we demonstrated that one YRP from each of both *Phlebotomus* species, *P. perniciosus* and *P. orientalis,* had high binding affinity to serotonin, biogenic amine which affects host's platelet aggregation and vasoconstriction. Contrarily, two YRP homologues from *S. schwetzi* do not possess binding function to any of the tested biogenic amines, suggesting their possible adaptation to reptile hosts.

In the last part of this Ph.D. thesis we revealed utilisation of recombinant YRP from *P. perniciosus* (PpeSP03B) as universal marker of exposure for dogs, which can be used in various endemic sites across the western half of Mediterranean basin.

ABSTRAKT

Flebotomové (Diptera, Phlebotominae), drobný krevsající hmyz, jsou známí především jako přenašeči protist z rodu *Leishmania*, které způsobují medicínsky i veterinárně důležité onemocnění – leishmaniózu. Během sání samice vypouštějí sliny do hostitele, což jim umožňuje snadnější sání krve. Biologicky aktivní látky obsažené ve slinách flebotomů mají protizánětlivé účinky a schopnost ovlivnit hemostatický systém hostitele, čímž zabraňují srážení krve. Sliny také ovlivňují imunitu hostitele v místě sání a zvyšují tak pravděpodobnost nákazy různými patogeny, které flebotomové mohou přenášet.

Slinné proteiny byly doposud zkoumány především u druhů flebotomů patřících do rodů *Phlebotomus* a *Lutzomyia*, zatímco flebotomové rodu *Sergentomyia* byli po dlouhou dobu přehlíženi. Proto jsme se v této disertační práci zaměřili na porovnání slinných proteinů druhů *Phlebotomus perniciosus* a *Phlebotomus orientalis* se slinnými proteiny druhu *Sergentomyia schwetzi*. Zmíněné druhy se liší nejen ekologií a zeměpisným rozšířením, ale i preferencí hostitele. Oba druhy rodu *Phlebotomus* upřednostňují jako hostitele savce většího či středního vzrůstu. *Phlebotomus perniciosus* saje především na králících, zajících a psech. *Phlebotomus orientalis* dává přednost sání na větších savcích, jako jsou krávy, kozy, ovce a případně i lidé. Oproti tomu zástupci rodu *Sergentomyia* preferují jako hostitele plazy, nicméně bylo pozorováno jejich sání i na savcích.

V první části této práce jsme se zaměřili na charakterizaci slinných proteinů *S. schwetzi* a jejich porovnání mezi dvěma liniemi tohoto druhu flebotoma, kdy první linie dlouhodobě sála na myších a druhá na gekonech. Pro zachycení a kvantifikaci možného přizpůsobení slin na rozdílné typy hostitelů jsme zvolili čtyři metodologické postupy: sekvenování transkriptomu, porovnání míry exprese transkriptů metodou RNA-seq, porovnání proteomů pomocí hmotnostní spektrometrie a porovnání enzymatických aktivit ve slinách.

Porovnání složení a vlastností slin mezi dvěma liniemi odhalilo signifikantně zvýšenou aktivitu hyaluronidázy a zvýšenou expresy PpSP15-like transkriptu u linie sající na myši. Oproti tomu ve slinách linie přizpůsobené sání na gekonovi jsme identifikovali šest obohacených proteinů v porovnání s linií sající na myších. Navíc se nám kromě již známých slinných proteinů podařilo objevit nový slinný enzym, ribonukleázu, která byla zatím popsána pouze u komárů

Druhá část této disertační práce byla věnována slinným proteinům skupiny Yellow-related (YRP), které mají u flebotomů schopnost vázat biogenní aminy hostitele. Metodou „microscale thermophoresis“ jsme testovali schopnost vazby různých biogenních

aminů u rekombinantních YRP proteinů ze slin druhů *P. perniciosus*, *P. orientalis* a *S. schwetzi* získaných ze savčího expresního systému. Výsledky ukázaly, že YRP proteiny se liší ve svých vazebných vlastnostech a váží jednotlivé biogenní aminy s různou afinitou. Jeden YRP protein, přítomný ve slinách *P. perniciosus* i *P. orientalis*, váže silnou vazbou serotonin, který hostiteli napomáhá vazokonstrikci a agregaci krevních destiček. Oproti tomu, ani jeden YRP ze slin *S. schwetzi* nevázal žádný z testovaných biogenních aminů, což je pravděpodobně důsledkem přizpůsobení *S. schwetzi* na plazí hostitele.

V poslední části této práce jsme ověřili a potvrdili využití jednoho z YRP proteinů ze slin *P. perniciosus*, konkrétně PpeSP03B proteinu, jako expozičního markeru pro poštípání psů tímto flebotomem v různých oblastech západního Mediteránu.

LIST OF ABBREVIATIONS

ADA	adenosine deaminase
ADP	adenosine diphosphate
Ae.	*Aedes*
Ag5r	antigen5-related proteins
An.	*Anopheles*
AMP	adenosine monophosphate
ATP	adenosine triphosphate
DTH	delayed type hypersensitivity
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FXa	coagulation factor Xa
IFN-γ	interferon-γ
IL	interleukin
L.	*Lutzomyia*
Le.	*Leishmania*
MW	molecular weight
OBP	odorant-binding protein
P.	*Phlebotomus*
S.	*Sergentomyia*
SGH	salivary gland homogenate
Th1	T-cell helper type 1
Th2	T-cell helper type 2
VL	visceral leishmaniasis
YRP	yellow-related protein

INTRODUCTION

Saliva affects the blood uptake of all blood feeding arthropods, including sand flies. Its compounds and properties play a crucial role in successful blood feeding and moreover they also influence potential pathogens which are transmitted by arthropods.

The main aim of this thesis is to compare salivary proteins of three sand fly species, particularly *Phlebotomus perniciosus*, *Phlebotomus orientalis* and *Sergentomyia schwetzi*. Each of these three sand flies prefers different vertebrate hosts for blood feeding and differs in their ecology and geographical distribution. In order to highlight all the ecological differences possibly affecting the host preferences, in first part of the thesis the brief analysis of ecology (preferred habitats and seasonality) is discussed for all three species. The characterisation of sand fly salivary proteins with an emphasis to *P. perniciosus* and *P. orientalis*, including properties, their blood feeding functions and immunogenicity for hosts, is the main element of the second part of the thesis. This thesis includes four publications published in peer-reviewed journals, in one I am the firs author (Polanska et al., 2020) and in three I am a co-author (Kostalova et al., 2017; Spitzova et al., 2020; Sumova et al., 2019).

1. Biology of sand flies with an emphasis on *S. schwetzi*, *P. perniciosus* and *P. orientalis*

Phlebotomine sand flies (Diptera, Nematocera, Psychodidae, Phlebotominae) are small blood-sucking insects. They belong to holometabolic insects, meaning that their development starts with the formation of an egg, which then leads through four instars of larvae, the pupal stage and eventually finishes with the adult fly. The larvae feed on a mixture of soil, excrements and fungi. Both sand fly females and males gain energy for their daily activities from sugar-feeding on plants or from feeding on honeydew-derived sugars from aphids or coccids. In addition to the sugar meal, female flies also need proteins and nutrients present in blood from vertebrate hosts to ensure egg development and maturation [reviewed in (Dvorak et al., 2018; Killick-Kendrick, 1999)].

To date, more than 800 sand fly species are recognized, with members of the *Phlebotomus* (Rondani) and *Sergentomyia* (França and Parrot) genera being present in the Old World and members of *Lutzomyia* (França), *Brumptomyia* (França and Parrot) and *Warileya* (Hertig) in the New World [reviewed in (Akhoundi et al., 2016)]. Hereinafter, only sand fly species of the genera *Phlebotomus* and *Sergentomyia* will be further discussed as only their salivary proteins are the topic of this thesis. More specifically, I

focused on the ecology and mainly on the feeding preferences of two *Phlebotomus* species belonging to the *Larroussius* (Nitzulescu) subgenus and one *Sergentomyia* (França and Parrot) species.

Overall, members of the *Sergentomyia* and *Phlebotomus* genera share a similar distribution in the Old World, being regions that comprise the Mediterranean and the Afrotropical area, the Middle East, and Oriental regions up to Asia. *Sergentomyia* spp. can, however, also be found in the Australasian region, and are more abundant in certain tropical areas [reviewed in (Akhoundi et al., 2016)].

1.1. Sand fly role in pathogens transmission

Phlebotomine sand flies are proven vectors of various pathogens like protists (*Leishmania* sp.), bacteria (*Bartonella baciliformis*) and various viruses. Their most important role as vector in human and veterinary medicine is *Leishmania* sp. transmission.

Phlebotomus perniciosus is a proven vector of *Le. infantum* in most of the countries in the Mediterranean area. *Leishmania infantum* causes visceral leishmaniasis (VL), cutaneous leishmaniasis and canine leishmaniasis [reviewed in (Maroli et al., 2013)]. *Phlebotomus orientalis* is a proven vector of *Le. donovani,* which causes VL, in Kenya, Ethiopia and Sudan [reviewed in (Elnaiem, 2011)]. *Sergentomyia* sand flies are vectors of reptile parasites from the genus *Trypanosoma* and *Leishmania* (Ashford, 1974; Lane, 1993; Maia and Depaquit, 2016). Human *Leishmania* sp. or its DNA [reviewed in (Maia and Depaquit, 2016)] were repeatedly isolated from these sand flies. However, human *Leishmania* sp. are not able to develop a transmittable infection in *S. schwetzi* (Lawyer et al., 1990; Sadlova et al., 2013).

1.2. Geographical distribution

Phlebotomus perniciosus (Newstead) is distributed mainly throughout Mediterranean Europe (Portugal, Spain, Andorra, France, Malta, Italy, Slovenia, Croatia, Montenegro) and North Africa (Morocco, Algeria, Tunisia). However, this sand fly species is also spreading to northern parts of Europe (the north of France, Switzerland and Germany) and to North Macedonia on the Balkan peninsula (ECDC July, 2019).

Phlebotomus orientalis (Parrot) is distributed throughout Central and East Africa, more specifically in Chad, Djibouti, Niger, Rwanda, Uganda, Sudan, South Sudan, Ethiopia, Kenya, Egypt, Saudi Arabia, Yemen, and it is also present on the Arabian Peninsula [reviewed in (Gebre-Michael et al., 2004; Lewis, 1982; Rueda et al., 2017)].

Sergentomyia schwetzi (Adler, Theodor and Parrot) belongs to the *Sergentomyia* (França and Parrot) subgenus and can, according to the morphological characteristics of the male genitalia, be divided into the "typical" form (Adler, Theodor and Parrot 1929) and the "atypical" form [e.g. (Abonnenc E., 1959; Lewis et al., 1969; Minter, 1963)]. It is interesting to know that these morphological forms can also differ by geographical distribution (Lewis et al., 1969). In general, *S. schwetzi* sand flies are mostly present from East Africa. to Central and West Africa (Congo, Egypt, Ethiopia, Kenya, Mali, Sudan, Senegal, Uganda) (Lewis et al., 1969; Seccombe et al., 1993) and are found to be the predominant sand fly species in the Atbara-River area in East Sudan (Lambert et al., 2002), in the Segen Valley in southern Ethiopia (Gebre-Michael and Lane, 1996), in the West Pokot area in Kenya (Mutinga et al., 1984), and in the Mont-Rolland district in Senegal (Senghor et al., 2011).

1.3. Ecology and feeding behaviour of *P. perniciosus*

Phlebotomus perniciosus favourable climate in European part of Mediterranean area corresponds to lower altitudes (Ballart et al., 2014; Hartemink et al., 2011) where the annual temperature is higher, relative humidity lower and less rainfall (Barón et al., 2011; Branco et al., 2013; Durán-Martínez et al., 2013; Risueño et al., 2017). This climate characterisation in Spain correlates with thermo- and meso-Mediterranean bioclimatic zones, where *P. perniciosus* was found in high numbers (Aransay et al., 2004; Ballart et al., 2014; Durán-Martínez et al., 2013; Gálvez et al., 2020). The higher altitudes in Spain belong to supra- and oro-Mediterranean zones where *P. perniciosus* occurs in lower densities (Aransay et al., 2004; Ballart et al., 2014; Durán-Martínez et al., 2013; Gálvez et al., 2020, 2010). However, sand flies of this species were found up to 1,548 m a. s. l. (Gálvez et al., 2010). On the other side of the Mediterranean Sea, in North Africa, *P. perniciosus'* presence was connected sub-humid and semi-arid bioclimatic zones in the northern part of Tunisia (Ghrab et al., 2006), in north Algeria (Bennai et al., 2018; Gherbi et al., 2020; Kabbout et al., 2016; Lafri et al., 2016; Ramdane and Berchi, 2018) and in central and northern Morocco (Boussaa et al., 2009; Kahime et al., 2015; Mhaidi et al., 2018; Zarrouk et al., 2016). Contrarily, this sand fly was less abundant in the areas with dry and hot climate, which corresponds to arid and Saharan zones in central and southern Tunisia (Ghrab et al., 2006), eastern Algeria (Zeroual et al., 2016) and southern Morocco (Zarrouk et al., 2016). However, due to higher irrigation of arid urbanized areas (Barhoumi et al., 2015), *P. perniciosus'* presence in this areas is on the rise (Zhioua et al., 2007).

The seasonal distribution of *P. perniciosus* is highly dependent on two aspects: the climatic conditions and the geographical latitude. The first *P. perniciosus* sand flies usually emerge around early April in the southern part of the Mediterranean area (Benabid et al., 2017; Lisi et al., 2014; Morillas Márquez et al., 1991). The abundance of sand flies during the season varies between locality and sampling year, and is largely influenced by weather conditions during the season (Cotteaux-Lautard et al., 2016; Rossi et al., 2008). The bi-modal and confluent bi-modal seasonality trend is caused by at least two emerging generations of sand flies, with the first generation – also called the "spring generation" – consisting of flies that emerged from larvae diapausing over the winter while the second generation is generated by adults that emerged from summer larvae. This *P. perniciosus* bi-modal phenology was shown in Algarve (Portugal), and a confluent bi-modal density in Madrid area (Spain), Rome province (Italy), and Lisbon (Portugal) (Alten et al., 2016), and in southern Tunisia (Benabid et al., 2017), northern Morocco (Talbi et al., 2015). If the weather is not warm enough or its more rainy during the season, only one abundance peak occurred, like in Italy (Rome) in 2002 (Rossi et al., 2008) and southern France (Marseille) in 2009 (Cotteaux-Lautard et al., 2016). Interestingly, the end of the sand fly season differs in various locations, does not correlate with latitude, or average annual or seasonal temperature, and is usually registered around September/November, or occasionally in December (Alten et al., 2016).

The habitat of *P. perniciosus* is quite variable. *Phlebotomus perniciosus* is present in both urbanized and sylvatic biotopes, but in these is less frequent (Branco et al., 2013; Carta et al., 2020; Kahime et al., 2015; Maroli et al., 1994). Interestingly, most of the studies reported high numbers of this sand fly in peri-urban areas, like solitary farm buildings and edges of villages, in South France (Cotteaux-Lautard et al., 2016; Peyrefitte et al., 2013), in central Portugal (Branco et al., 2013), on the island of Mallorca (Alcover et al., 2014), and northeast Spain (Ballart et al., 2014). *Phlebotomus perniciosus* was found in both peri-domestic and domestic biotopes in central and northern part of Morocco (Boussaa et al., 2009; Kahime et al., 2015; Mhaidi et al., 2018; Zarrouk et al., 2016). Contrarily, high densities of this sand fly were reported from urbanised biotopes, like Catania city (Sicily) (Lisi et al., 2014). Within these biotopes, the most favourable environments for *P. perniciosus* were shelters for domestic animals, stone walls, and drainage holes (Alcover et al., 2014; Branco et al., 2013; Gálvez et al., 2010; Kahime et al., 2015; Mhaidi et al., 2018; Prudhomme et al., 2015). Some of the studies, found correlation between occurrence of high numbers of *P. perniciosus* with specific flora – *Pinus*

halapensis and bushes creating garrigue shrubs (Alcover et al., 2014; Ballart et al., 2014; Branco et al., 2013) or *Quercus ilex* (Gálvez et al., 2010).

In conclusion, the density of *P. perniciosus* in a certain area is positively influenced by a warmer climate, lower relative humidity and lower altitudes (less than 800 m a.s.l.). In the continental Mediterranean area this corresponds to thermo-, meso- and supra-Mediterranean bioclimatic zones, while in North African countries, it is found to be highly abundant in sub-humid and semi-arid zones, which usually corresponds to higher altitudes (more than 900 m a.s.l.). However, due to increased irrigation its distribution is expanding to arid areas. The occurrence of *P. perniciosus* during the season has mostly bi-modal or confluent bi-modal character, but is highly affected by weather changes. Higher numbers of this sand fly species are usually found in peri-domestic environments and in village surroundings, although in some areas it was adapted to urban environments. Correlating the abundance of *P. perniciosus* with a specific flora needs to be further investigated, but the presence of pines, oaks and garrigue shrubs seem to be favourable for sand flies.

Sand flies from the genus *Phlebotomus* prefer to take blood from warm blooded animals (mammals and birds). More particularly, in the case of *P. perniciosus,* it is known that females prefer to take blood on medium sized mammals, although they have been exhibiting a more opportunistic behaviour in their host choice in the past years.

Even though, *P. perniciosus* is a vector of human VL, its feeding on human was not reported frequently. Some *P. perniciosus* females with engorged human blood were found in central Portugal (Branco et al., 2013), Spain and Menorca island (De Colmenares et al., 1995), south France (Cotteaux-Lautard et al., 2016), central Italy (Bongiorno et al., 2003), Sicily island (Abbate et al., 2020) and in northern Algeria (Bennai et al., 2018). Nevertheless the only report showing higher feeding preference on human host than other animals was from Tunisia (Remadi et al., 2020).

In most of the surveys, *P. perniciosus* females had been blood-feeding on various domestic animals including small ruminants, equines, pigs, and cows. This feeding preferences was observed in central Italy (Bongiorno et al., 2003) and Sicily island (Abbate et al., 2020), Spain and Menorca island (De Colmenares et al., 1995), south Portugal (Maia et al., 2015), northern Algeria (Bennai et al., 2018) and Tunisia (Remadi et al., 2020). But in some areas, like Sicily (Abbate et al., 2020) and the south of France (Cotteaux-Lautard et al., 2016) rabbits, wild rabbits and hares were found as the main sources of blood. These lagomorphs were depicted as *Le. infantum* reservoir hosts in

Madrid area (Molina et al., 2012). Therefore, they are one of the most important hosts for blood feeding also in south and central Portugal (including Madrid), which was confirmed both by analysis of engorged blood (Branco et al., 2013; González et al., 2017; Jiménez et al., 2013, 2014; Maia et al., 2015) and serology (Martín-Martín et al., 2014). Cats, various rodents and bats were also shown as possible but less frequent *P. perniciosus* hosts in Portugal (Branco et al., 2013; González et al., 2017; Jiménez et al., 2014, 2013; Maia et al., 2013; Pereira et al., 2019). On the other hand, chickens and birds were bitten very sporadically in all studied areas (Bongiorno et al., 2003; Branco et al., 2013; Cotteaux-Lautard et al., 2016; De Colmenares et al., 1995; Maia et al., 2015).

Similar to lagomorphs, dogs are the proven *Le. infantum* reservoir hosts in the Mediterranean basin. However, in some of the studies canine blood was detected in fed females (Abbate et al., 2020; Bongiorno et al., 2003; De Colmenares et al., 1995). Serological surveys that screen for the presence of *P. perniciosus* salivary-antibodies showed high titres in dogs from Madrid (Martín-Martín et al., 2014), north-eastern and southern Spain and Menorca (De Colmenares et al., 1995), north-eastern Spain, Mallorca (Velez et al., 2018; Willen et al., 2019) and southern and central Italy and Portugal (Kostalova et al., 2015; Vlkova et al., 2011).

According to these studies, it is clear that *P. perniciosus* is able to adapt to various warm-blooded hosts, with the main reason of its opportunistic behaviour being the availability of hosts in the sand fly area. Important to note, is that such studies are unquestionably influenced by the trapping spots (e.g. sheepshed, cowshed, stable, field …) (Bennai et al., 2018; Maia et al., 2013).

1.4. Ecology and feeding behaviour of *P. orientalis*

All areas where *P. orientalis* occurs are mainly associated with vegetation composed by *Acacia seyal* and *Balanites aegyptiaca* trees (Elnaiem et al., 1997; Quate, 1964), as is shown in studies from Sudan (Elnaiem et al., 1997, 1999a; Lambert et al., 2002; Mohammed et al., 2018; Widaa et al., 2012), South Sudan (Hoogstraal et al., 1962; Quate, 1964) and Ethiopia (Gebre-Michael et al., 2010; Lemma et al., 2014). In the study by Elnaiem et al., (1999b), was proposed that the abundance of *P. orientalis* in proximity to *A. seyal* relies on three factors: (i) on the density of the trees in the area, which affect the local microhabitat (humidity and temperature), (ii) its association with the preferred animal hosts and mound-building termites and (iii) the source of the sugar meal (Elnaiem et al., 1999a). Even though the connection of *P. orientalis* with woodlands seems to be tight, it

was shown that sand flies are also able to adapt to an agricultural area where there is only a minimum of trees (Gebre-Michael et al., 2010; Moncaz et al., 2013); implying that other factors are influencing the population density of *P. orientalis* in a specific area. One of these factors can be the type of soil. In all three countries mentioned above, *P. orientalis* abundance in an area was associated with specific black cotton soil (eutric vertisols) (Elnaiem et al., 1998; Gebre-Michael et al., 2004; Thomson et al., 1999). This soil type absorbs a lot of water during the wet season and then dries completely and deeply cracks during the dry season (Moncaz et al., 2014). Both temperature and humidity are more stable in these cracks, which causes them to be the ideal microhabitat for sand flies with copious breeding sites and special soil nutrients for the larvae (Elnaiem et al., 1998). However, in southern and north-eastern Ethiopia (Omo valley and Awash valley, respectively) different types of soil (e.g. vertic cambisols, eutric fluvicols) that also crack during the dry season dominate the environment (Gebre-Michael et al., 2004), and are also associated with *P. orientalis* occurrence.

An occurrence prediction modelling (based on Geographical Information System) performed by Gebre-Michael et al., (2004) identified that *P. orientalis* has a preference for the dry season with temperatures between 16-36 °C, a lower annual rainfall (180-1050 mm) and a lower soil moisture (of 67-108 mm). The high abundance of *P. orientalis* during the second half of the dry season and its subsequent decrease during the wet season was previously shown by several authors (Aklilu et al., 2017; Doha and Samy, 2010; Hoogstraal et al., 1962; Lambert et al., 2002; Mohammed et al., 2018; Quate, 1964; Yared et al., 2017). However, in certain areas the number of sand flies did not change between the seasons, e.g. Umsalala (Sudan) (Elnaiem et al., 1997). Even more so, close to Umsalala, in the Dinder NP, a higher abundance was reported during the end of the dry season and the beginning of the rainy season (Elnaiem et al., 1997). A similar phenology was observed in the Ethiopian highlands (Belessa), where *P. orientalis* was more abundant during the rainy season (Ashford et al., 1973).

The preference of *P. orientalis* for woodlands, forests of *A. seyal* and cracked black cotton soil, has also been shown to be connected with its feeding behaviour. Elnaeim et al., (1997) noticed man-biting behaviour only in foci of the Galegu camp, a place close to an acacia forest. Furthermore, even though Yared et al., (2019) shared no information about vegetation or soil in their study, they reported higher densities of *P. orientalis* in the periphery of villages and in the surrounding fields compared to inside the village around houses. A similar observation was made in Kafta Humera (Yared et al., 2017), in the

Tahtay Adiyabo area (Gebresilassie et al., 2015a) and in Libo-Kemeken (Aklilu et al., 2017), all located in north-west Ethiopia. Taken together, this suggests an exophilic and exophagic behaviour of *P. orientalis* – though one study showed more than 75 % of *P. orientalis* to be captured inside houses (Lambert et al., 2002).

It is believed that *P. orientalis* preferably takes blood form larger animals, like cows, horses and wild bigger ruminants which has been confirmed by studies from north-western Ethiopia, where *P. orientalis* females showed a preference for bovine blood, even more than human blood (Aklilu et al., 2018; Gebre-Michael et al., 2010; Lemma et al., 2014). In villages situated in northern Ethiopia, cattle was shown to serve as the dominant source of blood for sand flies, followed by humans (Kirstein et al., 2018), donkey, goat, sheep, dog and camel blood (Gebresilassie et al., 2015b). Also in central Ethiopia, bigger domestic ruminants (cows and camels) were the most preferred blood source for *P. orientalis* [Mammo 1999, as cited in (Gebre-Michael et al., 2010)]. However, a new study from the Kafta-Humera region (north-western Ethiopia) described *P. orientalis'* feeding behaviour to be more opportunistic and showed that the highest number of engorged females had fed on donkey followed by cows, and only a few had blood fed on humans, dogs and goats or sheep (Yared et al., 2019). A similar trend was observed in a serological study from three localities in north-western Ethiopia, revealing high titers of *P. orientalis* anti-saliva antibodies in the blood of various domestic animals (i.e. dogs, goats, sheep and donkeys) (Rohousova et al., 2015). Even more so, when the attractiveness of various mammals for sand flies was compared in the same region in Ethiopia, cows and donkeys were shown to be the most attractive for *P. orientalis*, followed by humans and other animals (i.e. sheep and goat). Dog and chicken baits only attracted very few sand flies (Gebresilassie et al., 2015c). Lastly, also the attractiveness of wild mammals was investigated for which baits of Ground squirrels (*Xerus rutilus*), gerbils (*Tatera robusta*), Cairo spiny mice (*Acomys cahirinus*) and hares (*Lepus* sp.) were used. The ground squirrel was shown to be the most attractive for *P. orientalis*, followed by hares, gerbils and spiny mice. It should be noted that all of these wild rodents and lagomorphs were much less attractive than bigger domestic animals (Gebresilassie et al., 2015c).

Similar experiments performed in South Sudan (Upper Nile area) Quate (1964) showed that *P. orientalis* females are able to feed on rats, the African grass rat, and only a few specimens bite skinks and lizards. Importantly, these feeding experiments were done unnaturally – sand flies were kept with the hosts in enclosed cages – the results should therefore be taken with caution. A later study on the attractiveness of various rodent baits

for *P. orientalis* in the same area did not confirm these previous findings as it showed only a low-level attraction of *P. orientalis* to baits with Natal multimammate mice and Kemp's gerbils (Turner and Hoogstraal, 1965). However, the most important finding in this locality is that human blood was found to be the most attractive and most common source of blood for *P. orientalis* (Hoogstraal et al., 1962; Quate, 1964).

Host attractiveness studies performed in eastern Sudan (Dinder National Park), compared the domestic dog, the Egyptian mongoose, the genet and the Nile rat. As expected, dogs were shown to attract sand flies the most, followed by the mongoose. The genet and rat attract less sand flies (Hassan et al., 2009). Only two studies in Sudan focused on the feeding preferences of *P. orientalis* or on host blood screening. Again, humans were shown to be frequently bitten by *P. orientalis* (Elnaiem et al., 1999b), results that were confirmed by a recent serological study that identified anti-*P. orientalis* saliva and anti-*P. orientalis* salivary proteins IgG antibodies in human sera from both Sudan and Ethiopia (Sumova et al., 2018).

1.5. Ecology and feeding behaviour of *S. schwetzi*

The all ecological aspects of *S. schwetzi* are difficult to assess as information is scarce. However, studies that focus on other sand fly species also partially report on the preferred habitat of this sand fly species. *Sergentomyia schwetzi* occurs in some areas in high numbers and sometimes co-occurs with other sand flies from the *Phlebotomus* genus. Quate (1964) described this sand fly species to be a forest sand fly present in woodlands in South Sudan, he showed that it also occurs in grasslands and villages. In Kenya (Kitui district), *S. schwetzi* was highly abundant in village huts, termitaries and tree holes (Heisch et al., 1956; Mutinga et al., 1986a). *Sergentomyia schwetzi* was also found in villages in north-east Congo (Trouillet et al., 1988). In Mali, *S. schwetzi* was shown to be the most abundant sand fly species in the Segou region (Anderson et al., 2011; Coulibaly et al., 2018). A study from two separate localities in south-central and southern Mali showed that *S. schwetzi* is the dominant species in both but occurs in lower numbers compared to the Segou region (Coulibaly et al., 2016). Similarly, a studies in Bamako city and its suburban area also showed lower numbers of *S. schwetzi* (Demba-Kodindo et al., 2015; Kone et al., 2016). Even though it appears that in Mali *S. schwetzi* is ubiquitously represented in all studied areas in high or medium amounts, the study from Mopti region in central Mali reported almost no sand flies of this species in the five screened villages (Berdjane-Brouk et al., 2012).

In the Mont Roland area in the Thies region in Senegal, *S. schwetzi* was caught in high numbers mainly in the periphery of villages that were situated in areas with sandy and sandy-clay soils. Contrarily, significantly lower numbers of this species were found in areas with lateric gravel soils (Senghor et al., 2016, 2011). Furthermore, most of *S. schwetzi* sand flies were captured outdoors in farming and peri-domestic areas; also gravid and fed females were most frequently captured outside – confirming an exophilic behaviour of *S. schwetzi* in this environment and locality (Senghor et al., 2016), which is similar to the Segou region in Mali (Anderson et al., 2011). On the contrary, an endophilic behaviour of *S. schwetzi* was shown by Lambert et al., (2002) in eastern Sudan (the Atbara-River area). The direct connection of the presence and abundance of *S. schwetzi* with some special biotopes or vegetation have not been shown yet. However, according to a study in southern Sudan (Dinder national park), *S. schwetzi* was present in high numbers in an *A. seyal* forest, and was shown to occur also in other biotopes like riverine vegetation (Elnaiem et al., 1999a). *Sergentomyia schwetzi* was also abundant in rodent burrows in the area of western Sudan (Mohammed et al., 2018) and western Senegal (Ba et al., 1998). All these data from different localities showed that *S. schwetzi* sand flies are not connected to a particular habitat, as already suggested by Quate (1964) and Elnaiem et al., (1997).

The abundance of *S. schwetzi* changes during the year according to the season. Usually, the abundance peak raises its top during the dry season (Ba et al., 1998; Lambert et al., 2002; Mohammed et al., 2018; Quate, 1964), although they also occur in lower numbers at the beginning of the rainy season (Quate, 1964). *Sergentomyia schwetzi* was found throughout the year in Kitui area in Kenya (Heisch et al., 1956).

Sand flies from the genus *Sergentomyia* are known for their preference to take blood from cold blooded animals, like reptiles (Ashford, 1974; Minter and Wijers, 1963; Seccombe et al., 1993). However, it has been shown repeatedly that members of this genus also take blood from mammals, including humans [reviewed in (Berdjane-Brouk et al., 2012; Maia and Depaquit, 2016)]. Even though most studies focus on sand fly species that are more important as vectors of human *Leishmania* sp., we can sometimes find information about *Sergentomyia* sand flies. According to Hiesch et al., (1956) in a study performed in Kenya, *S. schwetzi* was shown to feed most often on lizards, although man biting behaviour was also observed. This preference for cold blooded hosts was also shown by Mutinga et al., (1986a) in the Baringo District (Kenya) where sand flies were caught in termite hill ventilation shafts, and most of *S. schwetzi* females were engorged with lizard blood. Even more so, *S. schwetzi* showed a higher attraction to baits with lizards than to

those with mammal hosts (i.e. dogs, chickens, goats, rats) (Mutinga et al., 1986b). Another study in the same area tested the source of the blood meal from various sand flies, including *S. schwetzi,* by ELISA. Again, blood from lizard, but also humans, rabbits and chickens was found in *S. schwetzi*, suggesting it to behave as an opportunistic feeder (Ngumbi et al., 1992). Similarly, opportunistic feeding behaviour of *S. schwetzi* females were shown in the Sudan Upper Nile area (Quate, 1964). Moreover, host attraction experiments in the same area stipulated *S. schwetzi's* attraction to various African rodents, even though *S. schwetzi* females only fed on gerbils. Only some females were caught in human-baited nets even while feeding on human (Hoogstraal et al., 1962; Turner and Hoogstraal, 1965). The man-biting behaviour was also observed in the Mont-Rolland area in Senegal, where one third of tested fed females were fed on human blood and the rest on dogs, horses, cows, mice and some unspecified mammals (Senghor et al., 2016).

The possibility that *S. schwetzi* is also attracted to warm blooded animals was tested in Tahtay Adiyabo district (northern Ethiopia). Tent trap baits with different domestic animals or a human volunteer demonstrated that the most attractive animal for *S. schwetzi* are donkeys, followed by cows, goats, sheep, dogs, humans and chickens. However, Blood fed females were found only in baits with cows, followed by donkeys and goats (Gebresilassie et al., 2015c). In south-west Ethiopia (Omo river plains) *S. schwetzi* females were caught in human baits (Hailu et al., 1995). The possibility that mammals serve as host for *S. schwetzi* was shown in a field study in the Ethiopian district of Kafta-Humera, where Yared et al., (2019) collected sand flies from various environments in villages and in the surroundings and fields. The blood meal analysis from captured sand flies revealed that from all captured *S. schwetzi* 34 females had fed on cow, two on human, one on dog, two specimens had taken blood from more than one host (Yared et al., 2019). Finally, the attractiveness of sand flies to various animals was also tested in eastern Sudan. Here, *S. schwetzi* females were mostly attracted to animal-bait traps with domestic dogs, followed by bait traps with Egyptian mongoose (*Herpestes ichneumon*) and genet. Nile rats did not attract almost any females (Hassan et al., 2009). In conclusion, *S. schwetzi* feed mainly on cold-blooded animals, but it also bite mammals including humans.

2. Sand fly saliva

Sand fly saliva is produced by salivary glands, two "balloon-like" organs bounded by a single layer of epithelium and connected through the salivary duct to the mouth parts. Sand fly saliva is known to contain specific salivary proteins that differ between sex, age and

species of sand flies (Volf et al., 2000; Volf and Rohousova, 2001). Differences were found between various populations, between colonies of the same species that originated from distant localities, and even among long-term maintained colonies (Ramalho-Ortigão et al., 2015; Rohousova et al., 2012a; Volf et al., 2000). Saliva also facilitates the blood feeding process on vertebrate hosts as it contains pharmacologically active compounds that have immunomodulatory, anti-haemostatic and anti-inflammatory properties. Some of the salivary proteins are potent antigens that induce a humoral immune response in the host which is species-specific (Rohousova et al., 2005; Thiakaki et al., 2005; Volf and Rohousova, 2001). In repeatedly bitten hosts, IgG antibodies were shown to serve as reliable marker of exposure to sand fly bites that sensitively reflect contact of the host with this insect [reviewed in (Lestinova et al., 2017)].

While a sand fly specific antibody response shows us if the host has been exposed to sand fly bites, the host's cellular immune response is a crucial factor in the establishment of an actual *Leishmania* infection. In naïve hosts, who previously have not been repeatedly exposed to non-infected bites, sand fly saliva facilitates parasite survival by changing the environment from a pro-inflammatory to an anti-inflammatory one – leading to an inhibition of various protective functions of macrophages and a successful survival and spread of the parasite in the host. However, a completely different scenario is present in hosts who were regularly exposed to non-infectious sand fly bites. When these hosts are consequently exposed to infectious bites, the antigenic salivary proteins will be recognized by the hosts' immune response and a delayed-type hypersensitivity (DTH) reaction together with an increased IFN-γ/IL-12 production will protect the host [reviewed in (Lestinova et al., 2017)].

The skin composition, haemostasis, erythrocytes and some of the humoral and cellular immune responses differ among vertebrate hosts (Didisheim et al., 1959; Dodds and Matsushita, 2007; Doolittle, 2009, 2011; Lewis, 1996). These differences should also be extrapolated to quantitative or qualitative changes in the composition of sand fly saliva. However, studies focused on salivary adaptations to the preferred host are very limited for all blood feeding arthropods. The only discussed topic is the activity of the salivary enzyme apyrase, which causes hydrolysis of ATP and ADP. Adenosine diphosphate activates platelets of mammals causing platelet aggregation which together with other haemostatic processes stops the bleeding (Blanco and Blanco, 2017). However, platelets are replaced by thrombocytes in birds and reptiles, which are not activated by ADP (Schmaier et al., 2011; Soslau et al., 2005). The adaptation to possibly lower amounts of

ADP in avian and reptile blood could be a reason for the low anti-clotting and apyrase activities in *Culex quinquefasciatus* (Ribeiro, 2000) and *Dipetalogaster maxima* (Ribeiro et al., 1998), two arthropods that prefer to blood feed on birds and lizards, respectively. A similar adaptation of sand flies to reptiles was shown recently on the activity of *S. schwetzi* apyrase, which was lower compared to other sand flies' apyrases (Volfova and Volf, 2018).

3. Characterization of the main salivary protein families

All immunological processes mentioned in the previous chapter can be caused by a specific sand fly salivary protein. The characterisation of sand fly's salivary proteins can be done by various experimental approaches, one of them being transcriptome sequencing. The salivary gland transcriptomes, also called sialomes, of fourteen sand fly species belonging to two genera (*Phlebotomus*, *Lutzomyia*) has been published, up to date [reviewed according to (Lestinova et al., 2017; Oliveira et al., 2020)]. More than 250 salivary proteins were identified in these transcriptomes, all belonging to more than 30 salivary protein families (Coutinho-Abreu and Valenzuela, 2018). For certain sand fly species more sialomes were made, due to the different country of origin of the sand fly colony, e.g. *P. papatasi* from Israel (Valenzuela et al., 2001a) and Tunisia (Abdeladhim et al., 2012), *P. duboscqi* from Mali and Kenya (Kato et al., 2006), *P. perniciosus* from Italy (Anderson et al., 2006) and Spain (Martín-Martín et al., 2013), *P. orientalis* from two Ethiopian areas – Addis Zemen and Melka Werer (Vlkova et al., 2014). Various salivary proteins were identified and were, according to their characteristic (sequence, molecular weight, function), divided into main salivary protein families (Coutinho-Abreu and Valenzuela, 2018). The characteristics of these families are the content of next chapter, with a special focus on proteins from *P. perniciosus* and *P. orientalis*. Since the nomenclature of salivary proteins is rather confusing, the main salivary proteins from *P. perniciosus* and *P. orientalis* are listed in Tables 1 and 2. The *P. orientalis* Addis Zemen proteins' names (PorASPxx) will be mainly used in the text of this thesis.

Table 1: proteins identified in *P. perniciosus* sialome (Anderson et al., 2006; Martín-Martín et al., 2013) and transcriptome (Petrella et al., 2015)

P. perniciosus protein family	Sialome from colony originated in Italy [A]					Sialome from colony originated in Spain [B]			
	protein name	GenBank accession number		pred. MW	pI	protein name	GenBank accession number		pred. MW
		nucleotide	protein				nucleotide	protein	
Yellow-related	PpeSP03	DQ150621	ABA43049	41.8 kDa	6.0				
	PpeSP03B	DQ150622	ABA43050	42.7 kDa	8.6	PpeSP03B	HE974346	CCK33661	44.6 kDa
Antigen5-related	PpeSP07	DQ153101	ABA43055	29.6 kDa	9.1				
Lufaxin	PpeSP06	DQ153100	ABA43054	33.0 kDa	8.9	PpeSP06	HE970770	CCK18305	36.2 kDa
D7-related	PpeSP04	DQ150623	ABA43051	24.5 kDa	8.5	PpeSP04	HE980444	CCK73754	28.9 kDa
	PpeSP04B	DQ150624	ABA43052	26.9 kDa	8.7	PpeSP04B	HE980443	CCK73753	20.7 kDa
SP15	PpeSP02	DQ150620	ABA43048	14.8 kDa	8.7	PpeSP02	HE985074	CCM43814	17.1 kDa
						PpeSP02	HE985075	CCM43815	15.7 kDa
						PpeSP02	HE985076	CCM43816	17.1 kDa
						PpeSP02	HE985077	CCM43817	17.0 kDa
	PpeSP09	DQ153103	ABA43057	14.6 kDa	8.6	PpeSP09	HE966456	CCJ67632	16.7 kDa
	PpeSP11	DQ153105	ABA43059	13.2 kDa	9.0	PpeSP11	HE974348	CCK33663	15.3 kDa
ParSP25-like	PpeSP08	DQ153102	ABA43056	28.8 kDa	4.9	PpeSP08	HE974347	CCK33662	29.5 kDa
PpSP32-like	PpeSP05	DQ153099	ABA43053	27.8 kDa	10.4				
Apyrase	PpeSP01	DQ192490	ABB00906	35.5 kDa	9.3	PpeSP01	HE974344	CCK33659	37.7 kDa
	PpeSP01B	DQ192491	ABB00907	35.3 kDa		PpeSP01B	HE974345	CCK33660	37.3 kDa
Hyaluronidase						hyaluronidase [C]	KT160228	ALL27024	
Endonuclease	PpeSP32	DQ154099	ABA43064	41.4 kDa	4.4				
Phospholipase A2	PpeSP18	DQ154097	ABA43062	29.9 kDa	8.3				
Pyrophospha-tase						pyrophospha-tase [C]	KT160227	ALL27023	
Adenosine deaminase						adenosine deaminase [C]	KT160229	ALL27025	
7 kDa salivary protein SP12	PpeSP12	DQ153106	ABA43060	7.1 kDa	11.0				
10 kDa salivary protein SP13	PpeSP13	DQ153107	ABA43061	9.7 kDa	4.8				
2.7 kDa salivary protein	PpeSP15	DQ192489	ABB00905	2.7 kDa	10.6				
p119						p119	HE985078	CCM43818	17 kDa

[A] published in Anderson et al., (2006), [B] published in Martín-Martín et al., (2013),

[C] published in Petrella et al., (2015); empty – not detected or un-published

Table 2: proteins identified in *P. orientalis* sialome (Vlkova et al., 2014)

P. orientalis protein family	Sialome from colony originated in Addis Zemen					Sialome from colony originated in Melka Werer				
	protein name	GenBank accession number		pred. MW	pI	protein name	GenBank accession number		pred. MW	pI
		nucleotide	protein				nucleotide	protein		
Yellow-related	PorASP2	KC170933	AGT96427	41.5 kDa	6.1	PorMSP23	KC170966	AGT96460	41.6 kDa	6.1
	PorASP4	KC170934	AGT96428	42.3 kDa	8.1	PorMSP24	KC170967	AGT96461	42.3 kDa	8.1
Antigen5-related	PorASP74	KC170947	AGT96441	28.8 kDa	8.9	PorMSP6	KC170962	AGT96456	28.8 kDa	8.9
	PorASP76	KC170948	AGT96442	28.8 kDa	8.9	PorMSP8	KC170963	AGT96457	28.8 kDa	8.9
Lufaxin						PorMSP78	KC170976	AGT96470	18.8 kDa	8.4
D7-related						PorMSP28	KC170969	AGT96463	27.3 kDa	7.5
	PorASP48	KC170943	AGT96437	26.9 kDa	8.3	PorMSP38	KC170970	AGT96464	26.9 kDa	8.3
	PorASP46	KC170942	AGT96436	26.7 kDa	6.4	PorMSP43	KC170971	AGT96465	26.7 kDa	6.7
	PorASP122	KC170954	AGT96448	26.8 kDa	9.2	PorMSP67	KC170973	AGT96467	26.8 kDa	9.2
SP15	PorASP28	KC170938	AGT96432	14.5 kDa	8.9	PorMSP96	KC170978	AGT96472	14.5 kDa	8.9
	PorASP31	KC170939	AGT96433	14.3 kDa	8.7	PorMSP90	KC170977	AGT96471	14.3 kDa	8.7
	PorASP37	KC170940	AGT96434	14.9 kDa	8.8	PorMSP12	KC170964	AGT96458	14.9 kDa	8.8
	PorASP61	KC170944	AGT96438	13.9 kDa	9.1	PorMSP74	KC170974	AGT96468	13.9 kDa	9.2
	PorASP64	KC170945	AGT96439	14.7 kDa	8.0	PorMSP75	KC170975	AGT96469	14.7 kDa	8.0
ParSP25-like	PorASP106	KC170953	AGT96447	27.6 kDa	4.7	PorMSP65	KC170972	AGT96466	27.6 kDa	4.8
PpSP32-like	PorASP86	KC170950	AGT96444	25.0 kDa	10.1	PorMSP15	KC170965	AGT96459	25.0 kDa	10.2
	PorASP87	KC170951	AGT96445	25.0 kDa	10.5					
Apyrase	PorASP14	KC170936	AGT96430	35.1 kDa	9.0	PorMSP4	KC170961	AGT96455	33.2 kDa	8.9
	PorASP15	KC170937	AGT96431	35.3 kDa	9.2					
	PorASP11	KC170935	AGT96429	35.5 kDa	10.0	PorMSP3	KC170960	AGT96454	35.6 kDa	8.8
Hyaluronidase	PorASP112	KC170958	AGT96452	37.2 kDa	6.5	PorMSP108	KC170981	AGT96475	35.6 kDa	7.0
Endonuclease	PorASP139	KC170955	AGT96449	41.7 kDa	9.3	PorMSP101	KC170979	AGT96473	41.7 kDa	9.4
Phospholipase A2	PorASP80	KC170949	AGT96443	29.7 kDa	8.4	PorMSP129	KC170982	AGT96476	29.7 kDa	8.3
Pyrophospha-tase	PorASP262	KC170959	AGT96453	32.9 kDa	7.2					
SP16-like	PorASP150	KC170956	AGT96450	16.0 kDa	5.0	PorMSP162	KC170983	AGT96477	16.0 kDa	5.0
	PorASP98	KC170952	AGT96446	5.6 kDa	10.5	PorMSP104	KC170980	AGT96474	5.6 kDa	10.0
	PorASP68	KC170946	AGT96440	4.9 kDa	9.8	PorMSP196	KC170984	AGT96479	4.9 kDa	10.2
	PorASP40	KC170941	AGT96435	3.9 kDa	9.2	PorMSP169	KC170985	AGT96478	3.9 kDa	9.2

3.1. Yellow-related protein family

Sand fly yellow-related proteins (YRPs) contain a characteristic major royal jelly protein (MRJP) domain and belong to a large family of insects' MRJP/Yellow. They are named after the protein yellow from *Drosophila melanogaster* and the MRJP from honey bee jelly produced by nurse bees (Buttstedt et al., 2014). The YRPs are highly abundant in sand fly saliva and are present in all sand fly species studied, usually even represented by more than one homolog (Coutinho-Abreu and Valenzuela, 2018; Oliveira et al., 2020). The YRPs can be divided into two subfamilies. First there is the "42 kDa" subfamily which is characterised by a lower molecular mass (41.5 – 43.5 kDa) and a more acidic isoelectric point (pI), while the second "44 kDa" subfamily has a higher molecular mass (42.3 – 45.2 kDa) and a basic pI. Proteins originating from different species but that are part of the same subfamily cluster together, while protein paralogues likely originated by gene conversion (Abdeladhim et al., 2016). When considering the sialomes of *P. perniciosus* and *P. orientalis,* both contain two homologues of YRPs. The sequence similarity, MW and pI of these *P. perniciosus* and *P. orientalis* proteins divide them into two subfamilies: the 42 kDa subfamily comprising PpeSP03 and PorASP2 and the 44 kDa subfamily with PpeSP03B and PorASP4. (Anderson et al., 2006; Sima et al., 2016a; Vlkova et al., 2014).

The structure of YRPs with their specific six-bladed β-propeller that has a binding cavity inside a "tunnel shape", was first described on a crystal of a *Lutzomyia longipalpis* YRP (LJM11). The amino acid residues within the cavity are responsible for binding pro-haemostatic and pro-inflammatory biogenic amines (serotonin, histamine and catecholamines), which leads to vasodilatation, platelet activation and vascular permeability in the host. This amine-binding function was first described on recombinant YRPs of *L. longipalpis* (LJM11, LJM111, LJM17), with each of them having a specific affinity for different biogenic amines. The variation in the binding properties of these proteins most likely complement each other (Xu et al., 2011). This ability to bind biogenic amines was proposed for all sand fly YRPs based on a sequence alignment of this amine-binding cavity. The cavity was shown to have eleven conserved amino acids, responsible for binding of the amines. Furthermore, *in silico* 3D structural modelling also confirmed these findings (Sima et al., 2016a; Xu et al., 2011). Besides these conserved amino acids, the sequences of YRPs contain a four cysteine residue pattern, which are connected with structure stabilisation and right folding, by building disulfide bounds (Xu et al., 2011). Conserved cysteines are more frequent in soluble extracellular proteins and is presented in other salivary proteins (Coutinho-Abreu and Valenzuela, 2018).

It is well-established that sand fly YRPs generate high numbers of antibodies in repeatedly bitten hosts. Their strong antigenicity has been shown for various hosts such as mice (Martín-Martín et al., 2012; Rohousova et al., 2005), hamsters (Martín-Martín et al., 2012), rabbits (Rohousova et al., 2012b), dogs (Hostomska et al., 2008; Teixeira et al., 2010; Vlkova et al., 2011) and humans (Marzouki et al., 2011; Rohousova et al., 2005; Vinhas et al., 2007). Based on these studies, the antibody response against recombinant YRPs from various sand fly species has been tested as marker of exposure.

All three recombinant YRPs from *L. longipalpis* showed a different antigenicity for different hosts. The recombinant protein LJM17 was recognized by a broad spectrum of hosts, namely: chicken (Soares et al., 2013), dogs, foxes and humans (Teixeira et al., 2010). On the other hand, the protein LJM11 was only antigenic in dogs and humans, whereas the third YRP (LJM111) showed a very low antigenicity in humans (Teixeira et al., 2010). The antibody response against both LJM17 and LJM11 and their mixture were proposed as potential exposure markers due to their high antigenicity, their broad host specificity and their no cross-reactivity with anti-*L. intermedia* antibodies, a sympatrically occurring sand fly species (Souza et al., 2010; Teixeira et al., 2010). Despite these promising results, the antibody response against the recombinant YRP (LinB-21) from *L. intermedia* was unable to distinguish between positive (bitten) and negative (non-bitten) humans, rendering it not suitable as a marker for human exposure to *L. intermedia* (Carvalho et al., 2017). Although YRPs from *P. papatasi* are not suitable to test for human exposure (Marzouki et al., 2011), plasmids coding for *P. papatasi* YRPs (PpTSP42 and PpTSP44) were shown to stimulate the Th1 cellular immune response and the production of IFN-γ (Tlili et al., 2018). This specific Th1 immune response and the higher levels of IFN-γ produced, were shown to protect mice against *Le. major* infections [reviewed in (Lestinova et al., 2017)]. YRPs from *L. longipalpis* exhibited a similar protective effect against a *Le. major* infection when mice were pre-immunised with LJM11 (Gomes et al., 2012). These pre-immunised mice generated a Th1 immune response which together with a DTH reaction led to protection against *Le. major* (Abi Abdallah et al., 2014). Furthermore, the immune reaction against the LJM17 protein was shown to confer protection against *Le. infantum* in pre-immunised dogs (Collin et al., 2009).

The antibody response against the SGH of *P. orientalis* has been shown to be useful to measure exposure of Ethiopian domestic animals to bites of *P. orientalis* (Rohousova et al., 2015). Furthermore, the high correlation between the SGH of *P. orientalis* and its recombinant YRP (PorMSP24 ~ PorASP4) validated the antibody response against this

protein as a promising exposure marker for dogs, sheep and goats in Ethiopia (Sima et al., 2016b). Antigenic B-cell epitopes of the PorMSP24 were then synthesised as short peptides, and showed to be a good substitute for the recombinant protein in estimating dog and goat exposure (Sima et al., 2019). Moreover, the same *P. orientalis* recombinant YRP expressed in a mammalian expression system – in contrast to a bacterial expression system used in previous studies – was also shown to be a useful exposure marker for naturally exposed humans living in Ethiopia and Sudan. The antibody response against this protein showed a high correlation with the *P. orientalis* SGH. When this YRP was combined with another salivary recombinant Ag5r protein (PorASP74) from *P. orientalis* an even higher correlation coefficient was achieved, resulting in the antibody response against this antigen mixture to be proposed as a valid human exposure marker for *P. orientalis* bites (Sumova et al., 2018).

The main reservoir host of VL in the Mediterranean area are dogs. Therefore, from an epidemiological perspective, it is important to measure their exposure to *P. perniciosus* bites facilitated by a reliable exposure marker. Antibody responses against two YRPs of *P. perniciosus* (PpeSP03 and PpeSP03B) were shown to be able to reliably indicate exposure when sera of pre-exposed dogs (Vlkova et al., 2011), laboratory mice (Martín-Martín et al., 2012), hamsters (Martín-Martín et al., 2012; Volf and Rohousova, 2001) and rabbits were used (Volf and Rohousova, 2001). Even more so, a high correlation was found between the canine antibody response against PpeSP03B and the SGH of *P. perniciosus* (Drahota et al., 2014). Subsequent studies successfully employed the PpeSP03B is in measuring exposure in naturally exposed dogs, hares and rabbits in Spain (Martín-Martín et al., 2014; Velez et al., 2018). Furthermore, during a study in VL foci in Italy, anti-PpeSP03B antibodies in dogs imported in the area prior to the study were able to reflect the seasonal sand fly dynamics in a similar trend as the anti-SGH antibodies do. More specifically, the anti-PpeSP03B antibody levels rose during the summer months when sand fly density is at its highest and decreased during the winter months when sand flies are not active (Kostalova et al., 2015).

All previously mentioned studies paved the way for a new state-of-the-art application of this YRP of *P. pernicious*: an immunochromatographic test (ICT) that allows for rapid identification of dogs exposed to *P. perniciosus*. The resulting ICT highly correlated with standard serology assays (ELISA) (Willen et al., 2018). Follow-up studies were able to improve the specificity of the test from 86.8 % to 94.9 %, allow the use of whole canine

blood (Willen et al., 2019), and confirm its validity in field conditions (Burnham et al., 2020).

3.2. Lufaxin protein family

The salivary protein lufaxin (***Lu****tzomyia longipalpis* **Fa**ctor **X**a **In**hibitor) was first described in the sialome of *L. longipalpis* (Collin et al., 2012; Valenzuela et al., 2004). It has been described only in sand fly saliva and has a predicted MW of 32.4 kDa in *L. longipalpis* and between 32.3 – 34.5 kDa in other sand fly species (Oliveira et al., 2013). The sequence of all lufaxin proteins contain a specific pattern of five cysteine residues while the rest of the sequence is more variable (between 35 and 40 %) (Coutinho-Abreu and Valenzuela, 2018).

Lufaxin acts as an anticoagulant through tightly binding and consequently inhibiting the host's coagulation factor Xa. As a result, the prothrombinase complex cannot be formed, leading to an unrealized conversion of prothrombin to thrombin. Collin et al., (2012) characterised lufaxin as a slow-tight and non-competitive inhibitor of FXa. Moreover, FXa also influences protease activated receptors (PARs), presented on various cell types, which are involved in inflammatory processes. Therefore, lufaxin has also anti-inflammatory function (Collin et al., 2012). Furthermore, lufaxin was also shown to inhibit the alternative pathway of the complement cascade by binding to the C3b-B complex, eventually causing an inhibition of the C3 convertase formation (Mendes-Sousa et al., 2017).

Importantly, lufaxin has a potential to produce a strong Th1 cellular immunity and a DTH response in dogs, an immune response known for its protective effect against an infection with both *Le. major* and *Le. infantum* in various animal models (Gomes et al., 2008; Oliveira et al., 2008; Xu et al., 2011). This finding led to lufaxin proteins being studied as *Leishmania* transmission blocking vaccine candidates (Collin et al., 2009), which was further supported by the fact that it has a conserved sequence, conserved B-cell epitopes and that it is present in both Old and New World sand flies (Coutinho-Abreu and Valenzuela, 2018). However, despite these findings Xu et al., (2011) was unable to show a protective effect of *L. longipalpis* lufaxin against a *Le. major* infection in mice. A combination of lufaxin with KMP11, LeishF3+ and an adjuvant in virus-like particles could be a promising substitute as it was shown to generate higher antigen-specific cellular and humoral immune responses in uninfected mice (Cecílio et al., 2017) – encouraging results that need to be further investigated, especially its combination with *Leishmania* sp..

In both the sialome of *P. perniciosus* and *P. orientalis* only one homolog of the lufaxin protein was found (Anderson et al., 2006; Vlkova et al., 2014). The predicted MW of the *P. perniciosus* lufaxin is 33 kDa (Anderson et al., 2006), the one for the *P. orientalis* lufaxin is 18.8 kDa (Vlkova et al., 2014). Phylogenetic analyses showed that *P. perniciosus* lufaxins are closely related to *P. tobbi* (both from *Larroussius* subgenus), while they are closely related to other lufaxin proteins from *Adlerius* and *Larroussius* subgenus (Coutinho-Abreu and Valenzuela, 2018). This shows that the evolution of these proteins is in accordance with the classical sand fly phylogeny inferred from the small subunit nuclear ribosomal DNA (Aransay et al., 2000). Since the *P. orientalis* lufaxin is almost half the length of homologues in other sand flies (Vlkova et al., 2014), it has not been included in any of the phylogenetic analysis published so far. However, since *P. orientalis* lufaxin is highly related to the lufaxin of *P. perniciosus* with a 88 % sequence similarity and other lufaxins from *Larroussius* sand flies cluster together (Coutinho-Abreu and Valenzuela, 2018), it can be assumed that also the lufaxin from *P. orientalis* will cluster with the lufaxins of other *Larroussius* species.(Vlkova et al., 2014).

3.3. Antigen 5-related protein family

The antigen-5 related proteins (Ag5r) are found in various insect species and together with the cysteine-rich secretory proteins (CRISPs) and the plant pathogenesis-related protein 1 (PR-1) they form the CAP family (Gibbs et al., 2008). They have been described for the first time in venom of various ants and wasps [reviewed in (King and Spangfort, 2000)] and in saliva of various blood sucking insects (Ameri et al., 2008; Assumpção et al., 2011, 2008; Ribeiro et al., 2010a; Xu et al., 2011). Antigen 5-related proteins are present in the sialomes of all studied sand fly species and have a predicted MW around 28.8 – 31.2 kDa (Oliveira et al., 2006). Sequences of Ag5r generally consist of fourteen conserved cysteine residues and an almost 80 % sequence similarity is found between sand flies from the Old and New World (Coutinho-Abreu and Valenzuela, 2018). One homologue of the Ag5r protein was shown to be present in the sialome of *P. perniciosus* (Anderson et al., 2006), while two homologues were detected in the sialome of *P. orientalis* (Vlkova et al., 2014). The antigen 5-related proteins from these two members of the *Larroussius* subgenus are highly related in phylogenetic analyses, creating one cluster together with the *P. tobbi* Ag5r, and are closely related to other *Larroussius*, *Adlerius* and *Euphlebotomus* Ag5r proteins (Coutinho-Abreu and Valenzuela, 2018; Vlkova et al., 2014).

The function of Ag5r remained elusive for a long time. However, in recent years it was shown that Ag5r proteins possess diverse functions. For instance, the Ag5r protein from the horse fly *Tabanus yao* has a fibrin(ogen)olytic enzymatic activity and is able to inhibit platelet aggregation (Ma et al., 2009; Xu et al., 2008), whereas the Ag5r of kissing bugs has an antioxidative function (Assumpção et al., 2013). The Ag5r (IT5) from *Stomoxys calcitrans*, on the other hand, can bind immunoglobulins (Ameri et al., 2008) and inhibit the classical pathway of the complement system (Wang et al., 2009). Despite these findings, the function of Ag5r proteins in mosquitoes and sand flies remains unknown.

In sand flies, the Ag5r proteins are highly antigenic in various vertebrates (Hostomska et al., 2009; Rohousova et al., 2012a, 2012b; Vlkova et al., 2012, 2011). The antibody response against recombinant *L. intermedia* Ag5r protein (LinB-13) have been proposed as an exposure marker for sand fly bites in humans (Carvalho et al., 2017). However, the recombinantly expressed *P. perniciosus* Ag5r protein (PpeSP07) did not recognize sera from mice nor dogs that were previously bitten by *P. perniciosus* (Drahota et al., 2014). Also for the recombinantly expressed *P. orientalis* Ag5r (PorASP76) protein, no satisfying results were obtained when testing it as a marker of exposure to *P. orientalis* bites in Ethiopian domestic animals (Sima et al., 2016b). Latter, the second *P. orientalis* Ag5r protein (PorASP74) was expressed in a mammalian system, which resulted in it being a valid antigen for human exposure to *P. orientalis* when combined with the *P. orientalis* YRP (Sumova et al., 2018). A lack of glycosylation patterns and other post-translational modifications due to the expression of the recombinant proteins in a bacterial expression system might be the cause of its low antigenicity in study by Sima et al., (2016a) or the differences between two *P. orientalis* Ag5r homologues, even though they share 99 % identity.

3.4. Odorant-binding protein family

The family of odorant-binding proteins belongs to the odorant binding protein (OBP) superfamily that has a characteristic OBP domain. The number of copies of the OBP domain in the sequence, the molecular weight of the proteins and the species of origin all divide these proteins into subfamilies. Homologues of these proteins have also been found in the sialomes of other blood feeding insects e.g. mosquitoes [reviewed in (Valenzuela et al., 2002)], black flies (Andersen et al., 2009), and biting midges (Wilson et al., 2008). The sialomes of all sand flies contain two groups of OBPs: (i) long odorant binding proteins named D7 and (ii) short OBPs that are similar to *P. papatasi* SP15, and therefore

commonly named PpSP15-like proteins. Interestingly, another small group of OBPs (LJM19) was found only in *L. longipalpis* saliva (Ribeiro et al., 2010a).

The D7-related proteins (large OBPs):

The first subfamily of salivary OBPs are the D7-related proteins, found in all sand flies' sialomes studied so far and with a predicted MW of 25.3 – 28.1 kDa (Oliveira et al., 2013). A specific pattern of ten cysteine residues is present in their sequences. However, the unconserved part of their sequence is highly divergent, even among homologues from the same subgenus (Coutinho-Abreu and Valenzuela, 2018). Multiple homologues of D7-related proteins were found in the sialomes of *P. perniciosus* and *P. orientalis*. The sequence similarity between the homologues – even within the same species – is low (30 – 40 %) (Anderson et al., 2006; Vlkova et al., 2014). However, certain regions, mainly at the N-terminus, are more conserved (Anderson et al., 2006; Vlkova et al., 2014). Strong selective forces related to the function and/or immunogenicity of these proteins are hypothesized to lay at the base of the difference in phylogeny of these D7-related proteins and the classical sand fly phylogeny (Aransay et al., 2000; Coutinho-Abreu and Valenzuela, 2018).

The D7 proteins from *Aedes aegypti* and *Anopheles gambiae* were able to act as eicosanoids and biogenic amines binders (Calvo et al., 2006, 2009; Mans et al., 2007), while this function in sand fly's saliva is hold by YRPs. The inability of sand fly D7-related proteins to bind biogenic amines is due to a different arrangement of the C-terminal domain compared to the D7 proteins from *Ae. aegypti* and *An. gambiae* (Jablonka et al., 2019). Moreover, the *An. stephensi* D7 protein was shown to bind factor XII (FXII) and a high molecular weight kininogen which leads to an inhibition of the intrinsic coagulation pathway in the vertebrate host (Isawa et al., 2002). In sand flies the function of D7-related proteins was discovered recently when it was demonstrated that both D7-related proteins of *P. duboscqi* (PduM01) and *P. papatasi* (PptSP28) are able to inhibit platelet activation by binding thromboxane A2, confirming their role as anti-haemostatic molecules. Finally, they are able to bind cysteinyl leukotrienes (Jablonka et al., 2019), which are released by hosts' mast cells and may cause vasoconstriction, itching, pain and swelling (Boyce, 2005; Soter et al., 1983).

Importantly, D7-related proteins are also antigenic in vertebrate hosts. This antigenicity was proven for various combinations of sand fly and host; e.g. *P. papatasi* – human (Marzouki et al., 2011; Rohousova et al., 2005), *P. papatasi* – mice (Vlkova et al.,

2012), *L. longipalpis* – dogs (Bahia et al., 2006; Hostomska et al., 2008), *P. tobbi* – rabbit (Rohousova et al., 2012b). Even more so, the antigenicity of D7-related proteins was also shown for *P. perniciosus* with sera from naturally bitten hares and rabbits (Martín-Martín et al., 2014), experimentally bitten mice (Martín-Martín et al., 2015), experimentally bitten hamsters (Martín-Martín et al., 2012) and experimentally and naturally bitten dogs (Vlkova et al., 2011); and for *P. orientalis* and sera from naturally bitten dogs (Sima et al., 2016b). Due to their antigenicity their potential as exposure markers was explored. However, only some were found suitable for certain host. For example, the recombinant D7-related protein (LJL13) of *L. longipalpis* was specifically recognised by antibodies present in sera of dogs, but not by human sera (Teixeira et al., 2010). Similar results were obtained with, the recombinant D7-related protein from *P. papatasi* (PpSP30) (Marzouki et al., 2012) (Vlkova et al., 2011), and from *P. orientalis* (PorMSP67), the latter one showing promising results in a preliminary study with sera from Ethiopian goats, but with an insufficient correlation with the SGH (Sima et al., 2016b).

The PpSP15-like proteins (short OBPs):

The second subfamily of OBPs was named after the *P. papatasi* SP15 protein (Valenzuela et al., 2001b), and is characterized by the presence of a single OBP domain and a lower predicted molecular weight than the large OBPs (between 12.2 and 17.1 kDa)(Oliveira et al., 2013). The sequence of the PpSP15-like proteins contains five cysteine residues organized in a specific motif. Similar to the D7-related proteins, also the PpSP15-like proteins have a highly divergent sequence across sand fly subgenera and even between species (Coutinho-Abreu and Valenzuela, 2018). Three and six PpSP15-like protein homologues were identified in the *P. perniciosus* sialome according to Anderson et al., (2006) and Martín-Martín et al., (2013), respectively and five in the *P. orientalis* sialome (Vlkova et al., 2014). All PpSP15-like proteins from *P. perniciosus* and *P. orientalis* have a predicted MW (13 – 17 kDa) characteristic for this protein family (Coutinho-Abreu and Valenzuela, 2018; Vlkova et al., 2014). The phylogeny of PpSP15-like proteins is hard to reproduce, as their sequences are very variable due to multiplication of gene copies. However, sequences of *Larroussius* and *Adlerius* are usually divided into three clades (Coutinho-Abreu and Valenzuela, 2018; Vlkova et al., 2014).

Only the function of the *P. duboscqi* PpSP15-like proteins (PduM02, PduM03) has been elucidated. More specifically, they were shown to be able to bind anionic polymers like polyphosphate, dextrane sulphate and heparin (Alvarenga et al., 2013), which are

responsible for the activation of FXII and subsequent stabilisation of coagulation complexes (Didiasova et al., 2018). Hence the PpSP15 serve as anticoagulant molecule in sand fly saliva (Alvarenga et al., 2013).

When PpSP15 was inoculated in mice, followed by exposure to *Le. major* and *P. papatasi* SGH, the number of parasites and the lesion size were reduced, suggesting of a protective effect of the PpSP15 protein. A DTH response was demonstrated to lay at the base of this protective effect (Valenzuela et al., 2001a). This protective effect mediated by a DTH immune response and a Th1 polarisation was consequently shown for the PpSP15-like protein from *P. ariasi* (ParSP03) (Oliveira et al., 2006), the PpSP15-like protein (PsSP9) from *P. sergenti* (Gholami et al., 2019) and the *P. duboscqi* PpSP15-like protein (PduM02) (Oliveira et al., 2015, 2008). Interestingly, also a protective effect of a plasmid containing the PpSP15 cDNA together with cysteine proteinases expressed by *Le. tarantolae* was shown (Zahedifard et al., 2014). Up to date, the PdSP15 is the most promising candidate to be part of a vaccine against human cutaneous leishmaniasis (Oliveira et al., 2015).

Not only PpSP15-like proteins were tested as vaccine candidates: the *L. longipalpis* small OBP (LJM19) was successfully tested as a protective molecule against both *Le. donovani-infantum* complex and *Le. brasiliensis* (da Silva et al., 2011; Gomes et al., 2008; Tavares et al., 2011). Similar results were reached by vaccinating hamsters with either a plasmid coding for LJM19 and leishmania antigen KMP11 (da Silva et al., 2011) or the *Le. donovani* centrin gene knock-out parasites (Fiuza et al., 2016), which protected against *Le. infantum* and *Le. donovani*, respectively. Even more so, LJM19 also protected hamsters against a *Le. brasiliensis* infection when inoculated with the SGH of *L. intermedia* (Tavares et al., 2011). Based on these results, LJM19 is an important candidate as an anti-*Le. donovani-infantum* vaccine or vaccine adjuvant, especially as it has a protective effect against both *Le. donovani-infantum* and *Le. brasiliensis*.

Apart from using PpSP15-like proteins as an anti-*leishmania* vaccine, they are also shown to be highly antigenic. High titers of *P. papatasi* anti-PpSP15 antibodies were detected in repeatedly bitten mice (Vlkova et al., 2012) and humans (Marzouki et al., 2011). *Phlebotomus perniciosus* PpSP15-like was recognized by antibodies from sera of dogs (Vlkova et al., 2011) as well as rabbits and mice (Martín-Martín et al., 2015) and *P. tobbi* small OBP was antigenic for repeatedly bitten rabbits (Rohousova et al., 2012b). Despite these results, none of the immune responses against these proteins was shown to be a good marker of exposure for sand fly bites.

3.5. PpSP32-like protein family

The first member (PpSP32) of this protein family was discovered in *P. papatasi.* PpSP32-like proteins are sometimes called "silk proteins" according to the only detected homolog of PpSP32, which is a flagelliform silk protein from *Nephila clavipes* (Valenzuela et al., 2001b). The average molecular weight of these proteins varies from 22.5 to 34.9 kDa (Oliveira et al., 2013) and their isoelectric point is highly basic. The function of these proteins within sand fly saliva remains elusive. It has been suggested that it could bind the extracellular matrix of the host as it is similar to collagen-binding proteins from *Bacillus cereus* (Valenzuela et al., 2004). However, the *P. perniciosus* PpSP32-like protein is similar to collagen type VII, contradicting the previous hypothesis (Anderson et al., 2006). The sequence of this protein family does not include any specific domains, but they are rich in glycine residues organised in patterns similar to those in collagen type VII (Anderson et al., 2006). Representatives of the PpSP32 family were found in the sialomes of sand flies (Coutinho-Abreu and Valenzuela, 2018) but not in other blood feeding insects. Similar to OBPs, the sequence divergence of PpSP32-like proteins is one of the highest among the sand flies studied so far (Coutinho-Abreu and Valenzuela, 2018).

Even though the sequence of PpSP32-like proteins is not very conserved among all sand flies, the recombinant PpSP32 protein of *P. papatasi* was shown to be an ideal marker for humans naturally exposed to *P. papatasi* bites (Marzouki et al., 2015, 2012).

One homologue of the PpSP32-like protein was found in the transcriptomes of the salivary glands of both *P. perniciosus* and *P. orientalis* (Anderson et al., 2006; Vlkova et al., 2014). Both proteins from these *Larroussius* sand flies shared a similar predicted MW and an alkaline isoelectric point, and a very high sequence similarity (87 %), supporting their distribution in the phylogenetic tree of PpSP32-like proteins, where PpSP32-like proteins of *P. orientalis* and *P. perniciosus* cluster together (Coutinho-Abreu and Valenzuela, 2018).

3.6. ParSP25-like protein family

The ParSP25-like protein family is a small family of which the members are unique in the sialomes of Old World sand flies belonging to the *Larroussius* and *Adlerius* subgenera (Anderson et al., 2006; Coutinho-Abreu and Valenzuela, 2018). They were named after the first discovered 26.6 kDa protein in the sialome of *P. ariasi*. The sequence of the ParSP25 protein does not contain any specific domains, but its N-terminus is rich for negatively charged amino acids, resulting in a very acidic isoelectric point (4.8) (Oliveira et al., 2006).

The molecular weight of these proteins is predicted between 24.8 and 38.8 kDa (Anderson et al., 2006; Hostomska et al., 2009; Oliveira et al., 2006; Rohousova et al., 2012b; Vlkova et al., 2014). The divergence among homologous sequences from different sand fly species is very low (Coutinho-Abreu and Valenzuela, 2018). One ParSP25-like protein is present in the saliva of both *P. perniciosus* and *P. orientalis* (Anderson et al., 2006; Vlkova et al., 2014). Both of these proteins have a highly similar sequence (73 %), predicted MW and a very acidic pI (Anderson et al., 2006; Vlkova et al., 2014). According to phylogeny of available ParSP25-like proteins' sequences, the *P. perniciosus* and *P. orientalis* proteins are not directly related within one group (Coutinho-Abreu and Valenzuela, 2018).

The ParSP25 protein from *P. ariasi* was tested for its ability to induce either a cellular or humoral immune response in mice. A strong DTH reaction was observed when a plasmid containing this protein was injected in mice, which was, interestingly, not combined with a Th1 immune profile and infiltration of pro-inflammatory immune cells into the injection site, causing it not to be an optimal vaccine candidate (Oliveira et al., 2006).

The function of ParSP25-like proteins remains to be discovered. They have been repeatedly shown to be highly antigenic in the various hosts, such as repeatedly bitten mice, hamsters (Martín-Martín et al., 2012) and dogs (Vlkova et al., 2011) which recognized the *P. perniciosus* ParSP25-like protein (PpeSP08). Furthermore, the recombinant *P. orientalis* ParSP25-like protein (PorSP65) was chosen as one of the potential *P. orientalis* exposure markers for domestic animals from Ethiopia (Sima et al., 2016b), while this was not the case for the *P. perniciosus* ParSP25-like recombinant protein (rPperSP08) which was tested as an exposure marker for dogs (Drahota et al., 2014).

3.7. Other salivary proteins from *P. perniciosus* and *P. orientalis* sialomes

Certain proteins that do not belong to the large salivary protein families that were mentioned above were found in the sialomes of *P. perniciosus* and *P. orientalis*. One of these are the SP16-like proteins, found in *P. orientalis* (Vlkova et al., 2014). The proteins of this family have a predicted molecular mass between 14 and 16 kDa, and their function remains unknown. They have only been shown to be present in the saliva of Old World sand flies, particularly *P. arabicus*, *P. argentipes*, *P. sergenti*, and *P. papatasi* (Coutinho-Abreu and Valenzuela, 2018). Another protein with an unknown function and part of the ParSP17-like family was identified in the *P. perniciosus* sialome (PpeSP19) (Anderson et

al., 2006). Finally, two groups of proteins with a predicted low molecular mass (5.6 – 10 kDa and 2.7 – 5 kDa, respectively) were found in both the *P. perniciosus* and *P. orientalis* transcriptomes. Homologues of these proteins were also present in the sialomes of *P. tobbi* (Rohousova et al., 2012b) and *P. ariasi* (Oliveira et al., 2006), but their function remains unknown.

3.8. Enzymes present in sand fly saliva

Salivary enzymes are one of the main compounds of sand fly saliva, with some being more abundant than others. Their enzymatic activities play a crucial role in facilitating the sand fly blood feeding. In the following chapter the major sand fly salivary enzymes that were found in sand fly saliva up to date will be discussed.

3.8.1. Salivary apyrase

In general, apyrases are enzymes that hydrolyse nucleotide di- and thri-phosphates to orthophosphate and mononucleotides, and they can be divided into three families in animals: (i) 5'-nucleotidase family, (ii) homologues to the human B-cell antigen CD39, and (iii) *Cimex*-type apyrases. The latter family was first described in saliva of the bed bug *Cimex lectularius* (Valenzuela et al., 1998), and enzymes in this family are responsible for the ATP hydrolysis effect in sand fly saliva (Charlab et al., 1999; Valenzuela et al., 2001b). *Cimex*-type apyrases have been found in all published sand fly sialomes (Coutinho-Abreu and Valenzuela, 2018; Oliveira et al., 2020), and compared to the other families of apyrases they are strictly Ca^{2+} dependent and unable to cleave adenosine monophosphate (AMP) (Valenzuela et al., 1998, 2001b). Since adenosine diphosphate (ADP) is released from ruptured tissues and consequently stimulates platelet aggregation, its degradation facilitates sand fly blood feeding. Moreover, apyrases also have an anti-inflammatory function. The degradation of both ATP and ADP inhibits purinergic signalling pathways which are responsible for the activation of inflammatory cells and the production of pro-inflammatory mediators and cytokines [reviewed in (Francischetti, 2010; Gounaris and Selkirk, 2005)].

Besides their important enzymatic function, sand fly apyrases were also shown to be antigenic in various hosts. Apyrases from *P. arabicus*, *P. papatasi*, *L. longipalpis* and *P. duboscqi* were all shown to be antigenic in mice (Hamasaki et al., 2009; Hostomska et al., 2009; Rohousova et al., 2005; Vlkova et al., 2012). However, only the apyrases of *P. papatasi* and *L. longipalpis* reacted with human sera from individuals naturally exposed to these sand flies (Marzouki et al., 2011; Rohousova et al., 2005). Apyrases from

P. perniciosus (PpeSP01 and PpeSP01B) produced high amounts of antibodies in mice, hamsters, dogs and lagomorphs (Drahota et al., 2014; Martín-Martín et al., 2013, 2014, 2015; Volf and Rohousova, 2001). The recombinant apyrase (PorSP15) from *P. orientalis* possesses similar antigenic properties and was indicated as a potential exposure marker in Ethiopian dogs (Sima et al., 2016b).

Furthermore, also the potential of the apyrase enzyme as an anti-*Le. infantum* vaccine has been studied. However, both the *L. longipalpis* (LJL23) apyrase (Gomes et al., 2008) and the *P. papatasi* apyrase (PpTSP36) were unable to produce a protective DTH response in mice (Oliveira et al., 2008). Studies on the apyrase of *P. papatasi* did show a stimulated production of IFN-γ together with a Th1 polarisation in cell cultures isolated from humans pre-exposed to this sand fly, thereby creating an anti-*Leishmania* milieu indicative of a potential use of this apyrase as a vaccine candidate (Tlili et al., 2018). Contrarily, apyrases from *P. ariasi* (ParSP01) and *P. sergenti* (PsSP40, PsSP41, PsSP42) were able to produce a DTH response in mice (Gholami et al., 2019; Oliveira et al., 2006), but immunisation of mice did not result in an increase in the production of IFN-γ (Gholami et al., 2019).

The apyrase enzymatic activities were characterised for six *Phlebotomus* (Hamasaki et al., 2009; Ribeiro et al., 1989; Vlkova et al., 2014; Volfova and Volf, 2018), one *Lutzomyia* (Ribeiro et al., 1986; Volfova and Volf, 2018) and one *Sergentomyia* species (Volfova and Volf, 2018), respectively. The apyrases had activity in pH range 6.5 and 9 (Hamasaki et al., 2009; Ribeiro et al., 1986, 1989; Vlkova et al., 2014; Volfova and Volf, 2018). Both, the amino acid sequence and predicted MW (33 – 36 kDa) of these sand fly enzymes, are conserved (Coutinho-Abreu and Valenzuela, 2018; Oliveira et al., 2013; Vlkova et al., 2014).

The highest apyrase activity for both ATP and ADP was found in *P. argentipes*, followed by *P. papatasi* and *P. orientalis* together with *P. perniciosus* (Ribeiro et al., 1989, 1986; Vlkova et al., 2014; Volfova and Volf, 2018). A lower apyrase activity was shown in *S. schwetzi* saliva (Volfova and Volf, 2018), and the lowest was observed in *L. longipalpis* saliva (Ribeiro et al., 1986; Volfova and Volf, 2018). Compared to other sand fly species, *S. schwetzi* apyrase hydrolyses both ATP and ADP to a similar extent, possibly due to its adaptation to the evolutionary preferred host (reptiles) (Volfova and Volf, 2018). However, this functional variation is not distinguishable on the phylogeny of apyrases which follows the classical sand fly phylogeny (Aransay et al., 2000; Coutinho-Abreu and Valenzuela, 2018).

In the sialomes of *P. perniciosus* and *P. orientalis*, two and three homologues of apyrase were found, respectively (Anderson et al., 2006; Vlkova et al., 2014). Phylogenetic analyses revealed that they cluster into two paraphyletic clades (clade I.: PpeSP01 with PorASP14 and PorASP15; clade II.: PpeSP01 with PorASP11) within other *Larroussius-Adlerius* apyrases. The sequences of the apyrases of *P. perniciosus* and *P. orientalis* share a high degree of identity (94 – 99 %) within a clade, while their sequence identity is lower (66 – 68 %) between clades – a trend caused by a gene duplication during their evolution (Coutinho-Abreu and Valenzuela, 2018; Vlkova et al., 2014).

3.8.2. Salivary hyaluronidase

Hyaluronidase is another enzyme present in saliva of sand flies (Coutinho-Abreu and Valenzuela, 2018; Volfova and Volf, 2018) and other blood sucking arthropods (Campbell et al., 2005; Ribeiro et al., 2000a, 2004, 2010b). The salivary hyaluronidase belongs together with the mammalian and the Hymenopteran ones, to the endo-β-N-acetyl-hexosaminidases [reviewed in (Kreil, 1995; Stern, 2003)]. They are responsible for the degradation of hyaluronan (HA) and other glycosaminoglycans that are contained in the extracellular matrix (ECM) of the vertebrate host, which eventually facilitates feeding, particularly for insects with shorter mouthparts that have a pool-feeding strategy. While hyaluronidase increases the skin matrix permeability of the host and enlarges the intradermal hemorrhagic "feeding pool", it simultaneously promotes the spread of active salivary components and pathogens such as *Leishmania* sp. (Volfova et al., 2008). Moreover, cleaved HA triggers various immunological processes that affect macrophages (induction of iNOS and chemokine secretion), activate dendritic cells and stimulate T-lymphocyte proliferation [reviewed in (Mummert, 2005)]. When this effect was studied with the recombinant hyaluronidase of *L. longipalpis* (LuloHya), the subsequent immune reaction caused an acute haemorrhage, edema and inflammation on the ears of the tested mice, and provided a better milieu for a successful infection of *Leishmania* sp. (Martín-Martín et al., 2018).

The possibility of using *L. longipalpis* recombinant hyaluronidase (LuloHya) as an anti-*Le. major* vaccine was shown to depend on the production of specific anti-LuloHya antibodies, which completely block its enzymatic function (including the subsequent hosts' immune reaction) and eventually lead to a reduction in lesion size and parasite load, as was shown in a *L. longipalpis-Le. major* model (Martín-Martín et al., 2018).

The sequences of sand fly hyaluronidases are highly conserved (around 70 – 90 %) and only one homolog of this enzyme was found in each sand fly species (Coutinho-Abreu and Valenzuela, 2018). The low abundance of the transcript (Oliveira et al., 2020) together with the large size of the protein cause that they were detected only in eight sand flies' sialomes or transcriptomes (*P. arabicus*, *P. kandelakki*, *P. pernicious*, *P. orientalis*, *P. tobbi*, *L. longipalpis*, *L. olmeca*, *L. intermedia*) (Charlab et al., 1999; Coutinho-Abreu and Valenzuela, 2018; Petrella et al., 2015).

On the other hand, the hyaluronidase activity was measured and characterized in all ten species studied so far. A high activity was shown for *P. papatasi* (Cerna et al., 2002; Rohousova et al., 2012b; Volfova and Volf, 2018), *P. halapensis* (Cerna et al., 2002), *P. tobbi* (Rohousova et al., 2012b; Vlkova et al., 2014) and *P. perniciosus* (Rohousova et al., 2012b; Vlkova et al., 2014). A medium activity was detected in *P. arabicus* (Rohousova et al., 2012b), *P. duboscqi* (Cerna et al., 2002), and *P. argentipes* (Rohousova et al., 2012b) saliva, while *P. sergenti* (Cerna et al., 2002; Rohousova et al., 2012b), *S. schwetzi*, and *L. longipalpis* saliva had a low hyaluronidase activity (Cerna et al., 2002; Rohousova et al., 2012b; Volfova and Volf, 2018).

The predicted MW of sand fly hyaluronidases varies between 24 – 53 kDa (Abdeladhim et al., 2016; Charlab et al., 1999; de Moura et al., 2013; Hostomska et al., 2009; Vlkova et al., 2014). However, the apparent MW on protein-gel electrophoresis was usually found much higher (60 – 135 kDa), suggesting that hyaluronidase contains a high number of post-translational modifications, such as glycosylations (Cerna et al., 2002; Hostomska et al., 2009; Rohousova et al., 2012b). Interestingly, *S. schwetzi* hyaluronidase had an apparent MW of 43 kDa on protein-gel electrophoresis, possibly due to low glycosylation (Volfova and Volf, 2018). Importantly, the glycosylation of this enzyme (*L. longipalpis* hyaluronidase) was recently shown to be essential for the activity of hyaluronidase (Martín-Martín et al., 2018). Not only the glycosylation patterns but also the oligomerisation of the molecule affect the apparent MW of these enzymes. For example, hyaluronidases of *P. tobbi*, *P. duboscqi*, *P. halapensis* and *S. schwetzi* are active as monomers, while *P. sergenti* is active as a dimer, and *L. longipalpis* and *P. papatasi* hyaluronidases as both monomers and oligomers (Cerna et al., 2002; Rohousova et al., 2012b; Volfova and Volf, 2018). Even though the sequences of sand fly hyaluronidases show high levels of identity, their activity, MW, and molecule organization significantly differs between all sand fly species.

3.8.3. Other salivary enzymes

Apart from apyrase and hyaluronidase, other enzymes have also been detected in sand fly saliva that might have an influence on sand fly blood feeding. All enzymes mentioned below are low abundant transcripts (Oliveira et al., 2020) and were recognised only in the sialomes of some sand fly species (Coutinho-Abreu and Valenzuela, 2018).

Salivary 5'-nucleotidase, an enzyme first and only described in the *L. longipalpis* sialome (Charlab et al., 1999). It is able to hydrolyse the nucleotide AMP, and ADP or UDP-glucose, suggesting that it possesses also a phosphodiesterase activity. Compared to apyrase, the 5'-nucleotidase activity does not depend on the presence of divalent-cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}).

Another enzyme connected to the AMP hydrolysis pathway is the salivary **adenosine deaminase (ADA)**. Similarly to the 5'-nucleotidase, this enzyme was first described in *L. longipalpis* saliva (Charlab et al., 1999), after which transcripts were found in the sialome of *P. duboscqi* (Kato et al., 2006), *L. olmeca* (Abdeladhim et al., 2016) and *P. perniciosus* (Petrella et al., 2015), and the ADA catalytic activity was identified in *P. duboscqi* saliva (Kato et al., 2007).

The salivary apyrase, ADA and 5'-nucleotidase are all involved in vertebrate hosts' ATP-ADP-AMP-adenosine catalysis pathway. The salivary 5'-nucleotidase is responsible for degradation of AMP to soluble adenosine (Ribeiro et al., 2000b), which is then catalysed by salivary ADA to inosine (Charlab et al., 2000, 2001). Adenosine itself has a vasodilatory (Edlund et al., 1987) and anti-platelet-aggregation activity (Collis, 1989) and it participates in pain processes (Burnstock and Wood, 1996). Moreover, together with inosine, it induces mast cell degranulation, which provokes itching in the vertebrate host (Tilley et al., 2000). Inosine is also able to inhibit the production of pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-12...) and it activates anti-inflammatory ones, like IL-10 (Haskó et al., 2000). Some sand fly species, like *P. papatasi* and *P. argentipes*, lack both the transcripts and the enzymatic activity of ADA. However, large amounts of AMP and adenosine were detected in their saliva (Ribeiro et al., 1999; Ribeiro and Modi, 2001), suggesting that the hosts' balance of AMP at the sand fly bite site is affected by several mechanisms of salivary components that were independently invented throughout the individual sand fly species evolution.

Another enzyme present in sand fly saliva is **pyrophosphatase**, with transcripts present in Old World (*P. argentipes*, *P. duboscqi*, *P. arabicus*, *P. orientalis*, *P. perniciosus*, *P. kandelakki*) but not in New World sand fly species [reviewed in (Coutinho-Abreu and

Valenzuela, 2018)]. The sequence of pyrophosphatase showed a low rate of diversification between sand fly species. Its possible function might be connected with the hydrolysis of the phosphodiesterase bond which is present in dinucleotides, nucleotide sugars or nicotinamid adenine dinucleotide (NAD). Some dinucleotides are inflammatory or mediators of vasoconstriction (Flores, 1999; Gasmi et al., 1996; Schlüter et al., 1996), indicating that pyrophosphatase might serve as an anti-inflammatory and anti-vasoconstriction molecule. Unfortunately, its enzymatic activity has not yet been functionally characterised.

Transcripts of the salivary **phospholipase A2** were detected only in *Larroussius* and *Adlerius* sand fly species, more specifically in the sialomes of *P. perniciosus*, *P. orientalis*, *P. ariasi* and *P. argentipes*. The amino acid sequences of this enzyme are highly conserved among these sand fly species and include a signature of ten cysteines [reviewed in (Coutinho-Abreu and Valenzuela, 2018)]. The phospholipases A2 from *P. orientalis* and *P. perniciosus* have a similar MW (30 kDa) and show a high degree of amino acid sequence conservancy (99 %) (Anderson et al., 2006; Vlkova et al., 2014). The function of this enzyme remains unknown. Efforts to measure the phospholipase A2 activity in saliva of *P. arabicus* were unsuccessful (Hostomska et al., 2009).

Lastly, **Salivary endonuclease** was found in both Old World and New World sand fly species, with an exception of *P. sergenti* and *P. tobbi* [reviewed in (Coutinho-Abreu and Valenzuela, 2018)]. The enzyme has a low divergence of its amino acid sequence in all sand fly species studied so far. It possesses a specific domain (DNA/RNA non-specific endonuclease domain) and a specific pattern of ten cysteine residues (Coutinho-Abreu and Valenzuela, 2018; Valenzuela et al., 2004). When the sequence of this endonuclease from *P. orientalis* and *P. perniciosus* was compared, a high degree of identity (92 %), a similar predicted MW, and a strong evolutionary relationship was shown (Anderson et al., 2006; Coutinho-Abreu and Valenzuela, 2018; Vlkova et al., 2014). The salivary endonuclease of *P. perniciosus* reacted with sera of dogs exposed to *P. perniciosus* (Vlkova et al., 2011). Before the function of this enzyme was revealed, it was believed that it facilitates feeding together with hyaluronidase by permeabilization of the hosts' ECM (Ribeiro et al., 2010a). Later, an additional function of this endonuclease was revealed for the *L. longipalpis* homologue. This homologue is called Lundep (**Lu**tzomyia **N**ET **d**estroying **p**rotein) (Chagas et al., 2014) as it degrades neutrophil extracellular traps (NETs), thereby facilitating the survival of *Le. major* in neutrophils both *in vitro* and *in vivo* (Chagas et al., 2014). Moreover, Lundep was shown to be highly antigenic and after exposure of mice to

this molecule, the produced antibodies decreased the feeding success of the sand fly (Chagas et al., 2014). Even more so, immunizing mice with a combination of the recombinant Lundep and the *L. longipalpis* hyaluronidase resulted in a promising anti-*Le. major* protection (Martín-Martín et al., 2018).

OBJECTIVES

The main aim of this thesis was to compare the salivary proteins and enzymes of *Sergentomyia schwetzi* with those found in *Phlebotomus perniciosus* and *Phlebotomus orientalis*, with special attention to their adaptation to different vertebrate hosts. Furthermore, the function of one salivary protein family, the yellow-related proteins, was compared between these sand fly species and its use as an exposure marker against *P. perniciosus* was verified for dogs.

The main objectives of thesis were:

1. Characterise the salivary proteins and enzymes from *Sergentomyia schwetzi* saliva, compare their properties and function with their known homologues from *Phlebotomus perniciosus* and *Phlebotomus orientalis* with emphasis on their preferred hosts

2. Compare biogenic amine-binding properties of the recombinant yellow-related proteins from *Phlebotomus perniciosus*, *Phlebotomus orientalis* and *Sergentomyia schwetzi*

3. Study the antigenic properties of the *Phlebotomus perniciosus* yellow-related protein and its utilisation as a *P. perniciosus*-exposure marker in dogs

LIST OF PUBLICATIONS

1. ***Sergentomyia schwetzi*: Salivary gland transcriptome, proteome and enzymatic activities in two lineages adapted to different blood sources.**
   Polanska N, Ishemgulova A, Volfova V, Flegontov P, Votypka J, Yurchenko V, Volf P. *PLoS One*. 2020;15(3), e0230537. doi:10.1371/journal.pone.0230537

2. **Amine-binding properties of salivary yellow-related proteins in phlebotomine sand flies.**
   Sumova P, Sima M, Kalouskova B, Polanska N, Vanek O, Oliveira F, Valenzuela JG, Volf P. *Insect Biochemistry and Molecular Biology*. 2019; 115, 103245. doi:10.1016/j.ibmb.2019.103245

3. **Interactions between host biogenic amines and sand fly salivary yellow-related proteins.**
   Spitzova T, Sumova P, Volfova V, Polanska N, Poctova L, Volf P. *Parasites & Vectors*. 2020;13(1), 237. doi:10.1186/s13071-020-04105-2

4. **The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis.**
   Kostalova T, Lestinova T, Maia C, Sumova P, Vlkova M, Willen L, Polanska N, Fiorentino E, Scalone A, Oliva G, Veronesi F, Cristóvão JM, Courtenay O, Campino L, Gradoni L, Gramiccia M, Volf P. *Medical and Veterinary Entomology*. 2017;31(1), 88-93. doi:10.1111/mve.12192

Polanska N, Ishemgulova A, Volfova V, Flegontov P, Votypka J, Yurchenko V, Volf P. *Sergentomyia schwetzi*: Salivary gland transcriptome, proteome and enzymatic activities in two lineages adapted to different blood sources.
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RESEARCH ARTICLE

Sergentomyia schwetzi: Salivary gland transcriptome, proteome and enzymatic activities in two lineages adapted to different blood sources

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files. The Illumina raw sequencing reads from this study are uploaded to Sequence Read Archive (SRA) and are available via BioProject number PRJNA573465. All salivary transcripts and proteins are uploaded to NCBI GenBank (Accession Numbers MN605259 – MN605417 for mRNA and QHO60649 - QHO60807 for protein sequences)

Abstract

During the blood feeding, sand fly females inject saliva containing immunomodulatory and anti-haemostatic molecules into their vertebrate hosts. The saliva composition is species-specific, likely due to an adaptation to particular haemostatic pathways of their preferred host. Research on sand fly saliva is limited to the representatives of two best-studied genera, *Phlebotomus* and *Lutzomyia*. Although the members of the genus *Sergentomyia* are highly abundant in many areas in the Old World, their role in human disease transmission remains uncertain. Most *Sergentomyia* spp. preferentially attack various species of reptiles, but feeding on warm-blooded vertebrates, including humans and domestic animals, has been repeatedly described, especially for *Sergentomyia schwetzi*, of which salivary gland transcriptome and proteome is analyzed in the current study. Illumina RNA sequencing and *de novo* assembly of the reads and their annotation revealed 17,293 sequences homologous to other arthropods' proteins. In the sialome, all proteins typical for sand fly saliva were identified–antigen 5-related, lufaxin, yellow-related, PpSP15-like, D7-related, ParSP25-like, and silk proteins, as well as less frequent salivary proteins included 71kDa-like, ParSP80-like, SP16-like, and ParSP17-like proteins. Salivary enzymes include apyrase, hyaluronidase, endonuclease, amylase, lipase A2, adenosine deaminase, pyrophosphatase, 5'nucleotidase, and ribonuclease. Proteomics analysis of salivary glands identified 631 proteins, 81 of which are likely secreted into the saliva. We also compared two *S. schwetzi* lineages derived from the same origin. These lineages were adapted for over 40 generations for blood feeding either on mice (S-M) or geckos (S-G), two vertebrate hosts with different haemostatic mechanisms. Altogether, 20 and 40 annotated salivary transcripts were up-regulated in the S-M and S-G lineage, respectively. Proteomic comparison revealed ten salivary proteins more abundant in the lineage S-M, whereas 66 salivary proteins were enriched in the lineage S-G.

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No difference between lineages was found for apyrase activity; contrarily the hyaluronidase activity was significantly higher in the lineage feeding on mice.

Introduction

Phlebotomine sand flies (Diptera, Psychodidae) are bloodsucking insects and vectors of viruses, bacteria and protists, causative agents of several important diseases of humans and animals. Out of over 800 described sand fly species, about 100 (all belonging to the two genera: *Phlebotomus* and *Lutzomyia*) are proven or suspected vectors of medical importance [1,2]. Sand flies of the third genus, *Sergentomyia*, are highly abundant in many areas in the Old World, but their vectorial role in human diseases is uncertain. Most of them exhibit a preference to feed on cold-blooded vertebrates (such as reptiles and amphibians), yet feeding on warm-blooded vertebrates, including domestic animals and humans, was also described [3,4]. Traditionally, *Sergentomyia* sand flies were considered as insects with possible vector role in the transmission of reptile trypanosomatid parasites belonging to the genera *Trypanosoma* and *Leishmania* [5–7], but more recently they have been found positive for several human pathogenic viruses, namely Chandipura [8], Toscana [9], and Dashli virus [10], despite that *Sergentomyia* sand flies vectorial capacity is still under consideration.

During the blood feeding process, a female sand fly injects saliva into the host skin. Saliva has anti-haemostatic, immunomodulatory and anti-inflammatory properties, enabling the successful completion of the blood uptake by the sand fly (reviewed in [11]). Sand fly saliva can also facilitate transmission of the *Leishmania* parasites and enhance the *Leishmania* infection in the mammalian host [12,13]. While a number of reports focused on the composition and function of *Phlebotomus* and *Lutzomyia* saliva (reviewed in [11]), only a single study dealt with characterization of *Sergentomyia* salivary enzymes [14].

The salivary composition of blood-feeding insects differs, likely due to their adaptation to a specific haemostatic pathways of their preferred host [15]. The host's haemostatic system is a complex physiological process that halts bleeding at the site of injury (reviewed in [16]). The compounds involved in the host haemostasis vary across vertebrates [17]. For example, ADP was shown to activate mammalian platelets, but not thrombocytes of birds and reptiles [18]. Thus, biting insects adapted to feeding on birds or reptiles are expected to have lower level of apyrase activity for successful blood feeding on mammals [15]. This could explain why the triatomine bug *Dipetalogaster maxima*, which prefers to feed on lizards, exhibits very low salivary apyrase activities, compared to other triatomine bugs [19]. Similarly, the saliva of *Culex quinquefasciatus*, the species which appears to be adapted to mammalian hosts only recently, exhibits very low anti-clotting and apyrase activities, and, therefore, is not able to effectively prevent platelet aggregation caused by ADP [15]. In other bloodsucking insects, several studies have focused on comparison of salivary compounds between various insect families (reviewed in [20–22]), but not in respect to host preferences.

Here we present data on the salivary gland transcriptome, proteome and salivary enzyme activities of two *S. schwetzi* lineages come from a common origin, that were adapted to blood feed either on mice (S-M) or geckos (S-G), two hosts with a different body temperature and haemostatic mechanisms.

Materials and methods

Ethics statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/

2012 coll. on Protection of Animals against Cruelty in present statutes at large), which includes all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on Ethics of Laboratory Experiments of the Charles University (Prague) and were performed under permission of the Ministry of the Environment of the Czech Republic (number: MSMT-10270/2015-6) and the Certificate of Competency (number: CZ 02451) approved by the Ministry of Agriculture of the Czech Republic. Mice were housed in polypropylene breeding cage and geckos in terrarium. Both cages and terrarium were placed in a room with constant room temperature with a 12-hour-light/12-hour-dark cycle and all animals had free access to food and water. All efforts were made to minimize suffering of experimental animals within the study.

Sand flies

The colony of *Sergentomyia schwetzi* (Adler, Theodor & Parrot, 1929) was established from a pool of eggs obtained during field work in Ethiopia (Sheraro district, Tigray region) in 2011 and it was maintained under standard conditions as described previously [23]. The females from third generation were fed on two different hosts: BALB/c mice and the common leopard gecko, *Eublepharis macularius* (Blyth, 1854). Consequently, one lineage (S-G) was established from 191 female sand flies that were blood fed on the gecko, whereas the second lineage (S-M) derived from 91 females blood fed on BALB/c mice. Both lineages were maintained under the same conditions (but on different hosts) for over 40 generations (3 years) without any bottleneck effect and only then used for the experiments described below.

Sample preparation for illumina sequencing

In the sand fly's salivary glands the majority of mRNA is transcribed during first two days after emerging from pupae and therefore all transcriptomic studies are using 1–2 days old sand flies [24], from which salivary glands were dissected in sterile Tris buffer (20mM Tris, 150 mM NaCl, pH 7,8) and stored in batches of 60 salivary glands (originated from 40 to 50 individual females) in TRIzol (Thermo Fisher Scientific, Waltham, USA) at -80˚C until subsequent processing. Three samples from three following generations, each containing 180 salivary glands, were prepared for both lineages of *S. schwetzi*. In one day we dissected 60 to 120 salivary glands (one or two batches) from females emerged on that day or one day before in the same pot (one generation). For another batch we dissected newly emerged females from another pot (second generation). We repeated this procedure to reach required amount of salivary glands. Total RNA was extracted according to the manufacturer's protocol; its quality was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) and quantified on a NanoPhotometer (Implen GmbH, München, Germany). All six samples (three from S-G and three from S-M lineages) were sequenced at Macrogen (Seoul, Republic of Korea) on the Illumina HiSeq^{TM} 4000 platform (Illumina, San Diego, USA).

Assembly and annotation of the transcriptome

Raw reads were trimmed for sequencing adapters, ambiguity, quality and length in the CLC genomics workbench with standard settings (software versions 7.0.5 to 8.5.1, Qiagen, Hilden, Germany) as described previously [25,26]. Trimmed reads were stored in FastQ format after which a quality check was performed in FastQC [27].

All raw trimmed reads were *de novo* assembled by Trinity v2.2.0 [28,29] with the following settings:—min_kmer_cov 1;—min_contig_length 200. Additionally, *de novo* assembly was verified by mapping the raw trimmed reads back to the assembled contigs using Bowtie v1.1.2 [30] utility in the Trinity pipeline by running a perl script bowtie_PE_separate_then_join.pl

(settings: -aligner bowtie—-p 4—all—best–strata). The assembled contigs were then clustered, compared and filtered by CD-HIT-EST v4.6 [31] with an identity parameter 95%. Contigs, which passed the identity parameter, were screened for open reading frames (ORFs) and the single longest ORF was translated to its amino acid sequence using the TransDecoder v3.0.1 [29].

The protein sequences were annotated by finding their closest homologues in NCBInr protein database (National Center for Biotechnology Information non-redundant database) using BLASTp (basic local alignment search tool) with an e-value cut-off $\leq 10^{-5}$ [32]. All annotated protein sequences were manually sorted into seven groups according to the BLASTp hit organism: bacteria, fungi, plants, vertebrate, protozoa, other invertebrates, and arthropods. The sequences from the arthropod subset were divided into the following groups: sand flies, mosquitoes, other blood-feeding arthropods, and other (non-blood-feeding) arthropods. Further annotations of specific protein domains and families were performed against the Protein family database (Pfam v31.0) [33], the Protein domain families (ProDom v2006.1) [34], the gene ontology (GO) database [35], and the InterPro (classification of protein families) database using the InterProScan software [36]. The GO hits were annotated using the WEGO v2.0 [37,38]. The putative signal peptide cleavage sites in the proteins were predicted by SignalP v4.1 for all sequences [39], and the protein sub-cellular localization was predicted by TargetP v1.1 for arthropod sequences subset [40]. The theoretical molecular weight (Mw) and isoelectric point (pI) of the annotated proteins was predicted by ExPASy Compute pI/Mw [41]. Finally, the potential O-, N- and C-glycosylation sites of the selected proteins were predicted using the NetOGlyc v4.0 Server [42], NetNGlyc v1.0 Server [43] and NetCGlyc v1.0 Server [44].

Differential gene expression analysis

The RNA-seq analysis of all six transcriptomes (S-G 1–3, S-M 1–3) was performed in the CLC genomics workbench. The transcripts were statistically compared by an "Empirical analysis of differential gene expression" (EDGE) algorithm with default settings, p-value of ≤ 0.05, and FDR (false discovery rate) correction. The transcripts with salivary annotation were then sorted into four groups: i) transcripts with a fold change lesser than 1.5, ii) transcripts with a fold change greater than 1.5, iii) transcripts with a fold change greater than 1.5 and p-value lesser than 0.05. The fourth group of transcripts contains those, which passed the FDR correction regardless their annotation.

RT-qPCR gene expression analysis

Six independently pooled samples each containing 15 salivary gland pairs were prepared for each lineage. Salivary glands were dissected as above from 1- to 2-day-old sand fly females and stored in RNA*later* RNA Stabilization Reagent (Qiagen) at -80˚C for a maximum of 4 hours. Total RNA was isolated using the High Pure RNA Tissue Kit (Roche, Basel, Switzerland) following the manufacturer's protocol, but eluting RNA into 35 μl of PCR-clean water. The cDNA was then synthesized with a combination of anchored-oligo $(dT)_{18}$ and random hexamer primers using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions.

The RT-qPCR (reverse transcription quantitative polymerase chain reaction) primers sequences are listed in S1 Table. The expression of all transcripts was confirmed by PCR using the EmeraldAmp® GT PCR Master Mix (TaKaRax Bio, Inc., Kusatsu City, Japan) (S1 Table) and cDNA from either the S-G or S-M sand flies. PCR products were sequenced directly. All RT-qPCR reactions were performed with the SYBR Green (Bio-Rad, Hercules, USA) using

optimized conditions (S1 Table) on the iQ5 Multicolor Real – Time PCR Detection System (Bio-Rad) in the technical duplicates. Relative gene expression values were quantified according to the $2^{-\Delta\Delta CT}$ method [45] using actin and G3PDH (glycerol-3-phosphate dehydrogenase) as the reference transcripts and all relative gene expression values were calibrated to mean of C_T values measured for each transcript from S-G lineage. The R software [46] was used for data evaluation and visualization.

Phylogenetic analysis

Selected orthologues sequences were aligned by MAFFT (multiple alignment using fast fourier transform) with L-INS-I method [47]. The phylogenetically informative regions were selected using BMGE (block mapping and gathering with entropy) with BLOSUM30, and entropy threshold of 0.4 to 0.5 [48]. Maximum likelihood phylogenetic trees were built in the IQ-TREE software [49] using the ModelFinder [50] with corrected Akaike information criterion and an ultrafast bootstrap approximation with 1,000 replicates [51]. The trees were constructed using various protein substitution models: WAG (for D7-related and PpSP15-like proteins), WAG + I + G4 (for antigen 5-related proteins), WAG + F + I + G4 (for lufaxins and YRPs), WAG + F + G4 (for hyaluronidases), LG + F + R4 (for amylases), LG + F + I + G4 (for apyrases). Phylogenetic trees and the aligned sequences were edited and visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/) and Jalview 2 [52], respectively. Nodes supports in phylogenetic trees were indicated by the bootstrap values, while values higher or equal to 50% were displayed.

Proteomic analysis

In order to perform the proteomic analysis, 15 salivary glands from 5- to 7-day-old sand fly females were dissected and stored in 15 μl of 100 mM TAE buffer (Tris Acetate EDTA) with 1% SDC (sodium deoxycholate) at 4˚C. The dissection was performed between 5th and 7th day after emerging from pupae, as the full protein content in salivary glands is reached only after 4th day [24]. Three salivary gland samples from each lineage were analyzed on mass spectrometry in the OMICS Proteomics laboratory at BIOCEV (Vestec, Czech Republic), each of them in three technical replicates. All the data were analyzed and quantified with the MaxQuant software (v1.5.3.8) [53] with FDR set to 1% for both proteins and peptides and specified minimum length set to seven amino acids. Quantification was performed with the label-free algorithms described previously [53]. The data analysis was performed using Perseus v1.5.2.4 software [54]. Subsequently, the final data were filtered according to their quality and LFQ (log 2-transformed normalized label-free quantification) intensity (threshold 20). The significance of the protein enrichment was statistically evaluated by Student's T-test. The proteins with salivary annotation were divided into three groups according to difference of LFQ intensity (S-G vs. S-M samples) and Student's T-test q-value: i) proteins with difference of LFQ intensity lesser than 0.6, ii) proteins with difference of LFQ intensity greater than 0.6, and iii) proteins with difference of LFQ intensity greater than 0.6 with Student's T-test q-value lesser than 0.05.

For the proteome analysis by the SDS PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), the salivary glands from 5- to 7-day-old *S. schwetzi* females, of both S-G and S-M lineages, were dissected into Tris buffer and stored (-20˚C) until used. The samples, with an equivalent protein concentration (20 μg per well), were incubated (95˚C, 5 min, sample buffer with 2-mercaptoethanol) and electrophoretically separated at 4˚C using two SDS PAGE maxi gels on OmniPAGE Maxi Plus (Cleaver Scientific, Rugby, UK) with 10% and 15% acrylamide, respectively. Gels were stained for total proteins with Coomassie Briliant Blue R-250 (SERVA Electrophoresis GmbH, Heidelberg, Germany). Raw gels are displayed on S1 Fig.

Sample preparation for measuring enzymatic activities

For the enzymatic activity assays, the newly emerged females of both lineages were separated (to ensure age standardization) and maintained at 26˚C on 50% sucrose for 7 days. Dissected salivary glands were transferred to Eppendorf tubes containing either 0.02M TBS (tris-buffered saline), pH 7.6 with 0.005% Triton X-100 for apyrase, or 0.01M PBS, pH 7.2 for the hyaluronidase assays. Samples were stored at -80˚C in batches of 10 salivary gland pairs per 20 μl of buffer until used. Salivary gland homogenates (SGHs) were obtained by the disruption of tissue with a plastic pestle after one freeze-thaw cycle in liquid nitrogen. The resulted homogenate was diluted in an appropriate assay buffer to a working concentration and analyzed immediately. The protein concentrations of the SGHs were measured by the Qubit Fluorometer (Thermo Fisher Scientific) following the manufacturer's instructions.

Apyrase assay

The apyrase activity was measured using a colorimetric microassay based on the Fiske and Subbarow method [55] with slight modifications [56] on the Infinite M 200 Fluorometer (Tecan, Männedorf, Switzerland) at 665 nm. The concentration of the Pi was calculated from a standard curve with potassium dihydrogen phosphate. One unit of enzyme activity was defined as the amount of enzyme that releases one micromole of orthophosphate per minute from the nucleotide substrate at the specified assay conditions.

Hyaluronidase assays

The zymographic analysis of hyaluronidase activities were visualized by SDS PAGE electrophoresis in 10% polyacrylamide gels (0.75 mm thick) with copolymerized 0.002% HA (hyaluronic acid) as was described previously [14] with the modification in sample loading– 0.5 of a gland pair was loaded per lane. Raw gels are displayed on S1 Fig.

The hyaluronidase activities were quantified using a method of Frost and Stern [57] with previously described modifications [58]. Briefly: biotinylated HA (bHA) was covalently bound onto Covalink NH microtiter plates (NUNC) at the final concentration of 2 μg of bHA per well, coated for 45 min with 1% BSA in PBS and equilibrated with 100 μl of an appropriate assay buffer: 0.1M citrate-phosphate buffer (pH 4.0–7.0), and 0.1M sodium phosphate buffer (pH 7.0–8.0), all containing 0.1M NaCl and 0.1% Triton X-100. SGH samples were loaded in triplicates at a final concentration of 0.25 of a salivary gland pair per well, incubated for 45 min at 37˚C and the reaction was stopped by 6M guanidine (200 μl/well). Avidin-peroxidase (Sigma-Aldrich) was used at a final concentration of 0.2μg/well for 30 min and the color reaction was developed in a substrate buffer with o-phenylenediamine for 15 min in the dark. The plates were read at 492 nm. Bovine testicular hyaluronidase (Sigma-Aldrich) serially diluted in the assay buffer (pH 4.5) served as a standard and wells without bHA or without SGH were used as negative controls. Raw data were evaluated by the Measurement Parameters Editor Magelan 6 (Tecan). The measured activity was expressed as relative turbidity reducing units (rTRU) per pair of salivary glands.

Results

Illumina sequencing and read assembly

Total of 339,133,323 trimmed reads generated from six libraries of *S. schwetzi* salivary glands (S-M 1-3, S-G 1-3) were subsequently assembled into 88,676 contigs (N50 length 1,104 bp; median contig length 329 bp; average contig length 644 bp; total assembled bases 57,076,819). Out of these, 53,976,836 (16% of all reads) reads were matched back to assembled contigs in

proper pairs and 61,570 contigs were obtained after clustering similar sequences (with similarity threshold 95%). These were translated into 26,253 protein sequences. Among them, 5,927 ORFs were complete (from Met to stop codon), 9,970 ORFs were partial and 10,356 were identified as internal sequences. All protein sequences were annotated using their closest homologues by searching the NCBInr with 19,800 matched sequences and UniProt/SwissProt database with 14,676 matched sequences. The BLASTp against NCBInr results divided the final dataset into 7 groups: bacteria (1,134 sequences), plants (325 sequences), fungi (24 sequences), protozoans (194 sequences), vertebrates (727 sequences), invertebrates other than arthropods (103 sequences), and arthropods (17,293 sequences) (Fig 1A). In addition, 13,064; 175; and 12,194 sequences were matched in the Pfam, ProDom, and InterPro databases, respectively (Fig 1B), and 7,749 sequences obtained GO terms (S2 Fig). Putative signal peptide cleavage sites were predicted for 1,208 sequences, from which 474 had complete ORF.

Focusing on arthropod sequences only, four main groups were identified: sand flies (10,389 sequences), mosquitoes (3,151 sequences), other bloodsucking arthropods (189 sequences), and other arthropods (3,568 sequences) (Fig 1A). From the arthropods subset, the 4,714; 6,389, and 6,181 ORFs were identified as complete, partial, and internal ORFs, respectively. Within these protein sequences, 12,884; 11,293; 10,602; and 120 hits were matched with UniProt/SwissProt, Pfam, InterPro, and ProDom annotations, respectively. Total of 5,937 hits with various function were annotated with GO (S2 Fig), and 856 and 2,122 proteins were predicted to possess signal or targeting peptides (Fig 1B).

Identification of salivary protein families

In the salivary gland transcriptome of *S. schwetzi*, we found the full-length and/or partial sequences of main members of sand flies' salivary protein families, namely lufaxin, antigen 5-related proteins, yellow-related proteins, small odorant-binding proteins (PpSP15-like proteins), large odorant-binding proteins (D7-related proteins), silk proteins (PpSP32-like proteins), and ParSP25-like proteins. Furthermore, we documented the expression of the following sand fly salivary enzymes: apyrase, hyaluronidase, endonuclease, amylase, phospholipase A2, adenosine deaminase, pyrophosphatase, 5'nucleotidase and ribonuclease, and proteins: 71kDa-like protein, C-type lectin, ParSP80-like, SP16-like and ParSP17-like proteins and peptide homologous to Lol38.8. These data for proteins with complete ORFs are summarized in Table 1; the partial sequences of the detected salivary proteins are listed in S2 Table along with their annotations.

Antigen 5-related proteins

The antigen 5-related proteins (Ag5r) are proteins found in various insect species. In the analyzed transcriptomes, we identified two complete and five partial sequences of Ag5r proteins. Only the complete sequences were used in further analyses. Data on the homologues of *S. schwetzi* Ag5r proteins (SschwAg5r) are presented in Table 1 and S2 Table.

Both full-length proteins, SschwAg5r1 and SschwAg5r2 (sequence identity 70.5%), are 290 aa (amino acid) long with predicted Mw (without signal peptides) of 29.2 kDa and 30.2 kDa, respectively. In the sequence of SschwAg5r1, there were no N-glycosylation but 11 putative O-glycosylated sites, while in the sequence of SschwAg5r2, 2 and 5 putative N-glycosylation and O-glycosylation sites were predicted, respectively. The sequence identity of SschwAg5r with other sand fly Ag5r was between 67.6 and 60.2%. All Ag5r proteins share a motif of 14 Cys residues $CX_4CX_{9\text{-}13}CX_{9\text{-}10}CX_{61-63}CX_6CX_5CX_{70-71}CX_{17\text{-}18}CX_2CX_{15}CX_2CX_4CX_7C$ [59]. This pattern was slightly modified in the sequences of SschwAg5r1 and SschwAg5r2 (S3 Fig).

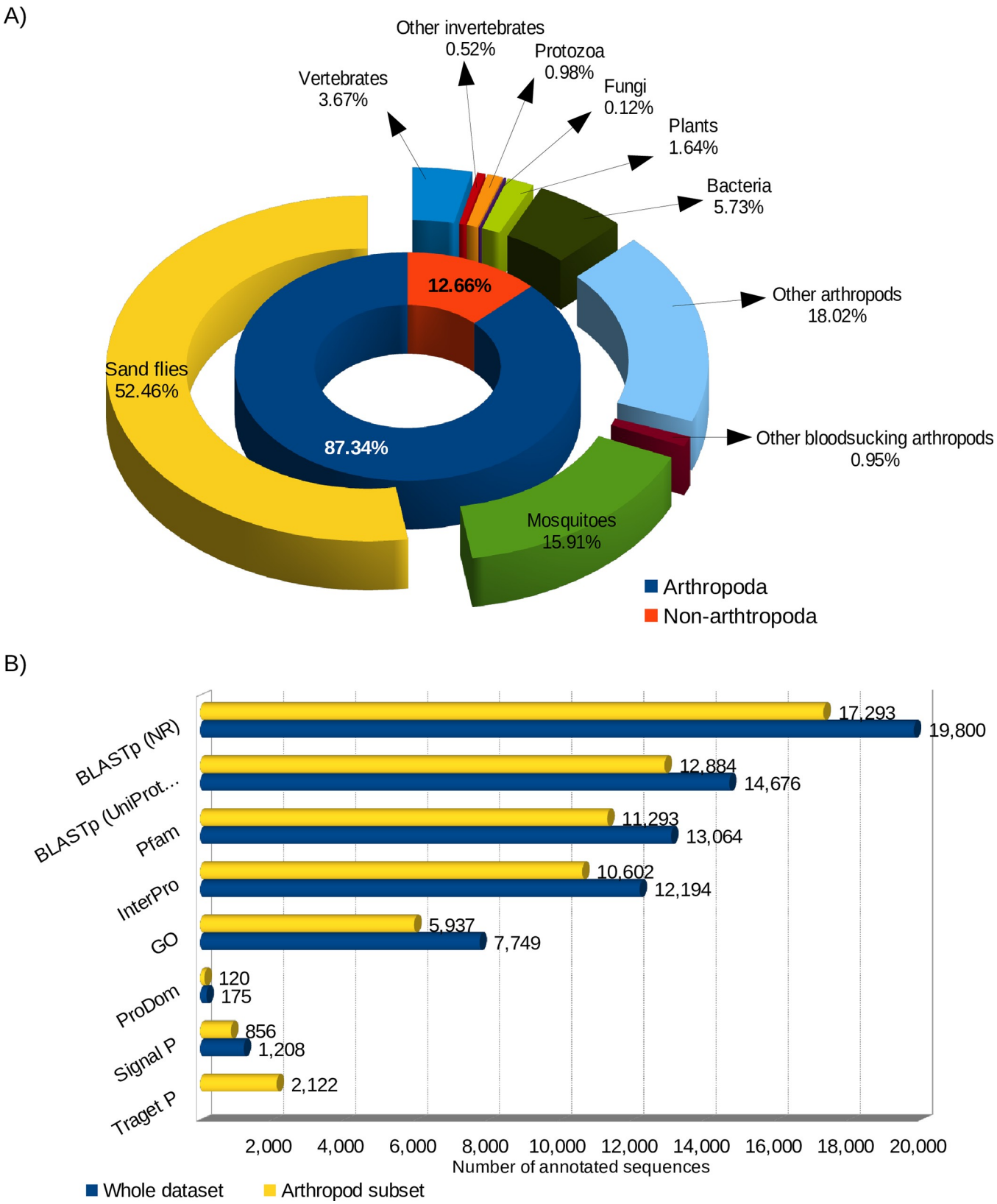

Fig 1. *S. schwetzi* salivary gland transcriptome annotation. (A) A chart with sequence annotation groups. Inner part represents distribution of sequences among arthropods (87% of all annotated sequences) and non-arthropods (13%). Outer part represents dividing sequences to 10 groups–sand flies (52.4%,

yellow), mosquitoes (16%, green), other blood-sucking arthropods (1%, maroon), other arthropods (18%, azure), bacteria (5.7%, dark green), plants (1.6%, lime), fungi (0.1%, purple), protozoa (1%, orange), other invertebrates (0.5%, red), and vertebrates (3.7%, blue). (B) Results of sequences annotation using different sequence databases. Numbers at the end of each bar indicate number of sequences with hit obtained using each database. Sequences annotated from whole transcriptome sequence dataset are shown in blue and sequences annotated from arthropod sequences subset in yellow, TargetP search was performed only for arthropod sequences subset.

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The phylogenetic analysis showed that SschwAg5r1 and SachwAg5r2 are sister to all sequences available from other sand fly species (Fig 2).

Lufaxin

Lufaxin proteins are sand flies' specific salivary anticoagulants (inhibitors of factor Xa). Five full-length sequences and one partial sequence of lufaxin homologues were identified in *S. schwetzi* transcriptome. The BLASTp hits and more annotations for each SschwLuf sequences are listed in Table 1 and S2 Table.

The amino acid sequences of SschwLuf are between 296 and 337 aa. The putative signal peptide cleavage site was predicted for all SschwLuf, except the SschwLuf5. The predicted molecular weight of SschwLuf varies between 31 kDa and 37.4 kDa and the putative O-glycosylation sites were detected for SschwLuf1 (5 sites), SschwLuf2 (5 sites), SschwLuf3 (6 sites), SschwLuf4 (1 site). The putative N-glycosylation sites were predicted only for SschwLuf4 (2 sites). Six cysteines typical for other sand fly lufaxins consensus sequence $CX_{21\text{-}29}CX_9CX_{22\text{-}35}CX_{118\text{-}125}CX_6C$ [60] were conserved across all SschwLuf sequences.

The sequence identity between SschwLuf proteins ranged between 30% and 37.6%, except the SschwLuf2 and SschwLuf3, which shared 97.7% sequence identity. Sequence identity with other known sand fly lufaxins varied from 30.3% to 46.8%. The cysteine motifs and putative glycosylations are shown in the S4 Fig. The phylogenetic analysis clustered SschwLuf sequences as two separate groups out from both Old World (OW) and New World (NW) sand flies. Further SschwLuf1 and SschwLuf5 were clustering as one group and SschwLuf2, SschwLuf3 and SschwLuf4 were generating the other group (Fig 3).

Odorant-binding proteins (OBPs)

Large odorant-binding proteins (D7-related). Large odorant-binding proteins (D7-related) were represented in *S. schwetzi* transcriptome by two full-length transcripts, SschwD7_1 and SschwD7_2, and 19 partial transcripts. The further annotations of all SschwD7 sequences are listed in Tables 1 and S1.

Both SschwD7 protein sequences possessed putative signal peptide cleavage sites. The amino acid sequence lengths were 242 aa for SschwD7_1 and 263 aa for SschwD7_2 and the predicted Mw of matured proteins were 26.4 kDa and 28.3 kDa, respectively. Both SschwD7 proteins contained one putative N-glycosylation site and SschwD7_2 had one putative O-glycosylation site (S5 Fig). The other sand flies D7-related proteins contain 10 conserved Cys residues in the pattern $CX_{25\text{-}27}CX_3CX_{44\text{-}46}CX_{49\text{-}50}CX_{6\text{-}12}CX_3CX_{30\text{-}32}CX_9CX_8C$ [59]. The Cys distribution pattern in SschwD7 was slightly modified (S5 Fig).

The sequence identity between the two SschwD7 was 30.2% and the identity with other sand fly D7-related proteins was between 24.8% and 37.9%. The SschwD7 proteins cluster with other *Phlebotomus* (*Adlerius*, *Larroussius*, and *Euphlebotomus*) proteins; however, the phylogenetic analysis did not give any significant support for closer relationship with these subgenera (Fig 4).

Small odorant-binding proteins (PpSP15-like). Small odorant-binding proteins (PpSP15-like) were detected in *S. schwetzi* transcriptome and presented by four complete

Table 1. Main salivary protein families identified in the transcriptome of *S. schwetzi*.

Protein family	Protein name	Mw [kDa]	pI	Best BLASTp match to NCBInr database				GenBank protein accession number
				Species of the best match	Accession number	E-value	Seq. identity [%]	
Antigen 5-related (Ag5r)	SschwAg5r1	29.2	8.7	*P. argentipes*	ABA12137	9E-118	62	QHO60649
	SschwAg5r2	30.2	9.0	*P. duboscqi*	ABI20191	2E-106	57	QHO60650
Lufaxin (Luf)	SschwLuf1	31	8.3	*P. papatasi*	AGE83098	3E-36	35	QHO60656
	SschwLuf2	35.3	8.7	*L. olmeca*	ANW11471	2E-41	32	QHO60657
	SschwLuf3	35.3	8.4	*L. olmeca*	ANW11471	2E-41	32	QHO60658
	SschwLuf4	37.2	5.9	*L. ayacuchensis*	BAM69113	5E-55	45	QHO60659
	SschwLuf5	37.4	7.9	*P. ariasi*	AAX55751	1E-70	45	QHO60660
Large odorant-binding proteins (D7-related)	SschwD7_1	26.4	9.1	*P. orientalis*	AGT96467	3E-44	37	QHO60662
	SschwD7_2	28.3	8.5	*P. perniciosus*	ABA43058	9E-43	35	QHO60663
Small odorant-binding proteins (PpSP15-like)	SschwSP15_1	14.8	7.7	*L. neivai*	JAV08238	1E-22	38	QHO60683
	SschwSP15_2	31.7	9.3	*L. ayacuchensis*	BAM69139	7E-24	38	QHO60684
	SschwSP15_3	14.5	9.0	*P. ariasi*	AAX55748	2E-25	39	QHO60685
	SschwSP15_4	15.0	9.3	*P. ariasi*	AAX55748	2E-21	35	QHO60686
Yellow-related proteins (YRPs)	SschwYRP1	40.9	5.0	*P. orientalis*	AGT96428	6E-100	44	QHO60691
	SschwYRP2	39.6	6.0	*P. ariasi*	AAX56360	1E-90	41	QHO60692
	SschwYRP3	43.8	8.4	*P. argentipes*	ABA12136	3E-79	40	QHO60693
Apyrase (Apy)	SschwApy1	35.3	8.4	*L. neivai*	JAV08627	4E-137	60	QHO60713
	SschwApy2	35.5	8.7	*L. ayacuchensis*	BAM69108	2E-105	47	QHO60714
	SschwApy3	35.9	9.0	*P. orientalis*	AGT96431	1E-99	47	QHO60715
Hyaluronidase (Hya)	SschwHya1	38.9	8.5	*L. longipalpis*	AAD32195	2E-133	56	QHO60718
5'-nucleotidase (s5nuc)	Sschw5nuc1	59.9	8.9	*L. neivai*	JAV08429	0	57	QHO60721
Adenosine deaminase (sADA)	SschwADA1	57.6	5.1	*P. perniciosus*	ALL27025	0	69	QHO60732
Amylase (sAmy)	SschwAmy1	53.9	6.2	*P. papatasi*	AGE83100	0	74	QHO60734
	SschwAmy3	52.7	6.2	*M. domestica*	XP_005185446	0	66	QHO60735
	SschwAmy2	54.1	5.3	*P. papatasi*	AGE83100	0	59	QHO60736
Endonuclease (sEnuc)	SschwEnuc1	52.3	8.7	*L. longipalpis*	AAS16916	2E-16	38	QHO60737
Phospholipase A2 (sPLA2)	SschwPLA2_1	42.1	8.2	*L. neivai*	JAV08461	0	82	QHO60752
Pyrophosphatase (sPP)	SschwPP1	47.1	7.4	*P. perniciosus*	ALL27023	0	75	QHO60755
71kDa-like	Sschw71kDa1	70.9	5.4	*L. longipalpis*	AAS16911	0	85	QHO60759
C-type lectin	SschwCTL1	19.1	5.0	*L. ayacuchensis*	BAM69191	2E-12	33	QHO60772
	SschwCTL2	15.9	5.5	*L. neivai*	JAV08591	8E-25	33	QHO60773
SP16-like	SschwSP16	17.6	5.6	*P. orientalis*	AGT96450	9E-17	34	QHO60782
ParSP60-like	SschwSP60-like	15.2	3.9	*P. argentipes*	ABA12152	6E-6	38	QHO60783
ParSP80-like	SschwSP80	16.2	5.5	*L. longipalpis*	AAS16917	2E-99	82	QHO60784
MBF2-like	SschwMBF2	15.3	5.8	*P. duboscqi*	ABI20163	5E-58	63	QHO60785
Secreted peptide 1	SschwPeptide1	7.7	5.0	*L. neivai*	JAV08913	8E-27	52	QHO60786
Secreted peptide 2	SschwPeptide2	7.8	4.8	*L. neivai*	JAV08913	1E-22	45	QHO60787

Complete ORFs of *S. schwetzi* annotated as main salivary protein and enzyme families. Protein family name, *S. schwetzi* protein name, putative matured protein features (Mw–molecular weight, pI–isoelectric point), BLASTp match to NCBInr protein database and NCBI GenBank protein accession numbers are listed.

https://doi.org/10.1371/journal.pone.0230537.t001

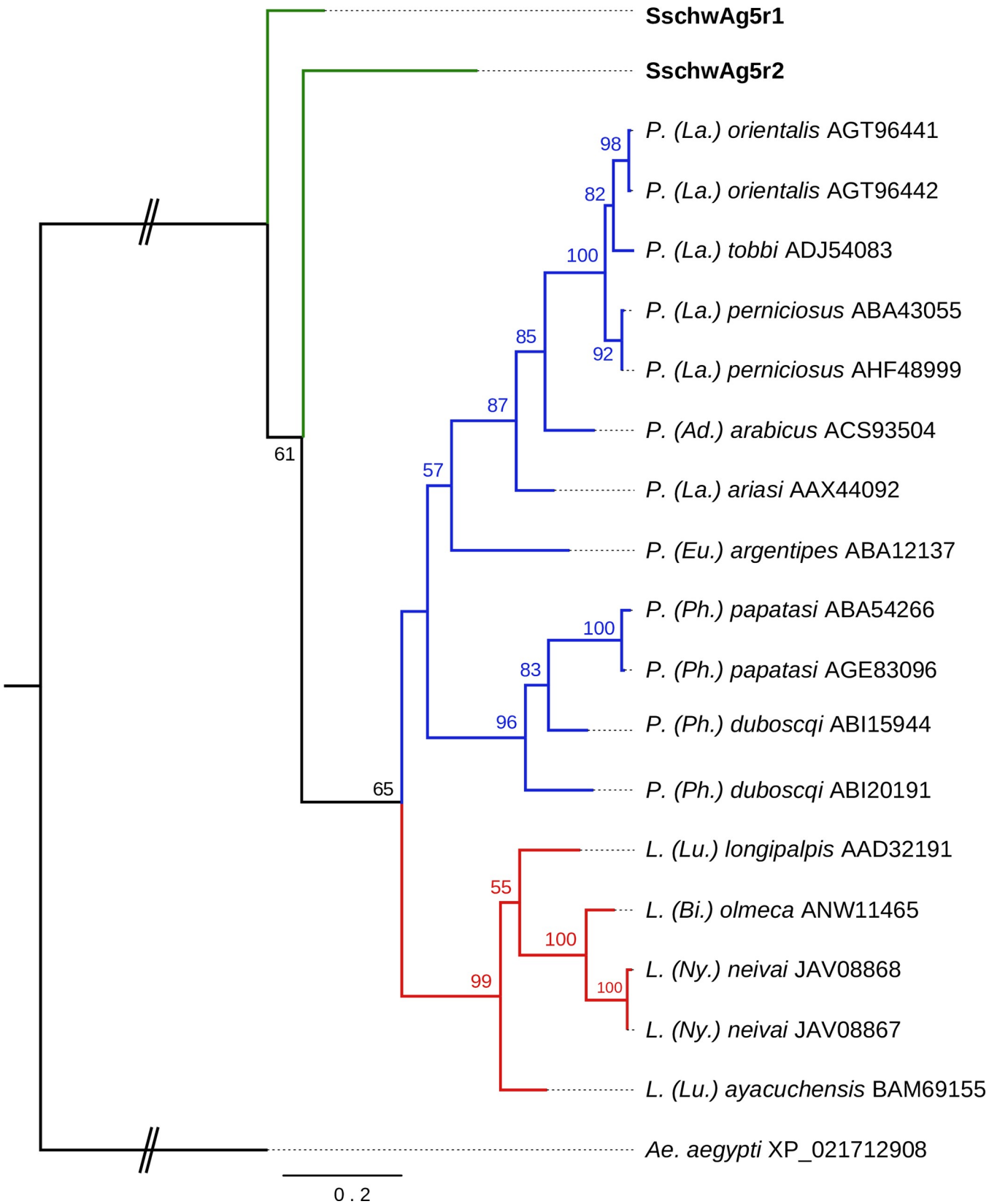
SschwAg5r1
SschwAg5r2
P. (La.) orientalis AGT96441
P. (La.) orientalis AGT96442
P. (La.) tobbi ADJ54083
P. (La.) perniciosus ABA43055
P. (La.) perniciosus AHF48999
P. (Ad.) arabicus ACS93504
P. (La.) ariasi AAX44092
P. (Eu.) argentipes ABA12137
P. (Ph.) papatasi ABA54266
P. (Ph.) papatasi AGE83096
P. (Ph.) duboscqi ABI15944
P. (Ph.) duboscqi ABI20191
L. (Lu.) longipalpis AAD32191
L. (Bi.) olmeca ANW11465
L. (Ny.) neivai JAV08868
L. (Ny.) neivai JAV08867
L. (Lu.) ayacuchensis BAM69155
Ae. aegypti XP_021712908
98
82
100
85
92
87
61
57
100
83
96
65
55
100
99
100
0 . 2

Fig 2. Phylogenetic comparison of antigen 5-related proteins. The phylogenetic tree with 20 sequences was based on MAFFT alignment with BMGE (threshold 0.4) containing 240 aa sites, 124 parsimony-informative sites, 49 singleton sites and 67 constant sites. The node values represent the percentage of bootstrap support for each branch (values equal or higher than 50% are shown). The position of sand flies of *Lutzomyia* genus is marked by red branches color, the sand flies of *Phlebotomus* genus are marked with blue branches color. The *S. schwetzi* proteins are marked by green branches color. For rooting the tree, the *Aedes aegypti* (XP_021712908) sequence was used (branch in black). The sand flies' subgenera are marked by shortcut in parentheses: *Ad.* - *Adlerius*, *La.* - *Larroussius*, *Eu.* - *Euphlebotomus*, *Ph.* - *Phlebotomus*, *Pa.*–*Paraphlebotomus*, *Lu.* - *Lutzomyia*, *Bi.* - *Bichromomyia*, *Ny.* - *Nyssomyia*.

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sequences (SschwSP15_1 –SschwSP15_4) and four partial sequences. The aa length of all sequences, putative Mw and pI for complete SschwSP15 sequences and other annotations are listed in Table 1 and S2 Table.

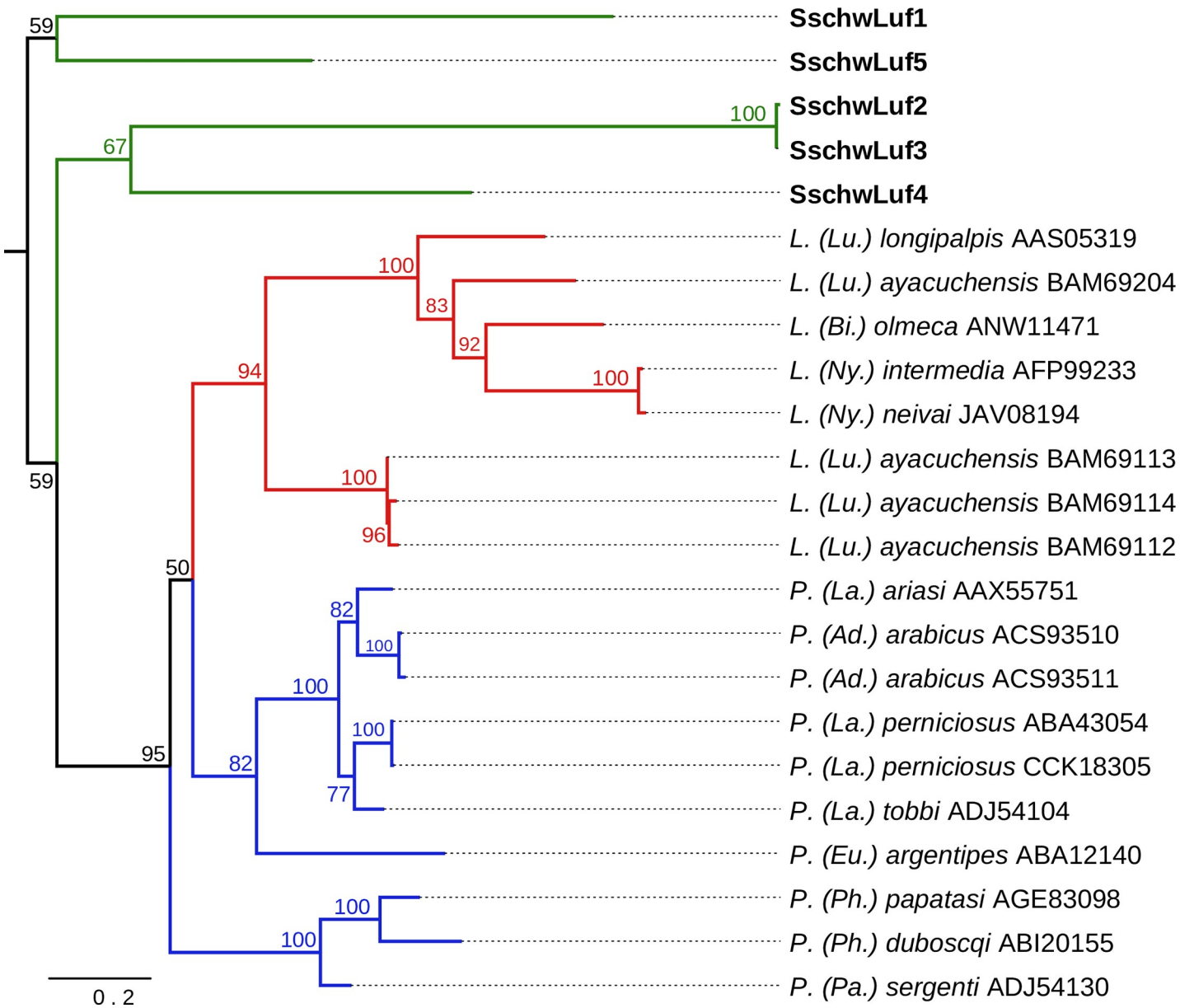
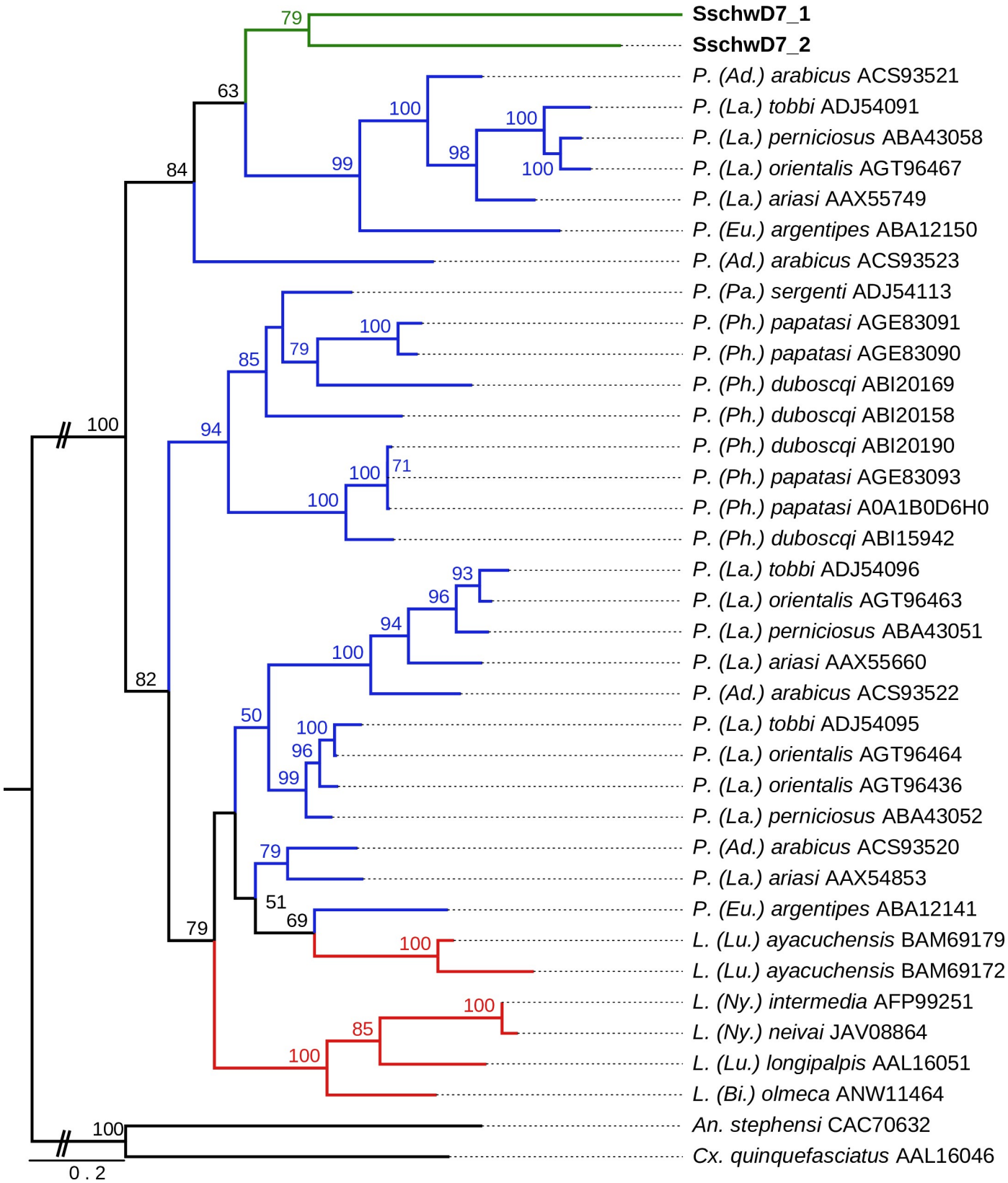


Fig 3. Phylogenetic comparison of lufaxin proteins. The phylogenetic tree with 23 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 259 aa sites, 213 parsimony-informative sites, 18 singleton sites and 28 constant sites. The tree is unrooted. For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g003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P. (La.) perniciosus ABA43058
P. (La.) orientalis AGT96467
P. (La.) ariasi AAX55749
P. (Eu.) argentipes ABA12150
P. (Ad.) arabicus ACS93523
P. (Pa.) sergenti ADJ54113
P. (Ph.) papatasi AGE83091
P. (Ph.) papatasi AGE83090
P. (Ph.) duboscqi ABI20169
P. (Ph.) duboscqi ABI20158
P. (Ph.) duboscqi ABI20190
P. (Ph.) papatasi AGE83093
P. (Ph.) papatasi A0A1B0D6H0
P. (Ph.) duboscqi ABI15942
P. (La.) tobbi ADJ54096
P. (La.) orientalis AGT96463
P. (La.) perniciosus ABA43051
P. (La.) ariasi AAX55660
P. (Ad.) arabicus ACS93522
P. (La.) tobbi ADJ54095
P. (La.) orientalis AGT96464
P. (La.) orientalis AGT96436
P. (La.) perniciosus ABA43052
P. (Ad.) arabicus ACS93520
P. (La.) ariasi AAX54853
P. (Eu.) argentipes ABA12141
L. (Lu.) ayacuchensis BAM69179
L. (Lu.) ayacuchensis BAM69172
L. (Ny.) intermedia AFP99251
L. (Ny.) neivai JAV08864
L. (Lu.) longipalpis AAL16051
L. (Bi.) olmeca ANW11464
An. stephensi CAC70632
Cx. quinquefasciatus AAL16046
0 . 2

Fig 4. Phylogenetic comparison of large odorant-binding proteins (D7-related). The phylogenetic tree with 38 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 213 aa sites, 185 parsimony-informative sites, 19 singleton sites and 9 constant sites. For rooting the tree, the *Anopheles stephensi* (CAC70632) and *Culex quinquefasciatus* (AAL16046) sequences were used (branches in black). For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g004

All full-length sequences contained putative signal peptide cleavage site. Twelve putative O-glycosylation sites were predicted for SschwSP15_2 and one site in the SschwSP15_3 sequence. No putative N- or C-glycosylation sites were detected.

The PpSP15-like proteins also contain 6 Cys residues in the $CX_{10}CX_3CX_{46}CX_{15}CX_8C$ pattern [59]. Changes in the SschwSP15_1, SschwSP15_2, SschwSP15_3, and SschwSP15_4 are depicted in the S6 Fig.

The sequence identity among SschwSP15 proteins was relatively high between SschwSP15_3 and SschwSP15_4 (87%) but relatively low between SschwSP15_1 and SschwSP15_2 (43.8%), SschwSP15_1 and SschwSP15_3 (43%) and SschwSP15_1 versus SschwSP15_4 (42.2%). The lowest sequence identity were predicted between SschwSP15_2 and SschwSP15_3 (32.8%) and SschwSP15_2 versus SschwSP15_4 (32.3%). In comparison with other sand fly PpSP15-like proteins the SschwSP15 proteins reached sequence identities between 23% and 40.7%.

Phylogenetic analysis of four SschwSP15 proteins and other sand fly PpSP15-like proteins revealed a high diversity among this protein family. *S. schwetzi* PpSP15-like proteins constitute a sister clade to *Lutzomyia* and part of *Phlebotomus* sequences (Fig 5).

Yellow-related proteins (YRPs)

Three full-length transcripts and 19 partial transcripts were identified as homologous to other known sand fly YRPs. The description of SschwYRP sequences and their annotation are shown in Table 1 and S2 Table.

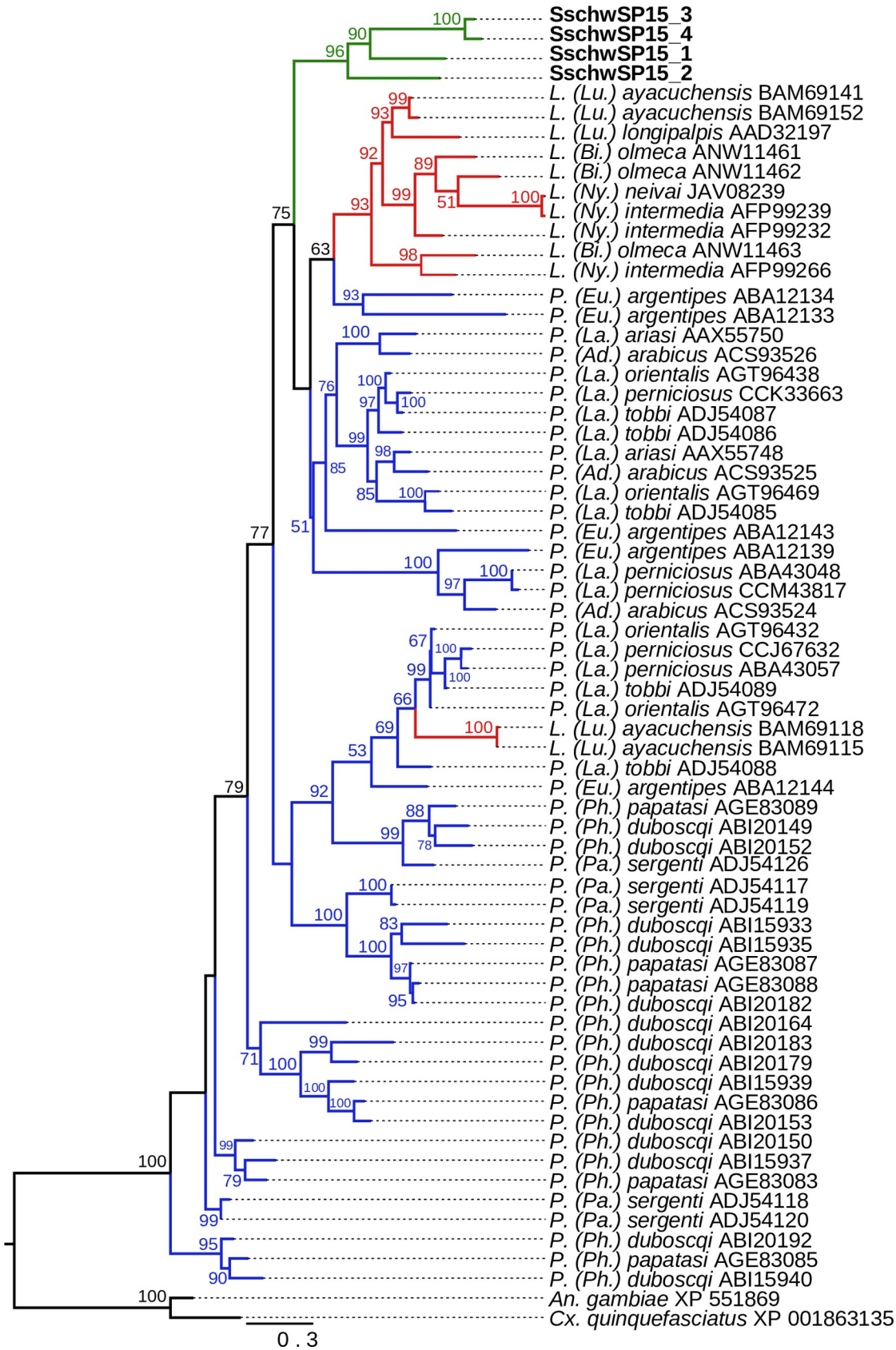
The protein sequence length was 387 aa for SschwYRP1, 370 aa for SschwYRP2 and 404 aa SschwYRP3. The predicted Mw of secreted SschwYRP1 was 40.9 kDa and 3 putative N-glycosylation sites were predicted. Mw of matured SschwYRP2 was lower (39.6 kDa) than other YRPs homologues, and no glycosylation site was presented in its sequence. Third protein, SschwYRP3, possessed 2 putative O-glycosylation sites and predicted Mw of 43.8 kDa (secreted variant). All sand fly YRPs sequences share similar Cys motif ($CX_{67\text{-}72}CX_{122\text{-}123}CX_{71\text{-}76}C$ modified according to [60]). Interestingly this sequence design was modified for all three SschwYRPs–SschwYRP1: $CX_{64}CX_{120}CX_{69}C$; SschwYRP2: $CX_{57}CX_{114}CX_{73}C$; SschwYRP3: $CX_{66}CX_{138}CX_{73}C$ (S7 Fig). The MRJP (Major royal jelly protein) domain, specific for insect YRPs, was present in all SschwYRPs. Further, the amino acid residues, responsible for binding biogenic amines, were highly conserved only for SschwYRP1. The amine-binding residues in other two SschwYRPs were more variable as it is visible in alignment in S7 Fig.

The sequence identity between SschwYRP1 and SschwYRP2 and between SschwYRP1 and SschwYRP3 was 39.4% and 41.4%, respectively, while the identity between SschwYRP2 and SschwYRP3 was 42.9%. Comparing with the other sand fly YRPs, SschwYRPs shared between 32.7% and 47.1% of the sequence.

Phylogenetic analysis of YRPs revealed SschwYRPs as the paraphyletic basal group to other sand fly YRPs. Within the SschwYRPs, SschwYRP2 and SschwYRP3 generated distinct group to SschwYRP1 (Fig 6).

Apyrase

Salivary apyrase was represented in the transcriptome by 3 full-length and 2 partial transcripts (Table 1, S2 Table).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SschwSP15_4
SschwSP15_1
SschwSP15_2
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L. (Lu.) ayacuchensis BAM69152
L. (Lu.) longipalpis AAD32197
L. (Bi.) olmeca ANW11461
L. (Bi.) olmeca ANW11462
L. (Ny.) neivai JAV08239
L. (Ny.) intermedia AFP99239
L. (Ny.) intermedia AFP99232
L. (Bi.) olmeca ANW11463
L. (Ny.) intermedia AFP99266
P. (Eu.) argentipes ABA12134
P. (Eu.) argentipes ABA12133
P. (La.) ariasi AAX55750
P. (Ad.) arabicus ACS93526
P. (La.) orientalis AGT96438
P. (La.) perniciosus CCK33663
P. (La.) tobbi ADJ54087
P. (La.) tobbi ADJ54086
P. (La.) ariasi AAX55748
P. (Ad.) arabicus ACS93525
P. (La.) orientalis AGT96469
P. (La.) tobbi ADJ54085
P. (Eu.) argentipes ABA12143
P. (Eu.) argentipes ABA12139
P. (La.) perniciosus ABA43048
P. (La.) perniciosus CCM43817
P. (Ad.) arabicus ACS93524
P. (La.) orientalis AGT96432
P. (La.) perniciosus CCJ67632
P. (La.) perniciosus ABA43057
P. (La.) tobbi ADJ54089
P. (La.) orientalis AGT96472
L. (Lu.) ayacuchensis BAM69118
L. (Lu.) ayacuchensis BAM69115
P. (La.) tobbi ADJ54088
P. (Eu.) argentipes ABA12144
P. (Ph.) papatasi AGE83089
P. (Ph.) duboscqi ABI20149
P. (Ph.) duboscqi ABI20152
P. (Pa.) sergenti ADJ54126
P. (Pa.) sergenti ADJ54117
P. (Pa.) sergenti ADJ54119
P. (Ph.) duboscqi ABI15933
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P. (Ph.) papatasi AGE83087
P. (Ph.) papatasi AGE83088
P. (Ph.) duboscqi ABI20182
P. (Ph.) duboscqi ABI20164
P. (Ph.) duboscqi ABI20183
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P. (Ph.) duboscqi ABI20153
P. (Ph.) duboscqi ABI20150
P. (Ph.) duboscqi ABI15937
P. (Ph.) papatasi AGE83083
P. (Pa.) sergenti ADJ54118
P. (Pa.) sergenti ADJ54120
P. (Ph.) duboscqi ABI20192
P. (Ph.) papatasi AGE83085
P. (Ph.) duboscqi ABI15940
An. gambiae XP 551869
Cx. quinquefasciatus XP 001863135
0 . 3

Fig 5. Phylogenetic comparison of small odorant-binding proteins (PpSP15-like). The phylogenetic tree with 67 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 213 aa sites, 87 parsimony-informative sites, 1 singleton sites and 6 constant sites. For rooting the tree, the *Anopheles gambiae* (XP_551869) and *Culex quinquefasciatus* (XP_001863135) sequences were used (branches in black). For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g005

The complete sequences were composed of 329 aa (SschwApy1), 333 aa (SschwApy2) and 339 aa (SschwApy3). One putative N-glycosylation site and one O-glycosylation site was predicted for SschwApy1 and SschwApy3, respectively. In the complete sequences of SschwApy the apyrase domain was detected with highly conserved Ca^{2+} binding sites. Nevertheless active site of enzyme was highly conserved only for SschwApy1 (S8 Fig). The sequence identity was 42.9% between SschwApy1 and SschwApy2, 39.8% between SschwApy1 and SschwApy3 and 47.9% between SschwApy2 and SschwApy3. The comparison of SschwApy sequences with other sand fly salivary apyrases revealed sequence identities between 38.9% and 50.8%.

Based on the phylogenetic analysis, visualized on Fig 7, three analyzed SschwApy sequences form a sister clade to all available sequences from *Phlebotomus* and *Lutzomyia* sand fly species. Furthermore the SschwApy2 and SschwApy3 were clustering as one group separated from SschwApy1.

Hyaluronidase

One full-length transcript of hyaluronidase, belonging to glycoside hydrolase family 56 –bee venom hyaluronidase, was detected in *S. schwetzi* transcriptome. The sequence contained 360 aa with the predicted Mw of 38.9 kDa for matured protein. Another two partial sequences of salivary hyaluronidase were identified (Table 1 and S2 Table).

In the complete sequence of SschwHya1 one putative O-glycosylation site was predicted, as well as 7 amino acid residues were putatively N-glycosylated. Further, the active sites of the enzyme were highly conserved in SschwHya1 (S9 Fig). The SschwHya1 shared sequence identity with other sand fly salivary hyaluronidases between 52.4% and 59.6%.

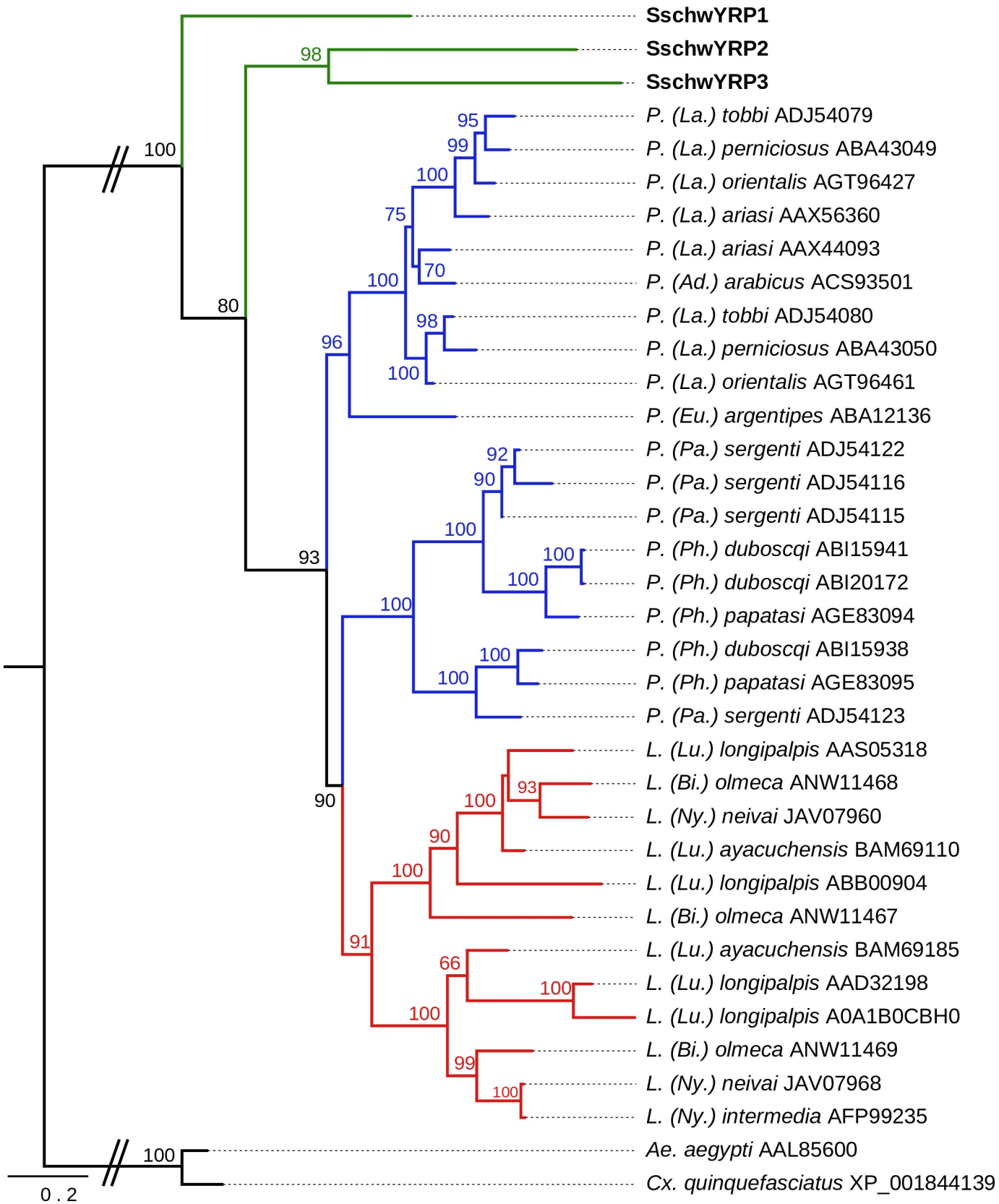
Phylogenetic analysis of other sand fly salivary hyaluronidases together with SschwHya1 showed that *S. schwetzi* hyaluronidase clustered as the sister group to hyaluronidase from the genus *Lutzomyia* (Fig 8).

Other salivary enzymes

Complete and partial sequences were detected also for 5'-nucleotidase (s5nuc), adenosine deaminase (sADA), amylase (sAmy), endonuclease (sEnuc), pyrophosphatase (sPP) and phospholipase A2 (sPLA2). All detected full-length transcripts contained putative signal peptide cleavage site and the further annotations of each sequence are listed in Tables 1 and S2.

One full-length transcript of 5'-nucleotidase and 10 partial sequences of this enzyme were identified in the transcriptome. The complete sequence (560 aa residues) contained the putative signal peptide cleavage site. The putative Mw of matured protein of Sschw5nuc1 was 59.9 kDa and one positive N-glycosylation site (Asn102) was found in the sequence. The sequence consisted of two main domains i) calcineurin-like phosphoesterase ApaH type with conserved active sites and metal binding sites and ii) C-terminal 5'-nucleotidase domain. Both glycosylation and active and metal binding sites were depicted on the alignment in S10 Fig. The sequence identity of Sschw5nuc1 with other sand fly s5nuc was between 57.3% and 57.5%.

The adenosine deaminase was identified in one full-length transcript. The 515 aa long sequence contained 5 putative O-glycosylation sites (highlighted in S11 Fig) and the matured protein had Mw 57.6 kDa. Sequence identity of SschwADA1 compared with other known sand fly sADA was between 65.8% and 69.4% in the MAFFT alignment. The SschwADA1


SschwYRP1
SschwYRP2
SschwYRP3
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P. (La.) perniciosus ABA43049
P. (La.) orientalis AGT96427
P. (La.) ariasi AAX56360
P. (La.) ariasi AAX44093
P. (Ad.) arabicus ACS93501
P. (La.) tobbi ADJ54080
P. (La.) perniciosus ABA43050
P. (La.) orientalis AGT96461
P. (Eu.) argentipes ABA12136
P. (Pa.) sergenti ADJ54122
P. (Pa.) sergenti ADJ54116
P. (Pa.) sergenti ADJ54115
P. (Ph.) duboscqi ABI15941
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L. (Ny.) neivai JAV07960
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L. (Bi.) olmeca ANW11467
L. (Lu.) ayacuchensis BAM69185
L. (Lu.) longipalpis AAD32198
L. (Lu.) longipalpis A0A1B0CBH0
L. (Bi.) olmeca ANW11469
L. (Ny.) neivai JAV07968
L. (Ny.) intermedia AFP99235
Ae. aegypti AAL85600
Cx. quinquefasciatus XP_001844139
0 . 2

Fig 6. Phylogenetic comparison of yellow-related proteins (YRPs). The phylogenetic tree with 36 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 359 aa sites, 312 parsimony-informative sites, 18 singleton sites and 29 constant sites. For rooting the tree, the *Aedes aegypti* (AAL85600) and *Culex quinquefasciatus* (XP_001844139) sequences were used (branches in black). For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g006

contained specific adenosine/AMP deaminase domain belonging to metal-dependent hydrolases family. The amino acid residues forming active site were highly conserved through the SschwADA1 sequence (S11 Fig).

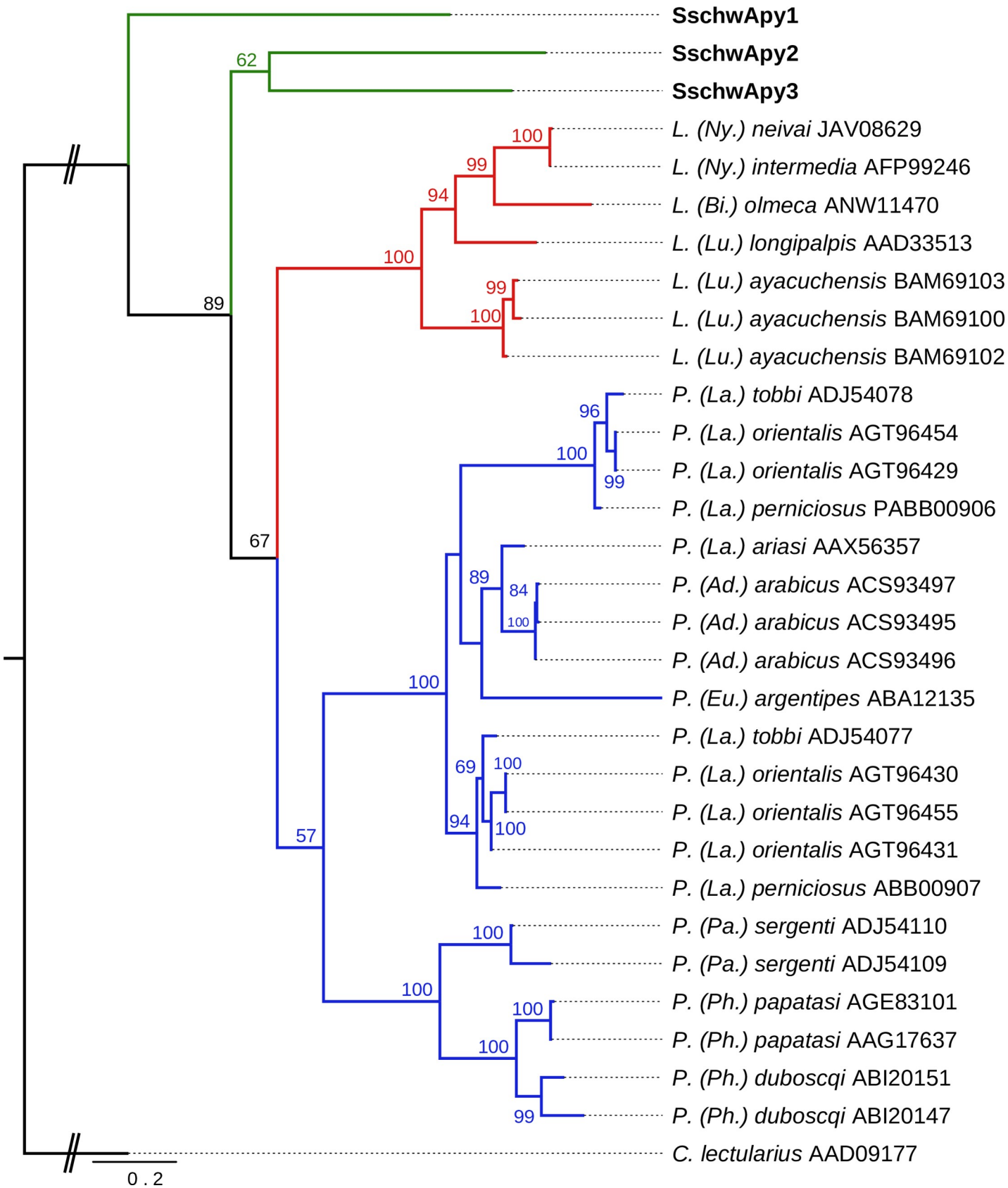
Three full-length transcripts of amylase were identified with protein sequence length 499 aa (SschwAmy1), 494 aa (SschwAmy2) and 509 aa (SschwAmy3), respectively. The predicted Mw of matured proteins were 53.9 kDa for SschwAmy1, 52.7 kDa for SschwAmy2 and 54.1 kDa for SschwAmy3. Both SschwAmy1 and SschwAmy3 had putative N-glycosylation sites (SschwAmy1 –Asn149, Asn416; SschwAmy3 –Asn159), while the sequence of SschwAmy2 possessed 6 putative O-glycosylation amino acid residues. According to MAFFT alignment the sequence identity between SschwAmy1 and SschwAmy2 was 59.2%, while between SschwAmy1 and SschwAmy3 was 67.7%. The sequence identity between SschwAmy2 and SschwAmy3 was lower (53.2%). In comparison with other sand fly amylases the SschwAmy reached sequence identities between 51.1% and 75.8%. All sequences of SschwAmy possessed glycoside hydrolase catalytic domain (family 13) and alpha-amylase C-terminal domain as other members of glycoside hydrolase superfamily. The active sites were conserved though the alignment as well as the Ca^{2+} binding sites. All the sequences annotations mentioned above are displayed on alignment in S12 Fig.

The phylogenetic analysis of putative salivary amylase, depicted on Fig 9, does not correspond with the taxonomy position of analyzed sand fly species. Three SschwAmy proteins do not form a monophyletic group and constitute a sister clades to other known sand fly sAmy from *P. papatasi*, *P. arabicus* and *L. longipalpis* and/or to the putative alpha amylase from *L. neivai* (JAV0853).

Another salivary enzyme, endonuclease, was found in one full-length sequence with 468 aa and 14 partial sequences. The predicted Mw of secreted protein was 52.3 kDa and 10 putative O-glycosylation sites were predicted for SschwEnuc1 (S13 Fig). Comparing SschwEnuc1 with other sand fly sEnucs, the SschwEnuc1 shared with them from 32.4% to 39.2% of the sequence identity, with low level of sequence conservation. The exception was the DNA/RNA non-specific endonuclease domain which was detected in the SschwEnuc sequence, and is specific for other sEnucs. Even though the domain is truncated from C-terminus, the amino acid residues forming active site and Mg^{2+} binding site were conserved, as is visible on alignment in S13 Fig.

The phospholipase A2 with sequence of 383 aa was found in the *S. schwetzi* transcriptome. The secreted protein had putative Mw 42.1 kDa and 4 putative O-glycosylation sites and two N-glycosylation sites, which were highlighted in S14 Fig, were predicted for the sequence. The sequence identity with other sand fly sPLA2 was between 30.5% and 31.2%, especially the central part of the alignment was unconserved but on the C-terminus the SschwPLA2_1 sequence possessed conserved phospholipase A2 domain, specific for insects (Phospholipase_A2_2), with catalytic sites and metal binding sites (S14 Fig).

The complete protein sequence (442 aa) of pyrophosphatase (sPP) was identified. The putative Mw of 47.1 kDa and the putative 5 O-glycosylation and 4 N-glycosylation sites were predicted for the sequence. Comparing SschwPP1 with other sand fly sPP sequences by MAFFT alignment showed a sequence identity between 60.6% and 71.9%. SschwPP1 sequence contained typical phosphodiesterase domain with the active site, as well as the Zn binding sites, which were highly conserved. The sequence characterizations mentioned above are highlighted on the alignment in S15 Fig.


SschwApy1
SschwApy2
SschwApy3
L. (Ny.) neivai JAV08629
L. (Ny.) intermedia AFP99246
L. (Bi.) olmeca ANW11470
L. (Lu.) longipalpis AAD33513
L. (Lu.) ayacuchensis BAM69103
L. (Lu.) ayacuchensis BAM69100
L. (Lu.) ayacuchensis BAM69102
P. (La.) tobbi ADJ54078
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P. (Ad.) arabicus ACS93497
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P. (La.) tobbi ADJ54077
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P. (Ph.) papatasi AAG17637
P. (Ph.) duboscqi ABI20151
P. (Ph.) duboscqi ABI20147
C. lectularius AAD09177
0 . 2

Fig 7. Phylogenetic comparison of salivary apyrase. The phylogenetic tree with 31 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 303 aa sites, 236 parsimony-informative sites, 33 singleton sites and 34 constant sites. For rooting the tree, the *Cimex lectularius* (AAD09177) sequence was used (branch in black). For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g007

Other salivary proteins

Other protein families rarely found in previously published sand fly salivary gland transcriptomes include 71kDa-like protein, protein with C-type lectin domain, ParSP80-like and SP16-like.

The 71 kDa-like protein family, so far detected only in NW sand fly species, and is annotated as angiotensin-converting enzyme. In *S. schwetzi* transcriptome one complete sequence and 12 partial sequences were detected (listed in Table 1 and S2 Table).

The complete sequence, Sschw71kDa1, possessed putative signal peptide cleavage site and had 626 aa, which resulted in Mw 70.9 kDa. The putative glycosylation sites displayed in S16 Fig, include one O-glycosylated residue, two N-glycosylated residues and one C-glycosylation residue. The domain typical for angiotensin-converting enzyme, which belonging to glu-zincin sub-group of metalloproteases, was detected in the sequence and the active site together with Zn binding site were highly conserved through the sequence (S16 Fig). According to MAFFT alignment Sschw71kDa shared identity with other *Lutzomyia* spp. homologues between 85.1% and 76.1%.

Two complete sequences of protein containing C-type lectin domain were identified (Tables 1 and S2 Table), both having a putative signal peptide cleavage site. The SschwCTL1 had 180 aa, its predicted molecular weight was 19.1 kDa with one putative N-glycosylation site (Asn36). The second protein, SschwCTL2, was 154 aa long and the molecular weight of its secreted form was 15.9 kDa with no putative glycosylation sites.

A single member of the SP16-like protein was detected. The complete sequence of SschwSP16 had 192 aa and a putative signal peptide cleavage site was identified in the sequence (Table 1 and S2 Table). The molecular weight of secreted protein was 17.6 kDa with no putative glycosylation site. In SschwSP16 no specific domains were detected. The sequence identity with other SP16-like proteins is quite low, between 29% and 38.8%.

One full-length transcript of a ParSP80-like protein was identified (Table 1, S2 Table). The SschwSP80 protein has 170 aa residues and putative signal peptide cleavage site followed by TRAP-delta (translocon-associated protein delta subunit) domain with no specific known function. The predicted molecular mass was 16.2 kDa and no putative glycosylation site was recognized. The SschwSP80 sequence identity with other sand fly homologues was detected between 82.4% and 74.6% according to MAFFT alignment.

Search through the transcriptomic data revealed two secreted proteins and two peptides which were homologous to infrequent sand flies' putative salivary proteins (Table 1, S2 Table). The sequence of SschwSP60-like was annotated as homologous to *P. argentipes* SP60 and it contained 162 amino acid residues including putative signal peptide cleavage site. The molecular mass of mature protein was predicted as 15.2 kDa, but this protein sequence showed high glycosylation– 19 amino acid residues as positive for O-glycosylation and two residues for N-glycosylation. No specific domain or pattern was predicted for SschwSP60-like protein. The second putatively secreted protein showed homology with *P. duboscqi* 14.5 kDa salivary protein. The specific domain–transcription activator MBF2 (multiprotein bridging factor 2)–was detected in the sequence of SschwMBF2. This protein contained a putative signal peptide and its matured version was predicted to have 15.3 kDa molecular weight. Serine 88 was positive for putative O-glycosylation. Furthermore, two putatively secreted peptides, homologous to one *L. neivai* salivary secreted peptide, were identified in the transcriptomic data. The

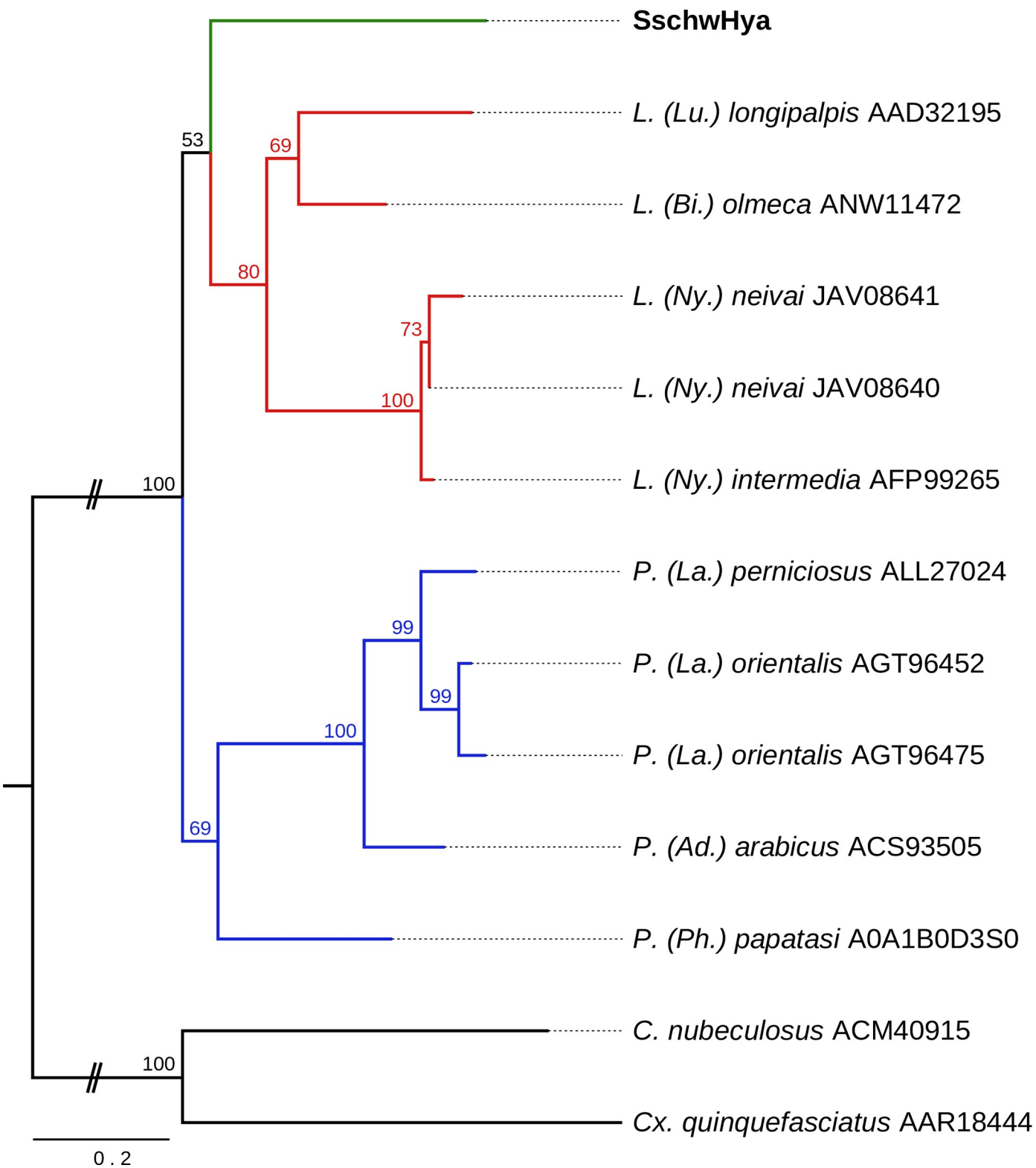

Fig 8. Phylogenetic comparison of hyaluronidase. The phylogenetic tree with 13 sequences was based on MAFFT alignment with BMGE (threshold 0.4) containing 270 aa sites, 154 parsimony-informative sites, 56 singleton sites and 61 constant sites. For rooting the tree, the *Culex quinquefasciatus* (AAR18444) and *Culicoides nubeculosus* (ACM40915) sequences were used (branches in black). For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g008

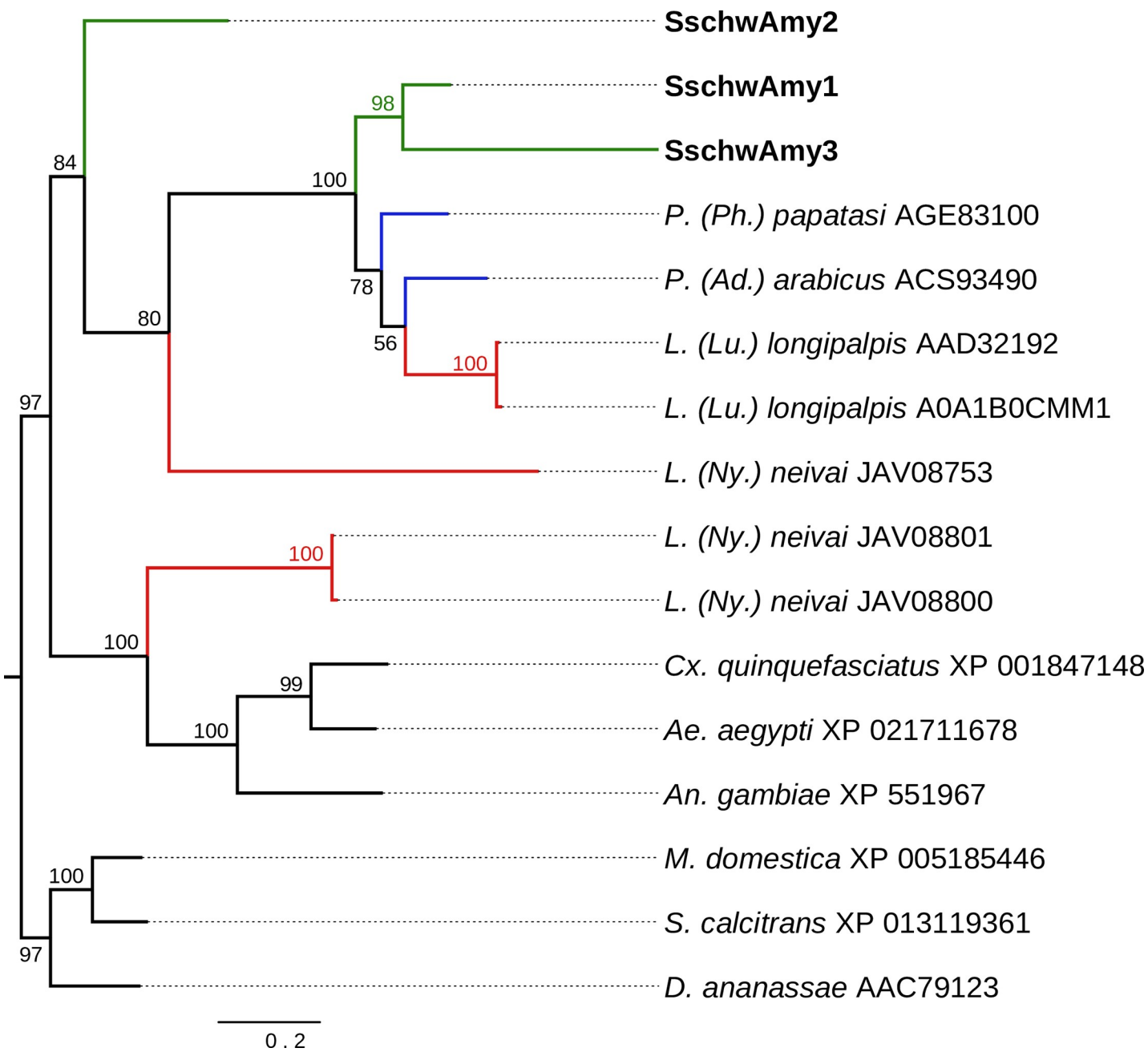

Fig 9. Phylogenetic comparison of salivary amylase. The phylogenetic tree with 16 sequences was based on MAFFT alignment with BMGE (threshold 0.5) containing 475 aa sites, 295 parsimony-informative sites, 51 singleton sites and 129 constant sites. By the black color are marked the additional sequences of sAmy from mosquito (*Culex quinquefasciatus* XP_001847148, *Aedes aegypti* XP_021711678, *Anopheles gambiae* XP_551967), drosophila (*Drosphila ananassae* AAC79123) and Muscidae (*Musca domestica* XP_005185446, *Stomoxys calcitrans* XP_013119361) species. Tree is unrooted. For more detail see Fig 2 legend.

https://doi.org/10.1371/journal.pone.0230537.g009

sequences of SschwPeptide1 and SschwPeptide2 were 89 aa long, contained putative signal peptide cleavage sites and had low molecular mass (7.7 kDa and 7.8 kDa). No specific domains or patterns were presented in the sequence.

Some of the salivary protein families were detected in *S. schwetzi* transcriptome only as partial sequences. The partial transcripts are listed with basic annotation in the S2 Table which includes the members of PpSP32-like (silk proteins), ParSP25-like, ParSP17-like, salivary

secreted ribonuclease (sRNase) and a conserved secreted peptide homologous to *L. ayacuchensis* Lol38.8.

Expression differences between sand flies fed on mice and geckos

Genes differentially expressed on the level of RNA between S-M and S-G lineages were identified and BLASTp annotated. From the whole annotated arthropod RNA-seq dataset, 150 transcripts of the salivary protein families were identified (S3 Table). The majority of the most abundant transcripts in both lineages (60 out of 70) were members of salivary protein families: YRPs, OBPs, lufaxins, Ag5rs, ParSP17-like and a PpSP32-like proteins, apyrase, hyaluronidase, endonuclease, protein with CTL domain and transcript homologous to Lol38.8.

First we analyzed only transcripts annotated as arthropod genes. The analysis step-by-step is displayed on S17 Fig. In the S-G lineage, 40 transcripts of the salivary protein families were up-regulated (EDGE fold change ≥ 1.5) (S17A, I Fig). The statistical evaluation of EDGE fold change (p-value < 0.05) of the up-regulated S-G transcripts did not revealed any salivary transcript (S17A, II Fig).

In the S-M lineage, the group of up-regulated transcripts (EDGE fold change at least 1.5) included 20 transcripts homologous to salivary proteins (S17B, I Fig). The statistical evaluation of EDGE fold change revealed two salivary sequences namely: SschwSP15_5 and SschwLuf3 (S17B, II Fig).

Later, we expanded the dataset and analyzed all differentially expressed transcripts (EDGE fold change ≥ 1.5, FDR corrected p-value < 0.05). This dataset contained eight differentially expressed transcripts (marked as DET1-8, Table 2). Only three of these sequences (DET2, DET6 and DET7) were annotated as arthropod sequences, while DET2 corresponded with protein SschwSP15_5. Other sequences were manually annotated using the online BLASTx algorithm, but the homology of DETs with known sequences was low (Table 2). While any of salivary transcripts was not up-regulated more than 30 times (either in the S-G or the S-M lineage), some of the DET transcripts were up-regulated for more than 1,000 times. According to RNA-seq analysis, seven transcripts (DET1-6, DET8) were up-regulated in the S-M lineage and one (DET7) in the S-G lineage.

Subsequently we confirmed RNA-seq results accuracy by RT-qPCR. Seven DETs were chosen for RT-qPCR tests (DET5 was excluded because it was annotated as a prokaryotic sequence). The RT-qPCR results confirmed significant up-regulations of three transcripts (DET1, DET2, and DET3) in the S-M lineage and up-regulation of DET7 in the S-G lineage (Fig 10). There was no significant difference detected in the expression of DET4, DET6 and DET8.

Salivary gland proteome

Mass spectrometry data analysis of the whole salivary glands identified 1,153 proteins. The quality-filtering of these data resulted in the final dataset of 631 proteins, selected for subsequent analysis. Out of these, 85 proteins were annotated as salivary (S18 Fig). The group of the top 54 enriched proteins (with intensity 1×10^9), contained members of the main salivary protein families, namely YRPs, OBPs, Ag5r, ParSP17, PpSP32, lufaxin, as well as members of typical salivary enzymes i.e. apyrase, endonuclease and adenosine deaminase. The proteomic data for salivary proteins including the LFQ intensities and their comparison between the two lineages are show in S4 Table.

The proteome analysis revealed 586 proteins shared by both lineages. Moreover, 43 proteins were detected in the S-M sialome exclusively, including one salivary SschwYRP20 protein. Two proteins were found only in the sialome of SG lineage–one salivary protein containing a

Table 2. Differentially expressed transcripts (DETs) annotation.

	GenBank Accession number	BLAST match to NR database					Nucleotide seq. length	Lineage up-regulation	EDGE test: Fold change	S-M lineage expression value (RPKM)	S-G lineage expression value (RPKM)
		Species of the BLAST match	Accession number	Annotation	E-value	Seq. identity [%]					
DET1	MN605409	*Rhagoletis zephyra*	XP_017480351	**uncharacterized protein**	3.3	50	218	S-M	1206	1120.53	0.88
DET2*	MN605297	*Phlebotomus orientalis*	AGT96472	SP15-like salivary protein	0.001	34	253	S-M	576	145.52	0.16
DET3	MN605411	*Acropora digitifera*	XP_015766698	uncharacterized protein	0.29	28	605	S-M	752	107.05	0.02
DET4	MN605412	No hits	-	-	-	-	239	S-M	393	56.01	0.03
DET5	MN605413	*Pseudomonas fulva*	AEF21400	**hypothetical protein Psefu_1424**	6.9	36	229	S-M	236	105.62	0.36
DET6	MN605414	*Lutzomyia longipalpis*	AAS17937	16.4 kDa salivary protein	0.15	32	265	S-M	64	98.29	1.53
DET7	MN605415	*Aedes aegypti*	XP_01655722	general odorant-binding protein 56a	4E-21	32	535	S-G	137	0.20	47.80
DET8	MN605416	*Lingula anatina*	XP_023930744	**macrophage mannose receptor 1-like**	0.18	39	317	S-M	474	108.65	0.12
Reference transcript 1	MN605417	*Lutzomyia neivai*	JAV13762	putative actin, partial	7E-220	100	1,131	non		136.6	158.5
Reference transcript 2	MN605410	*Lutzomyia neivai*	JAV12252	putative glycerol-3-phosphate dehydrogenase/ dihydroxyacetone 3-phosphate reductase	2E-100	98	1397	non		7.6	7,0

Annotation of differentially expressed transcripts from RNA-seq and reference transcripts, which were used for RT-qPCR. Table columns indicates: Name of transcript, NCBI GenBank nucleotide accession number, BLAST match to NCBInr database, nucleotide length of transcript, lineage in which was the transcript up-regulated, fold change difference in expression between lineages, average expression from three replicates for both S-M and S-G lineage.

*DET2 is SschwSP15_5

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CTL domain (SschwCTL4) and one with a C-terminal tandem repeat domain in type 4 procollagen (S18A Fig). When comparing the protein enrichment (LFQ intensities) between lineages, 359 and 227 proteins were found to be enriched in the S-M and S-G sialomes, respectively. Importantly, 10 of the 359 enriched proteins in the S-M sialome and 66 out of 227 enriched proteins in the S-G sialome were annotated as salivary. At a cut off 0.6 for the LFQ intensity, 113 proteins in the S-M sialome were found to be enriched at least 1.5 ×; none of them, however, were homologous to the salivary proteins. In contrast, 58 proteins in the S-G sialome were shown to be enriched at last 1.5 ×, of which 36 were identified as salivary. Finally, statistical evaluation of both sialomes revealed 35 proteins to be significantly enriched in the S-M sialome–all annotated as housekeeping proteins (Fig 11), compared to 8 significantly enriched proteins in the S-G sialome, of which six were annotated as salivary (Fig 11). The distributions of enriched proteins within the lineages and the analysis step-by-step are highlighted in S18B Fig. The putative actin and G3PDH (transcripts which were used as reference transcript for RT-qPCR) were used as the normalization control between the lineages. The LFQ intensities of both reference proteins were very similar for S-G (29.5 and 24.7 respectively) and SM (29.2 and 25.4 respectively) lineages.

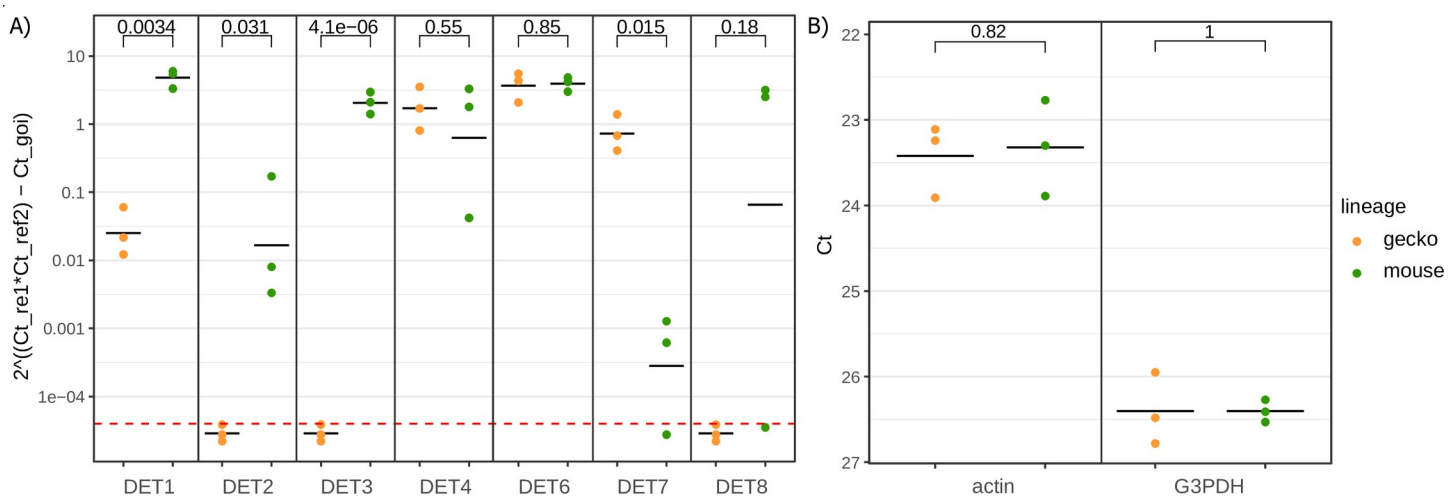

Fig 10. Differential gene expression in *S. schwetzi* lineages feeding on mice (S-M) and geckos (S-G). Differential expression of seven transcripts was evaluated by RT-qPCR method. (A) The obtained C_T values were relatively quantified according to the $2^{-\Delta\Delta CT}$ method [45] using actin and G3PDH as the reference transcripts. The relative gene expression values of all samples were calibrated to mean of C_T values measured for each transcript from S-G lineage. Each column of chart contains relative expression of one differential expressed transcript (DET1–8) for both S-G (orange points) and S-M (green points) cDNA, measured in three independent replicates. The mean of relatively quantified values is depicted by horizontal line in black color. The expression values below the red line were under the detection RT-qPCR limit (C_T = 40). The expression difference between S-G and S-M were compared by t-test and p-values are shown above the square bracket. (B) The C_T values for the reference transcripts (actin and G3PDH) for both S-G (orange points) and S-M (green points) cDNA, measured in three independent replicates.

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The electrophoretic separation of both SM and S-G salivary gland proteins revealed very similar profiles. The only visible difference was a 40-45 kDa band (red arrow on Fig 12), enriched in the S-G sample.

Apyrase assay

The apyrase activities were similar in the SGHs from both *S. schwetzi* lineages. The maximum of substrate hydrolysis at pH 8.5 was identical for both lineages and both substrates (S19 Fig). Mean ATPase and ADPase activities determined per a pair of salivary glands as well as per milligram of total proteins are summarized in Table 3.

Hyaluronidase activity

The analysis by SDS PAGE zymography on hyaluronan substrate gels revealed pronounced hyaluronidase activities in saliva of both lineages. Both enzymes were visualized as monomers, with Mw about 43–44 kDa in non-reduced environment, and remained active under reducing conditions, with estimated Mw at 50 kDa. However, the degradation of HA substrate was substantially higher in SGHs from the lineage maintained on mice (Fig 13A).

The assay on microtitration plates showed maximum of HA hydrolysis at pH 5.0 in both lineages studied however, significant differences were detected in measured enzyme activities. At the optimal pH, hyaluronidase activity of the S-M lineage was about 30% higher than the activity of the S-G lineage (Table 3). Moreover, elevated hyaluronidase activity in the S-M lineage was detectable within a broad pH range, from 4.0 to 7.5 (Fig 13B).

Discussion

This is the first study describing salivary components of phlebotomine sand fly of the genus *Sergentomyia*. Previously, sand fly salivary gland transcriptomes have been published for 13 sand fly species (reviewed in [11]), all made by sequencing phage cDNA libraries; that

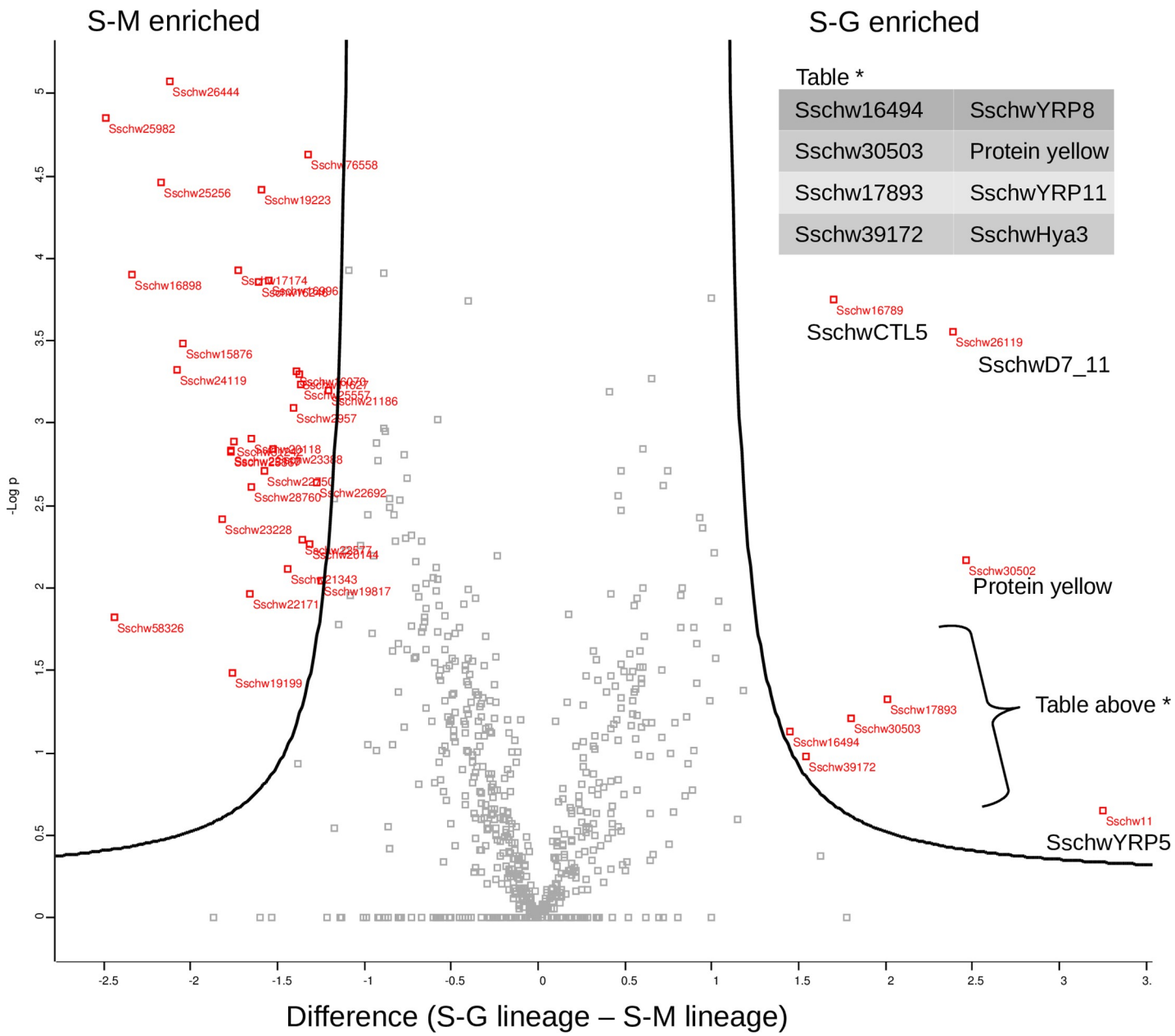

Fig 11. Volcano plot with protein enrichment from mass spectrometry analysis of *S. schwetzi* lineages feeding on mice (S-M) and geckos (S-G). Differences in protein enrichment between S-M and S-G lineage proteome with Student's t-test evaluation. In S-M lineage proteome there are 35 proteins significantly enriched. In S-G lineage proteome are 8 proteins significantly enriched (including six salivary proteins and two non-salivary putative proteins yellow-d).

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technology allowed identification of the low numbers of high quality transcripts and secreted full-length proteins (from 535 to 1,765 transcripts and from 15 to 64 full-length proteins per transcriptome) [13,59]. The only currently available *L.* (*Ny.*) *neivai* sialome and mialome sequences done on Illumina platform have not been published yet. Our BLASTp annotation showed that 51.5% (10,193 sequences) of *S. schwetzi* were homologous to *L.* (*Ny.*) *neivai* proteins.

Main salivary protein families detected in all sand fly species studied so far include yellow-related proteins, D7-related proteins, PpSP15-like proteins, lufaxin proteins, salivary apyrase,

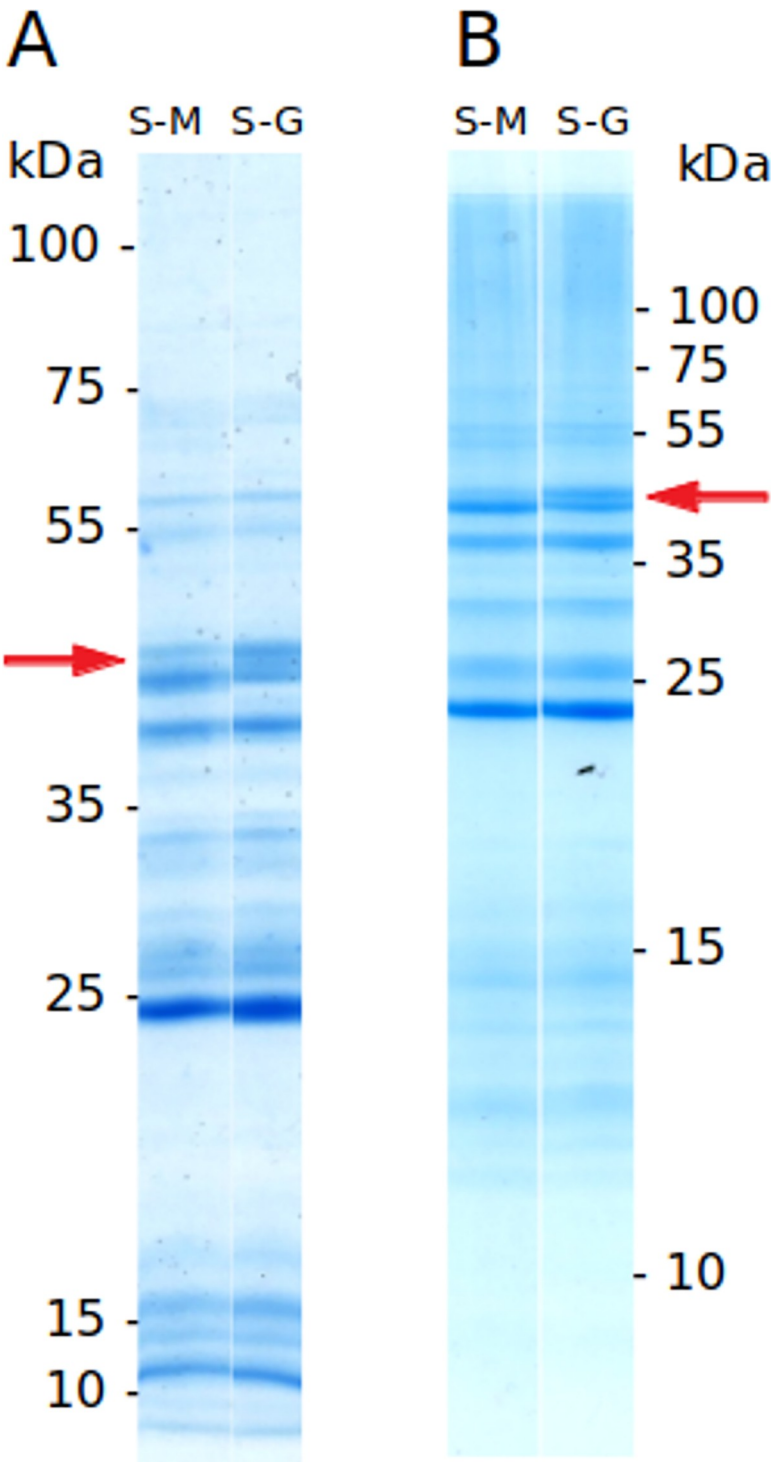

Fig 12. Salivary glands proteins from two *S. schwetzi* lineages maintained on different blood-meal sources geckos (S-G) and mice (S-M): SDS PAGE electrophoresis under reducing conditions. (A) separation on 10% polyacrylamide gel, (B) separation on 15% polyacrylamide gel. Molecular weights in kilodaltons are indicated and red arrow marks the band 40–45 kDa.

https://doi.org/10.1371/journal.pone.0230537.g012

Table 3. Salivary apyrase and hyaluronidase in two *S. schwetzi* lineages maintained on different blood-meal sources, geckos (S-G) and mice (S-M).

		S-G	S-M
Total protein in μg/gland pair*		0.44 ± 0.06	0.42 ± 0.06
Mean specific apyrase activity at 37˚C, pH 8.5*			
mUnits/pair of glands	ATPase	28.40 ± 4.62	29.50 ± 4.20
	ADPase	31.61 ± 4.84	34.26 ± 4.81
Units/mg of total protein	ATPase	64,55 ± 10.5	70.23 ± 10.0
	ADPase	71.84 ± 11.0	81.57 ± 11.5
ATPase/ADPase ratio		0.9	0.86
Mean specific hyaluronidase activity at 37˚C, pH 5.0*			
rTRU/pair of glands		0.3713 ± 0.07	0.5338 ± 0.1
rTRU/mg of total protein		0.844 ± 0.16	1.213 ± 0.24

* Results represent the mean ± SD of ten independent measurements

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and PpSP32-like proteins [59]. Here, in *S. schwetzi*, we demonstrated the presence of all these protein families. In addition, we found several proteins previously considered as specific for the genus *Phlebotomus* among sand flies [60]: particularly salivary pyrophosphatase, phospholipase A2, ParSP25-like proteins and SP16-like proteins. Interestingly, we also found a salivary ribonuclease (partial sequence, QHO60797), which was previously detected only in transcriptomes of mosquitoes salivary glands.

Salivary proteins or peptides previously identified exclusively in transcriptomes of NW sand flies include vasodilatory peptide maxadilan [61], salivary 5′-nucleotidase, an enzyme responsible for cleaving AMP to adenosine [62], SALO anti-complement proteins [63], the

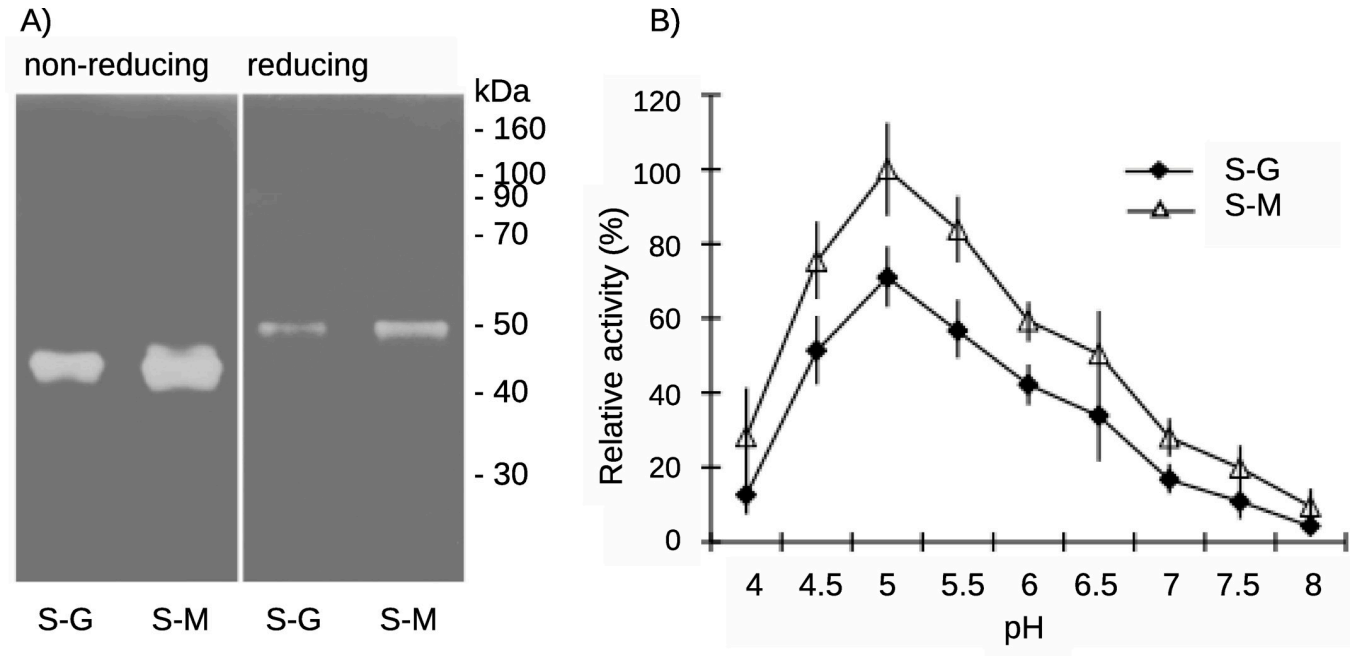

Fig 13. Visualization and comparison of the hyaluronidase activities in two *S. schwetzi* lineages maintained on different blood-meal sources, geckos (S-G) and mice (S-M). (A) Visualization and comparison by SDS PAGE zymography. SDS PAGE gel contained 10% polyacrylamide gel with incorporated 0.002% HA. SGHs of two *S. schwetzi* lineages maintained on different blood meal sources geckos (S-G) and mice (S-M) were tested under non-reducing and reducing conditions. (B) Comparison of the pH dependence of salivary hyaluronidase activity from S-G and S-M lineages. Results represent the mean ± SD of five independent measurements. The difference in relative activities for different buffer systems at pH 7 was lower than 1%.

https://doi.org/10.1371/journal.pone.0230537.g013

RGD-containing peptides with an anti-platelet aggregation function [64] and several protein families with an unknown biological function, like the C-type lectins, 71 kDa salivary protein, spider-toxin-like and ML domain proteins [59,62,65–67]. Even though the *S. schwetzi* is a sand fly species from OW, we identified in its salivary gland transcriptome salivary 5'-nucleotidase, 71 kDa salivary protein and the protein containing C-type lectin domain.

Three sequences found homologous to protein with C-type lectin domain (SschwCTL4, SschwCTL5 and SschwCTL6) which had high expression values in transcriptome (S3 Table), were detected also in the proteome (S4 Table). Usually, the proteins with C-type lectin domain are immunity-related molecules associated with an activation of complement system (reviewed in [68]). On the other hand, the snake venom C-type lectins showed anti-coagulation (by binding FX and FIX) and anti-thrombotic (by inhibition of collagen-induced platelet activation) functions [69]. However, the function of sand fly proteins with C-type lectin domain remains unknown.

The phylogenetic and sequence analysis showed that most of the *S. schwetzi* salivary protein sequences are more divergent, compared to the *Lutzomyia* and *Phlebotomus* proteins. However, the results of phylogenetic comparison of *S. schwetzi* salivary protein families (antigen 5-related proteins, lufaxins, YRPs, and apyrases) were in accordance with the established sand fly phylogeny [70,71]. The *S. schwetzi* OBPs showed high sequence divergence, which is together with high gene duplication rates, typical for the sequences belonging to D7-related and PpSP15-like families [60]. On the other hand, *S. schwetzi* salivary enzymes identified in the transcriptome were more conserved, likely due to their enzymatic function. The exception in this trend was found in the *S. schwetzi* endonuclease and phospholipase A2 sequences, which were longer than other sand flies' homologues. Other *S. schwetzi* proteins usually clustered as basal group to other sand fly proteins, and some of the *S. schwetzi* proteins created paraphyletic groups. This phylogenetic trees structure can be caused by selective pressure of different feeding preferences of *Sergentomyia* sand flies, which preferably bite reptiles in contrast to *Lutzomyia* and *Phlebotomus* sand flies, which feed mostly on mammals. Despite all *Sergentomyia* species studied prefer reptiles, species-specific differences were described [72]. In Ethiopia, *S. schwetzi* feed not only on reptiles but also on mammals, Gebresilassie *et al.* 2015 found engorged females on cow, donkey and goat [73], while Yared and colleagues detected human and canine blood in *S. schwetzi* [74].

In *S. schwetzi* transcriptome we identified one homologue of salivary hyaluronidase and three homologues of apyrase. The activities of both these enzymes and their pH optima were already described in *S. schwetzi* saliva by Volfova and Volf (2018) [14]. The sequence of SschwApy1 was conserved in all active sites, but the function of SschwApy2 and SschwApy3 can be questioned due to their amino acids replacements in the penultimate and last position of the active site sequence. Comparison of the salivary apyrase activities between S-G and S-M lineages did not show any significant differences. On the other hand, interesting differences were found in hyaluronidase activities using two methods (SDS PAGE zymography, microtitration plates assay). In both tests, the HA cleavage activity was significantly higher in S-M lineage, the quantitative assay on microtitration plates repeatedly showed about 30% difference in the hyaluronidase activity. The adaptation of sand flies to feed on mammals is associated with the length of their mouthparts and the depth of skin penetration due to the relatively thick and stiff epidermis of mammalian skin [75]. However, various species with a rather short labrum e.g. *P. argentipes* and some *Sergentomyia* species are able to obtain successfully blood from the mammal hosts [76]. Increasing the tissue permeability of mammalian skin, HA degrading enzymes might represent an efficient tool for sand flies originally adapted to non-mammalian hosts to get access to blood.

Yellow-related proteins (YRPs) of *Lutzomyia* and *Phlebotomus* genera are known to have amine-binding function [77,78]. The amine-binding affinity of YRPs depends on the conservation of sequence regions, especially on eleven amino acid residues, which are responsible for creating amine binding pocket and that are conserved across the *Phlebotomus* and *Lutzomyia* species [79]. However, the SschwYRPs sequences forming the amine binding pocket are more variable (S6 Fig), which may result in inability to bind biogenic amines. Thus, SschwYRPs functions would be interesting topic for future studies.

Besides the description of *S. schwetzi* salivary proteins, we focused also on the comparative transcriptomic and proteomic analysis of salivary glands from sand fly females from two lineages maintained on different vertebrate hosts, mice and geckos, animals with different haemostasis. For example, all vertebrates possess C3 component and factor B, which is necessary for creation of C3b-B proconvertase. Reptile complement shares several features with the mammalian one, like activation by both alternative and classical pathway (reviewed in [80]). On the other hand, all reptiles lack genes coding for FXI, which active form (FXIa) is responsible for activation of coagulation factors leading to transformation of prothrombin to thrombin (reviewed in [81]).

The RNA-seq analysis of *S. schwetzi* salivary gland transcriptome revealed two salivary transcripts, one belonging to lufaxin salivary protein families (SschwLuf3) and another to PpSP15-like salivary protein family (SschwSP15_5), which were significantly up-regulated in S-M lineage (S3 Table, S17 Fig). Enrichment of SschwLuf3 was confirmed by proteomic analysis but biological function of the molecule is questionable as its expression is relatively low in both lineages (S3 Table). The SschwSP15_5 transcript up-regulation in S-M lineage might be connected to variability of coagulation pathway between geckos and mice, but its function remains suspicious as its expression was very low (S3 Table).

In order to compare saliva composition between S-G and S-M lineages, we also did SDS-PAGE gels and mass spectrometry of salivary glands. The single visible difference between electrophoretograms of S-M and S-G lineages was found in the Mw between 40 and 45 kDa: the protein band was more pronounced in S-G sample (Fig 12). In this Mw range several sand fly salivary proteins including hyaluronidase, phospholipase A2, pyrophosphatase and YRPs. The best candidate is probably one of three SschwYRPs (SschwYRP5 with highest LFQ intensity value) as their enrichment in S-G lineage was confirmed by mass spectrometry analysis, see below (Fig 11).

The mass spectrometry analysis revealed six salivary proteins significantly enriched in S-G sialome (S18 Fig, S4 Table). Three of enriched proteins were homologous to sand flies' YRPs, but all of them were detected only as the partial sequences (SschwYRP5, SschwYRP8 and SschwYRP11). Last three enriched proteins were partial sequences homologous to sand fly hyaluronidase (SschwHya3), D7-related protein (SschwD7_11) and protein with C-type lectin domain (SschwCTL5) (Fig 11).

As we mentioned above, two different methods revealed higher hyaluronidase activity in the saliva of S-M lineage but the comparison by RNA-seq analysis did not reveal any significant differences in expression of the hyaluronidase transcripts. Therefore, there is a possibility of later expression of SschwHya1 than in first two days after adult emergence. However, the salivary proteins expression dependence on sand fly age was showed only for *P. papatasi* SP44 protein (YRP), while the expression of this protein was higher for younger and lower for older sand flies which were fed on sugar [82]. In contrast, all three homologues of hyaluronidase (one complete and two partial proteins) were more abundant in proteome of S-G lineage, where SschwHya3 was significantly enriched. This contrasting result can be due to coexistence of different homologues of the enzyme, the one enriched in S-G lineage has no or low activity and the second one is fully active. Thus, it is possible that the protein SschwHya3 which is

significantly enriched in S-G lineage, has less or no activity, and the active homologues of enzyme could be SschwHya1 or SscheHya2. Similar trend was recently revealed in salivary yellow-related proteins, where one homologue has high amine-binding activity and the other has very low activity [78]. Furthermore, the difference in hyaluronidase activity can be explained by different glycosylation of SschwHya homologues, but this we were not able to compare, because two of three SschwHya are only partial sequences. This crucial role of glycosylation for hyaluronidase activity was revealed for *L. longipalpis* hyaluronidase [83].

The SschwD7_11 was significantly enriched in S-G proteome (Fig 11) and its transcript was highly up-regulated in S-G transcriptome as well. The function of D7-related proteins, known also as large OBPs, was firstly proved for their mosquito homologues. The *An. stephensi* D7-related protein inhibits the *intrinsic* coagulation pathway by binding FXII and high molecular weight kininogen (HK), which are together with prekallikrein at the beginning of *intrinsic* coagulation pathway [84]. Other homologues of D7-related proteins, from *Ae. aegypti* and *An. gambiae*, are able to bind biogenic amines and eicosanoids [85–87]. Recently, the sand flies' D7-related were identified as binders of cysteinyl leukotrienes and thromboxane A2 [88]. Despite these new findings, we are not able to connect the SschwD7_11 up-regulation to its specific function on reptile haemostasis or coagulation.

In conclusion, our study significantly expands the knowledge on salivary proteins of neglected sand fly genus *Sergentomyia*. Thanks to phylogenetic and sequence analysis we found, that salivary proteins of *S. schwetzi* are more diverse from *Phlebotomus* and *Lutzomyia* homologues which can be due to adaptation to preferable vertebrate host–reptiles. To support this theory, we compared two *S. schwetzi* lineages adapted to different hosts (gecko and mice). This comparison revealed significantly higher hyaluronidase activity, which can be caused by different properties of mice's skin comparing to geckos'. Further we showed an up-regulated expression of transcripts for PpSP15-like protein and lufaxin in mice lineage, which might be due to different haemostasis of these two animals. Last but not least, the transcriptomic analysis also demonstrated unique salivary secreted ribonuclease, the enzyme previously found only in mosquitoes.

Supporting information

S1 Fig. Raw gels image for Figs 12 and 13.
(PDF)

S2 Fig. Functional gene ontology (GO) classification of the *S. schwetzi* salivary gland transcriptome. The percentage and distribution of top-level GO-terms are portrayed in the three categories: cellular component, molecular function and biological process. Transcripts annotated from whole transcriptome sequences dataset are indicated by blue bar (7,749 sequences) and transcripts annotated from arthropod sequences subset are in yellow (5,937 sequences).
(PDF)

S3 Fig. Multiple sequence alignment of sand flies' antigen 5-related proteins. Multiple sequence alignment of *S. schwetzi* antigen 5-related proteins with chosen sand flies' antigen 5-related proteins. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.ser–*P. sergenti*, P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.pap–*P. papatasi*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.aya–*L. ayacuchensis*, L. olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Conserved cysteines residues are highlighted in green, putative glycosylation sites in SschwAg5r sequence are highlighted in blue. Lines below the alignment indicate conserved cysteines residues by "$", glycosylation by "N" for N-

glycosylation and by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S4 Fig. Multiple sequence alignment of sand flies' lufaxin proteins. Multiple sequence alignment of *S. schwetzi* lufaxin proteins with chosen sand flies' lufaxin proteins. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.ser–*P. sergenti*, P.per–*P. perniciosus*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.pap–*P. papatasi*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.aya–*L. ayacuchensis*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Conserved cysteines residues are highlighted in green, putative glycosylation sites in SschwLuf sequences are highlighted in blue. Lines below the alignment indicate conserved cysteines residues by "$", glycosylation by "N" for N-glycosylation and by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S5 Fig. Multiple sequence alignment of sand flies' D7-related proteins. Multiple sequence alignment of *S. schwetzi* D7-related proteins with chosen sand flies' D7-related proteins. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.ser–*P. sergenti*, P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.pap–*P. papatasi*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.aya–*L. ayacuchensis*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Conserved cysteines residues are highlighted in green, putative glycosylation sites in SschwD7 sequences are highlighted in blue. Lines below the alignment indicate conserved cysteines residues by "$", glycosylation by "N" for N-glycosylation and by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S6 Fig. Multiple sequence alignment of *S. schwetzi* PpSP15-like proteins. Multiple sequence alignment of *S. schwetzi* PpSP15-like proteins with two chosen *P. papatasi* SP15-like proteins. Name of sequence include sand fly species shortcut (P.pap–*P. papatasi*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Conserved cysteines residues are highlighted in green, putative glycosylation sites in SschwSP15 sequences are highlighted in blue. Lines below the alignment indicate conserved cysteines residues by "$" and for SschwSP15_2 the duplication of cysteines motive by "^", glycosylation by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S7 Fig. Multiple sequence alignment of sand flies' yellow-related proteins. Multiple sequence alignment of *S. schwetzi* YRPs with chosen sand flies' YRPs. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.ser–*P. sergenti*, P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.pap–*P. papatasi*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.aya–*L. ayacuchensis*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Conserved cysteines residues are highlighted in green, putative glycosylation sites in SschwYRPs sequences are highlighted in blue, putative amine binding residues are highlighted in orange. Lines below the alignment indicate amine binding site by "A", conserved cysteines residues by "$", glycosylation by "N" for N-glycosylation and by "O" for O-

glycosylation and consensus sequence. Alignment was made by MAFFT with LINS-i method and visualized in Jalview.
(PDF)

S8 Fig. Multiple sequence alignment of sand flies' apyrases. Multiple sequence alignment of *S. schwetzi* apyrases with chosen sand flies' apyrases. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.ser–*P. sergenti*, P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.pap–*P. papatasi*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.aya–*L. ayacuchensis*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwApys sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", substrate binding site by "B", glycosylation by "N" for N-glycosylation and "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S9 Fig. Multiple sequence alignment of sand flies' hyaluronidases. Multiple sequence alignment of *S. schwetzi* hyaluronidase with sand flies' hyaluronidases. Name of sequence include sand fly species shortcut (P.tob–*P. tobbi*, P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ara–*P. arabicus*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwHya1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", glycosylation by "N" for N-glycosylation and "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S10 Fig. Multiple sequence alignment of sand flies' 5'-nucleotidases. Multiple sequence alignment of *S. schwetzi* 5'-nucleotidase with other sand flies' 5'-nucleotidases. Name of sequence include sand fly species shortcut (L.lon–*L. longipalpis*, L.nei–*L. neivai*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in Sschw5nuc1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", glycosylation by "N" for N-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S11 Fig. Multiple sequence alignment of sand flies' adenosine deaminases. Multiple sequence alignment of *S. schwetzi* and other sand flies' adenosine deaminases. Name of sequence include sand fly species shortcut (P.per–*P. perniciosus*, P.dub–*P. duboscqi*, L.lon–*L. longipalpis*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwADA1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", glycosylation by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S12 Fig. Multiple sequence alignment of sand flies' amylases. Multiple sequence alignment of *S. schwetzi* amylases and other sand flies' amylases. Name of sequence include sand fly species shortcut (P.ara–*P. arabicus*, P.pap–*P. papatasi*, L.lon–*L. longipalpis*, L.nei–*L. neivai*) and

GenBank accession number or UniProtKB accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwAmy sequences are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", glycosylation by "N" for N-glycosylation and "O" for O-glycosylation and consensus sequence. For easier visualization two parts of sequence *L. longipalpis* (A0A1B0CMM1) were hidden (highlighted by blue vertical lines with arrows, number of hidden aa is displayed below the alignment). Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S13 Fig. Multiple sequence alignment of sand flies' endonucleases. Multiple sequence alignment of *S. schwetzi* and other sand flies endonucleases. Name of sequence include sand fly species shortcut (P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*, P.arg–*P. argentipes*, L.lon–*L. longipalpis*, L.int–*L. intermedia*, L.olm–*L. olmeca*, L.nei–*L. neivai*) and GenBank accession number or UniProtKB accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwEnuc1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", glycosylation by "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S14 Fig. Multiple sequence alignment of sand flies' phospholipases A2. Multiple sequence alignment of *S. schwetzi* phospholipase A2 with other sand flies' phospholipases A2. Name of sequence include sand fly species shortcut (P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ari–*P. ariasi*, P.ara–*P. arabicus*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwPLA2_1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", glycosylation by "N" for N-glycosylation and "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S15 Fig. Multiple sequence alignment of sand flies' pyrophosphatases. Multiple sequence alignment of *S. schwetzi* pyrophosphatase with other sand flies pyrophosphatases. Name of sequence include sand fly species shortcut (P.per–*P. perniciosus*, P.ori–*P. orientalis*, P.ara–*P. arabicus*, P.dub–*P. duboscqi*, P.arg–*P. argentipes*) and GenBank accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in SschwPP1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&", glycosylation by "N" for N-glycosylation and "O" for O-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S16 Fig. Multiple sequence alignment of sand flies' 71 kDa-like proteins. Multiple sequence alignment of *S. schwetzi* 71 kDa-like protein with other sand flies 71 kDa-like proteins. Name of sequence include sand fly species shortcut (L.lon–*L. longipalpis*, L.aya–*L. ayacuchensis*, L. olm–*L. olmeca*) and GenBank accession number or UniProtKB accession number. Sequence conservation is depicted by shading of purple color. Active sites of enzyme are highlighted in orange, putative glycosylation sites in Sschw71kDa1 sequence are highlighted in blue. Lines below the alignment indicate active site of enzyme by "A", metal binding site by "&",

glycosylation by "N" for N-glycosylation, "O" for O-glycosylation, "C" for C-glycosylation and consensus sequence. Alignment was made by MAFFT with L-INS-i method and visualized in Jalview.
(PDF)

S17 Fig. RNA-seq transcriptome analysis of *S. schwetzi* salivary glands.
(PDF)

S18 Fig. Proteome analysis of *S. schwetzi* salivary glands.
(PDF)

S19 Fig. Comparison of ATPase and ADPase activities and their pH optima in two *S. schwetzi* lineages. Comparison of ATPase and ADPase activities and their pH optima in two *S. schwetzi* lineages maintained on different blood-meal sources, geckos (S-G) and mice (S-M). Results represent the mean of five independent measurements.
(PDF)

S1 Table. PCR and RT-qPCR primer sequences and reaction conditions.
(XLSX)

S2 Table. The annotation of *S. schwetzi* salivary proteins.
(XLSX)

S3 Table. Differential gene expression analysis (RNA-seq) of *S. schwetzi* transcripts.
(XLSX)

S4 Table. *S. schwetzi* salivary gland proteome analysis.
(XLSX)

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

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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 kratagonist 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 predicted 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.

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 20,000 and 56,700, in Mediterranean and East African regions, respectively (Alvar et al., 2012).

To facilitate successful blood feeding, sand fly female 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 flies employ anticoagulants which affect the components of coagulation cascade (Chagas et al., 2014; Collin et al., 2012) or inhibit the activators of coagulation (Alvarenga et al., 2013). Some sand fly saliva components such as maxadilan (Lerner and Shoemaker, 1992) and adenosine (Ribeiro et al., 1999) act directly as vasodilatators, while other proteins, such as salivary apyrase prevent the ATP-induced aggregation of platelets (reviewed in Lestinova et al., 2017).

Host haemostatic responses to insect bites are triggered also by biogenic amines such as serotonin, histamine or catecholamines. Serotonin is released by platelets and initiates vasoconstriction resulting in limitation of the flow of blood to the insects mouthparts

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

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(Ribeiro, 1995). Histamine is secreted upon the tissue damage from basophiles and mast cells granules and influences the hydrostatic pressure of capillaries and their permeability for plasma containing immune cells and other factors (Paesen et al., 1999; Ribeiro and Francischetti, 2003). Both serotonin and histamine have also roles in inducing itch and pain response to the insect bite (Julius and Basbaum, 2001; Yosipovitch et al., 2018). Other biogenic amines playing roles in host haemostatic responses belong to the catecholamine family. Norepinephrine stimulates vasoconstriction via adrenergic receptors in the vasculature (Calvo et al., 2009; Xanthos et al., 2008). When released by the local nerves in response to bleeding, epinephrine also initiates constriction of blood vessels and together with serotonin it potentiates platelet aggregation (Andersen et al., 2003; Francischetti, 2010).

Bloodsucking insects prevent haemostatic responses through binding biogenic amines in the pocket of hollow barrel structure forming the amine-binding proteins belonging into three different protein families (reviewed in Lestinova et al., 2017). Lipocalins serve this function in ticks and triatomine bugs (Andersen et al., 2003; Mans and Ribeiro, 2008; Sangamnatdej et al., 2002; Xu et al., 2013), while D7-related proteins are known kratagonists (= abundant proteins that arrest or seize their bioactive agonists; Ribeiro and Arca, 2009) in mosquitoes (Calvo et al., 2006; Jablonka et al., 2019; Mans et al., 2007). The amine-binding potential of sand fly salivary yellow-related proteins (YRPs) was firstly hypothesized 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 (MST) 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 estimate 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.

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 h 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 subcloned 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 YRPs 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

Table 1
Recombinant salivary yellow-related proteins.

Name	Species	MW (kDa)	ε ($M^{-1}cm^{-1}$)	GenBank ACCN	Parallel codes
rSP03	*P. perniciosus*	43.20	51,590	ABA43049	PpeSP03[1], Pper1[2]
rSP03B	*P. perniciosus*	44.26	51,590	ABA43050	PpeSP03B[1], Pper2[2]
rPorASP2	*P. orientalis*	42.96	53,080	AGT96427	Pori1[2], mYEL2[3]
rPorASP4	*P. orientalis*	43.74	53,080	AGT96428	Pori2[2], mYEL1[3], PorSP24[4]
LJM11	*L. longipalpis*	44.67	65,000	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 YRPs are indicated. [1] retrieved from Anderson et al. (2006); [2] Sima et al. (2016b); [3] Sumova et al. (2018); [4] Sima et al. (2016a).

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 h with a monoclonal anti-polyhistidine-peroxidase antibody (Sigma Aldrich) diluted 1:1000 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 vol 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 PepMap 300 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 1400 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 the 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 10–1000 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 Tools 2 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). So far, YRPs were found only in sand fly salivary proteomes. Presence of YRP in the midgut was once reported (Volf et al., 2002), but it seems that the protein was swallowed there with saliva. YRPs 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 validated 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 gland homogenates, 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 (S1 Table;Sumova, 2019 [dataset]). 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 flies 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 scavenge local biogenic amines. YRPs are therefore present in salivary glands in physiologically significant quantities to act as kratagonists.

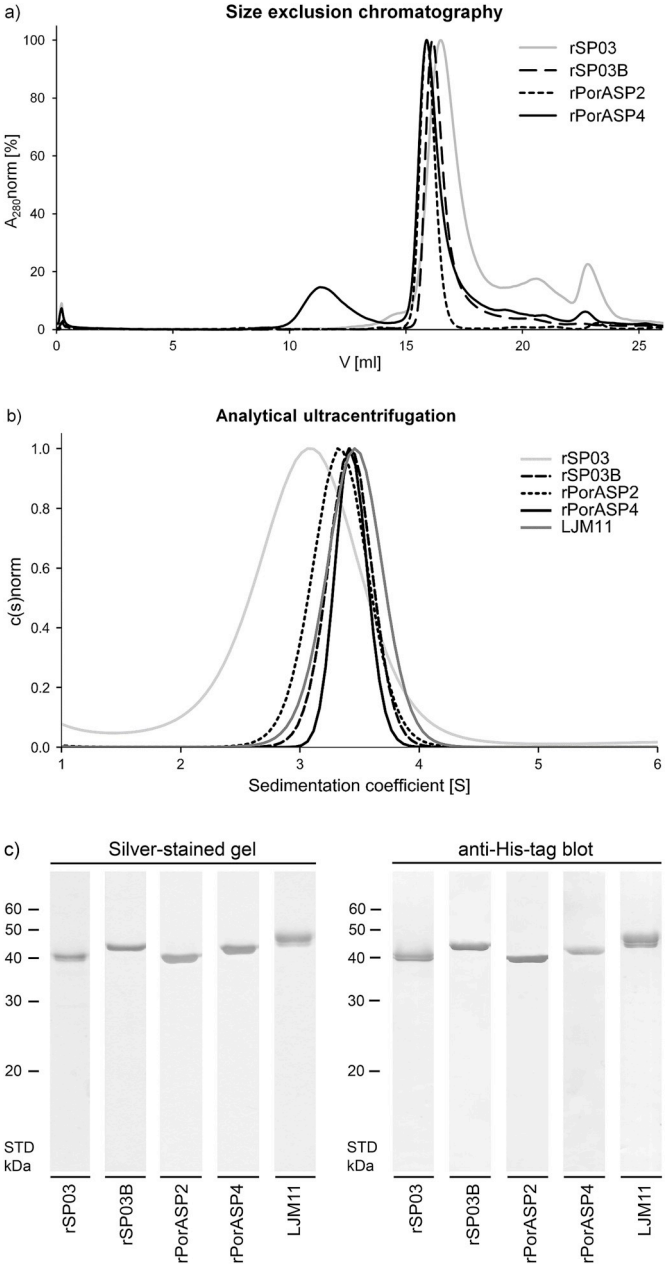

Fig. 1. Purification and quality control of recombinant proteins. a) Representative results of size exclusion chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare); peaks for each protein are shown with distinct type of line (see legend on the right). b) Recombinant YRPs were analyzed in analytical 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.

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 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.

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.

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 (Table 3, square brackets; 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 = 10–1000 nM), suggesting that both sand fly species 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 serotonin (rSP03B and rPorASP4) in sand fly saliva. On the contrary, all tested proteins bound histamine with affinity lower than 1 μM which was indicated as insufficient to prevent interaction of histamine with its physiological receptors (Mans et al., 2008; Xu et al., 2011). To our knowledge, there are recently no other histamine-binding candidates in sand flies. Sand flies D7-related proteins, whose counterparts serve this function in mosquitoes, were recently shown not to contain the essential amine-binding pocket (Jablonka et al., 2019).

The binding affinities for epinephrine, dopamine and octopamine varied among the individual YRPs. When comparing the two *Phlebotomus* species, the binding affinities were in *P. perniciosus* much lower for all these potential ligands. Observed species-specific differences in amine-binding properties could be hardly explained by different feeding behavior as both *Phlebotomus* species studied share similar host preferences. Despite the tight binding of dopamine and octopamine to *P. orientalis* YRPs, these ligands are improbable physiological targets for the salivary YRPs, as they do not play a major role in haemostasis or inflammation (Xu et al., 2011). Therefore, the interspecific difference in binding of these catecholamines is not supposed to have an application in physiological conditions. We may hypothesize that the only physiological activity of sand flies YRPs is to bind serotonin, while binding the other amines might be artifact of their similar structure. Therefore both sand fly species have one functional YRP which binds serotonin with very high affinity.

Table 2
Recombinant YRPs amino acid sequence identity.

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

Percent identity matrix of recombinant YRPs created in Clustal Omega (Sievers et al., 2011).

3.4. Amine-binding site

The YRPs amine-binding site was formerly determined from LJM11 crystals with added serotonin (Xu et al., 2011). The site was shown to be composed by 11 amino acids from which eight bound biogenic amines through van der Waals forces and hydrophobic interactions. Five of these amino acids are conserved in all YRPs modeled up to date. Three extra amino acids (Thr327, Asn 342 and Phe 344) of the LJM11 binding motif were able to bind serotonin not only by above mentioned non-covalent intermolecular interactions, but also by hydrogen bonds (Fig. 3), which are predicted to play major role in the binding event (Sima et al., 2016b; Xu et al., 2011).

We have modeled the major amino acids together with their hydrogen bond interactions in the amine-binding site of all YRPs tested (Fig. 3). In *P. orientalis* rPorASP4, the binding motif differed only in one amino acid (position 344; Gln instead of Phe) from *L. longipalpis* LJM11, though the amino acid sequence identity of these two proteins is only 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

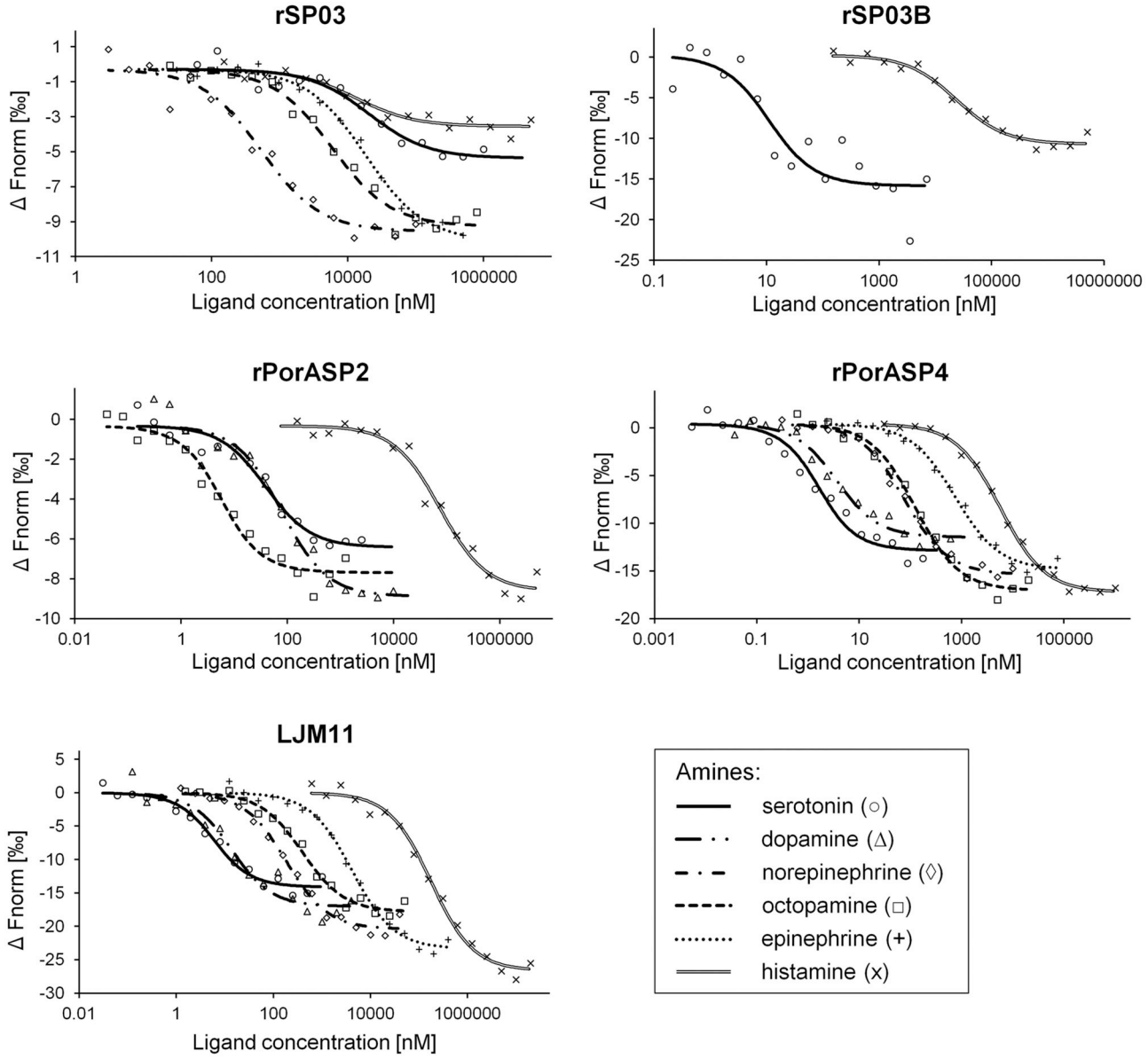

Fig. 2. MST showing binding of biogenic amines to each YRP. Graphs show the MST data fitted with Kd model binding curves for each YRP and all the biogenic amines it bound. For the amines for which no binding was detected, no graphical visualization is shown. Each curve and data point represents an average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

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 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 larger and therefore closer to the

Table 3
Amine-binding properties of recombinant YRPs.

YRP	Ligand	Kd (nM)	Kd CI	Amplitude	Signal to Noise
rSP03	Serotonin	20,558	± 7062	5.1	10.0
	Dopamine	> 20,000	NA	NA	NA
	Norepinephrine	534	± 131	9.3	13.1
	Epinephrine	18,418	± 1920	9.8	33.6
	Octopamine	5935	± 1160	9.0	17.1
	Histamine	13,138	± 4431	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	± 4602	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	5753	± 378	17.6	48.7
LJM11	Serotonin	4.5 [4.3]	± 1.1	14.1	15.5
	Dopamine	12.4 [12]	± 4.0	17.0	10.5
	Norepinephrine	182 [63]	± 24.6	20.4	23.8
	Epinephrine	4212 [454]	± 465	23.4	29.3
	Octopamine	396 [217]	± 61.9	17.8	20.6
	Histamine	178,801 [> 1000]	± 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. The dissociation constants measured previously for protein LJM11 by ITC (Xu et al., 2011) are presented in square brackets. NB – no measurable binding interaction, NA – not applicable.

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

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

3.5. 3D models and electrostatic potential of YRPs

L. longipalpis YRPs share a similar six-bladed β-propeller fold with all sand fly YRPs studied up to date, which were all shown by homology modeling to comprise a ligand-binding site within their barrel structure (Sima et al., 2016b). The electrostatic potential of the *P. perniciosus*, *P. orientalis* and *L. longipalpis* YRPs surface was compared on their 3D models (Fig. 4). The models of both entrance sides of the six-bladed β-propeller structure showed that the cavity of the channel has in all cases negative charge, which enables the binding of positively charged bioamines in all YRPs studied. This is in accordance with the negatively charged amine-binding site of structurally similar D7 proteins in mosquitoes (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 rSP03B 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.

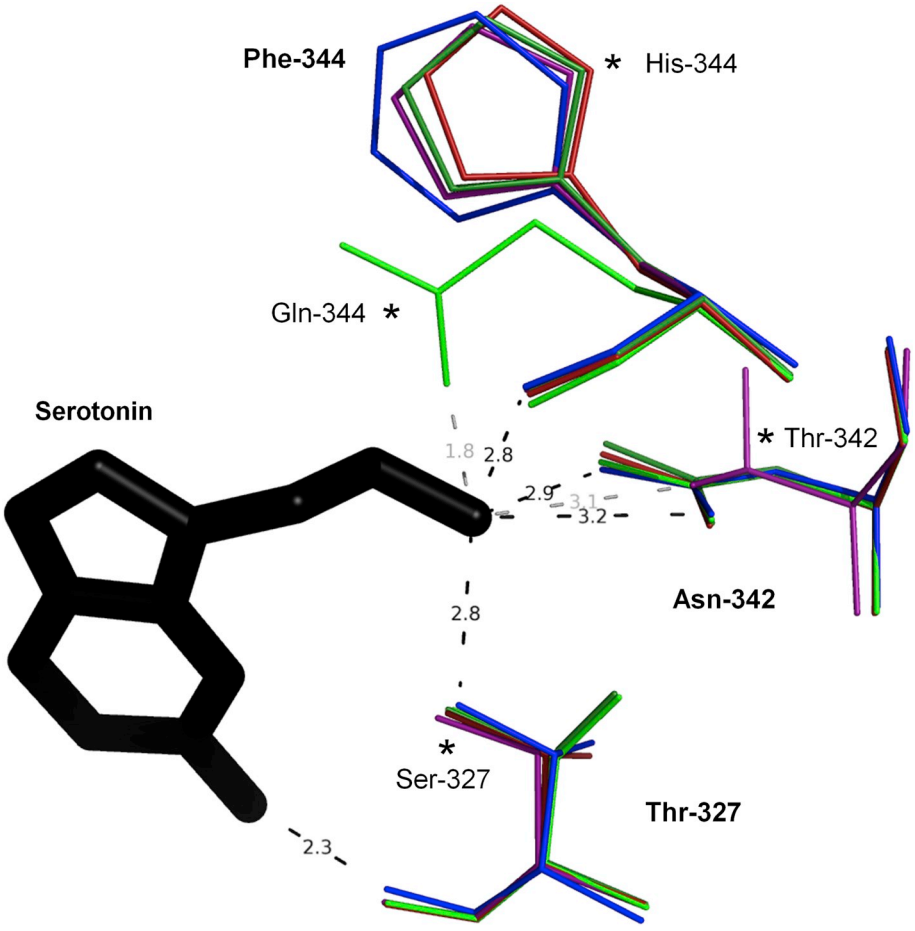

Fig. 3. Amine-binding pocket of YRPs. The model depicts the relevant amino acids of the serotonin binding site for both *P. perniciosus* (rSP03 - red, rSP03B - violet) and *P. orientalis* (rPorASP2 - dark green, rPorASP4 - light green) YRPs and *L. longipalpis* protein LJM11 (blue). Numbering of amino 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

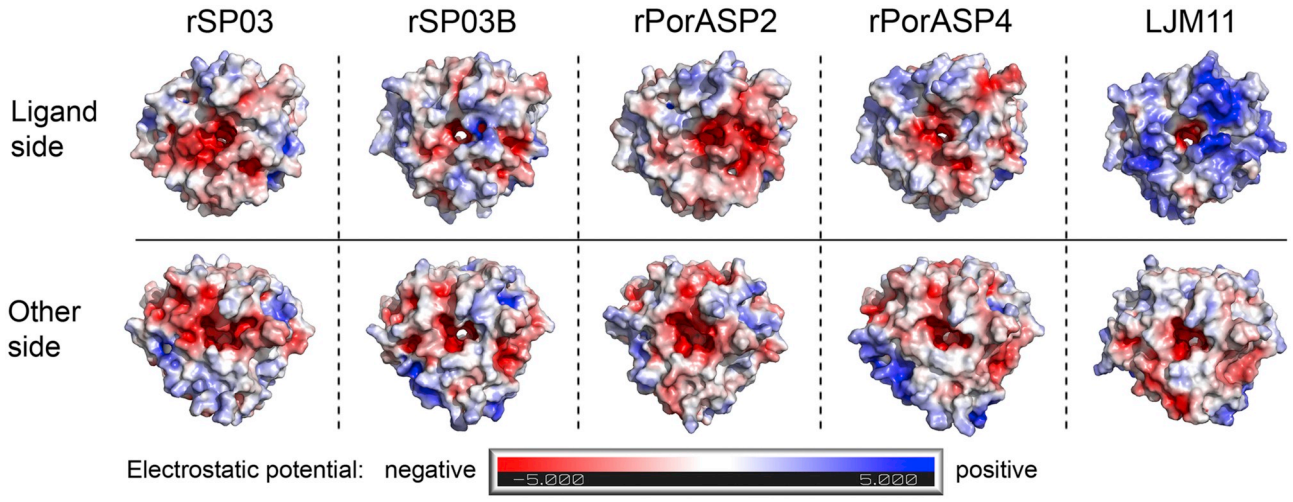

Fig. 4. Electrostatic potential at YRPs channel entrances. Models depict each recombinant YRP from side closer (ligand side) and further (other side) to the ligand-binding site inside the channel. Color and its brightness correspond to the scale of negative (red) to positive (blue) charge. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Interactions between host biogenic amines and sand fly salivary yellow-related proteins

Tatiana Spitzova*, Petra Sumova, Vera Volfova, Nikola Polanska, Luisa Poctova and Petr Volf

Abstract

Background: During blood feeding, sand flies inoculate salivary proteins that interact with the host haemostatic system. The blocking of biogenic amines such as serotonin and histamine helps to limit vasodilatation and clot formation, and thus enables the insect to finish the blood-feeding process. In sand flies, an amine-binding ability is known only for the yellow-related proteins of *Phlebotomus* and *Lutzomyia* vectors, but not yet for members of the genus *Sergentomyia*.

Methods: The ability of *Phlebotomus argentipes* and *Sergentomyia schwetzi* recombinant yellow-related salivary proteins to bind histamine and serotonin was measured by microscale thermophoresis. Both sand fly species were also fed through a chicken-skin membrane on blood mixed with histamine or serotonin in order to check the effects of biogenic amines on sand fly fitness. Additionally, fecundity and mortality were compared in two groups of *P. argentipes* females fed on repeatedly-bitten and naive hamsters, respectively.

Results: The *P. argentipes* recombinant yellow-related protein PagSP04 showed high binding affinity to serotonin and low affinity to histamine. No binding activity was detected for two yellow-related proteins of *S. schwetzi*. Elevated concentrations of serotonin significantly reduced the amount of eggs laid by *P. argentipes* when compared to the control. The fecundity of *S. schwetzi* and the mortality of both sand fly species were not impaired after the experimental membrane feeding. Additionally, there were no differences in oviposition or mortality between *P. argentipes* females fed on immunized or naive hamsters.

Conclusions: Our results suggest that in natural conditions sand flies are able to cope with biogenic amines or anti-saliva antibodies without any influence on their fitness. The serotonin binding by salivary yellow-related proteins may play an important role in *Phlebotomus* species feeding on mammalian hosts, but not in *S. schwetzi*, which is adapted to reptiles.

Keywords: *Phlebotomus argentipes*, *Sergentomyia schwetzi*, Serotonin, Histamine, Oviposition, Mortality, Yellow-related proteins, Anti-saliva antibodies

Background

Females of phlebotomine sand flies (Diptera: Psychodidae) feed on blood in order to complete egg development. During blood-feeding they inoculate salivary proteins into the skin that counteract the host haemostatic system [1]. Biogenic amines, such as, histamine and serotonin, are crucial molecules for host haemostasis. Histamine is commonly associated with an immediate-type hypersensitivity response (i.e. increased vascular permeability and vasodilatation) and chemoattractant activity. This amine is produced by a wide variety of cell types (e.g. mast cells, basophils) [2]. Serotonin plays a role in numerous physiological processes, among others as an inflammatory modulator, vasoconstrictor and contributor to clot formation. Circulating platelets are the main storage site for peripheral serotonin [3].

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Bloodsucking arthropods have developed various salivary molecules to cope with biogenic amines such as lipocalins in ticks [4] and triatomids [5] and D7 proteins in mosquitoes [5]. In sand flies, the ability to bind host biogenic amines has only been described for the family of salivary yellow-related proteins (YRPs) [6, 7]. Proteins of this family are found exclusively in insects and are abundant in phlebotomine sialomes with high variability in the number of YRPs among different sand fly species [1, 8]; they show a similar structure with some intraspecific modifications that influence the ligand-binding abilities [9].

In repeatedly-bitten hosts, sand fly saliva also stimulates the production of high levels of species-specific anti-saliva antibodies [1]. According to some authors, these antibodies could have an impact on sand fly fecundity and mortality [10–12]; however, other studies have not found any significant effects [13–15].

In this study, we focused on two sand fly species, *Phlebotomus argentipes* and *Sergentomyia schwetzi*. *Phlebotomus* (*Euphlebotomus*) *argentipes* is the most important vector of visceral leishmaniasis in Asia [16], with a mainly zoophilic feeding behaviour and a preference to feed on humans as the second choice [17]. This study of the amine-binding properties of its yellow-related protein adds to previously published data on other visceral leishmaniasis vectors in America, Europe and Africa [6, 7]. *Sergentomyia schwetzi* is the only representative of the genus *Sergentomyia* available in laboratory colonies worldwide [18]. *Sergentomyia* species prefer to feed on reptiles [19], and to our knowledge, this is the first study to describe the *S. schwetzi* salivary yellow-related proteins and their role in feeding processes.

The main aims of the study were (i) to compare the ability of *P. argentipes* and *S. schwetzi* yellow-related proteins to bind biogenic amines, particularly histamine and serotonin; (ii) to clarify if the fecundity and mortality of *P. argentipes* and *S. schwetzi* could be affected by biogenic amines present in blood using membrane feeding; and (iii) to study if high levels of anti-*P. argentipes* saliva antibodies in repeatedly-bitten hamsters interfere with *P. argentipes* fecundity and mortality.

Methods

Sand flies and laboratory rodents

Laboratory colonies of *P. argentipes* originating from India and *S. schwetzi* originating from Ethiopia were maintained in the insectary of the Department of Parasitology, Charles University, under standard conditions (at 26 °C, fed on 50% sucrose, with a 14 h:10 h light:dark photoperiod) as described by Volf and Volfova [20]. The hamsters used were 3-month-old Syrian hamsters (*Mesocricetus auratus*) kept in the animal facility of the Department of Parasitology, Charles University.

Expression of recombinant yellow-related proteins

For biogenic amine-binding experiments, one *P. argentipes* and two *S. schwetzi* salivary yellow-related proteins were expressed in a human cell line (Table 1). The gene constructs were prepared by isolating the total RNA from one-day-old females using a High Pure RNA Tissue Kit (Roche, Prague, Czech Republic), then the cDNA was synthesised with the anchored-oligo $(dT)_{18}$ primers using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's protocol. The requested transcripts were amplified from cDNA by PCR and subcloned into the pTW5sec expression plasmid, a derivative of pTT5 [21, 22]. Proteins expressed using this plasmid contain additional ITG- and -GTHHHHHHHHG amino sequences at their N- and C-termini, respectively. Proteins were then transiently expressed in the human embryonic kidney 293S (HEK293S) GnTI- cell line (ATCC CRL-3022), as previously described [6, 21, 23].

All recombinant yellow-related proteins were purified by IMAC chromatography using HiTrap Talon Crude columns (GE Healthcare, Prague, Czech Republic) followed by size exclusion chromatography using Superdex 200 Increase 10/300 GL column (GE Healthcare). Proteins were subsequently stored in phosphate-buffered saline (PBS; pH 7.5). Protein concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Prague, Czech Republic) at 280 nm and calculated using the theoretical molar extinction coefficients and molecular weights of the proteins (Table 1). The identity and purity of the proteins were further verified by mass spectrometry.

Microscale thermophoresis

Microscale thermophoresis (MST) was used to measure the binding affinities between recombinant yellow-related proteins and their potential ligands, serotonin and histamine. The MST affinity experiments were performed as described in [6] with minor modifications.

The highly pure recombinant yellow-related proteins were fluorescently labelled by a Monolith His-Tag Labeling Kit RED-tris-NTA 2nd Generation (Nanotemper, Munich, Germany) according to the manufacturer's

Table 1 Recombinant salivary yellow-related proteins

Name	Species	MW (kDa)	ε ($M^{-1}cm^{-1}$)	GenBank ID
PagSP04	*P. argentipes*	44.92	62020	ABA12136.1
SschwYRP1	*S. schwetzi*	42.51	57090	QHO60691.1
SschwYRP3	*S. schwetzi*	45.34	70103	QHO60693.1

Notes: List of recombinant yellow-related proteins based on the salivary proteins of *P. argentipes* and *S. schwetzi*. The name, species, molecular weight (MW), extinction coefficient (ε) and GenBank accession numbers are indicated

instructions. Fluorescent YRPs were then diluted to 40 nM concentration (corresponding to 1.73 µg/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 remove protein aggregates. Serotonin (Sigma-Aldrich, Prague, Czech Republic) and histamine (Sigma-Aldrich) were dissolved in MST buffer. For each tested recombinant YRP, a titration series with a constant concentration of fluorescently labelled YRP and an equal amount of a two-fold dilution series of a single unlabelled ligand were prepared in the MST buffer. Binding experiments were performed on a Monolith NT.115 PicoRed (Nanotemper).

Membrane feeding with histamine and serotonin

Phlebotomus argentipes and *S. schwetzi* females (5–7 days-old) were fed through a chick-skin membrane by the standard method described by Volf & Volfova [20]. From 100 to 120 female sand flies were used for each group. Histamine and serotonin were dissolved in 200 µl of physiological saline to concentrations of 0.3 mg/ml and 0.07 mg/ml, respectively, and mixed with 3 ml of defibrinated rabbit blood. In order to emphasize the effect of biogenic amines on the sand fly fitness, we decided to use elevated "non-physiological" concentrations for both amines [24, 25]. A blood mixture with saline only was used as a negative control. The experiment with serotonin and *P. argentipes* was repeated twice, to confirm differences between the experimental and naive groups. Engorged sand flies were maintained in cages under standard conditions until defecation.

Feeding on repeatedly exposed hamsters

Hamsters of both sexes were randomly assigned to two groups of 6 animals each. In the first group, anesthetized animals (ketamine 50 mg/kg and xylazine 2 mg/kg, intramuscularly) were exposed to 100–290 *P. argentipes* females six-times at 7–15-day intervals. The second group served as a negative control. One week after the last exposure, hamsters from each group were exposed to 100 *P. argentipes* females (5–7 days-old) for 45 min. Engorged sand flies were maintained in cages under standard conditions until defecation.

Sera were collected from anesthetized animals from both groups one week after the last exposure to sand flies and stored at − 80 °C until use.

Oviposition and mortality monitoring

After defecation (3 and 5 days after blood-feeding for *P. argentipes* and *S. schwetzi*, respectively), females were individually separated into small glass vials equipped with wet filter paper, closed with fine gauze and allowed to oviposit. All vials were placed into a single plastic box with its base filled with wet filter paper to ensure a uniform microclimate [18]. The humidity, mortality and occurrence of eggs were checked daily for the next 5 days, and laid eggs were counted at the end of the experiment.

Detection of anti-*P. argentipes* IgG

Anti-*P. argentipes* IgG were measured by an enzyme-linked immunosorbent assay (ELISA) as described in [26] with minor modifications. Briefly, microtiter plates were coated with salivary gland homogenate (SGH) (0.2 salivary gland per well) obtained as described in [26]. Hamster sera were diluted 1:100 in 2% (w/v) low fat dry milk with 0.05%Tween-20 (PBS-Tw), and secondary antibodies (anti-hamster IgG, AbD Serotec) were diluted 1:1000 in PBS-Tw. Each serum was tested in duplicate. Absorbance values were reported as optical densities (ODs) with a subtracted blank (value in the control wells).

Statistical analysis

Statistical analyses were carried out using R software (http://cran.r-project.org/). Differences in oviposition between groups were tested by fitting generalised linear models (GLM) with quasi-poisson distribution. Differences in mortality between groups were analysed by a 2-sample test for equality of proportions. A *P*-value of < 0.05 was considered to indicate statistical significance.

For the MST experiments, the Kd (dissociation constant) model binding curves were fitted to the average of three independent repetitions of each measurement. The Kd-values, confidence intervals, amplitudes and the signal-to-noise levels were calculated using the NanoTemper analytical software package.

Results

Ligand binding analysis using microscale thermophoresis

The amine-binding properties of *P. argentipes* and *S. schwetzi* YRPs measured by MST are visualized in Fig. 1, and the binding parameters are summarized in Table 2. *Phlebotomus argentipes* yellow-related protein PagSP04 bound serotonin with high affinity (Kd = 86.9 nM), while it had only a low affinity for histamine (Kd = 9.3 µM). On the contrary, neither of the *S. schwetzi* YRPs tested had detectable binding affinities for either biogenic amine.

Feeding on biogenic amines: effects on sand fly oviposition and mortality

Results from the experimental feeding of *P. argentipes* and *S. schwetzi* on histamine and serotonin are summarized in Table 3. In total, 358 *P. argentipes* and 178 *S. schwetzi* females were separated individually into

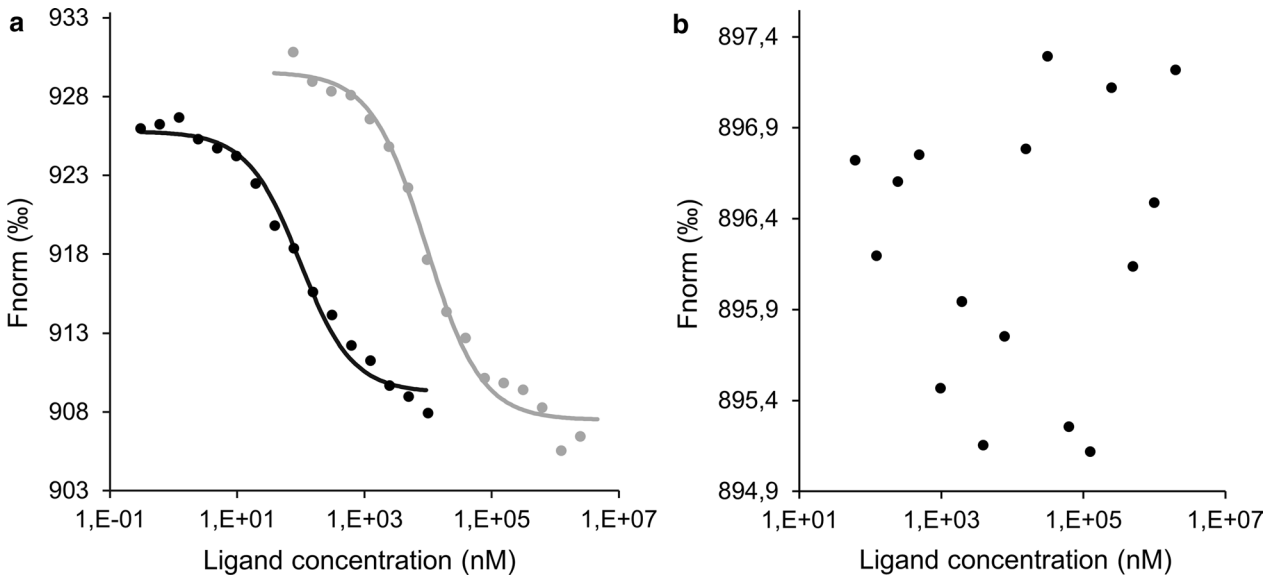

Fig. 1 Amine-binding properties of *Phlebotmus argentipes* and *Sergentomyia schwetzi* yellow-related proteins. **a** Kd model binding curves of *P. argentipes* yellow-related protein. The binding curves for serotonin and histamine are depicted in black and grey, respectively. Each curve and data point represent the average of three independent experiments. **b** An example of non-measurable binding interaction for *S. schwetzi* yellow-related proteins

Table 2 Amine-binding properties of recombinant yellow-related proteins

YRP	Ligand	Kd (nM)	Kd 95% CI	Amplitude	Signal to noise
PagSP04	Serotonin	86.9	72.5–101.3	16.6	20.8
	Histamine	9304.5	8042.9–10566.1	22.1	23.7
SschwYRP1	Serotonin	nb	na	na	na
	Histamine	nb	na	na	na
SschwYRP3	Serotonin	nb	na	na	na
	Histamine	nb	na	na	na

Notes: Dissociation constants (Kd; nM), Kd 95% confidence intervals (95% CI), amplitudes and the signal-to-noise ratios for *P. argentipes* and *S. schwetzi* yellow-related proteins

Abbreviations: nb, non-measurable binding interaction, na, not applicable

Table 3 Oviposition and mortality rates of *P. argentipes* and *S. schwetzi* females fed experimentally

	Histamine				Serotonin			
	P. argentipes		*S. schwetzi*		*P. argentipes*		*S. schwetzi*	
	Exp	Con	Exp	Con	Exp	Con	Exp	Con
Perctent ovipositing females[a]	90 (79/88)	89 (82/92)	92 (49/53)	96 (51/53)	96 (97/101)	96 (100/104)	85 (40/47)	88 (38/43)
Median no. of eggs per female (range) [IQR][b]	32 (1–58) [23–40]	31 (1–68) [15–41]	62 (1–128) [47–87]	84 (1–143) [68–101]	32 (1–62) [26–41]	43 (1–74) [33–49]	58 (1–108) [36–70]	70 (2–105) [50–84]
Percent mortality	26 (23/88)	18 (17/92)	72 (38/53)	62 (33/53)	36 (36/101)	27 (28/104)	49 (23/47)	49 (21/43)

[a] Calculated from total sum of blood fed females

[b] The difference between 25th and 75th percentile

Abbreviations: Exp, experimental group; Con, control group; IQR, interquartile range

glass vials to monitor oviposition and mortality. The prevalence of ovipositing *P. argentipes* and *S. schwetzi* females reached very similar numbers regardless of whether sand flies were fed on blood mixed with amines or blood mixed with physiological solution, with percentages ranging between 85–96%. However, the *P. argentipes* females fed on blood mixed with serotonin showed a significant decrease in the number of laid eggs compared to the control group ($t = -4.46$, $df = 195$, $P < 0.001$). The median numbers of eggs laid by the serotonin and control groups were 32 (range: 1–62) and 43 (range: 1–74), respectively. In the other experimental groups, the number of laid eggs was not significantly affected by the presence of biogenic amines in the blood meal.

No significant differences were detected in mortality rates between the experimental and the control groups (Table 3).

Feeding on repeatedly exposed hamsters: effects on sand fly oviposition and mortality

In total, 312 and 318 *P. argentipes* females fed on immunized or naive hamsters, respectively, were separated into glass vials for monitoring of oviposition and mortality. The prevalence of ovipositing females was 58% (181/312) for sand flies fed on immunized hamsters and 60% (192/318) for those fed on naive hamsters (Table 4). There was no significant difference between the numbers of eggs laid by females fed on immunized or naive hamsters. The median number of eggs laid per sand fly female was the same for both groups, 35, with a minimum of 1 egg per female for both groups and maxima of 72 and 74 eggs for the groups fed on immunized and naive hamsters, respectively (Table 4). Immunized hamsters showed very high levels of IgG antibodies against *P. argentipes* salivary proteins: the mean ODs for immunized and naive groups were 2.01 (95% CI: 1.78–2.25) and 0.04 (95% CI: 0.02–0.05), respectively.

At the end of the experiment (on day 8 post-blood meal) the mortality of sand fly females fed on immunized and naive hamsters did not differ ($\chi^2 = 2.85$, $df = 1$, $P = 0.09$), with mortality rates of 63% (197/312) and 70% (222/318), respectively (Table 4).

Table 4 Oviposition and mortality rates of *P. argentipes* females fed on host

	Immunized hamsters	Naive hamsters
Percent ovipositing females[a]	58 (181/312)	60 (192/318)
Median no. of eggs per female (range) [IQR][b]	35 (1–72) [16–48]	35 (1–74) [9–50]
Percent mortality	63 (197/312)	70 (222/318)

[a] Calculated from total sum of blood fed females

[b] The difference between 25th and 75th percentile

Discussion

Sand flies inoculate salivary molecules including yellow-related proteins into their host's skin in order to counteract the host haemostatic system and bind biogenic amines such as serotonin and histamine [6, 7]. *Phlebotomus argentipes* has only a single yellow-related protein, PagSP04 [27], and here we demonstrated that it acts as a poor binder of histamine but as a strong binder of serotonin. Similarly, strong affinities for serotonin and weak affinities for histamine have been shown for yellow related proteins of *P. pernicious*, *P. orientalis* and *L. longipalpis* [6, 7]. Our findings support the hypothesis that by binding serotonin, yellow-related proteins take part in counteracting the mammalian haemostatic system, especially platelet aggregation and vasoconstriction. On the other hand, the role of histamine at the site of bite is questionable, and it seems that in mosquito-induced itching in mice, histamine did not play a primary role [28]. In mosquitoes, D7 salivary proteins were shown to bind histamine in addition to other amines [29], but this has not yet been demonstrated for D7-related proteins in sand flies [30]. In sand flies, the D7 proteins are functionally and structurally similar to mosquito D7 proteins. However, the C-terminal domain of sand fly D7 protein is missing major elements of the putative ligand-binding pocket and therefore is not able to bind small molecule ligands [30].

The membrane feeding of *P. argentipes* on blood mixed with serotonin resulted in reduced fecundity (26% fewer eggs than the control group), which suggests that extremely elevated concentrations of serotonin negatively affect *P. argentipes* oviposition. In repeatedly-bitten hosts, however, serotonin concentrations are probably lower than our experimental concentration, and we expect that sand flies are able to cope with these lower concentrations. This corresponds with the results of experimental feeding on hamsters immunized by repeated sand fly bites: *P. argentipes* females did not show any difference in mortality and numbers of laid eggs when experimental and control groups were compared, despite the high levels of anti-saliva antibodies in repeatedly-bitten hamsters.

So far, studies focused on the effects of anti-saliva antibodies on various biological aspects of sand flies have failed to yield consistent results. Ghosh et al. [11] reported that feeding of *P. argentipes* on immunized hamsters led to a gradual decrease of feeding attraction, while mortality increased during subsequent bites. Although hamsters were exposed to sand flies using a

similar immunization scheme as in our experiment (to about 90–150 females twice a week followed by a two-week interval, for a total of six exposures), antibody titres detected by those authors were not high. This was explained by low concentrations of each protein fraction in whole saliva, so antibodies developed against these proteins could not have reached high levels [11]. However, in laboratory and in field conditions it was already proved that animals repeatedly exposed to sand flies revealed increased levels of anti-saliva IgGs when compared to the control group [1]. In our experiments, hamsters were exposed to about 100–290 *P. argentipes* females six times at 7–15-day intervals, and antibody titres were very high compared to the control group. In *L. longipalpis*, Vilela et al. [12] reported that females fed on animals immunized by repeated bites obtained lesser amounts of haemoglobin, laid fewer eggs and had higher mortality than females fed on naive animals [12]. On the contrary, Tripet et al. [14] showed that egg production by *L. longipalpis* is not affected by feeding on immunized hosts, and studies on *P. duboscqi* and *P. perniciosus* also did not observe any differences in oviposition or mortality between experimental and control groups of sand flies [13, 15]. Moreover, it is known that sand fly colonies thrive even on laboratory hosts that have been repeatedly exposed to sand flies [20]. Taken together, the effects of anti-saliva antibodies on sand fly physiology are not clear. A more promising approach to altering vector fecundity and mortality might be the immunization of hosts with body tissues, such as whole gut extracts or midgut chitinase [31, 32].

We successfully expressed and purified two yellow-related proteins in *S. schwetzi*, but the ligand binding analysis did not show any affinity to serotonin or histamine. As the feeding preferences of *S. schwetzi* are distinctly different than in *P. argentipes* and other sand fly species studied previously [6, 7], the different properties of this reptile-biting species are not surprising. Adaptations to feeding on either warm-blooded vertebrates or cold-blooded vertebrates [19] may result in different properties for salivary proteins, as demonstrated recently for the relatively low enzymatic activities of apyrase and hyaluronidase in *S. schwetzi* saliva [33]. Unlike in *P. argentipes*, the non-physiologically high concentration of serotonin did not have any effect on *S. schwetzi* fitness. The degradation of serotonin is connected with oxidative stress [34], similarly to heme detoxification [35]. The midgut epithelium of blood-sucking insects is protected from these toxins by the peritrophic matrix (PM) [35], which differs between *S. schwetzi* and *P. argentipes* in morphology and duration: in *S. schwetzi* the PM is thicker and has a prolonged persistence [36], and thus could block the unfavourable effects of serotonin on oviposition.

Surprisingly little is known about the presence of biogenic amines in reptiles. So far, circulating serotonin has been described in three reptilian species, two of them with true or partial endothermy (the leatherback sea turtle, *Dermochelys coriacea*, and the American alligator, *Alligator mississippiensis*). These findings support the hypothesis that circulating serotonin might have emerged with endothermic vertebrates [37]. A study carried out on the common snapping turtle (*Chelydra serpentina*), showed that the release of histamine from basophils takes 40–60 minutes, regardless of antigen concentrations [38]. In contrast, the histamine release from human basophiles is usually completed within several minutes [39, 40]. Due to the fact that sand flies can finish a blood meal within several minutes [14, 41], it is possible that the neutralization of histamine in cold-blooded animals is not necessary.

Conclusions

We confirmed the high affinity of salivary yellow-related proteins to serotonin in *P. argentipes*, a vector known for its mammalian host preference. This interaction may play a role in the neutralisation of serotonin at the site of the bite and thus facilitate successful blood-feeding. The production of high levels of specific antibodies in hosts repeatedly exposed to *P. argentipes* did not lead to a deterioration of sand fly fitness, suggesting a minor effect of anti-saliva antibodies on sand fly feeding processes. No affinity of the yellow-related proteins to biogenic amines was demonstrated in the reptile biter *S. schwetzi*, and this may reflect the adaptation to cold blooded vertebrates. However, further studies are needed to unravel the role of *Sergentomyia* yellow-related proteins.

Abbreviations

PagSP04: *P. argentipes* yellow-related recombinant protein; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; YRP: yellow-related protein; PBS: phosphate-buffer saline; MW: molecular weight; MST: microscale thermophoresis; ELISA: enzyme-linked immunosorbent assay; SGH: salivary gland homogenate; OD: optical density; Kd: dissociation constant; CI: confidence interval; SschwYRP: *S. schwetzi* yellow-related recombinant protein.

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Authors' contributions

TS, PS, VV and PV designed the study. TS, PS, VV, NP and LP performed the experiments. TS and PS analyzed and interpreted the data. TS, PS and PV wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Data supporting the conclusions of this article are included within the article. The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Animals were maintained and handled in the animal facility of Charles University in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of Charles University and were performed under permit no. MSMT-10270/2015-6 of the Ministry of the Environment of the Czech Republic.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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SHORT COMMUNICATION

The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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Abstract. The frequency of sandfly–host contacts can be measured by host antibody levels against sandfly salivary proteins. Recombinant salivary proteins are suggested to represent a valid replacement for salivary gland homogenate (SGH); however, it is necessary to prove that such antigens are recognized by antibodies against various populations of the same species. *Phlebotomus perniciosus* (Diptera: Psychodidae) is the main vector of *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) in southwest Europe and is widespread from Portugal to Italy. In this study, sera were sampled from naturally exposed dogs from distant regions, including Campania (southern Italy), Umbria (central Italy) and the metropolitan Lisbon region (Portugal), where *P. perniciosus* is the unique or principal vector species. Sera were screened for anti-*P. perniciosus* antibodies using SGH and 43-kDa yellow-related recombinant protein (rSP03B). A robust correlation between antibodies recognizing SGH and rSP03B was detected in all regions, suggesting substantial antigenic cross-reactivity among different *P. perniciosus* populations. No significant differences in this relationship were detected between regions. Moreover, rSP03B and the native yellow-related protein were shown to share similar antigenic epitopes, as canine immunoglobulin G (IgG) binding to the native protein was inhibited by pre-incubation with the recombinant form. These findings suggest that rSP03B should be regarded as a universal marker of sandfly exposure throughout the geographical distribution of *P. perniciosus*.

Key words. *Leishmania infantum*, *Phlebotomus* spp., antibody response, dog, markers of exposure, Mediterranean region, salivary proteins, sandflies.

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Leishmaniasis is a widely distributed disease caused by *Leishmania* protozoans and transmitted by phlebotomine sandfly vectors. During blood feeding, sandflies inoculate saliva into the host. Bitten hosts then develop a species-specific antibody response against salivary antigens that reflects the intensity of sandfly exposure and thus provides a useful marker of exposure to generate epidemiological data (Vlkova *et al.*, 2011; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015).

Large-scale serological studies using total sandfly salivary gland homogenate (SGH) are currently impractical because it is difficult to dissect the high numbers of sandflies necessary to obtain sufficient amounts of SGH. Another potential complication refers to variability in the protein composition of sandfly saliva, which has been found to fluctuate depending on physiological factors such as sandfly age and diet (Volf *et al.*, 2000; Prates *et al.*, 2008). Studies in Old World sandfly species also revealed a certain degree of intra- and inter-population variability in protein and mRNA levels (Rohousova *et al.*, 2012; Ramalho-Ortigão *et al.*, 2015). Therefore, salivary recombinant proteins have been suggested to represent valid replacements for the whole salivary gland protein cocktail, and some have already been validated in the field (Drahota *et al.*, 2014; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015). The use of specific recombinant salivary antigen circumvents the necessity for the laborious maintenance of sandfly colonies, and potentially provides a more refined way to minimize antigenic cross-reactivity with taxonomically close sandfly relatives. A useful recombinant salivary protein would demonstrate antigenicity comparable with that of SGH, share similar antigenic epitopes with the native proteins, and demonstrate similar antigenic patterns throughout the geographical distribution of a particular sandfly vector.

This study follows the canine longitudinal study conducted in southern Italy by Kostalova *et al.* (2015), which described the dynamics and diagnostic potential of antibodies recognizing *Phlebotomus perniciosus* (*Larroussius* subgenus) salivary recombinant proteins in dogs following natural exposure to sandflies over 2 years. Factors such as salivary antigens, age and expected sandfly dynamics were considered as variables and were therefore carefully evaluated. The most reactive and reproducible antigen was found to be the 43-kDa yellow-related recombinant protein (rSP03B) from *P. perniciosus* saliva. In view of these promising results, the rSP03B antigen was tested in canine sera samples collected cross-sectionally in canine leishmaniasis (CanL) endemic settings in Italy and Portugal. The study evaluated levels of individual canine antigenic responses to *P. perniciosus* rSP03B compared with *P. perniciosus* SGH, and the degree of similarity in these antigenic associations, across endemic canine populations in Portuguese and Italian foci in order to assess the universal use of rSP03B as a marker of natural sandfly exposure. Previous research had confirmed the presence of two native yellow-related proteins in *P. perniciosus* salivary gland transcriptome and proteome (Anderson *et al.*, 2006). Therefore, the antigenic similarity of rSP03B to its native form was studied and the specificity of the anti-rSP03B immunoglobulin G (IgG) antibody response was confirmed by the inclusion of 42-kDa yellow-related recombinant protein (rSP03).

Canine sera originated from three regions: (a) Campania ($n = 118$), a traditional high-risk area for CanL in southern continental Italy (Oliva *et al.*, 2006); (b) Umbria ($n = 96$), an inland area of central Italy recently recorded as a medium- to high-risk area for CanL (Di Muccio *et al.*, 2012), and (c) the metropolitan Lisbon region ($n = 341$), which is well known as a CanL endemic locality on the west coast of Portugal (Cortes *et al.*, 2012). In all three areas, *P. perniciosus* is the only or principal vector of CanL (Bongiorno *et al.*, 2003; Rossi *et al.*, 2007; Alten *et al.*, 2016). *Phlebotomus perfiliewi* (*Larroussius* subgenus), another vector of *Leishmania infantum*, was found to be abundant in some areas in Umbria (Maresca *et al.*, 2009). However, *P. perfiliewi* is found in association with large animals (cattle and equine species) in rural habitats (Bongiorno *et al.*, 2003). Dogs examined in Umbria included urban pets and animals hosted in kennels, but all lived in populated areas including residential zones surrounding urban centres, which represent typical habitats for *P. perniciosus* (Maroli *et al.*, 1994). Additionally, sampled dogs may have been exposed to sandflies from other subgenera occurring in study localities (Cortes *et al.*, 2007; Rossi *et al.*, 2007; Maresca *et al.*, 2009). A previous study by Volf & Rohousova (2001) suggested there was no cross-reaction of *Larroussius* species with other sandflies present in these study regions, namely *Phlebotomus papatasi* (*Phlebotomus* subgenus), *Phlebotomus sergenti* (*Paraphlebotomus* subgenus) and members of the genus *Sergentomyia*.

Single sera samples from Campania and Umbria were purposely selected from archived samples collected in 2007–2013 to represent the period from July (i.e. at least 2 months after the beginning of the sandfly season) to October (i.e. the end of the sandfly season). The selected sera were collected from dogs ranging in age from 1.5 to 13 years. The dogs from both Italian regions represented a mixture of hunting breeds and mongrels. Single sera samples from the metropolitan Lisbon region were randomly collected from kennelled dogs (mostly mongrel) at the beginning of the sandfly season in May 2012. These dogs ranged from young (6–12 months) to more senior (> 7 years) dogs.

Samples from Campania consisted of stored sera sent by veterinary clinics to the Istituto Superiore di Sanità for routine serological diagnosis of suspected CanL in owned dogs. Sera from Umbria were collected from healthy dogs that were enrolled on a voluntary basis in the Perugia University CanL surveillance programme. Blood sampling was performed in accordance with the Italian guidelines for animal welfare, following owners' consent, and did not include additional or unnecessary invasive procedures. The collection of sera in the metropolitan Lisbon region was ethically approved by the board of the Institute of Hygiene and Tropical Medicine, New University of Lisbon (IHMTUNL) (authorization no. 8 2011-PI) in compliance with Portuguese legislation for the protection of animals (Law 113/2013).

Anti-*Leishmania* IgG in canine sera from Campania and Umbria was detected with an in-house indirect fluorescent antibody test (IFAT) using *L. infantum* promastigotes as antigen, as described in Gradoni & Gramiccia (2008). Samples showing an IFAT titre of 1 : 40 or greater were considered to indicate exposure to *Leishmania*. Immunoglobulin G antibodies against *Leishmania* in canine sera from the metropolitan Lisbon region were detected using an enzyme-linked immunosorbent assay

(ELISA) kit (Bordier Affinity Products SA, Crissier, Switzerland) according to the manufacturer's guidelines (Maia *et al.*, 2010). The result was considered positive when the absorbance of the analysed sample was higher than the absorbance of the weak positive control serum provided with the kit.

A longterm established laboratory colony of *P. perniciosus* originating from Spain (Murcia) was reared under standard conditions as described in Volf & Volfova (2011). Salivary glands, rSP03B (GenBank accession no. DQ 150622) and rSP03 (GenBank accession no. DQ 150621) from *P. perniciosus* were obtained for this study as previously described (Kostalova *et al.*, 2015) and used as antigens for testing the canine sera.

Antibodies against *P. perniciosus* SGH and rSP03B protein were measured by ELISA as described by Kostalova *et al.* (2015). Each serum was tested in duplicate. Test absorbance values were reported as optical densities (ODs) with subtracted blanks (the ELISA plate background mean absorbance value measured in control wells).

Western blot analysis was used to confirm the similarity of antigenic epitopes between the native yellow-related protein found in *P. perniciosus* SGH and the corresponding recombinant protein rSP03B. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of SGH (equivalent to 4 µg total salivary proteins per lane) and rSP03B (2 µg per lane) was run on a 12% gel and blotted onto the nitrocellulose membrane using the iBLOT instrument (Invitrogen Corp., Carlsbad, CA, U.S.A.). Membrane with separated proteins was cut into strips and blocked in 5% milk diluted in Tris-buffered saline with 0.05% Tween 20 (Tris-Tw) overnight at 4 °C. For the inhibition test, three Italian canine sera possessing high levels of anti-*P. perniciosus* IgG against SGH and rSP03B were pooled. The positive serum pool was diluted 1 : 50 in Tris-Tw and split into halves. The first half was incubated for 2 h on a shaker with rSP03B (20 µg/mL) and the second half was incubated without rSP03B. Negative control sera (canine sera from a non-endemic locality) were diluted 1 : 50 in Tris-Tw and incubated without rSP03B on a shaker for 2 h. In the next step, part of the positive sera pool, incubated either with or without rSP03B protein, and part of the negative control sera was incubated with strips of separated *P. perniciosus* SGH. The same procedure was repeated for strips containing rSP03B, except that sera were diluted 1 : 100 in Tris-Tw. After 1 h, all strips were rinsed in Tris-Tw and subsequently incubated for 1 h with peroxidase-conjugated anti-dog IgG (1 : 3000) (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.). The colour reaction was developed by substrate solution containing 3,3′-diaminobenzidine (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). Furthermore, in order to confirm the specificity of the western blot analysis, the same procedure was repeated for rSP03 protein.

Statistical analyses were carried out using R software (http://cran.r-project.org/) and STATA Version 13.1 (Stata Corp., College Station, TX, U.S.A.). Correlations were analysed using the Spearman rank correlation test and medians were compared between groups using a Wilcoxon rank sum test. Optical density values were logarithmized (natural logarithm) for better readability. Statistical analyses of the relationships between SGH and rSP03B OD values among the canine populations were statistically tested by fitting Poisson general linearized models (GLMs) with an ln link function as the right-skewed frequency distributions were found not to follow a negative binomial distribution (deviance goodness-of-fit $\chi^2 > 56.2$; $P = 1$, d.f. = 549, for each antibody). The full Poisson GLMs included interaction terms to test differences between the regions, both in terms of baseline anti-rSP03B value (intercept where anti-SGH equals 0) and the relationship between antibodies against SGH and rSP03B (slopes). A P-value of < 0.05 was considered to indicate statistical significance.

The use of *P. perniciosus* rSP03B as an epidemiological tool was tested for investigations of canine exposure to sandfly bites in geographically distinct localities in which *P. perniciosus* is the prevalent phlebotomine vector. The recombinant protein rSP03B used in this study was obtained from the salivary glands of *P. perniciosus* in a laboratory-reared colony originating from Murcia in Spain, and was used as an antigen in the serology of dogs living in the Campania and Umbria regions of Italy and in the metropolitan Lisbon region in Portugal.

Levels of canine IgG antibodies reacting with SGH and rSP03B were measured by ELISA. Positive but variable correlations between antibody responses to SGH and rSP03B antigens were observed in sera from all three localities [Campania: $r = 0.73$, 95% confidence interval (CI) 0.62–0.82 ($P < 0.001$); Umbria: $r = 0.56$, 95% CI 0.38–0.71 ($P < 0.001$); metropolitan Lisbon: $r = 0.81$, 95% CI 0.76–0.84 ($P < 0.001$)] (Fig. 1). Table 1 summarizes the OD values for each region and indicates that OD frequency distributions were over-dispersed. To query possible differences in the relationships between SGH and rSP03B antibody responses between geographical regions, the equality of the population-specific regression slopes was tested by fitting a Poisson model. No significant differences were detected (population × antigen interaction terms: $Z > -0.85$, $P > 0.365$). Relative to the metropolitan Lisbon region, both the Campania and Umbria populations tended to produce higher baseline antibody responses against rSP03B, although these differences failed to reach significance at the 5% level (Campania: $Z = 1.66$, $P = 0.097$; Umbria: $Z = 1.95$, $P = 0.051$). One plausible explanation for the putative differences in baseline rSP03B antibody levels among populations is that the populations differ in their condition or past history of infections and that these differences affect general immunological responses to certain antigens, and/or that sandfly biting pressure differs across these populations. The seasonal exposure of dogs to sandflies has been found to lead to antibody response fluctuations related to the period of activity and abundance of vectors (Vlkova *et al.*, 2011; Kostalova *et al.*, 2015). Secondly, as age is a frequent covariate of cumulative exposure used to model cross-sectional age-related prevalence data of *Leishmania* infection (Courtenay *et al.*, 1994), the average older dog is expected to have experienced more sandfly seasons (Kostalova *et al.*, 2015). Dogs from Campania and Umbria were sampled from July (i.e. during the period of highest sandfly abundance in Italy). All of the animals tested from these two regions had experienced at least two consecutive transmission seasons. Sera from dogs in the metropolitan Lisbon region were sampled in May, which is the beginning of the sandfly season, and were sourced mainly from dogs aged > 1 year. Thus these dogs had experienced at least one transmission season. According to reactivity data shown by Kostalova *et al.* (2015), dogs will be reactive to saliva at the beginning of the transmission season if they have already been 'primed' in the

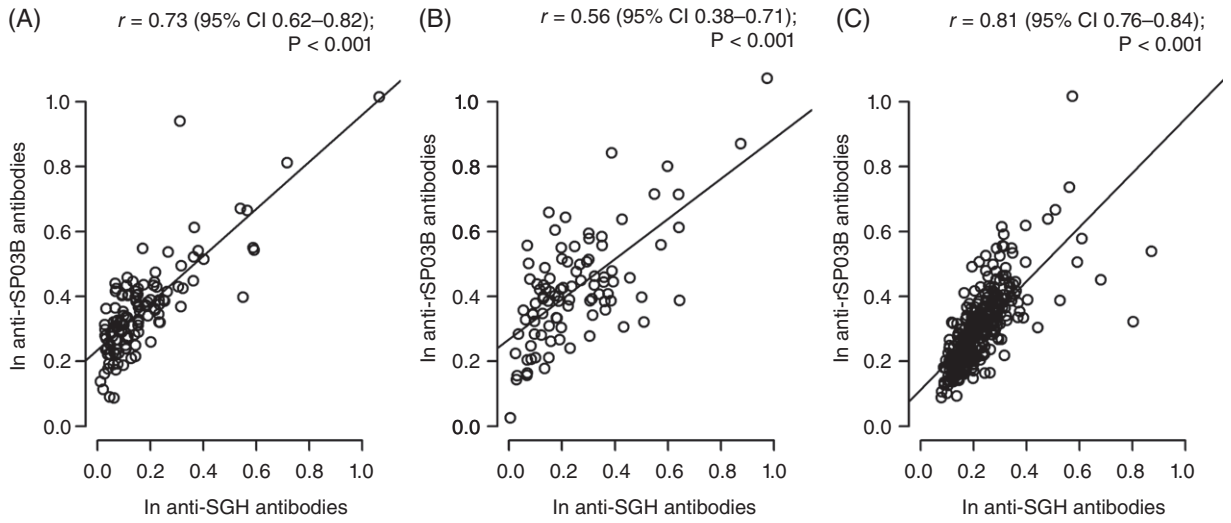

Fig. 1. Correlations between antibodies recognizing salivary gland homogenate (SGH) and rSP03B in dogs naturally bitten by *Phlebotomus perniciosus* in (A) Campania, (B) Umbria and (C) the metropolitan Lisbon region. Correlations were ascertained using Spearman rank correlation. *r*, correlation index; 95% CI, 95% confidence interval.

Table 1. Summary of optical density (OD) values recorded by enzyme-linked immunosorbent assay (ELISA) using *Phlebotomus perniciosus* salivary antigens.

			OD values	
Antigen	Region	Dogs, *n*	Median (IQR)	Min–max
SGH	Campania∗	118	0.131 (0.073–0.241)	0.011–1.899
	Umbria†	96	0.218 (0.133–0.409)	0.005–1.652
	Lisbon‡	341	0.221 (0.165–0.311)	0.081–1.390
rSP03B	Campania∗	118	0.407 (0.311–0.516)	0.091–1.761
	Umbria†	96	0.495 (0.386–0.649)	0.026–1.925
	Lisbon‡	341	0.323 (0.234–0.436)	0.092–1.766

∗Southern Italy.
†Central Italy.
‡Metropolitan Lisbon region (Portugal).
IQR, interquartile range; SGH, salivary gland homogenate.

previous season. These results indicate substantial salivary antigen cross-reactivity amongst *P. perniciosus* populations from Campania, Umbria and the metropolitan Lisbon region. Strong antigenic cross-reactivity between populations of the same sandfly species was similarly observed between two geographically distant colonies of *Phlebotomus orientalis* (*Larroussius* subgenus) in Ethiopia (Vlkova *et al.*, 2014), and among colonies of *P. sergenti* originating from Israel and Turkey (Rohousova *et al.*, 2012).

The similarity of antigenic epitopes between native yellow-related proteins in Spanish *P. perniciosus* SGH and rSP03B was demonstrated by an inhibition test (Fig. 2). For this analysis, sera of dogs from Campania and Umbria with high levels of specific antibodies were selected and pooled. The inhibition test showed that all IgG antibodies specific for the native yellow-related protein bind to the recombinant form during pre-incubation of the sera, which resulted in the complete disappearance of the corresponding band on western blotting (Fig. 2). This demonstrated that rSP03B shares antigenic epitopes with the native yellow-related protein contained within *P. perniciosus* saliva and presumably identifies the proportion of bitten dogs in a manner similar to the use of SGH. By contrast, when the inhibition test was performed with rSP03 protein, intended to confirm the specificity of the western blot analysis, no band appeared and no inhibition was observed (Fig. 2). Therefore, rSP03 is considered to be a non-immunogenic antigen. These results show that the band observed in western blotting with SGH as antigen corresponds to the native 43-kDa yellow-related protein and that the anti-SP03B IgG antibodies are highly specific for the tested rSP03B protein.

Italy and Portugal are generally assumed to show endemic CanL transmission (Oliva *et al.*, 2006; Cortes *et al.*, 2012; Di Muccio *et al.*, 2012). In this study, CanL seropositivity ranged from 5% to 30%, with the lowest prevalence in Umbria and the highest in Campania (Table 2). The use of antibodies against sandfly salivary proteins as risk markers of *L. infantum* infection has been tested earlier for SGH (Vlkova *et al.*, 2011), as well as for salivary recombinant proteins, among which rSP03B proved to be a powerful marker of host exposure to sandflies (Kostalova *et al.*, 2015). Therefore, the present study analysed the relationship between anti-*P. perniciosus* antibodies and *Leishmania* serological status. When using rSP03B antigen, significantly higher levels of specific IgG in *Leishmania*-seropositive dogs [median = 0.346, interquartile range (IQR) 0.257–0.536] than in *Leishmania*-seronegative dogs (median = 0.320, IQR 0.229–0.422) were found only in the metropolitan Lisbon region (Wilcoxon rank sum test, $W = 5391.5$, $P = 0.025$). In Campania, the differences in antibodies against rSP03B between *Leishmania*-seropositive (median = 0.457, IQR 0.357–0.550) and *Leishmania*-seronegative (median = 0.379, IQR 0.303–0.499) dogs were marginally significant (Wilcoxon rank sum test, $W = 1123.5$, $P = 0.053$). Previous studies on the relationship between anti-*P. perniciosus* antibodies and seropositivity to *L. infantum* show variable correlations. In Kostalova *et al.*

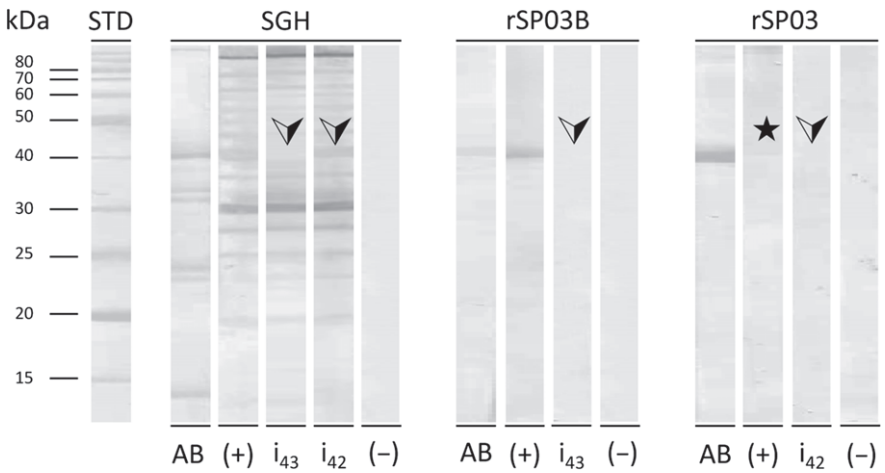

Fig. 2. Western blot analysis of salivary gland homogenate (SGH), rSP03B and rSP03 and inhibition test. A mixture of canine sera positive to *Phlebotomus perniciosus* SGH was pre-incubated with rSP03B or rSP03 and then tested in western blotting against SGH. Arrows indicate the points at which inhibition should take place. The star indicates the position of rSP03. STD, standard; AB, strip stained by Amido black; (+), positive control strip; i_{43}, inhibition strip for rSP03B; i_{42}, inhibition strip for rSP03; (−), negative control strip.

Table 2. Frequencies of *Leishmania* seropositivity and seronegativity in dogs from different regions.

			Anti-*L. infantum* IgG positive/total animals sampled, *n* (%)		
Diagnostic method	Cut-off	Serological status∗	Campania	Umbria	Lisbon
IFAT	1 : 40	Positive	35/118 (30%)	5/96 (5%)	—
		Negative	83/118 (70%)	91/96 (95%)	—
ELISA	0.26	Positive	—	—	46/341 (13%)
		Negative	—	—	295/341 (87%)

∗As determined by the IFAT titre or ELISA cut-off.
IgG, immunoglobulin G; IFAT, indirect fluorescent antibody test; ELISA, enzyme-linked immunosorbent assay.

(2015), a positive association was observed between levels of canine IgG antibodies against sandfly saliva and active CanL infection in dogs sampled longitudinally over 2 years. By contrast, the study by Vlkova *et al.* (2011) described a negative correlation between levels of specific IgG2 and risk for *Leishmania* infection. Comparisons between studies are difficult following observations that anti-saliva antibodies wax and wane with sandfly exposure and seasonality (Kostalova *et al.*, 2015). In actively infected dogs, anti-*Leishmania* antibodies tend to persist after an initial increase, whereas in exposed resistant animals they tend to fluctuate or convert to negative (Oliva *et al.*, 2006). As studies tend to be cross-sectional and use different approaches to determine *Leishmania* infection status, cross-study comparisons are difficult. Although longitudinal studies have already demonstrated the potential usefulness of the sandfly saliva antigenic response in dogs as a marker for *Leishmania* infection (Kostalova *et al.*, 2015; R. J. Qinnell, personal communication, 2016), the possibility of using sandfly salivary recombinant proteins in a similar way in cross-sectional surveys still needs to be validated.

In conclusion, this study showed that *P. perniciosus* rSP03B, the 43-kDa yellow-related recombinant protein, possesses the same antigenic epitopes as its native form in salivary glands, and binds similarly in canine sera from foci in Italy and Portugal. Therefore, it could serve as a universal marker of sandfly exposure across the entire geographical distribution of *P. perniciosus*, even in dogs of various breeds and ages.

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SUMMARY AND CONCLUSION

The main aim of this thesis involved the characterization of the sialome of *S. schwetzi* and comparison with the sialomes of other *Phlebotomus* and *Lutzomyia* sand fly species. This was then followed by the investigation of the binding affinities of a major protein family (YRP) present in all sand fly species. The binding affinity of these YRP to biogenic amines was tested for both *S. schwetzi* and two other palearctic and medically-important sand fly species – *P. orientalis* and *P. perniciosus*. Finally, we studied also the immunogenic properties of the YRP of *P. perniciosus*, highlighting its use as a universal exposure marker in various endemic sites across the Mediterranean basin. The results of these objectives are summarized below.

Sergentomyia salivary components have been neglected for a long time. There is only a single recent study on the enzymatic activities in *S. schwetzi* saliva (Volfova and Volf, 2018). Therefore, we performed a large transcriptomic, proteomic and enzymatic analysis of *S. schwetzi* saliva to address the differences with other *Phlebotomus* and *Lutzomyia* sand flies; the results are summarised in **Polanska et al., (2020)** published in **PLoS One**.

The analysis of *S. schwetzi* transcriptome revealed proteins that belong to all main sand fly salivary protein families. Some proteins were found in multiple homologues compared to previously published sialomes (Coutinho-Abreu and Valenzuela, 2018; Polanska et al., 2020), possibly caused by sensitivity of the next generation sequencing (NGS) method used in our study (the Illumina platform). This NGS allows for a deeper sequencing and will therefore also include low copy transcripts and consequently render more and higher quality transcripts [reviewed in (Zhou et al., 2010)]. On the other hand, the higher number of homologues of salivary proteins can also naturally occur by gene conversion and multiplication, like it was observed for e.g. YRPs, D7-related proteins and Ag5r (Coutinho-Abreu and Valenzuela, 2018). Most of the salivary proteins from *S. schwetzi* had more divergent amino acid sequences (23 – 50 % sequence identity) compared to *Phlebotomus* and *Lutzomyia* proteins (Polanska et al., 2020), with the Ag5r proteins and certain enzymes having the highest sequence identity (52 – 69 %).

Phylogenetic analyses showed that most of the *S. schwetzi* proteins (i.e. YRPs, Ag5r, lufaxin, apyrase) create separate basal groups to other *Phlebotomus* and *Lutzomyia* proteins (Polanska et al., 2020), which is in accordance with the established sand fly phylogeny (Aransay et al., 2000). Apart from the OBPs and hyaluronidase, all *S. schwetzi* proteins inclined to paraphyly, most probably caused by gene conversion (Polanska et al., 2020).

In the *S. schwetzi* sialome we detected some homologues from protein families known previously as specific for *Phlebotomus* (pyrophosphatase, phospholipase A2, ParSP25-like protein, SP16-like protein) or *Lutzomyia* species (71 kDa salivary protein, protein with C-type lectin domain, 5'-nucleotidase) [reviewed in (Coutinho-Abreu and Valenzuela, 2018)]. The 71 kDa salivary protein found in saliva of *S. schwetzi* is highly identical to those present in the sialomes of *L. longipalpis*, *L. olmeca* and *L. ayacuchensis* (going from 76.1 % up to 85.1 %) (Polanska et al., 2020). These 71 kDa salivary proteins are similar to the angiotensin converting enzyme (ACE) (Polanska et al., 2020; Valenzuela et al., 2004), responsible for hydrolysis of inflammatory or haemostatic peptides (angiotensin, bradykinin) [reviewed in (Gaddam et al., 2014)]. Besides these, also a partial transcript of secreted ribonuclease was found in *S. schwetzi* saliva, an enzyme previously found only in mosquito saliva (Calvo et al., 2010; Ribeiro et al., 2007).

In addition, we also studied differences between two lineages of this sand fly species. Both lineages of *S. schwetzi* had the same origin, but for more than thirty generations were adapted to different hosts for blood feeding. The first lineage fed on mice (a warm-blooded animal), and the second lineage fed on geckos (a cold-blooded animal), the latter being a more natural host for *Sergentomyia* sand flies. The distinctions were examined by various approaches: enzymatic assays were used to measure the activities of salivary apyrase and hyaluronidase, the RNA—seq method to measure mRNA expression levels and mass spectrometry to compare the proteome.

A significantly higher hyaluronidase activity (30 % difference) was observed in the *S. schwetzi* lineage that was adapted to mice. However, this difference was not detected in the mRNA expression levels nor did this reflect in the protein abundance in the proteome. Interestingly, one hyaluronidase identified only as a partial transcript was significantly more abundant in the proteome of the gecko lineage. However, this hyaluronidase homologue might not possess the enzymatic activity (Polanska et al., 2020). We suggested that the higher hyaluronidase activity observed in the mice lineage could be caused by its adaptation to mammalian skin, which is thicker than a reptile's one. The mouthparts of sand flies that feed on reptiles are shorter than those of mammal feeders (Lewis, 1987). A thicker mammalian skin might require more active hyaluronidase to increase the permeability of the skin and facilitate sand fly feeding. Degrading the ECM in the mammalian host by hyaluronidase may therefore decrease the tissue barrier for feeding.

The PpSP15-like transcript was only detected at partial transcript length, but was significantly up-regulated in the *S. schwetzi* lineage that was adapted to mice. However, its

expression was low in saliva of both lineages, so its biological function in the *S. schwetzi* mice lineage saliva was dubious. All the other significantly up-regulated transcripts were also only at partial length and had low annotation support, which made it impossible to predict their function (Polanska et al., 2020).

Mass spectrometry of both *S. schwetzi* lineages, identified a significant enrichment of the D7-related protein, hyaluronidase, a protein with C-type lectin domain and three YRPs in the lineage adapted to feed on geckos. A subsequent comparison of this protein enrichment using SDS-PAGE showed that only one protein band (between 40 and 42 kDa) was more apparent on the electrophoretogram of the gecko lineage. We cannot exclude the possibility that this band comprises more salivary proteins, however, most likely it corresponds YRPs, since YRPs are abundant proteins and three of them were enriched in the proteomic analysis of the gecko lineage (Polanska et al., 2020).

Sand fly YRPs are one of the most abundant proteins in sand fly saliva and are known to bind biogenic amines in the vertebrate host (Xu et al., 2011). Eleven amino acids in their sequence are responsible for this binding, of which three create the direct hydrogen bonds with the amines (Xu et al., 2011). Among these amino acids the various substitutions occurred in sand flies' YRPs (Sima et al., 2016a). These together with variable hydrophobicity and the charge of the residues surrounding the entrance of the binding cavity could contribute to the different binding abilities of each YRP (Sima et al., 2016a).

In our study by **Sumova et al., (2019),** published in **Insect Biochemistry and Molecular Biology,** we confirmed these predicted differences in bioamine-binding ability of YRPs of *P. perniciosus* and *P. orientalis*. Five recombinant YRPs (two from *P. perniciosus,* two from *P. orientalis* and LJM11 from *L. longipalpis)* were prepared in human embryonic kidney cells and used for bioamine-binding assays measured by micro scale thermoforesis (MST). This novel method enables to detect the dissociation constants (Kd) of the YRP-bioamine bond. Our MST results were comparable with those obtained by isothermal titration calorimetry (ITC), previously used by Xu *et al.* (2011) to measure bioamine-binding abilities of *L. longipalpis* LJM11.

According to their Kd the YRPs were divided into four groups: i) high affinity binders (Kd = 1.1 – 10 nM), ii) medium affinity binders (Kd = 10 – 1,000 nM), iii) low affinity binders (Kd = 1,000 – 10,000 nM) and iv) poor affinity binders (Kd ≤ 10,000 nM). Recombinant proteins from both *P. perniciosus* (PpeSP03B) and *P. orientalis* (PorASP4) bound serotonin with a high affinity and histamine with a low affinity. Furthermore, PorASP4 bound dopamine with a high affinity and catecholamines with medium affinity.

The second recombinant protein form *P. orientalis*, PorASP2, bound one of the catecholamines – octopamine – with high affinity, serotonin and dopamine with medium affinity, and histamine with poor affinity. For the other catecholamines (norepinephrine and epinephrine) no measurable binding was shown. The YRP PpeSP03 of *P. perniciosus* showed medium and low affinity binding to norepinephrine and octopamine, respectively, and poorly bound the other bioamines. We proposed that the amount of PpeSP03B and PorASP4 present in sand fly saliva, together with their high ability of binding to serotonin, is physiologically relevant to neutralize the natural serotonin function and might lead to an inhibition of vasoconstriction and platelet aggregation in the sand fly bite site and hence facilitate sand fly feeding (Sumova et al., 2019).

A third study (**Spitzova *et al.* 2020,** published in **Parasites & Vectors)** used the MST to measure the binding affinity of YRPs of *S. schwetzi* and *P. argentipes* to serotonin and histamine. We expressed two recombinant YRPs from *S. schwetzi* (SschwYRP1 and SschwYRP2) and one from *P. argentipes* (PagSP04) in human embryonic kidney cell lines. *P. argentipes* YRP bound serotonin with a high affinity and histamine with a low affinity. Interestingly, both *S. schwetzi* YRPs did not bind any of these bioamines (Spitzova et al., 2020). The inability to bind bioamines can be caused by amino acid changes in the binding cavity, as previously mentioned. The sequence analysis of *S. schwetzi* YRPs supported this hypothesis. Moreover, the *S. schwetzi* YRPs sequences also differ in length compared to YRPs of other sand fly species, which might affect their tertiary structure (Polanska et al., 2020).

Besides the physiological function (binding of hosts' bioamines) the sand fly YRPs were shown to induce humoral immune response in the sand fly hosts. The antibodies against sand fly saliva (or single salivary protein) produced by repeatedly bitten host were shown to be sand fly species-specific and they reflect host's exposure to sand fly bites. Therefore, some of the salivary proteins can be used as sand fly exposure markers [reviewed in (Lestinova et al., 2017)].

The recombinant YRP (PpeSP03B) of *P. perniciosus* has previously been shown to be a good marker of exposure for dogs (Kostalova et al., 2015). In our study by **Kostalova et al., (2017)** published in **Medical and Veterinary Entomology**, we further investigated the potential of the PpeSP03B to be used as a marker of exposure across multiple foci of canine leishmaniasis in the Mediterranean basin. The protein sequence used for production of recombinant PpeSP03B was derived from mRNA isolated from saliva of *P. perniciosus* (laboratory colony originated in Spain). The blood sera were collected from dogs coming

from two different localities in Italy (Campania and Umbria) and one in Portugal (Lisbon). A positive correlation was observed in all studied localities between the canine antibody response against the *P. perniciosus* SGH and the recombinant PpeSP03B. Moreover, by performing an inhibition immunoblot we were able to show that the antigenic epitopes are highly similar for both the natural and recombinant PpeSP03B (Kostalova et al., 2017). Based on the results published in this study, the PpeSP03B was proposed as a universal exposure marker for dogs to *P. perniciosus*, which subsequently led to its usage in multiple follow-up studies (Burnham et al., 2020; Maia et al., 2020; Velez et al., 2018; Willen et al., 2018, 2019).

To conclude, we characterised salivary proteins from *S. schwetzi* by various methods. The transcriptomic study revealed a presence of proteins belonging to the main sand fly salivary protein families, but their sequences showed a higher divergence compared to those from *Phlebotomus* and *Lutzomyia*. We identified a novel sand fly salivary enzyme – a secreted ribonuclease – which had not been described in sialomes of sand flies up to date. By comparing the enzymatic activities, mRNA expression and protein abundances from saliva of two *S. schwetzi* lineages adapted to feeding either on mice or gecko, we have tried to reveal the adaptation to hosts with different haemostasis and immunity. A higher hyaluronidase activity was detected in the lineage adapted to feeding on mice. As the exact functions of some salivary proteins remains elusive and more information about reptile haemostasis and immunity is lacking, it is challenging to explain the differences in mRNA expression levels and protein abundances. Interestingly, we showed that *S. schwetzi* YRPs do not bind serotonin nor histamine. Contrarily, a high binding affinity for serotonin was shown for the YRPs from *P. perniciosus* and *P. orientalis*, possibly affecting the physiological condition of the host which facilitates sand fly blood feeding. Finally, we proved that the recombinant YRP of *P. perniciosus*, PpeSP03B, can be used as a universal canine marker of exposure to *P. perniciosus* bites in the western part of Mediterranean area.

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