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Mgr. Magdaléna Jančářová

Critical factors affecting pathogen development in sand flies

Kritické faktory ovlivňující vývoj patogenů ve flebotomech

Ph.D. thesis / Disertační práce

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

Co-Supervisor / Konzultant: doc. RNDr. Jan Votýpka, Ph.D.

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

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I declare that Magdaléna Jančářová substantially contributed to the experimental work in the all projects presented in her thesis and she had a principal role in writing of one publication and one manuscript included in this thesis.

Prohlašuji, že se Magdaléna Jančářová významně podílela na experimentální práci všech projektů zahrnutých do této disertační práce a že je hlavní autorkou textu u jednoho publikovaného článku a také u manuskriptu, které jsou součástí této práce.

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Abstract

This thesis deals with barriers and factors critical for development of viruses, leishmania and gregarines in sand flies.

First, we focused on life cycle of sand fly-borne phleboviruses, especially possible routes of sand fly infection. As a laboratory model we chose Massilia virus (MASV), species closely related to Toscana virus, which is main causative agent of summer meningitis in Mediterranean area. We tested different ways of infection by MASV in various developmental stages of *Phlebotomus perniciosus*; infection of (i) first (L1) and fourth (L4) instar larvae through larval food, (ii) females by blood meal, (iii) both sexes by sugar meal. Infection of L1 and L4 by larval food and subsequent transstadial MASV transmission to adults were not efficient; from 875 adults only three were MASV-positive. Infection through bloodmeal led to high infection rate before defecation, nevertheless, post defecation the infection rate declined and only 5 out of 27 females were MASV-positive. The most efficient infection way was through the sugar meal: 72% of females (88 out of 122) and 51% of males (58 out of 113) were detected as MASV-positive. Moreover, both males and females infected by this way released MASV particles into the drop of sugar which stayed infectious for next 24 hours for other naïve sand flies; almost 30% *P. perniciosus* became infected after feeding on this sugar with regurgitated virus. We suppose that common feeding of infected and uninfected sand flies on the same sugar meal could be important part of *Phlebovirus* circulation in the nature.

Sand flies are well-known vectors of leishmania and sand fly peritrophic matrix (PM) was proposed as important barrier for leishmania development in some sand fly species, especially *Sergentomyia schwetzi.* We experimentally confirmed this theory by addition of *Beauveria bassiana* chitinase into infectious bloodmeal of *S. schwetzi*. In chitinase-treated *S. schwetzi* the PM was disrupted earlier and *Leishmania major* and *Leishmania donovani* had enough time to escape into ectoperithrophic space and develop mature infection with metacyclic forms and colonization of the stomodeal valve. In control group, no leishmania were able to survive defecation of bloodmeal remnants and all infections were lost.

As shown in mosquitoes, ambient temperature during both larval and adult life affects vector competence. We tested impact of different larval rearing temperature (27°C and 32°C) on susceptibility of *Phlebotomus sergenti* females to *Leishmania tropica*. Larvae kept at higher temperature developed faster and produced smaller females, nevertheless infection rate or intensity of infection *L. tropica* did not differ between groups maintained at different temperature. Interestingly, increase of temperature during larval development eliminated gregarines *Psychodiella sergenti*; all sand flies emerged from larvae and pupae maintained at 27°C were infected with

gregarines, with the mean number of gamonts per individual 29.5. In contrast, only in three adults out of 120 developed from larvae and pupae kept at 32°C were found positive for gregarines.

Finally, leishmania and gregarines may naturally co-occur in sand flies. In mosquitoes it was shown that the presence of gregarines affects development of other pathogens. Therefore, we decided to test whether the presence of gregarine *Ps. sergenti* in sand flies *P. sergenti* affects development of *L. tropica*. However, we did not find any significant differences in intensity of infection and infection rate of *L. tropica* between females infected and non-infected by gregarines.

Abstrakt

Tato práce se zabývá bariérami a kritickými faktory při vývoji virů, leishmanií a gregarin během vývoje ve flebotomovi.

Nejdříve jsme se zaměřili na vývojový cyklus virů přenášených flebotomy, jelikož dodnes není jasné, jak tyto viry dokážou kolovat v přírodě. Pro studium této problematiky jsme si zvolili Massilia virus (MASV), který je blízce příbuzný Toscana viru, hlavnímu původci meningitidy v Mediteránu během letních měsíců. Zkoušeli jsme různé způsoby nákazy Massilia virem u různých vývojových stádií flebotoma druhu *Phlebotomus pernicisous* a to infekci: (i) prvního a čtvrtého larválního instaru z larvální potravy, (ii) samic krví a (iii) obou pohlaví cukrem. Infekce larev z potravy a následný transstadiální přenos MASV na dospělce byl neefektivní; celkem se z larev vyvinulo 875 dospělců a pouze tři z nich byli MASV pozitivní. Po nákaze samic z krve jsme sice na počátku získali vysoké procento infikovaných jedinců, nicméně po defekaci došlo k eliminaci infekce a počet infikovaných samic poklesl na 5 z 27. Jako nejúčinnější způsob infekce MASV jsme prokázali nákazu z cukru; 72 % samic (88 ze 122) a 51 % samců (58 ze 113) bylo MASV pozitivní. Navíc, takto infikovaní flebotomové kontaminovali virem cukr, který díky tomu sloužil jako zdroj nákazy pro naivní jedince během následujících 24 hodin; téměř 30 % flebotomů se nakazilo. Domníváme se, že cukr, na němž společně sají infikovaní a neinfikovaní jedinci, může být u flebovirů v přírodě důležitým zdrojem infekce.

Flebotomové jsou významnými přenašeči leishmanií a peritrofická matrix flebotoma byla u některých druhů, zejména *Sergentomyia schwetzi*, považována za důležitou barieru pro vývoj leishmanií. Tuto teorii jsme potvrdili experimentálně přidáním chitinázy *Beauveria bassiana* do krve s leishmaniemi. Díky tomu došlo u *S. schwetzi* k vytvoření slabší PM, která se dříve rozpadla a *Leishmania major* a *Leishmania donovani* tak měly dostatek času uniknout do ektoperitrofického prostoru, vyvinout pozdní infekci s metacykly a kolonizovat stomodeální valvu. U kontrolní skupiny byly leishmanie eliminovány během defekace.

U komárů bylo dříve prokázáno, že teplota během larválního vývoje i během dospělosti může ovlivnit vektorovou kompetenci. Proto jsme testovali vliv různých teplot (27 °C and 32 °C) během larválního vývoje *Phlebotomus sergenti* na následnou vnímavost dospělců vůči nákaze *Leishmania tropica*. Larvy se ve vyšší teplotě vyvíjely rychleji a produkovaly menší dospělce, ale procento infikovaných samic a intenzita infekce *L. tropica* se mezi experimentálními skupinami nelišily. Vyšší teplota ale překvapivě vedla k eliminaci gregarin; všichni flebotomové, kteří se vyvinuli z larev chovaných v 27 °C byli nakaženi a průměrný počet gamontů na jedince byl 29.5, zatímco z dospělců, kteří se vylíhli z larev držených ve vyšší teplotě, byli nakaženi pouze tři ze 120.

V přírodě se mohou gregariny přirozeně vyskytovat ve flebotomech nakažených leishmaniemi. U komárů bylo prokázáno, že gregariny mohou ovlivňovat vývoj ostatních patogenů

přítomných ve vektorovi. Proto jsme se rozhodli otestovat, zda může mít přítomnost gregariny *Psychodiella sergenti* vliv na vývoj *L. tropica* ve flebotomech *P. sergenti.* Nicméně jsme prokázali, že procento infikovaných samic i intenzita infekce byly stejné, bez ohledu na přítomnost či absenci gregarin.

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1 Introduction

Insect-borne pathogens have to overcome various barriers during their development inside the vector; these are not just the physical barriers (i.e. peritrophic matrix, midgut wall or basal lamina), but also activity of immune system, natural gut microflora or the presence of another pathogens. Moreover, the ability to overcome these barriers could be influenced by environmental factors, most important of which is definitely ambient temperature.

The interaction between vector and pathogen is complex and different barriers play a role in various pathogen-vector pairs. Here, I described factors affecting development of arboviruses, leishmania and gregarines in sand flies. Moreover, I also included chapter about viruses (potentially) transmitted by sand flies, even if it is not directly related to my work, because studies on this topic are very limited (Depaquit et al., 2010; Alkan et al., 2013). For leishmania and gregarines, however, there are recent reviews published by our team (Dostalova and Volf, 2012; Lantova and Volf, 2014; Dvorak et al., 2018) and there is no reason to deal with these topics again. This thesis includes three publications in peer-reviewed journals and one manuscript (Jancarova et al., 2015; Jancarova et al., 2016; Sadlova et al., 2018; Jancarova et al., manuscript).

1.1 Barriers in insect vector for arboviruses

Vector competence for arboviruses is influenced by both extrinsic and intrinsic factors. The extrinsic factors involve density and composition of vectors and hosts populations and also environmental conditions. They affect if vector will find and contact appropriate vertebrate host of the virus (Hardy et al., 1983; Alto et al., 2005; Muturi et al., 2012; Christofferson and Mores, 2016). Intrinsic factors are those that affect preferences of vector and its susceptibility to infection and consequent ability to transmit the virus horizontally or vertically. Important are for example pathways of immune response, tissue barriers (Hardy et al., 1983; Sim et al., 2014; Franz et al., 2015) or natural gut microflora, which influence vector indirectly through stimulation of immune system and resource competition or directly by microfloral metabolites (Xi et al., 2008; Ramirez et al., 2012; Ramirez et al., 2014; Jupatanakul et al., 2014). Of course, various factors can interact with each other; for example, the temperature as extrinsic factor could influence vector physiology, which may affect e.g. virus replication and dissemination and finally vector competence (Hardy et al., 1983; Brubaker and Turell, 1998; Dohm et al., 2002; Richards et al., 2007; Westbrook et al., 2010; Christofferson and Mores, 2016).

Extrinsic incubation period (EIP) was described by Carter (1901) and represents duration when vector start to be infectious after its exposition to arbovirus (Carter, 1901; Bauer and Hudson, 1928). For productive infection of the mosqutioes by arboviruses following steps are necessary: (i)

infection of the mesenteron and spread infection through its epithelium, (ii) dissemination of virus particles to secondary tissues and its multiplication, (iii) infection of salivary glands and release of virus into saliva. During this development arboviruses have to surmount few barriers in the vector for successful transmission to vertebrate host, namely: (i) the midgut infection barrier (MIB), (ii) midgut escape barrier (MEB), (iii) salivary gland infection barrier (SGIB), and (iv) salivary glands escape barrier (SGEB) (Kramer et al., 1981; Hardy et al., 1983; Franz et al., 2015). These defined barriers do not necessarily have to be perceived only as physical obstacles, like peritrophic matrix, but can be formed also by other factors summarized bellow.

1.1.1 Tissue barierrs

After blood ingestion, midgut epithelium produces peritrophic matrix (PM) type I, which is acellular layer formed mainly by chitin, glycoproteins and proteoglycans. Peritrophic matrix protects midgut against mechanical damage, pathogens and toxins. In sand flies it is shaped within 6-12 hours after blood feeding and fully matured about 24 hours. Permeability of insect PM for virus is not clear, but PM contained pores of different size, which often ranges around 7-8 nm (Terra, 1990; Peters, 1992; Lehane, 1997; Pruzinova et al., 2015; Franz et al., 2015). However, size of Massilia virus (MASV) particles is around 80-120 nm (Charrel et al., 2009), so this virus probably cannot pass through PM and virions which remain in blood bolus surrounded by PM are defecated. According to some authors it means that only viral particles reaching midgut epithelial cells before formation of PM can infect these cells (Franz et al., 2015).

The way how virus enters into the midgut cells of the vector is still not clear, however, it is believed that arboviruses infect midgut cells mainly through receptor-mediated endocytosis. Some proteins supposingly involved in the interaction virus-cell were identified, for example, deletion in E2 glycoprotein of Sindbis virus (SINV) decreased its infectivity for midgut of *Aedes aegypti* (Myles et al., 2003). Chu et al. (2005) showed that antibodies against both 70 and 90 kDa membrane proteins of C6/36 cells significantly decreased binding and entry of West Nile virus (WNV), Japanese encephalitis virus (JEV) and Dengue-2 virus (DENV-2) into cells. Probably, these mebrane proteins are components of a receptor complex for binding of flaviviruses to vector cells (Chu et al., 2005). All four serotypes of DENV attached to 67 and 80 kDa protein on C6/36 cells and antibodies against these proteins decreased virus binding (Mercado-Curiel et al., 2006). DENV-2 used also prohibitin as a receptor on cell surface in *Aedes,* but not *Culex* (which is refractory to this virus). Moreover, this interaction is DENV-2 specific because antibodies against prohibitin did not affect entry of JEV (Kuadkitkan et al., 2010). These findings suggest that interaction between virus and cell surface is usually highly specific.

In case of non-susceptible vector this barrier will not allow the virus to infect midgut cells or, if the virus infected the cell, it will be not able to replicate or disseminate to other cells (Franz et al., 2015).

Replication in midgut cells is followed by dissemination to secondary tissues and final infection of salivary glands. Again, it is unclear how viruses leave the midgut cells. On the hemolymph side of midgut epithelium is basal lamina (BL); non-cellular, porous, proteinaceous and selectively permeable structure which is predominantly composed from collagen IV and laminin. The BL serves as border between gut and hemocoel and also as a scaffold for midgut part, where the cells are renewed during development. Additionally, BL works as filter and protects against pathogen passage into hemocoel (Yurchenco and Julian, 1994; Sasaki et al., 2004; Passarelli, 2011). Thickness and construction of BL are variable even within a species, as Grimstad and Walker (1991) showed. The BL in small *Aedes triseriatus* females had 3-6 layers and mean thickness 0.14 μm, while in large females BL had 9-16 layers mean thickness 0.24 μm. These females subsequently differed in infectious parameters after oral infection by La Crosse virus (LACV). Dissemination rate was 100% and 69% and trasmission rate was 90% and 42% in small and large females, respectively. It means that thinner BL probably allows faster release of virus and thereby increases vector competence (Grimstad and Walker, 1991).

Houk et al. (1981) described that BL of *Culex tarsalis* female is penetrated by particles up to the size 5-8 nm. Therefore, it is not clear how viruses, measuring around 100 nm, escape from the midgut to the haemocoel; various authors published several more or less likely theories. Dong et al. (2017) suggested that expansion of midgut and BL after blood feeding, associated with temporal degradation of collagen IV, allow to virus go through BL. Authors injected intrathoracally Chikungunya virus (CHIKV) into *Ae. aegypti* females and following day offered them sugar or blood meal and subsequently compared BL permeability for virions. In sugar fed females the virions did not penetrate BL and infect mesenteron but in blood fed females virions passed through BL and replicated in midgut cells. At 12-36 hours after blood feeding the amount of collagen IV was temporally decreased, collagenase activity increased and BL seems to be shredding 24-36 hours after blood feeding (Dong et al., 2017). Similarly, virions of WNV were observed within BL of *Culex pipiens quinquefasciatus* three days after oral infection (Girard et al., 2005). Other studies suggested that viruses use tracheal system for dissemination into hemocoel. Salazar et al. (2007) observed that presence of DENV-2 virions in tracheal system correlated with dissemination of this virus from midgut. Similarly, other authors detected Rift Valley fever virus (RVFV) or SINV in trachea of mosquito vectors (Bowers et al., 1995; Romoser et al., 2005). Third theory suggests virus dissemination through cardia. Oviedo et al. (2011) found correlation between cardial infection and dissemination in highly susceptible strain *C. tarsalis* infected by different doses of Western equine encephalomyelitis virus (WEEV) (but not for less susceptible strains). Girard et al. (2004) described that infection of cardia

C. pipiens quinquefasciatus by WNV concured with infection of salivary glands and authors hypothesise that in special case when cardia is infected from luminal side of anterior midgut, it can facilitate escape of virus from midgut.

Overcoming of midgut escape barriers in susceptible vector can be dose-dependent, even in some natural vector-virus combinations. *Culex tarsalis* females were infected by $10^{1.7}$, $10^{4.1}$ and $10^{6.1}$ PFU of WEEV and in 21%, 3% and 0% individuals, respectively, virus replicated in mesenteron but failed to disseminate to other tissues (Kramer et al., 1981). Similar phenomenon was observed also by Weaver et al. (1984) using Venezuelan encephalitis virus in *Culex taeniopus*. *Culex pipiens quinquefasciatus* females were infected by three infection doses of WNV: 5.8 (high), 4.4 (medium), or 3.7 (low) logs PFU/mL. Infection by low dose completely failed while females infected by medium dose were positive but the virus never disseminated. Only infection by high dose led to dissemination of infection to the body (Richards et al., 2007). Girard et al. (2004) hypothetized that there is a threshold for virus dose to overcome midgut barrier but it seems that this phenomenom depends on vector-virus combination. Oviedo et al. (2011) did not observed significant differences between groups of *C. tarsalis* infected by various doses of WEEV. Similarly, dissemination of CHIKV from *Ae. aegypti* midgut did not correlate with viral titer (Dong et al., 2016).

It is generally assumed that viruses need to replicate in secondary tissues for efficient infection of salivary glands and also need a vehicle to reach the salivary glands (Hardy et al., 1983; Girard et al., 2004). In *C. pipiens quinquefasciatus* orally infected by WNV, dissemination from the midgut was detected on day 2 after infection but first signs of salivary infection were noted on day 5. This three days difference supported theory about multiplication in the secondary tissues (Girard et al., 2004). Hardy et al. (1983) hypothetized that this secondary amplification is needed only in case of oral infection by low infection dose. However, when arboviruses escape from gut they can be detected first in the fat body, nervous system, muscles, tracheal system, Malphigian tubules, hemocytes and etc. (Girard et al., 2004; Girard et al., 2005; Salazar et al., 2007) and then the virus have to achieve salivary glands. The vehicle for transport of virus through hemocoel into salivary glands seems to be hemocytes. Parikh et al. (2009) injected SINV into *Ae. aegypti* and demonstrated high infection rate of hemocytes and virus replication inside these cells. 24 hours after injection, 100% of the hemocytes were infected. Authors observed similar phenomenom also in other three mosquito's vectors, however, it should be considered that they use unnatural way of infection (Parikh et al., 2009).

Finally, virus have to reach salivary glands, infected them (SGIB), multiply and finally have to be released into saliva (SGEB) (Hardy et al., 1983). Molecular mechanisms of these barriers are not known yet. Kramer et al. (1981) demonstrated that salivary gland infection is dependent on virus dose and time. Authors orally infected *C. tarsalis* by three different doses of WEEV and SGIB most

often occurred in females ingested with the lowest dose of virus. However, prevalence of this phenomenom declined during the time, but it was still visible 23 days after infection. Viral titer in hemolymph, ganglia and rest of the body (without mesenteron) was significantly lower in females manifested SGIB than in females transmitted infection by bite to vertebrates. However, SGIB were manifested in each tested group, it means that also other factors have to play role (Kramer et al., 1981). Other studies described positive correlation between viral titer and virus transmission. Fu et al. (1999) demonstrated during the experiments with midges orally infected by bluetongue virus that only individuals with viral titer higher than 10^3 TCID50 released virus into their saliva. Similar phenomenom was also demonstrated by Gargan et al. (1983) in combinations *C. pipiens*-RVFV: females which were able to transmit virus to hamsters contained 100-fold more virus then non-transmitted ones.

Not in all individuals in which virus infected salivary glands (overcome SGIB) is also subsequently released to saliva (means to overcome SGEB). Dong et al. (2016) infected HWE and ORL strains of *Ae. aegypti* by CHIKV; all females had positive salivary glands but only 60% of HWE and 65% ORL females contained virus in saliva. Infected females differ by histology, apoptosis was visible also in lobes of their salivary glands while in non-infected ones the apoptosis were detected only in salivary ducts (Dong et al., 2016). Similarly, Girard et al., (2005) noted WNV-induced degeneration in salivary glands of *C. pipiens quinquefasciatus* and Girard et al. (2007) observed that during the aging the number of salivary glands with apoptotic cells increased in mosquitoes infected by WNV while number of females with positive saliva decreased (83% at 14 days post blood meal (PBM), 53% at 21 PBM, 39% 25–28 PBM). Maybe apoptosis is necessary for releasing the virus into saliva but it leads to decreased production of saliva (Girard et al., 2005; Ciano et al., 2014).

Molecular interactions between arbovirus and vector in salivary glands are more or less unknown. Conway et al. (2016) proved that D7 protein in salivary glands extracts of *Ae. aegypti* is upregulated after infection DENV-2. Its recombinant form decreased amount of DENV-2 in both *in vivo* (mouse) and *in vitro* (cells) systems and binding assay experiments indicate that D7 protein can interact with virions and also its envelope proteins (Conway et al., 2016).

Except of horizontal transmission between vertebrates and vector, some arboviruses can be transmitted also vertically from females to progeny and/or transsexually between males and females (Tesh et al., 1972; Tesh and Chaniotis, 1975; Tesh and Modi, 1987; Tesh et al., 1992; Comer et al., 1990). During these ways, arboviruses have to overcome some barriers same as during horizontal transmission, but also some others. Moreover, these ways are not mutually exclusive and some arboviruses can be transmitted by both, horizontal and vertical transmission (Diallo et al., 2000; Joshi et al., 2002; Flores et al., 2010), however these additional barriers are not included in this text.

1.1.2 Immune system

Vector immune system is also one of the key barriers in the fight against arboviruses infection. In addition to immune pathways, such as Toll, Imd, JAK-STAT and RNA interference (RNAi), also processes like apoptosis and autophagy can be involved in certain virus-host combination (reviewed by Sim et al., 2014). Different components of the vector immune system are efficient at different phase of infection. During the initial stage of infection of *Anopheles gambiae* by O'nyong-nyong virus (ONNV), Imd and JAK-STAT pathways and Leu-rich repeat immune factors have antiviral function, whereas exogenous siRNA pathway control virus later, during systemic infection (Carissimo et al., 2015). However, these facts cannot be generalized. Contrary to previous study, in combination of DENV-*Ae. aegypti,* Toll pathway plays important role during the initial phase of infection; depletion of negative regulator Cactus led to declined virus titer in midgut, while Toll pathway suppression resulted in higher titer (Ramirez and Dimopolous, 2010). Jupatanakul et al. (2017) constructed *Ae. aegypti* mutants overexpressing components of JAK-STAT pathway in fat body after blood feeding which inhibited infection by DENV-2 and -4, but it did not influence susceptibility to Zika virus (ZIKV) or CHIKV. These results also indicate that the immune system of vector and its pathways cope with different viruses by various ways (Jupatanakul et al., 2017).

Moreover, virus can manipulate the immune response of the insect. Ramirez and Dimopolous (2010) observed suppression expression of some immune molecules in initial phase of DENV infection in *Ae. aegypti*. These informations indicate complexity of arbovirus/vector interactions. Moreover, as discussed below, immune response against arboviruses is also connected with gut microflora, coinfection and temperature.

1.1.3 Coinfection

During its development in insect vector virus can coexist and interact also with other potentially present pathogens/parasites, however these phenomenons are not well explored. Theoretically, three situations can happen: (i) no interaction, (ii) facilitative effect, when at least one pathogen will profit from this co-occurrence and (iii) competition; and vector immune system plays an important role during these events.

Vazeille et al. (2016) tested DENV-1 and DENV-4 coinfections in two *Ae. aegypti* populations. Whereas during single infection both viruses possessed same dissemination and transmission rates, during coinfection only DENV-4 was found in saliva (Vazeille et al., 2016). Contrary, Rückert et al. (2017) infected *Ae. aegypti* by CHIKV, DENV-2 and ZIKV as single infection or double/triple coinfections. In certain combinations some infections parameters were influenced but, importantly, females were able co-transmitting all virus combinations together. Similar results observed Vazeille

et al. (2010) with DENV-1 and CHIKV in *Aedes albopictus*. It means that at least in some vector-arboviruses combinations, the vector can be infected by more viruses and subsequently co-transmitt these viruses to another host. This fact has a very important epidemiological consequences.

Except of coinfection, when viruses enter into the vector together, there is a phenomenom of superinfection, when viruses infected vector at different time. Pesko and Mores (2009) tested effect of superinfections by St. Louis encephalitis virus and WNV in *C. quinquefasciatus*. Both viruses exhibited significantly lower infection and dissemination rates, when infected as a second one (Pesko and Mores, 2009). However, important is the timing of the second infection. Sundin and Beaty (1988) demonstrated in *Ae. triseriatus* that if the second infection occurred within 24 hours, superinfection established in the most females. But, if second infection came later than seven days from first one, females were refractory.

Phenomenon of superinfection was tested also in combination of virus and fungus. Dong et al. (2012) infected *Ae. aegypti* by fungus *Beauveria bassiana* two days before infection by DENV-2. Fungus presence significantly reduced virus loads in the midgut, probably through activation Toll and JAK-STAT pathways, which could be potentially used in future for control strategies against spread of DENV (Dong et al., 2012).

1.1.4 Gut microbiome

Microbiome naturally living in the gut of the vector can influence virus infection directly by the metabolites (Ramirez et al., 2014) and resource competition or indirectly through immune system stimulation (Xi et al., 2008; Ramirez et al., 2012; Ramirez et al., 2014). *Chromobacterium* sp., part of natural gut microflora in *Ae. aegypti*, reduced susceptibility of females to DENV-2 and also exerted anti-viral activities *in vitro* independent on mosquitoes, therefore it seems that this effect is caused by bacterial metabolites (Ramirez et al., 2014). Similarly, *Proteus* sp. and *Paenibacillus* sp. decreased the level of DENV-2 infection (Ramirez et al., 2012). Joyce et al. (2011) isolated bacteria from field-caught *Ae. albopictus* midgut and incubated them with LACV before infection of Vero cells. Half of isolated bacteria significantly decreased infectivity of the virus (Joyce et al., 2011). Microbiota can compete with arboviruses for cholesterol and lipids as these molecules are important for entrance of arboviruses into cells, intracellular replication and exit (Lu et al., 1999; Lee at al., 2008)

As for the indirect effect, reduction of gut microbiota by antibiotics in *Ae. aegypti* led to two times higher virus titer of DENV-2 virus in midgut than in non-treated mosquitoes and aseptic females also exhibited lower expression of some immune genes as defensin, cecropin, attacin and gambicin. These results suggested that Toll pathway stimulated on basal level by gut microflora has also antiviral activity (Xi et al., 2008). Ramirez et al. (2012) observed a similar phenomenon,

moreover, they also proved that infection of *Ae. aegypti* by DENV significantly decreased bacteria numbers in the midgut. It means that there is a reciprocal interaction between immune system of the vector, its gut microbiom and virus (Ramirez et al., 2012). Zink et al. (2015) showed increase of bacterial diversity seven days after infection of *C. pipiens* by WNV, which was also correlated with up-regulation of immune response such as RNAi, Toll, and JAK-STAT pathways. Nevertheless all of these results suggest that the vector natural gut microbiome is also component of insect defense but exact mechanisms still have to be found.

On the other hand, some bacteria, such as *Serratia odorifera*, increased susceptibility of the vector to the virus. Co-feeding of *S. odorifera* together with DENV-2 to antibiotic-treated *Ae. aegypti* females led to higher infection and dissemination rate compared with females co-fed with different bacteria or virus alone (Apte-Deshpande et al., 2012). Carissimo et al. (2015) showed that presence of natural gut microflora is needed for full midgut infectivity of ONNV in *An. gambiae*. However, mechanisms of this effects are unknown.

Intracellular bacteria of genus *Wolbachia* negatively influenced infection parameters (infection, dissemination and transmission rate) and caused prolonged extrinsic incubation period of CHIKV in *Ae. aegypti* (Aliota et al., 2016). They also negatively influenced infection parameters of DENV (Moreira et al., 2009; Blagrove et al., 2012), WNV (Glaser and Meola, 2010) or ZIKV (Dutra et al., 2016) in different vectors.

1.1.5 Temperature

Vectors are ectothermic animals and temperature during both larval and adult lifes represents an important factor in vector competence. However, results gained by various authors are contradictory. Dodson et al. (2012) showed that different temperature during larval development of *C. tarsalis* did not have a significant impact on the WNV infection, dissemination or transmission rates in adults. Similar data were presented by Brubaker and Turell (1998) for RVFV in *C. pipiens*. In contrary, *Ae. albopictus* females from larvae reared at 18°C exhibited higher infection rate by CHIKV than females from larvae reared at 24 or 32°C (Westbrook et al., 2010). Finally, an opposite effect was observed in *Ae. aegypti* infected by SINV: females from larvae kept at higher temperature were more susceptible to the virus (Muturi and Alto, 2011). Similary in adults, some studies demonstrated higher vector competence in females reared at lower temperatures (Kramer et al., 1983; Adelman et al., 2013), but others showed the opposite (Richards et al., 2007; Kilpatrick et al., 2008; Mackenzie-Impoinvil et al., 2015).

In addition, some authors evaluated the impact of fluctuating temperature as it should reflect better the conditions in nature. Lambrechts et al. (2011) studied effect of diurnal temperature

range (DTR) on DENV infection in *Ae. aegypti*: females were held at 26°C (DTR = 0°C), or at DTR = 10°C and at DTR = 20°C. During high temperature fluctuation (DTR = 20°C), females showed higher mortality but also higher resistance to infection. Nevertheless, according to termodynamic model this rule is valid only if mean temperature is higher than 18°C, with mean temperature below this threshold transmission of DENV increases, together with DTR (Lambrechts et al., 2011).

Temperature also affects the immune system of the vector but the interaction is very complex as individual parts of immune response have various thermal optima. In *Anopheles stephensi* treated by *Escherichia coli* the peak of melanisation, phagocytosis, production of defensin and expression of cecropin was detected at 18°C, whereas highest expression of nitric oxide synthase was observed at 30°C (Murdock et al., 2012). Maintenance of mosquitoes at lower temperature (18°C) impaired RNAi and females of *Ae. aegypti* and *Ae. albopictus* were more susceptible to CHIKV and Yellow fever virus respectively compared to mosquitoes held at 28°C (Adelman et al., 2013). As different compounds of immune system are efficient against various viruses (Jupatanakul et al., 2017) and different temperatures stimulate also distinct part of immune system (Murdock et al., 2012; Adelman et al., 2013) it is impossible to generalize the effect of temperature for various pathogen-vector pairs.

1.1.6 Arboviruses transmitted by sand flies

Sand flies transmit arthropod-borne viruses belonging to three genera: (i) *Phlebovirus* (family Bunyaviridae) (Depaquit et al., 2010), (ii) *Orbivirus* (family Reoviridae), (iii) *Vesiculovirus* (family Rhabdoviridae) and, in addition, (iv) potentially also the representatives of genera *Flavivirus* (Fontenille et al., 1994; Moureau et al., 2010) and *Negevirus* (Vasilakis et al., 2013).

(i) *Phlebovirus*

Phleboviruses are enveloped and negative-sense single stranded RNA viruses with trisegmented genome. Segment L encodes the RNA-dependent RNA polymerase, segment M (medium) encodes virions envelope glycoproteins Gn and Gc and segment S code N nucleocapsid protein. Moreover, both M and S segments encode non-structural proteins (reviewed by Alkan et al., 2013; Horne and Vanlandingham, 2014).

According ICTV (International Committee on Taxonomy of Viruses) (Plyusnin et al., 2011), the genus *Phlebovirus* contains nine viral species (Bujaru, Candiru, Chilibre, Frijoles, Punta Toro, RVFV, Salehabad, Sandfly fever Naples and Uukuniemi) and about 33 tentative species. Most of them are transmitted by phlebotomine sand flies, except of RVFV and Uukuniemi virus, which are vectored preferably by mosquitoes and ticks, respectively (Hubalek and Rudolf 2012; Alkan et al., 2013; Horne

and Vanlandingham, 2014). The following text, unless stated otherwise, deals only with sand fly-transmitted viruses.

The life cycle of sand fly-borne phleboviruses in nature is not well understood. There is no evidence supporting the existence of human or animal vertebrate reservoirs; vertebrates are considered as dead-end hosts without an important role in the circulation of the virus while sand flies are proposed by some authors as primary reservoirs (Tesh and Chaniotis, 1975; Alkan et al., 2013).

Phleboviruses cause human diseases with variety of clinical syndromes from mild febrile to neuroinvasive disease and usually two species (Sandfly fever Naples and Toscana virus) and one tentative species (Sicilian virus) are associated with human illness (Alkan et al., 2013). Diseases caused by Sandfly fever Naples and Sicilian virus are clinically indistinguishable and are also called as three-day or papatacci fever. The illness lasts 2-3 days and it manifests by flu-like symptoms as fever, headache, retro-orbital pain, malaise and myalgia. These diseases are mild but during the illness, people feel strongly affected (Depaquit et al., 2010; Alkan et al., 2013).

Toscana virus (TOSV) seems to be the most important *Phlebovirus* as it manifests with variety of symptoms, from non-symptomatic forms through febrile illness to CNS disease, and it is the major causative agent of meningitis during warm seasons in Mediterranean area. Among viruses transmitted by sand flies it is the only one which exhibits neurotropic activity (Charrel et al., 2005; Charrel et al., 2012). It circulates on large geographic area, from Turkey to Morocco (Charrel et al., 2012; Es-Sette et al., 2015) and is usually connected with the presence of suspected vectors *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*. TOSV was detected also from *Sergentomyia minuta* in Marseille (Charrel et al., 2006), *Phlebotomus tobbi* in Cyprus (Ergunay et al., 2014), *Phlebotomus longicuspis* and *Phlebotomus sergenti* in Morocco (Es-Sette et al., 2014; Es-Sette et al., 2015), but role of these species in the virus circulation remains unknown. Moreover, oral infection of sand flies by TOSV infected blood seems to be rather unsuccessful. Tesh et al. (1992) failed with infection dose 6.0 $\log_{10}$ PFU per ml, Maroli et al. (1993) had to used $10^{8.3}$ $TCID_{50}$ per ml and Tesh and Modi (1984) also showed that *P. perniciosus* was not very susceptible to oral infection by TOSV. It suggests that potential vertebrate reservoir has to exhibit high viremia for sand flies infection (Tesh and Modi, 1984).

Studies in humans are focused on detection of TOSV in patients or testing seroprevalence in population (Echevarría et al., 2003; Hemmersbach-Miller et al., 2004; Bichaud et al., 2011; Sakhria et al., 2013; Fezaa et al., 2014). Hemmersbach-Miller et al. (2004) claim that TOSV viraemia is transient and takes 24-36 hours. Anti-TOSV antibodies were found also in various domestic animals, for example in dogs, goats, sheep, cats, cows, pigs, horses. TOSV RNA was isolated from dogs, goat and cats (Navarro-Marí et al., 2011; Dincer et al., 2015; Alwassouf et al., 2016), but live virus was obtained only from the brain of bat *Pipistrellus kuhli* (Verani et al., 1988) and any vertebrate reservoir

or the source of infection for sand flies was not confirmed yet. Experimental studies proved transstadial, transovarial (Tesh and Modi, 1987; Tesh et al., 1992; Maroli et al., 1993) and venereal (Tesh et al., 1992) transmission of TOSV between *P. perniciosus* sand flies. However, these routes alone are not efficient enough for maintenance viruses in nature. Maroli et al. (1993) showed that orally infected females were not able to transmit infection into progeny during the first gonotrophic cycle but only during the second one. Question is, however, which proportion of females survive until the second egg batch. Moreover, number of transovarially infected progeny gradually decreased in each subsequent generation, Tesh and Modi (1987) showed 86% transovarially infected progeny in F5 generation and 5.9% in F12. Females never transmit infection on all progeny; reason is not known (Tesh and Modi, 1987). The rate of venereal transmission from infected males of *P. perniciosus* to females during copulation was 4.5% (Tesh et al., 1992).

(ii) *Orbivirus*

Orbivirus is one from 15 genera belongs to family Reoviridae. This genus includes 33 virus species and its members are transmitted between vertebrates by haematophaguos arthoropods, mainly mosquitoes, biting midges, ticks, but also by phlebotomine sand flies. Orbiviruses are group of non-enveloped, icosahedral viruses with genome contains ten linear, variously sized double stranded RNA (dsRNA) segments which encodes seven structural (VP1-VP7) and at least three non-structural proteins (NS1-NS3). The most investigated virus from this group is probably Bluetongue virus and about other orbiviruses exist only limited amount of information (Verwoerd et al., 1972; Roy, 1996; Silva et al., 2014; Attoui and Jaafar, 2015).

Sand flies transmit Changuinola virus (CGLV) serogroup consisting of 12 distinct serotypes according International Committee on Taxonomy of Viruses (Almeirim, Altamira, Caninde, Gurupi, Changuinola, Irituia, Jamanxi, Jari, Monte Dourado, Ourem, Purus and Saraca viruses) but probably exist more of them. Viruses from this taxon were recorded in Panama, Costa Rica and tropical regions of South America (Silva et al., 2014; Medlin et al., 2016). Sand flies are considered as main arthropod vector of CGLV serogroup because it was isolated from both females and males, which, moreover, suggest transovarial transmission (Tesh et al., 1974; da Rossa et al., 1984). However, this serogroup was detected also in mosquitoes, rat *Oryzomys goeldi*, armadillo and sloths, where the last were implied as a primary vertebrate host (Seymour et al., 1983; da Rossa et al., 1984; Medlin et al., 2016). This virus grew in insect cell lines, particularly sand fly (LL-5), *Culicoides sonorensis* (KC) and mosquitoes (C6/36) cells and also on Vero cells. After intracerebral inoculation it was also pathogenic for newborn mice and hamsters but human health importance of this group is not documented very well, there is only a single symptomatic case described: CGVL was isolated from blood of febrile

entomologist in 1966 in Panama (Tesh et al., 1974; da Rossa et al., 1984; Silva et al., 2014; Attoui and Jaafar, 2015).

(iii) *Vesiculovirus*

Bullet shaped virions of *Vesiculovirus* contains single stranded and linear negative sense RNA genome (Letchworth et al., 1999). According Wunner et al. (1995) at least 28 representatives from this genus infect vertebrates and invertebrates. Some of them, particularly Vesicular stomatitis virus (VSV with two main serogroups: Indiana VSV-IN and New Jersey VSV-NJ), Vesicular stomatitis virus-Alagoas, Cocal, Chalcaqui, Chandipura, Isfahan and Piry viruses, are probably transmitted by sand flies and are also infectious for humans and domestic animals (Letchworth et al., 1999; Maroli et al., 2013). Cocal and Alagoas virus are related to VSV-IN and it has been tentatively classified as subtype 2 and 3 of VSV-IN respectively (Pauszek et al., 2008; Pauszek et al., 2011; Hubalek et al., 2014).

The life cycle of this virus group is not entirely clear. Suspected vectors are black flies (Mead et al., 2004), mosquitoes (Mavale et al., 2005) or biting midges (Nunamaker et al., 2000) and also non-biting arthropods, but only sand flies have been confirmed biologically, at least for VSV (Comer and Tesh., 1991; Letchworth et al., 1999). The most supporting evidence are: (i) isolation of VSV from field-collected males and females sand flies (Tesh et al., 1971; Tesh et al., 1974; Tesh et al., 1987; Comer et al., 1992), (ii) oral infection of sand flies by VSV, its replication and transmission by bite (Tesh et al., 1971; Tesh et al., 1972; Tesh and Chaniotis, 1975; Tesh et al., 1987; Comer et al., 1990; Weaver et al., 1992), (iii) transovarial transmission to progeny (Tesh et al., 1972; Tesh and Chaniotis, 1975; Tesh et al., 1987; Comer et al., 1990), which can again transmit virus by bite on animals (Tesh et al., 1972), and finally, (iv) infected phlebotominae sand flies have been detected also in absence of clinical case of domestic animals or humans while other arthropods were found infected only during epidemics (Tesh et al., 1987; Letchworth et al., 1999).

Despite all the evidence listed above, there are uncertainties in circulation of VSV. Comer et al. (1990) proved oral infection of *Lutzomyia shannoni* by VSV-NJ, replication of virus in vector and also transmission (i) by bite on rodents and (ii) transovarial on F1 generation. However, minimal infection dose for establishment of infection in sand flies was 10^6 PFU of VSV-NJ virus per ml (Comer et al., 1990). Similarly, *Lutzomyia trapidoi* became infected after feeding on hamster with virus titer in blood at least $10^{4.5}$ PFU per ml (Tesh et al., 1971). Serological studies proved infection of different animals and humans in the field by VSV (Hanson and Brandly, 1957; Tesh et al., 1987, Webb et al., 1987) but experimental studies in vertebrates showed no or low viremia lasts for a short time (Hanson and Brandly, 1957; Tesh et al., 1970; Webb et al., 1987; Howerth et al., 2006; Mesquita et al., 2017). So, what is the source of infection for sand flies between outbreaks of this disease? One possibility is that there exists a susceptible animal non-detected reservoir with enough high and long

viremia (Tesh et al., 1971). Another option is the fluid in vesicular lesions which contain more virus than blood (10^{4-6} PFU of virus per ml) (Hanson and Brandly, 1957; Comer et al., 1990). Finally, some authors hypothetizate about plants (Johnson et al., 1969; Tesh et al., 1970; Tesh et al., 1972) which sand flies use as source of sugar meal.

Vesicular stomatitis viruses (VSV-Alagoas, VSV-Indiana, VSV-New Jersey) are endemic in New World (Canada, United States, Mexico, Brazil, Panama, Colombia, Venezuela, Ecuador) and cause stomatitis disease in cattle, pigs, horses and also in humans (reviewed by Maroli et al., 2013; Hubalek et al., 2014). In infected animals this disease manifests as lesions on the lips, gums, tongue, teats and coronary band and it is unrecognizable from foot-and-mouth disease. Disease in human could be subclinical or occurs like fever and acute flu-like illness which can be accompanied by lesions in the tongue, oral mucosa or pharynx. It usually passes during 3-6 days without any complications, but rarely in children encephalitis may appear. Except of transmission by insect vector, humans can be infected by direct contact with virus or its aerosol through nasopharyngeal route or maybe through conjunctiva (Hanson and Brandly, 1957; Patterson et al., 1958; Letchworth et al., 1999).

Chandipura and Isfahan viruses are endemic in the Old World. Chandipura virus (CHPV) affects often children under fifteen years and it is agent of several outbreaks of acute encephalitis in India characterized by quick beginning of fever, effecting of central nervous system and various rate of mortality (Rao et al., 2004; Chadha et al., 2005; Gurav et al., 2010; Rajasekhran 2014; Dwibedi et al., 2015). The virus was repeatedly isolated from *Phlebotomus* and *Sergentomyia* in India during outbreaks of disease (Dhanda et al., 1970; Rao et al., 2004; Geevarghese et al., 2005; Sudeep et al., 2014) and transovarial and veneral transmission of CHPV was proven experimentally by intrathoracic inoculation in *Phlebotomus papatasi* (Tesh and Modi., 1983; Mavale et al., 2006), however, this way of infection is not natural and allowed to avoid the midgut tissue barrier. Mavale et al. (2007) proved oral infection and dissemination of CHPV in *Phlebotomus argentipes*, moreover, females infected by intrathoracal inoculation were able infect mice by bite. However, another potential vectors are mosquitoes; *Ae. aegypti* was infected by feeding on viremic mouse and transovarialy transmitted the infection into F1 generation. Out of India this virus was isolated in west Africa from sand flies (Fontenille et al., 1994) and hedgehog *Atelerix albiventris* (Kemp et al., 1974).

Isfahan virus was first time isolated in Iran from two pools of *P. papatasi* females and quite high prevalence of neutralizing antibodies against this virus were detected in humans and gerbils. Surprisingly, all tested domestic animals were negative for presence of this virus despite they serve regularly as a blood source for *P. papatasi*. Therefore, ecology, life cycle and health importance of Isfahan virus remain unknown and authors suggested circulation between sand flies-gerbils-humans (Tesh et al., 1977).

(iv) *Flavivirus*

Flaviviruses are enveloped viruses with positive-sense and single-stranded RNA genome encodes three structural (C-capsid, M-membrane, E-envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Many arthropod vectors transmit representatives of this group and many flaviviruses infect also vertebrates. Geographical distribution of flaviviruses is widespread and currently they are divided into four main groups: (i) the mosquito-borne flaviviruses (MBFVs), (ii) the tick-borne flaviviruses (TBFVs), (iii) no-known-vector flaviviruses (NKVs), and (iv) no-known-vertebrate host flaviviruses (Rice et al., 1985; Blitvich and Firth, 2015).

The role of sand flies in life cycle of flaviviruses is not clear and further studies are needed. However, some detection of flaviviruses from field collected males and females are described. Saboya virus was isolated from phlebotominae sand flies in Senegal (Fontenille et al., 1994; Traoré-Lamizana et al., 2001). However, this virus was also detected in mosquitoes, ticks, rodents and chiropters (Traoré-Lamizana et al., 2001; Konstantinov et al., 2006; Alkan et al., 2015) but it has never been isolated from humans, so its health importance is unknown. Moureau et al. (2010) received two sequences of flaviviruses from two pools of *P. perniciosus* males from Algeria and RNA of flaviviruses were also isolated from sand flies from Portugal (Alkan et al., 2015). Finally, Alkan et al., (2015) described Ecuador Paraiso Escondido virus from *Psathyromyia abonnenci* in Ecuador. This virus infected C6/36 cells, where it also replicated and produced cytopathic effect (CPE), but failed to infect vertebrate cells or suckling mice (Alkan et al., 2015).

Negevirus

Negevirus is a recently described taxon of insect-specific viruses isolated from mosquitoes and phlebotomine sandflies. Representatives of this group were isolated from insect in both tropical and temperate regions of America, Europe, Africa, Australia and also Asia. Virions are spherical shape and contain non-segmented, positive-sense and single stranded polyadenylated genom. Position of *Negevirus* in system is not clear, according phylogeny and genomic analysis, this group is most related but still relatively distant to mite-transmitted plant viruses of the genus *Cilevirus* (Vasilakis et al., 2013; Carapeta et al., 2015; O'Brien et al., 2017; Nunes et al., 2017). Kallies et al. (2014) showed that taxon *Negevirus* formed two monophyletic groups on genus level: *Nelorpivirus* and *Sandewavirus*.

In sand flies, Loreto virus was isolated from *Lutzomyia* spp. in Peru, 1977. This virus was later isolated also from *Anopheles albimanus* and *Culex* sp. in the same country (Vasilakis et al., 2013; Nunes et al., 2017). It looks like that negeviruses are not species-specific but have a broad host range among Diptera species. The most of negeviruses were detected in hematophagous insects but it can

be biased by sampling during arboviruses surveillance studies (Vasilakis et al., 2013; Nunes et al., 2017).

Life cycle of this group in nature is not clear. Infected insect cells produced CPE but infection of vertebrate cells or laboratory animals failed (Vasilakis et al., 2013; O'Brien et al., 2017). It seems that the most probable mode of transmission is transovarial way as Kawakami et al. (2016) isolated representatives of *Negevirus* (Okushiri virus) from field-caught larvae of *Aedes*. Moreover, negeviruses are most closely related to plant viruses, so plants could be included also in natural cycle because both adult sexes of mosquitoes and sand flies feed on a natural sugar sources, such as sap of plants or honeydew (Killick-Kendrick, 1999; Vasilakis et al., 2013; Kawakami et al., 2016; Nunes et al., 2017). Other hypotheses about circulation of negeviruses involve parasitic aquatic mites of mosquito adults or infections of larvae through plant detritus and microbes (Nunes et al., 2017).

1.2 Barriers in sand flies for *Leishmania*

Leishmania are digenetic protozoan parasites circulating between vertebrate host and phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae). The genus *Leishmania* was divided into three subgenera: *Leishmania*, *Viannia*, *Sauroleishmania*, which differ by development in vector. The following text deals only with suprapylarian subgenus *Leishmania* (reviewed by Lainson and Shaw, 1987).

Vector infection begins by ingestion of mammalian blood which contains macrophages with infectious amastigotes. Midgut extension stimulates midgut cells to produce the PM type I, which surrounds the blood meal. In endoperitrophic area, amastigotes transform to procyclic promastigotes, which intensively replicate. During PM disruption, these forms transform to long nectomonads, which attach to midgut cells to prevent elimination from sand fly midgut during defecation. Long nectomonads transform to short ones, also call as leptomonads, which again undergo multiplication. During the late phase of infection leptomonads migrate to cardia region and to stomodeal valve and serve as precursor for metacyclics and maybe also for haptomonad promastigotes attached to the stomodeal valve. Metacyclics serve as an infectious form transmitted to vertebrate during the next blood feeding (reviewed by Kamhawi 2006; Dostalova and Volf, 2012).

It is obvious that leishmania have to surmount several critical moments during its development from amastigotes to metacycles forms in sand flies such as (i) blood digestion by sand fly proteases (Secundino et al., 2010), (ii) defecation of remnants after blood digestion (Pimenta et al., 1997; Myskova et al., 2007; Sadlova and Volf, 2009; Sadlova et al., 2013; Sadlova et al., 2018), (iii) presence of gut microbiom (Kelly et al., 2017; Louradour et al., 2017) (iv) activity of immune system (Boulanger et al., 2004; Diaz-Albiter et al., 2012; Telleria et al., 2012; Telleria et al., 2013) and (v)

transmission to the host through stomodeal valve (Schlein et al., 1992; Rogers et al., 2002; Volf et al., 2004; Rogers et al., 2008).

1.2.1 Digestive enzymes

Various authors have contrasting opinion about the effect of sand fly digestive enzymes on *Leishmania*. While Pimenta et al. (1997) suggest that proteases have direct negative effect on leishmania and the most sensitive forms to proteolytic damage are transitional stages, amastigotes transforming to promastigotes (Pimenta et al., 1997), recent paper by Pruzinova et al. (2018) found promastigotes as the most sensitive form. Authors showed, that leishmania are not destroyed directly by digestive enzymes but rather by products that occur during blood digestion. During *in vitro* experiments, in both, susceptible and refractory sand fly-leishmania combinations, leishmania mortality correlated with degree of blood digestion; the highest mortality was noted at the end of blood meal digestion (Pruzinova et al., 2018). Secundino et al. (2010) proved that promastigotes resistance to digestive enzymes is given by proteophosphoglycans.

Interestingly, several authors reported that leishmania modulate sand fly digestive enzymes, particularly trypsin and chymotrypsin. Borovsky and Schlein (1987) described that leishmania modulated activities of these two enzymes and delayed the peak of their production. Similarly, Telleria et al. (2010) observed displacement trypsin peak activity from 12 hours post blood meal to 24 hours in *Lutzomyia longipalpis* infected by *Leishmania infantum chagasi*. However, the above-mentioned studies (Borovsky and Schlein, 1987; Telleria et al., 2010) were done with promastigotes-initiated infection, which is not a natural situation. Nevertheless, Dillon and Lane (1993) proved significant changes in midgut protease activity *P. papatasi* after *Leishmania major* amastigote-initiated infections. Similarly, in *P. argentipes* trypsin and chymotrypsin activities were significantly decreased 24 and 36 hours after *Leishmania donovani* amastigote-initiated infection compared to uninfected ones (Verma et al., 2017). Contrary, Pimenta et al. (1997) and Secundino et al. (2010) did not observed any difference in total midgut trypsin activity between bloodfed noninfected control and leishmania-infected sand flies.

Mechanism how can leishmania manipulate with production and activity proteolytic enzymes remains rather unknown. Serine peptidase inhibitors were found in *L. major* (Eschenlauer et al., 2009) and *L. donovani* (Alam et al., 2016), and Verma et al. (2017) showed that ISP2 of *L. donovani* reduced significantly both trypsin and chymotrypsin and SP1 chymotrypsin activity in bloodfed midgut lysates. Also *L. donovani* ISP2 knockdown significantly decreased and ISP2 overexpression significantly increased viability of *L. donovani* in *P. argentipes* during first 16 hours after infection compared with WT. It was proved that expression profile of leishmania ISPs vary between stages

(Eschenlauer et al., 2009; Verma et al., 2017), ISP1 were noted only in promastigotes *L. donovani*, whereas ISP2 were presence in different level during transition from amastigotes to promastigots (Verma et al., 2017). In *L. major* recombinants ISP1 and ISP2 inhibited peptidase activity of the midgut extract of *P. papatasi in vitro* (Morrison et al., 2012), but no differences were found in development of ISP1 and ISP2 knockouts, addbacks and wild type in *P. papatasi* (Volf, personal communication).

1.2.2 Peritrophic matrix

After bloodmeal ingestion, midgut cells start to produce PM type I, as a response to the midgut extension. This membrane is acellular structure formed mainly by chitin, glycoproteins and proteoglycans with pores of different size, often ranging around 7-8 nm. PM protects midgut against mechanical damage, pathogens, toxins, chemicals and also provides compartmentalization of digestion on ecto and endoperitrophic area (Terra, 1990; Peters 1992; Lehane, 1997). Dynamics of PM production is species-specific and Sadlova and Volf (2009) divided sand flies into two groups: with quick maturation (about 12 hours PBM) and slow PM formation (24-48 hours PBM). At the end of bloodmeal digestion, PM is degradated by sand fly chitinases (Ramalho-Ortigao and Traub-Csekö, 2003; Ramalho-Ortigao et al., 2005).

Role of PM seems to be crucial during leishmania development as the long persistence of intact PM represent a barrier which prevented escape of leishmania from bolus and colonisation of midgut (Walters et al., 1992; Pimenta et al., 1997). The original hypothesis by Schlein et al. (1991) about the role of sand fly chitinase in penetration of PM has never been confirmed and seems to be highly unlikely. Although leishmania produce chitinase (Schlein et al., 1991; Shakarian and Dwyer, 2000; Joshi et al., 2005), it probably plays a role only in destruction of stomodeal valve (Schlein et al., 1992; Volf et al., 2004; Rogers et al., 2008), because activity of leishmania chitinases was strongly inhibited by haemoglobin (Schlein et al., 1992; Schlein and Jacobson, 1994) and recent studies showed that leishmania are waiting for PM breakup caused by sand fly chitinases (Ramalho-Ortigao and Traub-Csekö, 2003; Ramalho-Ortigao et al., 2005; Coutinho-Abreu et al., 2010; Sadlova et al., 2013).

Escape of leishmania from endoperitrophic space before defecation into the midgut represents “window of opportunity” (Rogers et al., 2008). In *Sergentomyia schwetzi* PM remained intact almost until defecation and serve also as barrier for signal molecules trigger transformation, which seems to be key factor responsible for *S. schwetzi* refractoriness to different leishmania species (Sadlova and Volf, 2009; Sadlova et al., 2013).

1.2.3 Attachment to midgut

Nectomonads have to attach to midgut tissue to avoid the defecation after blood meal digestion. Kamhawi (2006) and Volf and Myskova (2007) divided sand flies to specific and permissive vectors. Specific vectors are able to transmit only a limited number of leishmania species because these sand flies posses midgut receptors for species-specific leishmanial lipophosphoglycan (LPG). Typical example is *P. papatasi*, which have PpGalec, midgut receptor specifically binding side chains of *L. major* LPG (Kamhawi et al., 2004) or *Leishmania turanica* (Volf et al., 2014). Another typical specific vector is *P. sergenti* transmiting only *Leishmania tropica* and not other Old World species (Kamhawi et al., 2000; Jecna et al., 2013). In addition, Di-Blasi et al. (2015) proved that also leishmania flagellar protein FLAG1/SMP1 had a role during initial phase of infection of *L. major* in midgut *P. papatasi*.

Permissive vectors support development of more leishmania species, which obviously use LPG-independent mechanism for its attachment (Myskova et al., 2007; Svárovská et al., 2010). *Leishmania major lpg1*$^{-}$ mutants, which lack LPG, did not develop in *P. papatasi* (Myskova et al., 2007; Jecna et al., 2013), but fully established infection in permissive vectors *Lu. longipalpis*, *Phlebotomus arabicus* (Myskova et al., 2007), *P. perniciosus* and *P. argentipes* (Svárovská et al., 2010). Correlation was found between permissivity and O-glycosylated proteins with N-acetylgalactosamine (GalNAc) in the midgut (Volf and Myskova, 2007; Myskova et al., 2007) and recently the midgut mucin-like protein was suggested as a receptor for parasite binding in *Lu. longipalpis* (Myskova et al., 2016).

1.2.4 Stomodeal valve

Stomodeal valve (SV) is located between midgut and foregut, its purpose is to prevent backflow of ingested food during feeding and potentially regurgitation from the intestine. It is generally assumed that in sand flies this valve is also involved in deposition of ingested food: blood is directed to the midgut whereas sugar meal goes first into the crop (Tang and Ward, 1998; Tang and Ward, 1998b).

Colonization of SV is necessary for succesfull leishmania transmission and there is a generally accepted model for the passage of parasites throughout this barrier. Haptomonads colonize SV, where attached themselves through hemidesmosome-like plaques to chitinious part of valve and destroy it, presumably by chitinases (Schlein et al., 1992; Rogers et al., 2002; Volf et al., 2004; Rogers et al., 2008). Moreover, anterior midgut is fill by promastigote secretory gel (PSG) produced probably by leptomonads. The gel, together with leishmania, blocks the midgut and keeps SV opened. Permanently opened valve leads to regurgitation of midgut content and transmission of metacycles into host during feeding (Schlein et al., 1992; Volf et al. 2004). Infected females also have a difficulty

to engorge blood and bite repeatedly (Rogers et al., 2002). All of these facts together facilitate leishmania transmission to the vertebrate host.

1.2.5 Microbiome

Phlebotomine sand flies occur in diverse habitats from deserts to rainforests and during their development live in different environments and ingest different meal. Females lay eggs into suitable organic rich substrate, which serve as food for following four larval stages, whereas both females and males feed on a natural sugar sources, such as honeydew or sap of plants. Above that, females take a blood of various vertebrates (Killick-Kendrick, 1999; Maroli et al., 2013). Environment, type of meal and also fact, that some bacteria could be transmitted transstadially from larvae to adults, are reflected by composition of midgut microbiome (Volf et al., 2002; Gouveia et al., 2008; Hillesland et al., 2008; Mukhopadhyay et al., 2012) and subsequently may affect the vector competence to pathogens (Xi et al., 2008; Ramirez et al., 2012; Ramirez et al., 2014; Carissimo et al., 2015).

In *Leishmania* several recent studies have addressed the effect of sand fly midgut microbiome. Kelly et al. (2017) studied interactions between *Lu. longipalpis*, *L. infantum* and natural gut microflora. The highest richness in gut microflora were noted in females which took only a sugar meal, while in blood fed females bacterial diversity decreased, but then returned back to the original levels after defecation. However, in leishmania-infected females the reduction in microbial richness continued and was linked to increasing numbers of leishmania. Nevertheless, elimination of microbiome by antibiotics after leishmania infection significantly reduced grow of leishmania parasites and their metacyglogenesis (Kelly et al., 2017). Similar phenomenon was observed in study with *Phlebotomus duboscqi*/*L. major*. Treatment of sand flies by antibiotics during and after leishmania infection (blood and sugar meal) or after infection (sugar meal), led to drastic reduction in intensity of infection and numbers of metacyclics 9-14 days postinfection, which was then reflected by the decreased ability of females transmit infection to vertebrates. Adding of antibiotic-resistence bacteria into blood at least partially rescued development of leishmania in vector (Louradour et al., 2017). Similar effect was observed by Carissimo et al. (2015), where presence of natural gut microflora in *An. gambiae* was necessary for full midgut infectivity of ONNV.

Different theories exist for this phenomenon, however, the one about key nutrients provided by bacteria is not supported by experimental data: supernatant from bacterial cultures did not rescue leishmania development in antibiotics treated sand flies (Louradour et al., 2017). Kelly et al. (2017) hypothetized that bacteria in midgut provide signals necessary for leishmania differentiation by changing of pivotal nutrients concentration or through impact on sand fly cells and thus on

immune system. It seems that deprivation in adenosin, respectively purines, may stimulate metacyclogenesis (Serafim et al., 2012).

Both genders of sand flies feed naturally on sugar meal which serve as a source of energy for all: sand flies, leishmania and microbiome. Louradour et al. (2017) suggested that bacteria decrease osmolyte concentration by consumption of sucrose to acceptable level for leishmania. Three different genera of bacteria previously isolated from *P. duboscqi* were able, at least partially, to save leishmania survival and development in sand flies treated by antibiotics. These results also indicated that this effect is more general and rather connected with bacterial colonization, than effect of some specific bacterial species (Louradour et al., 2017).

On the other hand, prefeeding of *Lu. longipalpis* by *Asaia* sp. or *Ochrobactrum intermedium* or combination of yeast *Pseudozyma+Asaia* four days before infection *Leishmania mexicana* significantly decreased intensity of infection and last two treatments also declined infection rate (Sant'Anna et al., 2014). Bacteria infection trigger different parts sand fly immune system *in vitro* (Tinoco-Nunes et al., 2016) and also *in vivo* (Boulanger et al., 2004; Diaz-Albiter et al., 2012; Telleria et al., 2013). Theoretically, increasing number of bacteria in midgut after blood feeding (Pumpuni et al., 1996; Volf et al., 2002) can stimulate immune system, which can lead also to negative effect on leishmania, at least in some vector/bacteria/leishmania combination. Such effect was observed in *Ae. aegypti*, where natural gut microbiome stimulated immune system, leading to antiviral activity (Xi et al., 2008).

Nevertheless, it should be noted that parts of sand fly microbiome are not only extracellular bacteria (Sant'Anna et al., 2012; Kelly et al., 2017; Louradour et al., 2017) but it can be also *Wolbachia*, as intracellular endosymbiont (Azpurua et al., 2010; Vivero et al., 2017), and eukariotic organisms as fungi and yeasts (McCarthy et al., 2011; Akhoundi et al., 2012; Maleki-Ravasan et al., 2014). Their role is unknown, nevertheless Azpurua et al. (2010) found field-caught *Lu. trapidoi* coinfected by *Leishmania* sp. and *Wolbachia*, so at least in this combination *Wolbachia* does not inhibit leishmania development.

1.2.6 Immune response

So far, very few studies have addressed the immune response of sand flies against *Leishmania* sp. The first sand fly antimicrobial peptide studied was defensin (Boulanger et al., 2004). Authors described differences in defensin expression pattern in *P. duboscqi* infected by promastigotes and amastigotes of *L. major*. In amastigote-initiated infections Boulanger et al. (2004) found that the expression levels of defensin were increased only at day 10 postinfection; it was four times higher compared to non-infected females (Boulanger et al., 2004). In *Lu. longipalpis* defensin expression was

increased at 48 hour post blood meal and then slowly declined regardless of whether the females were infected with *L. mexicana* promastigotes or not. Nevertheless at 144 hours PBM defensin expression significantly decreased in *Leishmania* infected females. Authors hypothetized that expression of defensin is correlated with increasing numbers of bacteria after bloodfeeding and presence of *Leishmania* competitively reduced bacteria amount and thanks to that also defensin expression (Telleria et al., 2013). Differences between studies could be given by different sand fly-leishmania combination or experimental design; Boulanger et al. (2004) used natural amastigote infection, whereas Telleria et al. (2013) used promastigotes.

Boulanger et al. (2004) proved a negative effect of recombinant sand fly defensin on promastigotes *L. major in vitro*, however how it works *in vivo* is still unknown. Nevertheless, recombinant defensin from plants had also anti-leishmania activity, inhibited promastigote replication and dislocation of plasmma membrane, which finally caused cytoplasmic fragmentation and occurrence of multiple cytoplasmic vacuoles (Souza et al., 2013; do Nascimento et al., 2015).

Two other studies on leishmania interaction with sand fly immune system have been done by Diaz-Albiter et al. (2012) and Telleria et al. (2012). Telleria et al. (2012) monitored expression of Caspar, negative regulator of Imd pathway, in *Lu. longipalpis* after amastigote infection by *L. mexicana*. Caspar expression were significantly lower three and six days after infection, compared with uninfected bloodfed females. This time correlates with leishmania leaving from endoperitrophic space and colonization of the midgut. Silencing of Caspar by RNAi prior to blood feeding reduced *L. mexicana* numbers in *Lu. longipalpis* five days post infection. Similar effect was visible in promastigote-initiated infections in combination *L. infantum*-*Lu. longipalpis*, parasite growth was reduced and infection rate was decreased (from 85% to 45%). These data suggest that leishmania probably activate Imd pathway but this activation is not efficient enough to disrupt transmission (Telleria et al., 2012). Diaz-Albiter et al. (2012) studied midgut production of reactive oxygen species (ROS) during infection of *Lu. longipalpis* by *L. mexicana* amastigotes. Results implied that leishmania did not activate production of ROS, nevertheless feeding on H_2O_2 or silencing catalase (the anti-oxidant) had negative impact on leishmania survival (Diaz-Albiter et al., 2012).

1.2.7 Coinfection

Sand flies were experimentally coinfected by different strains or even different leishmania species, which also lead to hybridization and such hybrids may differ from the parental lines in virulence or transmission potential (Akopyants et al., 2009; Volf et al., 2007; Sadlova et al., 2011). *Leishmania infantum*/*L. major* hybrids were able to develop and colonize the stomodeal valve in *P. papatasi*, unlike to *L. infantum* (Volf et al., 2007) which has very important epidemiological consequences as

P. papatasi is widespread and antropophilic vector. Chajbulinova et al. (2012) tested coinfection of *P. papatasi* by *L. major* and *L. turanica*, parasites co-circulating in same area in nature: both species were able to develop in sand fly midgut together, without any visible competition. In this combination no hybrids were observed (Chajbulinova et al., 2012).

Sand flies are also vector of viruses (chapter 1.1.6) which co-circulate in the same area as leishmania (reviewed by Moriconi et al., 2017) (Fig. 1) and potentially coinfect the same vector. However, there is very little information about these interactions. Bichaud et al. (2011) tested human sera from Marseille and proved epidemiological link between Toscana virus and *L. infantum* (both having the same vector, *P. perniciosus)*: leishmania-positive individuals are at greater risk of being infected by TOSV and vice versa. Nevertheless, it is not clear if this is because a bite of coinfected vector or because repeated bites of different vectors with a single infection (Bichaud et al., 2011). Dincer et al. (2015) found coinfection of *L. infantum* and Toscana virus in two dogs in Turkey, Ergunay et al. (2014) demonstrated common coocurance of these pathogens in *P. tobbi* in Cyprus. Es-sette et al. (2014) proved also cocirculation TOSV and *L. tropica* through *P. sergenti* in Morocco and suggested transovarial way of TOSV transmission because of positive pool of males.

Nevertheless, consequences and impact of TOSV and leishmania coinfections in sand flies or vertebrates are unclear. One of the reason is that TOSV life cycle in nature remains unknown (more detailed in chapter 1.1.6), which substantially complicates any experiments. This is why we decided to study infection routes and development of MASV in sand flies (Jancarova et al., manuscript).

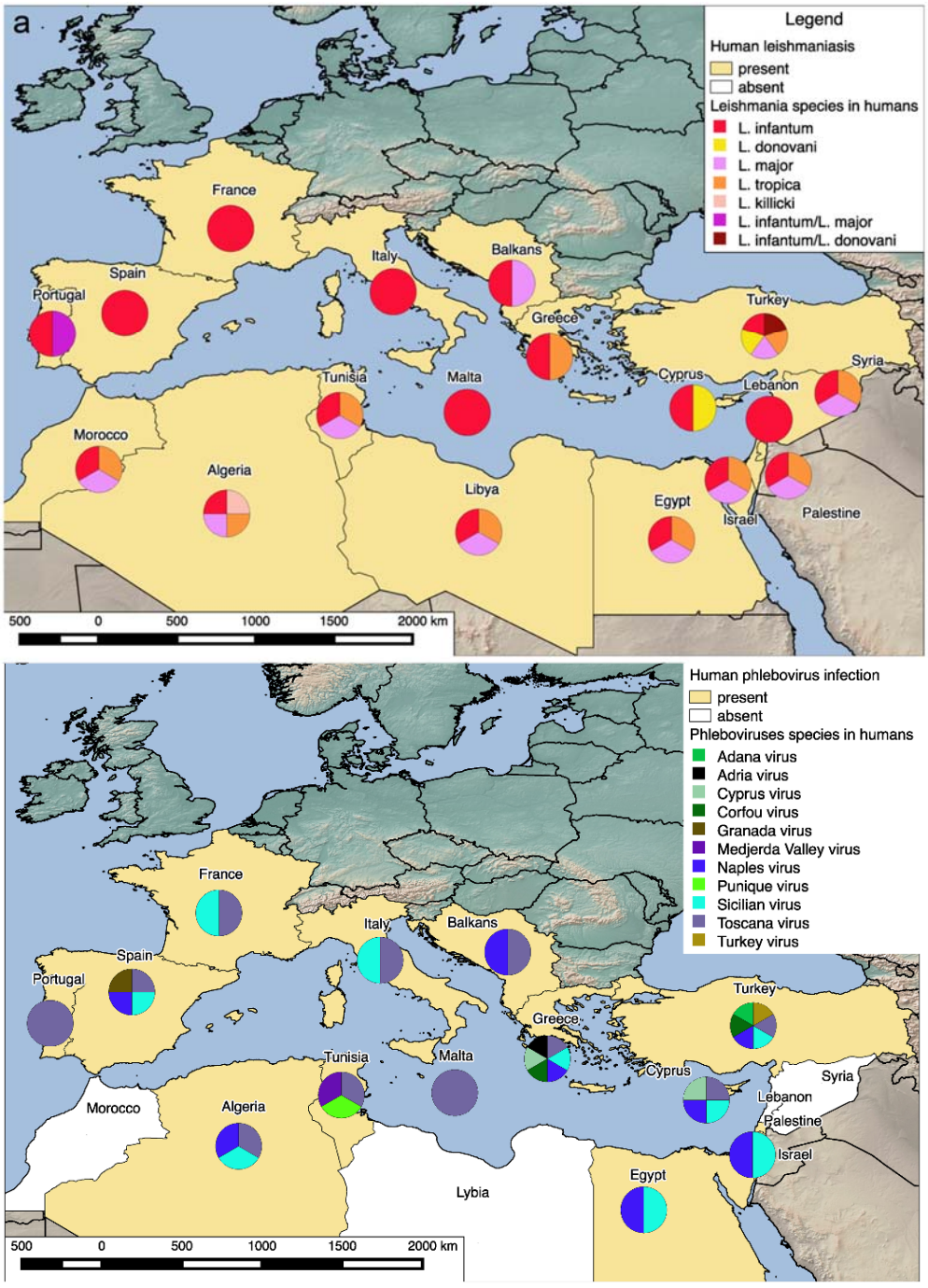

Fig 1. Taken from Moriconi et al. (2017): a) Autochthonous human leishmania infection in the Mediterranean detected by serological tests and/or *Leishmania* isolation and/or PCR. b) Autochthonous human phlebovirus infection in the Mediterranean detected by serological tests and/or *Phlebovirus* isolation and/or PCR

It was also suggested that leishmania help to vector and partially protect them against other pathogens. Sant'Anna et al. (2014) gave bacteria *Seratia marcescens* to *Lu. longipalpis* already infected by *L. mexicana*. Presence of leishmania significantly increased survival after exposition to *S. marcescens* (56% vs 11% six days after coninfection).

Except of human pathogens, sand flies also harbour its own parasites, particularly gregarines, which may coexist with leishmania infection, see chapter 1.3.

1.2.8 Temperature

Ambient temperature influences various aspects of sand fly life, such as developmental rate and time, metabolic processes, longevity and mortality (Ghosh et al., 1992; Kasap and Alten, 2005; Benkova and Volf, 2007; Chelbi and Zhioua, 2007; Hlavacova et al., 2013). Each sand fly developmental stage possessed various thermal sensitivities as was shown in colony *P. papatasi*: minimal temperature for eggs was 11.6°C, for larvae 19.8°C, for pupae 17.2°C, and temperature necessary for full development from eggs to adults was 20.3°C (Kasap and Alten, 2005). Not only different developmental stages but also different species have distinct thermal optima (Guzmán and Tesh, 2000; Prudhomme et al., 2015). In addition, ambient temperature can affect vector competence; this has been demonstrated in combination of mosquitoes-arbovirus (Westbrook et al., 2010; Muturi and Alto, 2011; Dodson et al., 2012; Adelman et al., 2013) or mosquitoes-*Plasmodium* (Paaijmans et al., 2011).

As the effect of temperature differs in various vector-pathogen combinations, we decided to test the effect of different larval rearing temperatures (27°C versus 32°C) on susceptibility of *P. sergenti* females to *L. tropica*. In sand flies, there are only two studies on the effect of ambient temperature on *Leishmania* development. *Leishmania peruviana* successfully developed in the permissive vector *Lu. longipalpis* only at 20°C, while at 26°C infection rate and intensity of infection rapidly declined. Lower temperature delayed female defecation of bloodmeal and authors hypothetized that it gave more time to leishmania to establish in the midgut. In *P. perniciosus* and *Lu. longipalpis* lower temperature decreased intensity of *L. infantum* infection after blood feeding, but finally it did not affect later phase of infection (Hlavacova et al., 2013). Similarly, Sadlova et al. (2013) did not find any effect of temperature (21°C vs 26°C) on development *L. infantum* in *Lu. longipalpis*.

1.3 Gregarines

Gregarines are typical pathogens of insects. Originally, gregarines of sand flies and mosquitoes were included in one genus *Ascogregarina* but Votypka et al. (2009) reclassified this taxon and moved sand fly gregarines into a new genus *Psychodiella*. During our experiments, we used *Psychodiella sergenti*, specific parasite of *P. sergenti* (Lantova et al., 2010). The life cycle starts, when the first instar larvae swallow oocysts containing sporozoites, which are then released into the midgut lumen, attach to midgut epithelial cells and develop into trophozoites. Later, trophozoites de-attach from midgut cells

and form gamonts. In fourth instar larvae, gamonts undergo sexual development in the midgut lumen, during process called such as syzygy, and form gametocysts. In adults, gamonts occur only in body cavity and syzygy take place exclusively in females after blood feeding. Subsequently gametocysts attach to accessory glands and release oocysts into their lumen. Finally, during laying eggs, oocysts contaminate eggs chorion and larvae are infected perorally during or soon after hatching (Lantova et al., 2010; Lantova et al., 2011; Lantova and Volf, 2012). *Psychodiella sergenti* is the only one sand fly species known to develop sexual development exclusively in adult females after bloodfeeding (rewieved by Lantova and Volf, 2014).

Effect of *Ps. sergenti* on its natural host *P. sergenti* was assessed by Lantova et al. (2011). Gregarines significantly increased mortality of immature stages and this phenomenon has been highlighted under stressful conditions, such as keeping larvae in higher density. Moreover, infection by this parasite also led to higher mortality of males and non-blood fed females (but not the blood fed ones). Authors hypothesized that blood meal, such as food rich for nutrients, helps to females surmount negative effect of gregarines (Lantova et al., 2011). Similarly, Wu and Tesh (1989) found that *Psychodiella chagasi* negatively influenced survival of *Lu. longipalpis*.

Critical phase for development of gregarines in the sand fly is the pupation of the insect host (Lantova et al., 2011). Larvae of Diptera defecate content of intestine shortly before pupation and during pupation there is massive restructuralization of gut and other tissue (Hakim et al., 2010; Fernandes et al., 2014). Another limiting factor is ambient temperature. Kolman et al. (2015) *in vitro* tested impact of temperature on viability of *Blabericola migrator* and *Blabericola cubensis*, parasites of cockroaches. No gametocysts finished development or produced oocysts at 10°C or 40°C. Oocysts viability was highest at 22°C and with higher temperature markedly decreased to 2% and 5% at 35°C for *B. migrator* and *B. cubensis* respectively (Kolman et al., 2015). Therefore, we studied the effect of rearing temperature on development of *Ps. sergenti* in its natural host, *P. sergenti.*

Sand fly immune system seems to play an important role in gregarine life cycle, but information about these interactions are scarce. Warburg and Ostrovska (1989) described encapsulation of *Ps. chagasi* gametocysts in *Lu. longipalpis*, nevertheless, this reaction seems to be rather beneficial for gregarines. Encapsulation increased press inside gametocysts and significantly improved release of oocysts on accessory glands lumen (Warburg and Ostrovska, 1989). There seems to be a certain balance between sand flies and gregarines, which probably developed during coevolution.

The impact of vector coinfection by gregarines and other pathogens was not studied in sand flies yet but some studies were done in mosquitoes. Chikungunya virus could be transmitted through oocysts *Ascogregarina culicis* on *Ae. aegypti* and moreover gregarines allowed to the virus to survive drying (Mourya et al., 2003). Contrary, coinfection of *Ae. triseriatus* by *Ascogregarina barretti* and

LACV did not cause any changes in infection rate of virus (Miller and DeFoliart, 1979). Other studies were focused on coinfection of gregarines and *Dirofilaria immitis* in mosquitoes.

Sneller (1979) demonstrated that *Ae. aegypti* infected by *As. culicis* showed higher mortality during coinfection with *D. immitis* compared to gregarine-free group. Moreover, presence of gregarines decreased infection rate and infected females possessed lower number of infectious L3 larvae of *D. immitis* (Sneller, 1979). On the other hand, Beier (1983) observed that common infection of *As. barreti* and *D. immitis* in *Ae. triseriatus* did not affect the infectious parameters of filariae in vector. According to this author, differences between studies are probably caused by the fact that *Ae. aegypti* is not the natural vector of *D. immitis* (Beier, 1983).

Interestingly, presence of gregarines could be beneficial for both, host and coinfected pathogens. Larvae of *Ae. albopictus* were infected by *Ascogregarina taiwanensis* and subsequently kept under low and high nutrients diet until adulthood, when gregarine-positive and negative females were infected by *D. immitis* through a bloodmeal. Coinfected females developed from larvae kept under high nutrient conditions survived significantly better after blood feeding and more females possessed infective L3 larvae than in only filaria-infected females. In females developed from larvae under low nutrients diets, authors did not observe any differences in these parameters (Comiskey et al., 1999).

Based on the contrasting information provided above, we decided to test whether gregarines affect development of *L. tropica* in its natural vector *P. sergenti*.

2 Objectives

Aims of this thesis were to contribute to the study of barriers and critical factors which affect development of pathogens transmitted by sand flies, namely viruses, leishmania and gregarines.

The main objectives of the study were:

1. clarify the life cycle of sand fly-borne viruses by study different ways of infection *P. perniciosus* by MASV

2. verify the hypothesis that peritrophic matrix of *S. schwetzi* represents the main barrier for *Leishmania* development

3. test if ambient temperature during larval stage affects vector competence of *P. sergenti* females to *L. tropica*

4. study whether presence of gregarine *Ps. sergenti* in sand fly *P. sergenti* influences development of *L. tropica*

3 Publications

Jancarova M., Bichaud L., Hlavacova J., Priet S., Spitzova T., Volf P., Charrel R. (manuscript): Experimental infection of sand flies by Massilia virus and viral transmission by co-feeding on sugar meal

Sadlova, J., Homola, M., Myskova, J., **Jancarova, M.**, & Volf, P. (2018). Refractoriness of *Sergentomyia schwetzi* to *Leishmania* spp. is mediated by the peritrophic matrix. *PLoS Neglected Tropical Diseases*, *12*(4), e0006382.

Jancarova, M., Hlavacova, J., Votypka, J., & Volf, P. (2016). An increase of larval rearing temperature does not affect the susceptibility of *Phlebotomus sergenti* to *Leishmania tropica* but effectively eliminates the gregarine *Psychodiella sergenti*. *Parasites & Vectors*, *9*(1), 553.

Jancarova, M., Hlavacova, J., & Volf, P. (2015). The development of *Leishmania tropica* in sand flies (Diptera: Psychodidae): A comparison of colonies differing in geographical origin and a gregarine coinfection. *Journal of Medical Entomology*, *52*(6), 1378–1380.

Jancarova M., Bichaud L., Hlavacova J., Priet S., Spitzova T., Volf P., Charrel R. (manuscript): Experimental infection of sand flies by Massilia virus and viral transmission by co-feeding on sugar meal

Experimental infection of sand flies by Massilia virus and viral transmission by co-feeding on sugar meal

Authors: Magdalena Jancarova, Laurence Bichaud, Jana Hlavacova, Stephane Priet, Tatiana Spitzova, Petr Volf, Remi N. Charrel

Corresponding author: Remi N. Charrel: remi.charrel@univ-amu.fr

Abstract:

Massilia virus (MASV) belongs to phleboviruses and is closely related to Toscana virus, causative agent of human neuroinvasive disease. Circulation of phleboviruses in nature is poorly understood, experimental studies demonstrated that transovarial and sexual transmission are not enough efficient for maintenance of virus in nature and there is no convincing evidence for vertebrates as reservoirs of the virus. We studied various transmission routes of MASV isolated from *Phlebotomus perniciosus* and its development in various sand fly species. In *P. perniciosus* 4 types of infection were compared: in larval food to the first instar larvae (L1) or to the fourth instar larvae (L4), by blood meal to adult females and by sugar meal to adults of both sexes. From 875 adults emerged from infected L1 and L4 only three were positive. In females infected by bloodmeal the infection rate was high before defecation, then it decreased and MASV was detected in 5 out of 27 post defecation. Surprisingly, the most efficient route of infection was through sugar meal: 72% of females (88 out of 122) and 51% of males (58 out of 113) became virus-positive. Moreover, these sand flies regurgitated virus particules into a drop of sugar which remained infectious for naïve sand flies for at least 24 hours. Almost 30% of *P. perniciosus* (both males and females) get MASV from the sugar with expectorated virus. We suppose that transmission by co-feeding of infected and uninfected sand flies on a sugar source may represent an important part of the life cycle of MASV. Sugar meal infection was tested also in other sand flies species belonging to the three different genera: *Phlebotomus orientalis*, *Phlebotomus papatasi*, *Phlebotomus sergenti*, *Phlebotomus argentipes*, *Sergentomyia schwetzi* and *Lutzomyia longipalpis*. In males, no significant differences were found in intensity of infection and infection rates. In females *P. perniciosus* was the only species in which the infection rate grew steadily for the whole time of experiment duration.

INTRODUCTION

Phlebotomine sand flies (Diptera: Phlebotominae) occur in a wide variety of habitats, from deserts to rainforests. They lay eggs into substrate rich in organic content. Four larval stages are followed by pupae and adults. Both adult sexes feed on a natural sugar sources, such as sap of plants or honeydew, but females feed also on blood of various vertebrates to obtain proteins required for egg development. Two genera, *Phlebotomus* in the Old World and *Lutzomyia* in the New World, frequently bite humans. Consequently, members of these two sand fly genera are known vectors of human or animal pathogens, namely *Leishmania* parasites, bacteria (e.g. *Bartonella bacilliformis*), and viruses belonging to genera *Phlebovirus*, *Vesiculovirus* and *Orbivirus* (reviewed by Killick-Kendrick, 1999; Maroli et al., 2013; Alkan et al., 2013).

According International Committee on Taxonomy of Viruses (ICTV) (Plyusnin et al., 2011), the genus *Phlebovirus* includes nine viral species (Bujaru, Candiru, Chilibre, Frijoles, Punta Toro, Rift Valley fever, Salehabad, Sandfly fever Naples and Uukuniemi) and 33 tentative species. Most of them are transmitted by phlebotomine sand flies, however Uukuniemi virus and Rift Valley virus are vectored preferably by ticks and mosquitoes, respectively (Hubalek and Rudolf, 2012; Alkan et al., 2013; Horne and Vanlandingham, 2014).

Phleboviruses cause human diseases with variety of clinical syndromes from mild febrile to neuroinvasive disease. Among those circulating in Mediterranean area, Toscana virus (TOSV) seems to be the most important one. Toscana virus manifests with variety of symptoms from non-symptomatic forms through febrile illness to CNS disease and it is the major causative agent of meningitis during warm seasons (Charrel et al., 2005; Charrel et al., 2012). It circulates on large geographic area, from Turkey to Morocco (Charrel et al., 2012; Es-Sette et al., 2015) and is usually connected with the presence of vectors *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*. Moreover TOSV was isolated also from *Sergentomyia minuta* in Marseille (Charrel et al., 2006), *Phlebotomus tobbi* in Cyprus (Ergunay et al., 2014), *Phlebotomus longicuspis* and *Phlebotomus sergenti* in Morocco (Es-Sette et al., 2014; Es-Sette et al., 2015), but role of these species in virus circulation remains unknown.

Massilia virus (MASV) was recently described and has been classified in the Sandfly fever Naples species, together with TOSV, of which it is closely related in terms of physical properties and antigenic properties although it is genetically distinct. MASV was first isolated

in 2005 from *P. perniciosus* trapped in Marseille and Nice, two cities of southeastern France. Subsequently, MASV was detected and isolated in several pools of *P. perniciosus* collected in France and a close relative (named Granada virus) was isolated in Spain. Interestingly, MASV was always associated with *P. perniciosus* which is also a main vector of TOSV (Charrel et al., 2009).

The circulation of sand fly-borne phleboviruses in nature is poorly understood. There is no convincing evidence supporting the existence of human or animal vertebrate reservoirs; vertebrates are considered as dead-end hosts without significant role in life cycle of the virus while sand flies are suggested as primary reservoirs by some authors (Tesh and Chaniotis, 1975; Alkan et al., 2013). Experimental studies demonstrated transovarial (Tesh and Modi, 1987; Tesh et al., 1992; Maroli et al., 1993) and sexual (Tesh et al., 1992) transmission of TOSV between sand flies. However, the transovarial and sexual transmission are not enough efficient for maintenance viruses in nature (Tesh and Modi, 1987; Tesh et al., 1992) and non-viraemic transmission known in ticks (Labuda et al., 1993), mosquitoes (Higgs et al., 2005) and black flies (Mead et al., 2000) was not demonstrated yet in sand flies. Therefore, some other alternative routes or different combinations of transmission could be expected.

Here we study MASV as safety laboratory model for investigation of unknown transmission routes of phleboviruses. Moreover, we studied various aspects of MASV biology and its infectivity for seven sand fly species belonging to three different genera, *Phlebotomus, Lutzomyia* and *Sergentomyia*.

MATERIAL AND METHODS

Sand fly colonies: Seven sand fly colonies tested were reared at Laboratory of Vector Biology, Charles University in Prague for many generations. The colony of the New World sand fly *Lutzomyia longipalpis* was established from females caught in Brazil. Six Old World colonies were represented by *P. perniciosus* (originated from Spain), *Phlebotomus papatasi* and *P. sergenti* (both from Turkey), *Phlebotomus orientalis* and *Sergentomyia schwetzi* (both from Ethiopia), *Phlebotomus argentipes* (from India). Unless otherwise specified, sand flies were maintained under standard conditions as described previously (Volf and Volfova, 2011). Before the experiments each colony was tested for presence of MASV and all were negative.

Virus strain.

All experiments were performed using Massilia virus strain UVE/MASV/2009/FR/M43(Ref-SKU #001V-02369https://www.european-virus-archive.com/virus/massilia-virus-strain-uvemasv2009frm43). Each vial contained 0.2mL which corresponded to $3.32x10^6$ TCID50.

Different infection routes for MASV infection in sand flies.

Phlebotomus perniciosus was used to study and compare the effectiveness of infection in 4 types of sand fly developmental stages through different pathways: i) in larval food to the first instar larvae (L1), ii) in larval food to the fourth (last) instar larvae (L4), iii) by blood meal to adult females and iv) by sugar meal to adults of both sexes.

For infections of L1 and L4, the lyophilized virus diluted in 500 µl of distillated water ($6.64x10^6$ TCID50/mL) was mixed with the same volume of larval food which consists of composted rabbit chow and rabbit feces (for details see Volf and Volfova, 2011).

L1 larvae kept in breeding pots were given this mixture on day 0 and day 2 of the experiment (D0 and D2). Then, 20 larvae were collected at various intervals (D2, D5, D12 and D19) and individually stored in 70% ethanol (Merck, Millipore). From the same breeding pot, 20 pupae were collected on D26, and freshly emerged adults on twelve different intervals (D33, D35, D37, D38, D40, D42, D45, D47, D49, D52, D54, D56), all specimens were again stored individually in 70% ethanol.

Actively feeding L4 were infected by larval food by a single infected food (D0) as described above. Ten larvae and ten pupae were collected on days D2 and D5, respectively. All adults

emerging from the same container were collected at seven different intervals (D12, D14, D16, D19, D21, D23, D26) and individually stored in 70% ethanol.

For blood meal infections of adult *P. perniciosus* females the lyophilized virus was diluted in 200 µl of sterile distillated water ($3.32x10^6$ TCID50) and mixed with 1.8 ml of heat-inactivated rabbit blood ($1.66x10^5$ TCID50/mL) and fed to approximately 150 females through a chick-skin membrane using a glass feeder (similar to *Leishmania* experimental infections described by Volf and Volfova, (2011)). Fully engorged females were separated into a new cage and kept at 26°C. Females collected on days D0, D5, D7, D9 (10, 10, 10 and 7 females, respectively) were stored individually in 70% ethanol.

For sugar meal infections, groups of freshly emerged adults, 200 males and 200 females, were separated into two cages and offered by a mixture of 500 µl of sugar with lyophilized virus ($6.64x10^6$ TCID50/mL). The sugar solution was the same as used for maintenance of sand fly colonies (50% brown sugar in sterile distilled water prepared as described by Volf and Volfova, 2011). The fresh mixtures of sugar with MASV were soaked into a small piece of cotton wool placed on a small glass Petri dish and offered to sand flies twice, on D0 and D2. About ten males and ten females were then collected at 11 time intervals (D2, D5, D7, D9, D12, D14, D16, D19, D21, D23 and D26) and stored as described above.

MASV detection in experimentally infected sand flies

All samples of various developmental stages (larvae, pupae and adults) were processed at UMR190, "Emergence des Pathologies Virales", Aix-Marseille Université in Marseille. Before processing, L1 and L4 were washed in physiological solution to remove larval feces and particles of infectious food. Each sample (larva, pupa or adult) was homogenized individually in 600 µl of Eagle minimal essential medium (EMEM) supplemented with 7% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine (200 mM) using a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) in the presence of a 3-mm tungsten bead. The resulting homogenate was centrifugated at 5000 g for 5 min to separate supernatant. 200 µl of supernatant was processed further and rest was stored at -80°C. Viral nucleic acid was extracted by the Virus Extraction minikit (Qiagen) by BioRobot EZ1-XL Advanced (Qiagen) and eluted into 90 µl. Five microliters of this solution were used for real-time RT-PCR performed by SuperScript® III Platinum® One-Step qRT-PCR Kit w/ROX (Invitrogen) according to manufacturer's protocol on a CFX96 real-time system (Bio-Rad): (i) 48°C for

30 min, (ii) 95°C for 2 min, (iii) 95°C for 30s, (iv) 60°C for 1 min; steps (iii) and (iv) were repeated 45x. Primers and probes designed for the nucleoprotein gene specific for MASV were described previously by Charrel et al. (2009).

Co-feeding experiments

We investigated the hypotheses if infected sand flies spit the virus during sugar feeding (first series of experiments) and then if virus particles are infectious to other sand flies (Experiment 2). For this second experiment we invented the term "co-feeding on sugar" to distinguish it from co-feeding on the host known from other virus-vector interactions (Labuda et al., 1993; Higgs et al., 2005)

In the first series of experiments, 100 females and 100 males were infected by sugar feeding as described above (on days D0 and D2). From day D4, groups of infected sand flies were offered a non-infected sugar meal in various intervals post-infective feeding. A small drop of sugar on parafilm in glass Petri dish was placed into the cage with 100 infected sand flies for 24 hours (high humidity in the cage prevents desiccation). Then, viral nucleic acid was extracted from sugar drop by QIAamp Viral RNA Mini Kit (Qiagen) and submitted to RT-PCR as described above. Experiment was done three times.

In the second experiment, groups of 300 females and 300 males were separated into two cages (cage A♀ and A♂) and infected by sugar meal at D0 and D2. From D6 to D14 we gave them almost every day a small glass Peter dish covered with parafilm with a drop of non-infected sugar meal on top of it. Next day we removed the sugar meal, took a 140 µl for real-time RT-PCR and transferred the rest to another cages (B♀ and B♂) with non-infected females and males to test, if spitted MASV is infectious for other sand flies. Sand flies from cages B♀ and B♂ were removed at D13, D16 and D21 of experiment, stored in 70% ethanol individually and examined for presence of MASV as described above. The scheme of experiment is depicted on Figure 1.

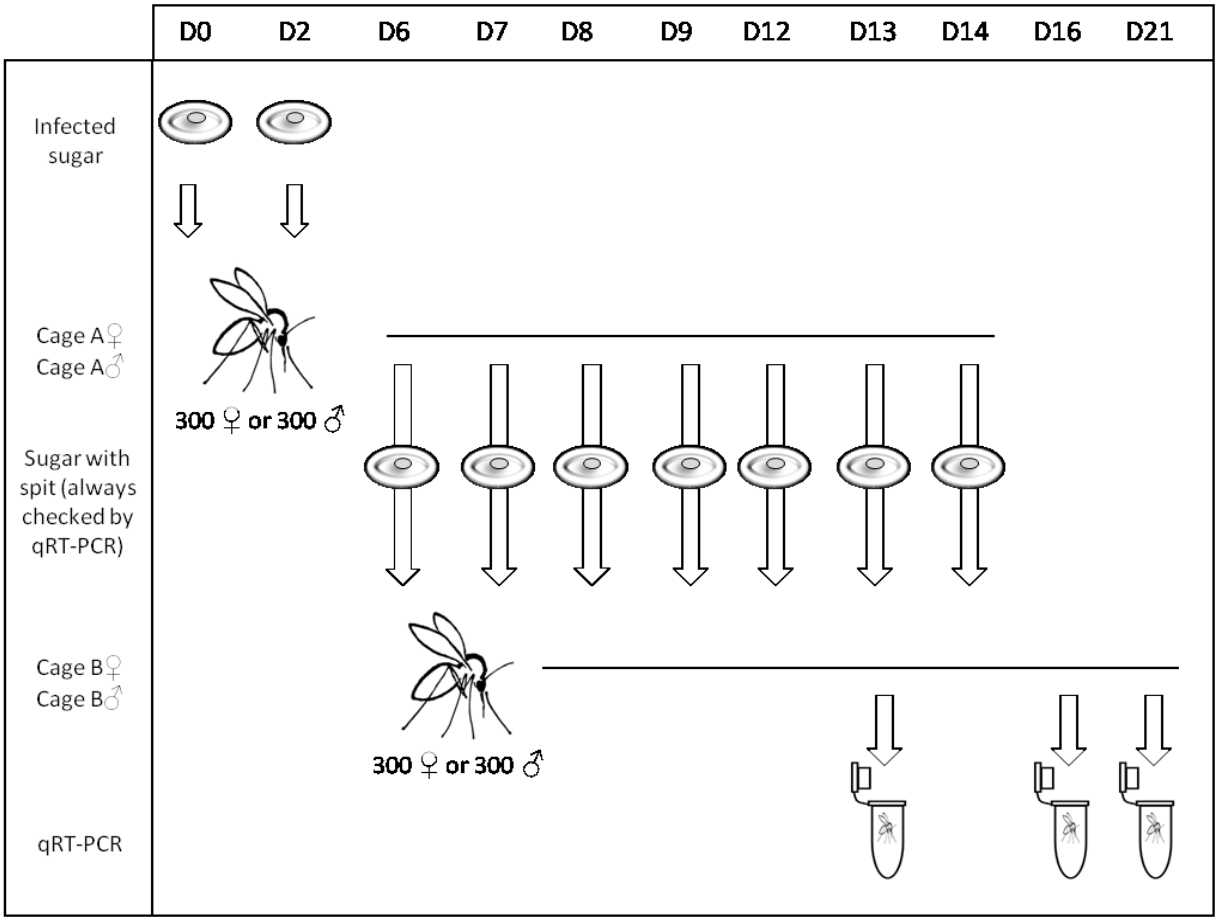

Figure 1: Schema of co-feeding experiment on sugar meal

Susceptibility of various sand fly species to MASV

Seven species belonging to all three sand fly genera *Phlebotomus, Lutzomyia* and *Sergentomyia* were used: *P. perniciosus* (positive control), *P. orientalis*, *P. papatasi*, *P. sergenti*, *P. argentipes, S. schwetzi* and *L. longipalpis*. For each species, to 250 females and 250 males were offered an infected sugar meal on D0 and D2. Then, up to 10 males and 10 females were collected up to eight time intervals (D2, D4, D7, D9, D11, D14, D16, D21) and stored individually in 70% ethanol. The experiment was repeated with another batch of sand flies and virus.

Tissue specificity of MASV

Females of three species belonging to three sand fly genera were chosen: *P. perniciosus*, *S. schwetzi* and *L. longipalpis*. 200 females of each species were infected by sugar meal at D0 and D2. Then, at three time intervals (D4, D7, D10), 10 females were carefully dissected to obtain salivary glands, midgut and the rest of body separately from each individual female.

During dissection, salivary glands and midgut were washed in drop of sterile physiological solution to prevent contamination by another tissue. All samples were homogenized in 20 µl of EMEM by automatic homogenizer than 120 µl of EMEM medium was added. Viral nucleic acid was extracted from the entire sample (140 µl) and RT-PCR for detection of MASV was performed as described above.

Statistical analysis

Statistical analyses were carried out using R software (http://cran.r-project.org/). Association between infection and the infection route (blood, sugar) was analysed using Chi^2 test. The relationship between virion loads and time and infection rate and time among the tested colonies were tested by fitting Generalised linear models (GLM) with quasi-poisson distribution and with quasi-binomial distribution, respectively. Used models included interaction terms to test differences between the colonies. The relationship between virion loads in different tissues (salivary glands, gut and rest of the body) and time among the tested colonies were analysed by fitting GLMs with quasi-poisson distribution. Used models included interaction terms to test differences between colonies. A p-value of <0.05 was considered to indicate statistical significance.

RESULTS

Infection of *P. perniciosus* L1 using larval food

Larvae of the first instar (L1) were infected by virus suspension mixed with larval food. Out of 78 infected L1 collected from D2 to D19, MASV was detected only in 6 larvae collected at D5. Forty larvae collected from D12 to D19 were negative. Low positivity (5%) was detected in pupae, one out of 20 collected on D26 was found positive for MASV. Even lower positivity was found in adults: among the 796 adults emerged from infected L1 between D33 to D56 (399 females and 397 males together), only one male (D35) was positive for MASV.

Infections of *P. perniciosus* L4 by larval food

Larvae of the fourth instar (L4) were infected by virus suspension mixed with larval food. All 30 L4 of *P. perniciosus* collected at D0 and D2 were negative. From 10 pupae sampled at D5, four were positive. Out of 79 adults emerged from D12 to D26, two were positive for MASV at D14 and 1 at D16.

***Phlebotomus perniciosus* females infected by bloodmeal**

Phlebotomus perniciosus females were infected by mixture of MASV and blood. Females defecated blood meal remains on day 4. Therefore, we may distinguish between non-defecated females (D0) and defecated ones (D5, D7 and D9). As expected, ten non-defecated females D0 had a high rate of infection (90%). In defecated females the infection rate was lower (18.5%), the MASV was detected in 5 females out of 27 on D5, D7 and D9 (positivity 20% (2/10), 20% (2/10) and 14%, respectively (1/7)).

***Phlebotomus perniciosus* males and females infected by sugar meal**

Adults of both genders were infected by MASV through sugar meal. In total, 72.1% (88/122) females collected from D2 to D26 were positive for MASV. The infection rate of females was more or less stable (between 70-100%) till D19 and then declined to 27-53%. Slightly lower infection rate was found for males, only 51.3% (58/113) of males were positive. The highest infection rates (80-100%) were found from D5 to D12, while later (D16-D23) the infection rate of males was only about 20-30% (Fig. 2). When MASV infection rate between females and males was compared, significantly lower infection rate was showed for males ($p=0.03$). However, the progress of the infection rate was similar for both genders ($p=0.2$) (Fig. 2).

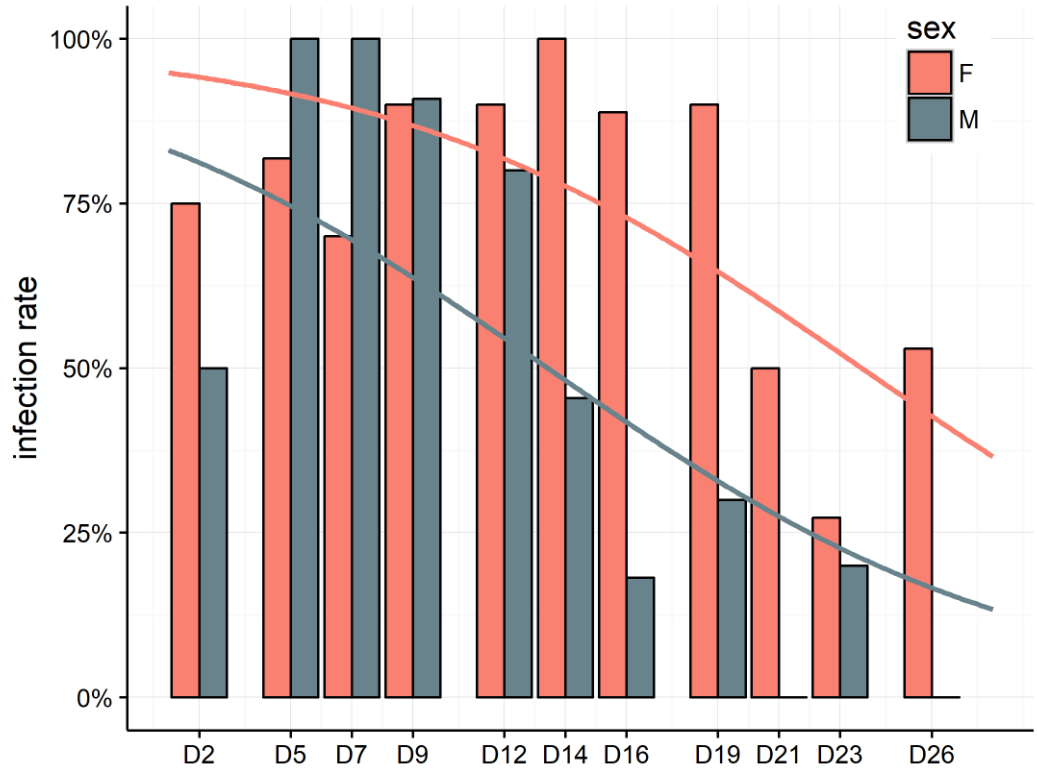

Figure 2: Infection of female and male *P. perniciosus* by MASV using sugar-meal.

When we compared the infection rate of females *P. perniciosus* infected through sugar meal and blood meal, females were significantly more infected by MASV through sugar meal (chi^2=13.07, df=1, p-value=0.0003). This is the reason why we used this type of infection in next experiments. Moreover infections through sugar meal permit infection of both genders.

Co-feeding experiments

In all three repeats of Experiment 1 both females and males regularly spit MASV virus into the sugar meal during the whole experiment (Table 1). Interestingly, in Experiment 2, MASV spit into the sugar by females and males kept in cages A was infectious for other sand flies in cages B. Positive for MASV were 23%, 17% and 3.5% females and 30%, 7% and 0% of males collected from cages B at D6, D9, D14, respectively (Table 2).

Table 1: Detection of MASV from sugar. Detection of MASV from sugar collected between days D4 to D21 from cage with infected females/males *P. perniciosus* during three experiments. + means presence of virus, - absence of virus.

	D4	D5	D6	D7	D10	D11	D12	D13	D14	D17	D18	D19	D20	D21
♀Group1	+	+	+	+	-	-								
♀Group2	+	+	+	+	-	+								
♀Group3	+	+	+	+	+	+	-	+	+	-	-	+	+	-
♂Group1	+	+	+	+	-	+								
♂Group2	+	+	+	+	-	+								
♂Group3	+	+	+	+	+	+	-	+	-	-	-	+	-	+

Table 2: Infection rate of females/males infected by "co-feeding on sugar". Infection rate of females/males *P. perniciosus* infected by sugar meal transported from cage with infected females/males (A) by MASV into cage with non-infected females/males (B). - mean absence of virus.

	D7	D8	D9	D12	D13	D14	D15	D16	D21
♀Detection of MASV in transported sugar (number of viral particles)	**440**	**-**	**192**	**199**	**-**	**3**			
Infected females/tested in cage B					**7/30 (23%)**			**5/30 (17%)**	**7/200 (3,5%)**
♂Detection of MASV in transported sugar (number of viral particles)	**1919**	**-**	**2258**	**142**	**-**	**1**			
Infected males/tested in cage B					**9/30 (30%)**			**2/30 (7%)**	0/92 (0%)

Susceptibility of various sand fly species to MASV

The susceptibility to MASV was compared in *P. perniciosus* and other six sand fly species belonging to three genera. Results are summarized in Figure 3, 4 and Figure S1, Tables S1 and S2 provided in supplementary data. The experiment was repeated twice for each species and for each gender. Results were merged for the analysis. MASV was found in both genders of all tested species during whole life but infection rate and virus loads differ between species. Also longevity of various sand fly species kept in laboratory conditions differ. Males live shorter time than females, therefore there is lack of data for males of some species at higher time intervals (*P. orientalis, P. argentipes* and *S. schwetzi*).

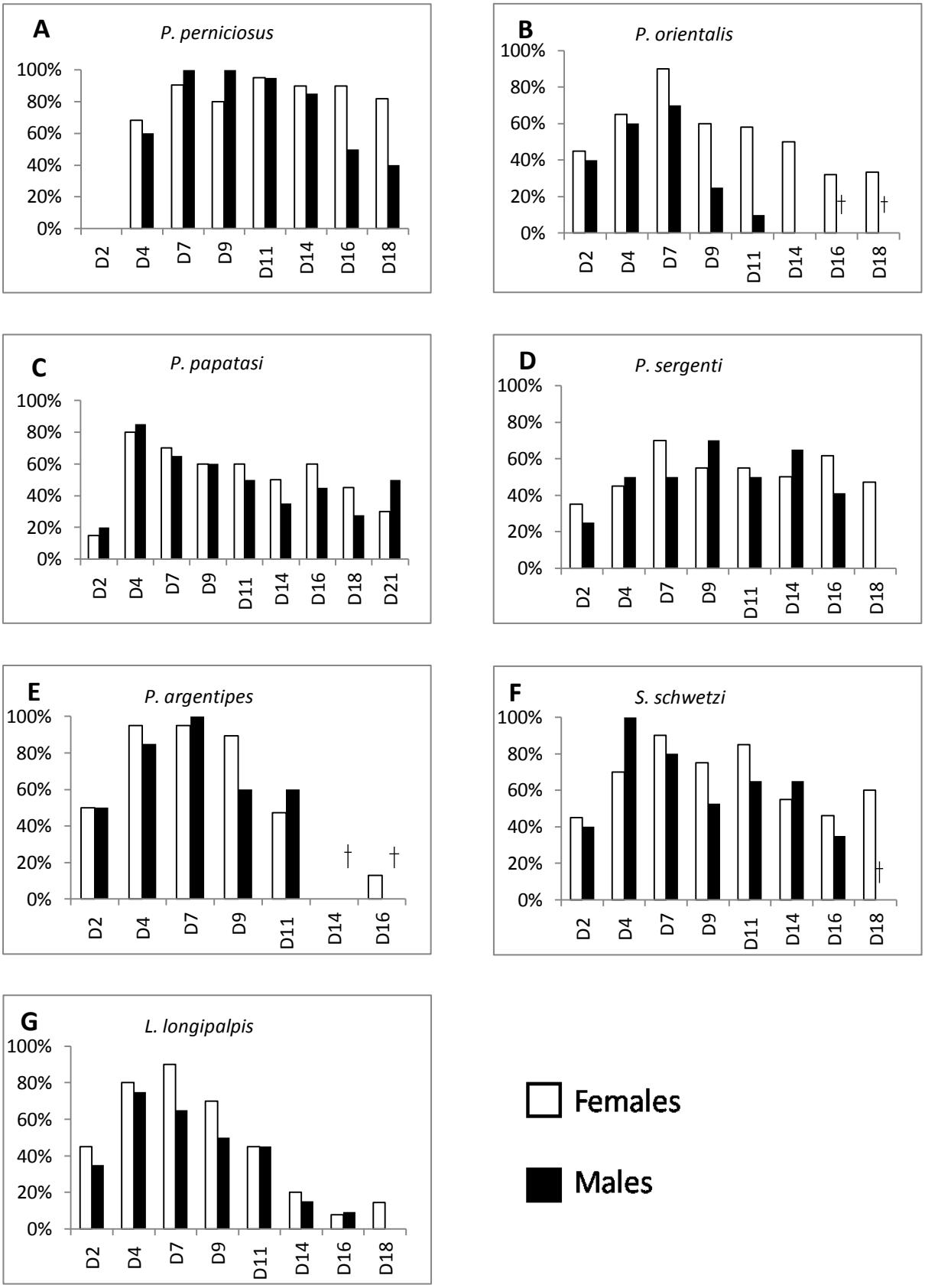
A
P. perniciosus
100%
80%
60%
40%
20%
0%
D2
D4
D7
D9
D11
D14
D16
D18
B
P. orientalis
D21
C
P. papatasi
D
P. sergenti
E
P. argentipes
F
S. schwetzi
G
L. longipalpis
Females
Males

Figure S1: Susceptibility of females and males of different sand fly species to MASV: (A) *P. perniciosus*, (B) *P. orientalis*, (C) *P. papatasi*, (D) *P. sergenti*, (E) *P. argentipes*, (F) *S. schwetzi*, (G) *L. longipalpis*. † no living males were available by these intervals.

The differences in virus loads and infection rates between the species were analyzed separately for females and males. *Phlebotomus perniciosus* was used as the reference due the fact that MASV was always isolated from *P. perniciosus*, so we consider it as a natural host/vector.

There were no significant differences in viral loads and progress of infection intensity between males of *P. perniciosus* and other species tested. However, for females the significant differences in viral loads were achieved for *P. sergenti* and *P. argentipes* (Fig. 3). *Phlebotomus sergenti* had significantly higher amount of virions than *P. perniciosus* females ($p<0.001$), however the infection intensity decreased much more quickly than in *P. perniciosus* ($p=0.02$). The similar case was also for *P. argentipes* females, higher virus loads ($p=0.03$), but quick decrease of infection intensity ($p=0.05$) when compared to *P. perniciosus*.

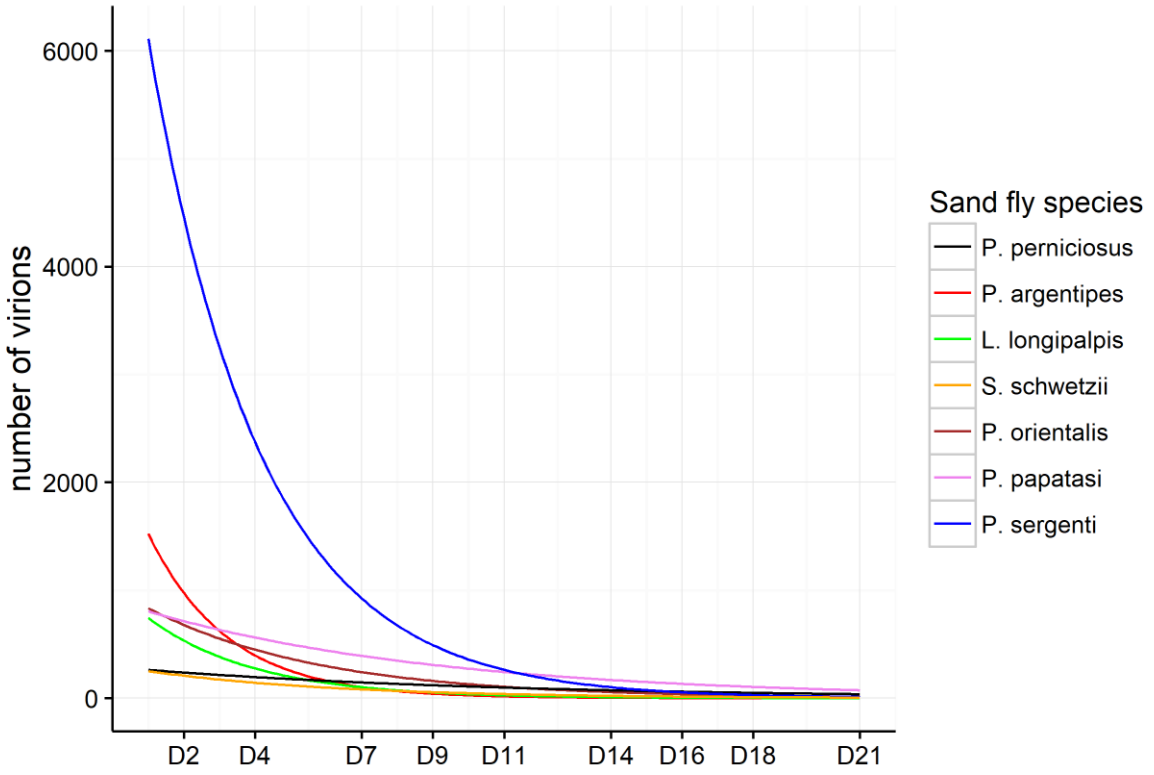

Figure 3: Fitted lines for viral loads in phlebotomine females from different colonies (fitted lines are calculated from the original data (Table S1) according model)

In males, no significant differences in infection rates were found between 7 species tested. In females, most species tested showed higher infection rate at the beginning of the experiment when compared to *P. perniciosus*, this difference was significant for *P. argentipes* (p=0.02) and *L. longipalpis* (p=0.03) (Fig. 4). Importantly, all colonies showed significant gradual decrease in infection rate during experiment when compared to *P. perniciosus* (*P. argentipes* p=0.004, *L. longipalpis* p=0.002, *P. orientalis* p=0.01, *P. papatasi* p=0.03, *S. schwetzi* p=0.04), marginally significant decrease was showed only for *P. sergenti* (p=0.08) (Fig. 4). Importantly, *P. perniciosus* was the only species in which the infection rate grew steadily for the whole time of experiment duration.

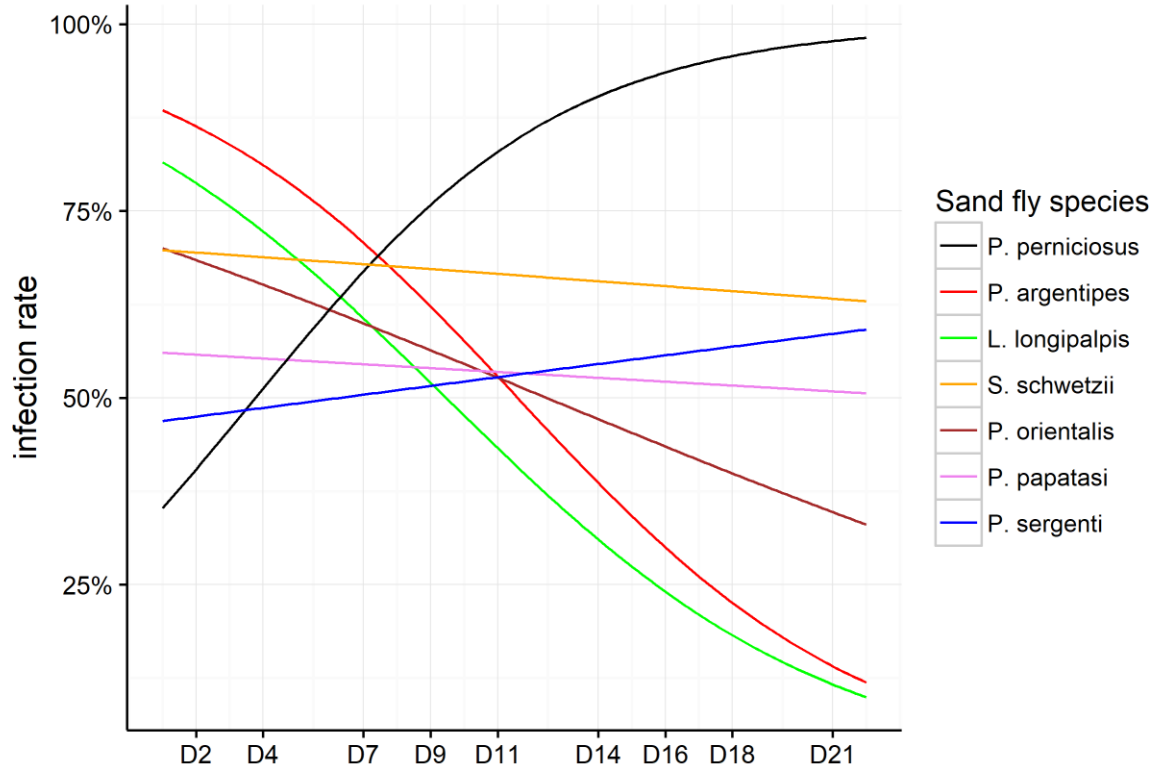

Figure 4: Fitted lines for infection rate in phlebotomine females from different colonies (fitted lines are calculated from the original data (Table S1) according model)

Table S1: Susceptibility of females of *P. perniciosus*, *P. orientalis*, *P. papatasi*, *P. sergenti*, *P. argentipes*, *S. schwetzi* and *L. longipalpis* to MASV, - mean absence of virus

Females		**D2**	**D4**	**D7**	**D9**	**D11**	**D14**	**D16**	**D18**	**D21**
P. perniciosus	infected/total	0/20	15/22	19/21	16/20	19/20	18/20	18/20	18/22	
	% infected	0	68.2	90.5	80	95	90	90	82	
	mean number of viral particules	**-**	**149**	**600**	**89**	**105**	**27**	**8**	**5**	
P. orientalis	infected/total	9/20	13/20	18/20	12/20	11/19	10/20	9/28	2/6	
	% infected	45	65	90	60	57.9	50	32.1	33.3	
	mean number of viral particules	**555**	**600**	**283**	**125**	**51**	**87**	**42**	**13**	
P. papatasi	infected/total	3/20	16/20	14/20	12/20	12/20	10/20	12/20	9/20	3/10
	% infected	15	80	70	60	60	50	60	45	30
	mean number of viral particules	**1385**	**139**	**134**	**49**	**366**	**75**	**53**	**443**	**57**
P. sergenti	infected/total	7/20	9/20	14/20	11/20	11/20	10/20	8/13	8/17	
	% infected	35	45	70	55	55	50	61.5	47.1	
	mean number of viral particules	**5714**	**769**	**786**	**874**	**324**	**130**	**38**	**64**	
P. argentipes	infected/total	10/20	19/20	19/20	17/19	9/19	0/8	1/8		
	% infected	50	95	95	89.5	47.4	0	13		
	mean number of viral particules	**1051**	**277**	**136**	**56**	**16**	**-**	**5**		
S. schwetzi	infected/total	9/20	14/20	18/20	15/20	17/20	11/20	6/13	6/10	
	% infected	45	70	90	75	85	55	46.2	60	
	mean number of viral particules	**230**	**95**	**95**	**84**	**35**	**16**	**5**	**15**	
L. longipalpis	infected/total	9/20	16/10	18/20	14/20	9/20	4/20	1/13	1/7	
	% infected	45	80	90	70	45	20	7.7	14.3	
	mean number of viral particules	**622**	**129**	**179**	**34**	**12**	**24**	**5**	**3**	

Table S2: Susceptibility of males of *P. perniciosus*, *P. orientalis*, *P. papatasi*, *P. sergenti*, *P. argentipes*, *S. schwetzi* and *L. longipalpis* to MASV, - mean absence of virus

Males		**D2**	**D4**	**D7**	**D9**	**D11**	**D14**	**D16**	**D18**	**D21**
P. perniciosus	infected/total	0/10	14/24	21/21	20/20	20/21	17/20	10/20	8/20	
	% infected	0	60	100	100	95	85	50	40	
	mean number of viral particules	**-**	**94**	**418**	**153**	**82**	**29**	**2**	**5**	
P. orientalis	infected/total	8/20	12/20	14/20	5/20	2/20	0/10			
	% infected	40	60	70	25	10	0			
	mean number of viral particules	**103**	**106**	**112**	**13**	**20**	**-**			
P. papatasi	infected/total	4/20	17/20	13/20	13/20	10/20	7/20	9/20	5/18	5/10
	% infected	20	85	65	60	50	35	45	27.8	50
	mean number of viral particules	**162**	**525**	**271**	**81**	**36**	**37**	**78**	**273**	**430**
P. sergenti	infected/total	5/20	10/20	10/20	14/20	10/20	13/20	7/17	0/1	
	% infected	25	50	50	70	50	65	41.2	0	
	mean number of viral particules	**302**	**271**	**433**	**160**	**116**	**66**	**86**	-	
P. argentipes	infected/total	10/20	17/20	20/20	9/15	3/5				
	% infected	50	85	100	60	60				
	mean number of viral particules	**258**	**110**	**110**	**43**	**26**				
S. schwetzi	infected/total	8/20	20/20	16/20	10/19	13/20	13/20	7/20		
	% infected	40	100	80	52.6	65	65	35		
	mean number of viral particules	**134**	**56**	**28**	**12**	**36**	**14**	**11**		
L. longipalpis	infected/total	7/20	15/20	13/20	10/20	9/20	3/20	1/11	0/10	
	% infected	35	75	65	50	45	15	9.1	0	
	mean number of viral particules	**320**	**135**	**38**	**12**	**114**	**46**	**4**	**-**	

Tissue specificity of MASV

Females of three sand fly species were dissected 4, 7 and 10 days after infection by MASV through sugar meal and the virus was detected and quantified in salivary glands, midgut and the rest of body (Fig. 5). Tissue specificity of MASV was analyzed separately for each organ between females of *P. perniciosus* as "reference colony" and *S. schwetzi* and *L. longipalpis*.

Massilia virus disseminated to salivary glands in *P. perniciosus* and *S. schwetzi* only. Low proportion (10-20%) of females dissected on D4 and D7 were found positive and there was no significant difference in intensity of infection. In *L. longipalpis*, no positive salivary glands were found throughout the experiment.

On day 4, all *P. perniciosus* and *S. schwetzi* females harbour MASV in the midgut and in the rest of body. On the other hand, the infection rate of *L. longipalpis* females was lower, 75 and 80% for midgut and rest of body, respectively (Fig. 5). On day 10, MASV infection rates of the rest of body did not differ between species, 70-80% positivity was found for all species tested. However, pronounced differences were found in MASV presence on day 10 in midguts. Infection rates of *P. perniciosus* and *S. schwetzi* midguts were 75 and 50%, respectively, while only a single positive midgut (10%) was found in *L. longipalpis* (Fig. 5).

Intensity of infection in guts was lower for *L. longipalpis* and *S. schwetzi* than in *P. perniciosus*, however these differences were marginally significant (both p=0.07). In the rest of the body, significantly lower viron loads were found for *S. schwetzi* (p=0.03) and marginally for *L. longipalpis* (p=0.07) when compared to *P. perniciosus*. There was also marginally significant difference in progress of the infection in the rest of the body between *P. perniciosus* and *S. schwetzi*. The amount of virions decreased slower in *S. schwetzi* than in *P. perniciosus* females (p=0.07) (Table S3).

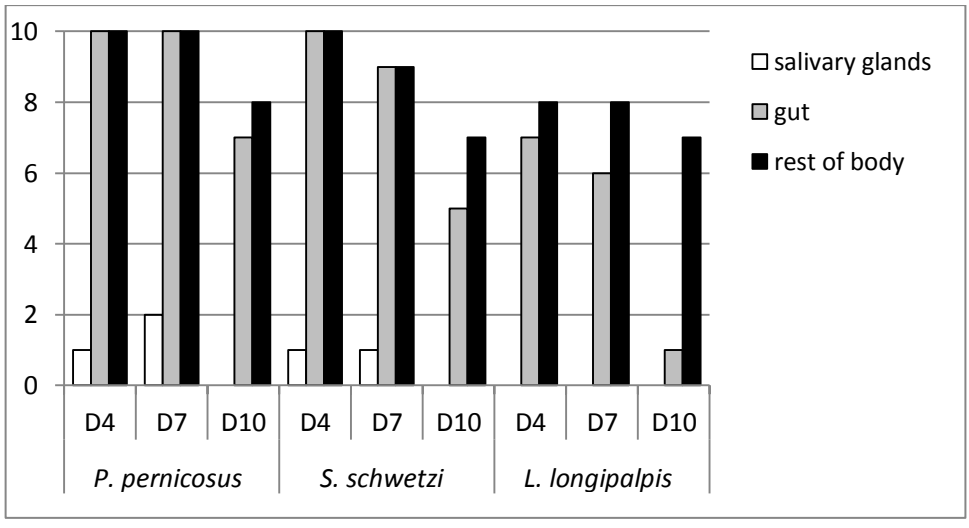

Figure 5: Tissue specifity and infection rate in *P. perniciosus*, *S. schwetzi* and *L. longipalpis*.

Table S3: Tissue specificity of MASV in three selected species**.** Intensity of infection in gut, salivary glands and rest of body in females *P. perniciosus*, *S. schwetzi* and *L. longipalpis*. - mean absence of virus

		D4	D7	D10
P. perniciosus	Mean number of viral particles in salivary glands	**12**	**12**	**-**
	Mean number of viral particles in gut	**1007**	**48**	**17**
	Mean number of viral particles in rest of body	**6354**	**496**	**227**
S. schwetzi	Mean number of viral particles in salivary glands	**11**	**7**	**-**
	Mean number of viral particles in gut	**262**	**30**	**32**
	Mean number of viral particles in rest of body	**1081**	**345**	**412**
L. longipalpis	Mean number of viral particles in salivary glands	**-**	**-**	**-**
	Mean number of viral particles in gut	**176**	**51**	**11**
	Mean number of viral particles in rest of body	**1202**	**506**	**71**

DISCUSSION

The main aim of this study was to investigate the transmission routes of phleboviruses, particularly MASV, virus closely related to TOSV. Although MASV is classified as BSL2 agent, its human pathogenicity seems more limited, if any, than that of other phleboviruses, particularly TOSV. Thus, MASV is a good candidate to be used as a surrogate of TOSV (Charrel et al., 2009).

Pathways of infection and developmental stage of insect can significantly affect tissue tropism of virus, immune response and finally outcome of infection (Möckel et al., 2011; Ferreira et al., 2014; Stevanovic and Johnson, 2015). For productive infection of the vector by arbovirus it is necessary: (i) infection of the midgut and spread infection through its epithelium, (ii) dissemination of virus particles to secondary tissues and amplification, (iii) infection of salivary glands and release of virus into saliva (Hardy et al., 1983; Franz et al., 2015).

In our experiments, transstadial transmission of MASV from larvae infected at first or fourth instar through larval food was not efficient. In nature, sand fly larvae feed on organic matters (Killick-Kendrick, 1999), thus, they develop efficient system for control and eventually elimination of bacteria, fungi and other pathogens from the gut. For example, Sudeep et al. (2015) failed to obtain transstadial transmission by feeding second instar larvae of *Culex quinqefasciatus* by fragmented larvae infected by West Nile virus. On other hand vertical transmission from mosquitoes and sand flies females to the next generation was repeatedly proven and virus was detected in larvae during whole development until adults (Tesh and Modi, 1987; Diallo et al., 2000; Wasinpiyamongkol et al., 2003; Castro et al., 2004; Saiyasombat et al., 2011). Tesh and Modi (1987) demonstrated that TOSV was transmitted in *P. perniciosus* during 13 successive generations; however, infection was established from oocytes, not by oral infection of larvae where virus has to overcome a number of barriers.

In Diptera, majority of extracellular bacteria present in the gut of larvae are not able to survive metamorphosis and only some of them are passed transtadially from larvae to adults (Volf et al., 2002; Chavshin et al., 2015). Diptera larvae defecate midgut content shortly before pupation and there is massive restructuralization of gut during pupation, including disintegration of larval midgut epithelial cells (Hakim et al., 2010; Fernandes et al., 2014). This process results in massive clearance midgut from bacteria (and possibly also from virus). According to some authors, bacteria *Escherichia coli*-GFP and *Pseudomonas*-GFP, survive pupation of *Anopheles* mosquitoes hidden in the Malphigian tubules which open back to

midgut lumen after eclosion of adults (Chavshin et al., 2013; Chavshin et al., 2015). We cannot exclude the possibility that similar mechanism is used by MASV and this may result in low efficiency of transstadial transmission of MASV. Anyway, infection of larvae by MASV through larval food was not efficient and if this route of infection occurs in nature, it is probably very rare or supplementary phenomenon, which does not participate significantly on maintenance of MASV in nature.

In contrast, more promising results were obtained by infections of adult sand flies: females by blood meal and both genders by sugar meal. In blood fed females, however, most MASV infection was eliminated by defecation, thus infection rate in females post defecation was very low. Important role in the defecation of virus from blood fed females may represent peritrophic matrix (PM), which protects midgut against mechanical damage, pathogens and toxins. This membrane is acellular structure mainly formed by chitin, glycoproteins a proteoglycans with pores of different size, which often ranges around 7-8 nm (Terra, 1990; Peters 1992; Lehane, 1997). Size of MASV particles is around 80-120 nm (Charrel et al., 2009) so virus cannot pass through intact PM. In sand flies, PM is formed within 6-12 hours post blood meal (PBM) and is fully developed about 24 hours PBM (Pruzinova et al., 2015). Only viral particles located close to midgut epithelial cells after blood feeding and before formation of PM can infect these cells through microvilli (Franz et al., 2015).

Moreover, elimination of MASV from gut could be affected by natural gut microflora, either indirectly though stimulation of immune system or directly by microfloral metabolites. In *Aedes aegypti* elimination of gut microbiom by antibiotics led to two time higher titer of Dengue virus in midgut than in non-treated mosquitoes and aseptic individuals showed lower expression of some immune genes like defensin, cecropin, attacin and gambicin. These results suggested that natural gut microflora stimulate Toll pathway on basal level which also exhibit antiviral activity (Xi et al., 2008; Ramirez et al., 2012). *Chromobacterium* naturally occurring in *Ae. aegypti* decreased susceptibility of mosquitoes to Dengue virus and exhibit anti-viral activities *in vitro* (Ramirez et al., 2014).

Interestingly, MASV infection of females and males by sugar meal was much more efficient than by blood meal. As far as we are aware, this is first record of infection of vector by arbovirus through sugar meal. Although infection rate of males was lower than in females, the progress of the infection rate was similar for both genders. It can be given by various volume of the sugar meal take by male and female. Tang and Ward (1998) showed that on the beginning of sugar feeding of *L. longipalpis* small amount of sugar is passed directly to the thoracic midgut which leads to closure of stomodeal valve and rest of sugar is deflect to crop.

This means that infection is initiated either from this "first drop" or from the sugar meal temporarily stored in the crop.

Both males and females repeatedly spited MASV into sugar solution during feeding from D4 until D21. Similar results were observed also for viruses transmitted by *Culex annulirostris* and *Culex gelidus* (Hurk et al., 2007). Interestingly, despite MASV infection was found in *P. perniciosus* salivary glands till D7 post infection, but virus particles were expectorated into sugar solution until D21. Moreover virus regurgitated into sugar meal by sand flies was still infectious after transport to another cage with naive females and males. These findings suggest that virus is not released only through saliva but also by regurgitation from alimentary canal. Additionally, data suggest that MASV survives for at least 24 hours in wet conditions, like plant nectar without an insect or vertebrate host. Almost 30% of *P. perniciosus* (both males and females) get MASV from the sugar with expectorated virus. Therefore, we suppose that transmission by co-feeding of infected and uninfected sand flies may represent an important part of the life cycle of MASV.

As little is known about specificity of phleboviruses to different sand flies, we tested MASV infection in seven species of three sand fly genera, *Lutzomyia, Phlebotomus* and *Sergentomyia*. In males, no significant differences were found in virus loads and infection rates between various sand fly species. On the contrary, in females virus loads and infection rates differed between species. In contrast to other sand fly species the infection rate in *P. perniciosus* grow steadily until D15 and then remain stable until the end of the experiment. The fact that infection rate is increasing even after the females were denied to feed on infectious sugar meal (given only at D0 and D2 of experiment) could be explain by co-feeding. Our results indicate that MASV survived in all tested species of sand flies for weeks but could not efficiently replicate. Results from infection by blood and sugar meal contradict the theory that the sand fly can serve as primary reservoir of phleboviruses as some authors like for example Alkan et al. (2013) assumed, at least in case of MASV.

In the conclusion, we showed that MASV is not transmitted effectively between different *P. perniciosus* development stadia or through blood meal in *P. perniciosus* adults. Our results support the theory, that vertebrates represent dead-end hosts without important role in virus life cycle (Tesh and Chaniotis, 1975). Transmission through blood feeding between vector and vertebrates can be supplementary way of circulation of MASV, respectively all phleboviruses, but different mechanism than insect-vertebrate cycles have to be hypothesized. The most efficient way of infection was through sugar meal. Although virus does not replicate well in *P. perniciosus* or another sand fly species, infected sand flies can

regurgitate virus particles into source of sugar during feeding which is then infectious for another naïve individuals. This way of transmission could be then responsible for the maintenance of circulation of phleboviruses in the natural conditions.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Refractoriness of *Sergentomyia schwetzi* to *Leishmania* spp. is mediated by the peritrophic matrix

Jovana Sadlova*, Miroslav Homola, Jitka Myskova, Magdalena Jancarova, Petr Volf

Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

* sadlovaj@natur.cuni.cz

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Abstract

Background

The peritrophic matrix (PM) is an acellular chitin-containing envelope which in most blood sucking insects encloses the ingested blood meal and protects the midgut epithelium. Type I PM present in sand flies and other blood sucking batch feeders is secreted around the meal by the entire midgut in response to feeding. Here we tested the hypothesis that in *Sergentomyia schwetzi* the PM creates a physical barrier that prevents escape of *Leishmania* parasites from the endoperitrophic space.

Methodology/Principal findings

Morphology and ultrastructure of the PM as well the production of endogenous chitinase in *S. schwetzi* were compared with three sand fly species, which are natural vectors of *Leishmania*. Long persistence of the PM in *S. schwetzi* was not accompanied by different morphology or decreased production of chitinase. To confirm the role of the PM in refractoriness of *S. schwetzi* to *Leishmania* parasites, culture supernatant from the fungus *Beauveria bassiana* containing chitinase was added to the infective bloodmeal to disintegrate the PM artificially. In females treated with *B. bassiana* culture supernatants the PM was weakened and permeable, lacking multilayered inner structure; *Leishmania* colonized the midgut and the stomodeal valve and produced metacyclic forms. In control females *Leishmania* infections were lost during defecation.

Conclusions/Significance

Persistence of the PM till defecation of the bloodmeal represents an important factor responsible for refractoriness of *S. schwetzi* to *Leishmania* development. *Leishmania major* as well as *L. donovani* promastigotes survived defecation and developed late-stage infections only in females with PM disintegrated artificially by *B. bassiana* culture supernatants containing exogenous chitinase.

Author summary

Phlebotomine sand flies are the main vectors of *Leishmania* parasites. However, only about ten percent of the described sand fly species are proven or suspected vectors. Several factors controlling vector competence act during the early phase of infection preceding defecation of bloodmeal remnants. Sand flies of the genus *Sergentomyia* including *S. schwetzi* were repeatedly suggested to be involved in *Leishmania* transmission in Africa. Here, we tested the hypothesis that *S. schwetzi* is refractory to all *Leishmania* species tested due to the long persistence of the peritrophic matrix, the chitinous envelope which surrounds ingested blood within the sand fly midgut. Addition of exogenous chitinase to the *S. schwetzi* infectious bloodmeal led to disintegration of the peritrophic matrix which allowed *Leishmania* parasites to escape into the midgut and produce mature infections with colonization of the stomodeal valve and generation of infective metacyclic forms. Parasites in control flies were not able to escape from the peritrophic matrix and were lost with the defecation of blood remnants. The study strongly suggests that in *S. schwetzi* the peritrophic matrix forms an important barrier for the development of *Leishmania* parasites.

Introduction

Sand flies (Diptera: Psychodidae) are the vectors of *Leishmania* species (Kinetoplastida: Trypanosomatidae) parasitizing humans. Over 800 species of sand flies have been described to date but only 98 species are proven or suspected vectors of human leishmaniases [1,2]. Development of *Leishmania* infections in the sand fly vectors is a complex, often species-specific process (reviewed by [3–5]). Some sand fly species can be infected with various *Leishmania* species (permissive vectors); other species are considered specific (restrictive) vectors which are only able to harbour the *Leishmania* species that they transmit in nature (e.g. *Phlebotomus papatasi* and *L. major/L. turanica)* [4,6,7].

Factors controlling vector competence act during the early phase of infection preceding or accompanying the defecation of the bloodmeal remnants. Blood digestion and early development of parasites occur inside the peritrophic matrix (PM), an acellular chitin-containing envelope which protects the midgut epithelium against damage and compartmentalizes the midgut into endo- and ectoperitrophic spaces [8]; in some hematophagous insects the PM performs also a central role in heme detoxification [9]. In sand flies, the PM was suggested to protect parasites against the action of digestive enzymes during the early hours post blood feeding [10]. Inside the endoperitrophic space surrounded by the PM, *Leishmania* amastigotes ingested with the bloodmeal transform to procyclic promastigotes. They must overcome the activity of the digestive enzymes, replicate and then escape to the ectoperitrophic space. This escape coincides with the transformation of procyclic forms to elongated nectomonads [11] which attach to the midgut epithelium to avoid expulsion from the midgut during defecation of undigested blood remnants. Failure of either (i) resistance to harmful environment caused by blood digestion, (ii) escape from the endoperitrophic space or (iii) attachment to the midgut epithelium leads to loss of infection in incompatible sand fly-*Leishmania* species combinations. In competent vectors, a substantial part of the parasite population survives defecation and colonizes the midgut. Finally the infection spreads anteriorly to be transmitted to the next host during subsequent blood meals [12].

Sand flies of the genus *Sergentomyia* are proven vectors of reptile *Leishmania* species, non-pathogenic to humans, which exhibit hypopylarian development patterns in their insect

vectors (developing in the hindgut) with transmission occurring by predation of the infected flies by lizards. However, *Sergentomyia* species are not fully restricted to feeding on reptiles and at least some species feed on humans and/or mammalian reservoirs of *Leishmania* pathogenic to humans. Therefore, they have been suspected as vectors in some visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) foci where *Sergentomyia* spp. were abundant and found to harbour *Leishmania* (reviewed by [13]). Recently, a vectorial role was strongly suggested for three *Sergentomyia* species including *S. schwetzi* in the Mont-Rolland area in Senegal [14]. However, experiments showed that three species of *Leishmania* pathogenic to humans (*L. donovani*, *L. infantum* and *L. major*) did not survive defecation of bloodmeal remnants in *S. schwetzi* [15–17]. It was proposed that the crucial aspect mediating the refractoriness of *S. schwetzi* was the relative timing of degradation of the PM and defecation [17] i.e. the extremely long persistence of the PM in *S. schwetzi*, in comparison with three species of the genus *Phlebotomus* [18].

The aim of the current study was to compare the morphology and ultrastructure of the PM and activity of endogenous chitinase in *S. schwetzi* with three *Phlebotomus* species and to demonstrate that persistence of the PM represents an important mechanism of refractoriness of *S. schwetzi*. We showed that the PM of *S. schwetzi* shares similar morphological features with *Phlebotomus* species transmitting *Leishmania*. The role of the PM in refractoriness of *S. schwetzi* was demonstrated experimentally; the artificial disintegration of the PM using culture media from *B. bassiana*, rich in chitinase activity, enabled the development of mature infections of both *L. major* and *L. donovani* in *S. schwetzi* females.

Methods

Sand flies and *Leishmania*

Laboratory colonies of *Sergentomyia schwetzi* (from Ethiopia), *Phlebotomus orientalis* (from Ethiopia), *P. argentipes* (from India) and *P. papatasi* (from Turkey) were maintained in the insectary of the Charles University in Prague under standard conditions (at 26 ˚C fed on 50% sucrose with a 14 h light/10 h dark photoperiod) as described previously [19]. *Leishmania donovani* (MHOM/ET/2010/GR374) transfected with green fluorescent protein (GFP) were cultured in M199 medium (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma), 2% sterile urine, 250 μg/mL amikacin (Amikin, Bristol-Myers Squibb) and 150 μg/mL selective antibiotic G418 (Sigma). *Leishmania major* LV561 (LRC-L137; MHOM/IL/1967/Jericho-II) transfected with GFP protein were cultured in the same medium without selective antibiotics.

Origin of exogenous chitinases

Chitinases from *Streptomyces griseus* and *Trichoderma viride* (both from Sigma, Cat. No C6137 and C8241, respectively) were diluted in PBS buffer and stored at -20 °C. *Beauveria bassiana* CCF 4422, obtained from the Culture Collection of Fungi (CCF, Dept. of Botany, Faculty of Science, Charles University, Prague, Czech Republic) was grown at 26 °C in a medium composed of (w/v) glucose 5%, Neopeptone (BD) 5%, Yeast extract (Sigma) 1%, NaCl (0.25%) in distilled water (modified after [25]). After three weeks of cultivation, the whole volume was centrifuged at 250 g for 10 min, the supernatant was sterilized using 0.22 μm millipore filter units (Millex-GP), concentrated by centrifugation through 30 kDa filters (Amicon) and stored at -80 °C.

Sand fly infections

Leishmania promastigotes from log-phase cultures (day 3–4 post inoculation) were resuspended in heat-inactivated rabbit blood (MVDr. Zdenek Petr, Czech Republic) and mixed 1:1

with a supernatant from *B. bassiana* culture containing chitinase (preparation described above) to obtain final concentration $1x10^6$ promastigotes/mL and 0.07 U of exochitinase activity/mL. Control flies were fed on the same *Leishmania* suspension in blood mixed with the fresh culture medium or heat-inactivated culture supernatant from *B. bassiana*. Sand fly females (5–9 days old) were infected by feeding through a chick-skin membrane (BIOPHARM, Czech Republic) on the suspension. Engorged sand flies were maintained in the same conditions as the colony. Females were dissected in several time intervals post bloodmeal (PBM), the density and location of *Leishmania* infections in their digestive tract was examined by fluorescence microscopy. Parasite loads were graded according to [20] as light (< 100 parasites per gut), moderate (100–1000 parasites per gut) and heavy (> 1000 parasites per gut). Experiments with each *Leishmania*–sand fly combination were repeated four to six times.

Morphometry of parasites

Midgut smears of sand flies infected with *Leishmania* parasites were fixed with methanol, stained with Giemsa, examined under the light microscope Olympus BX51 and photographed with an Olympus D70 camera. Body length, flagellar length and body width of parasites were measured using Image-J software. Four morphological forms were distinguished as described in [21]: procyclic promastigotes (PP), elongated nectomonads (EN), metacyclic promastigotes (MP) and short promastigotes (SP). Haptomonads were not distinguished as these forms remain attached on the gut and their numbers are often underestimated on gut smears. In total, 200 promastigotes from four females/smears were measured for each *Leishmania* species.

Light microscopy of the PM

Bloodfed females were dissected at 10 intervals after feeding on anesthetized BALB/c mice, starting immediately post bloodmeal (PBM) and at each of the following times: 1, 3, 6, 12, 24, 48, 72, 96 and 120 h PBM. Dissections were carried out in the isotonic saline solution with brief washing of the gut in distilled water in order to better separate the PM [22]. For each sand fly species and time interval, at least 20 females were analysed. Slides were observed under an Olympus BX51 microscope with Nomarski contrast and photographed with an Olympus D70 camera and software. The colour of the PM and presence of the anterior plug (AP) and posterior ending of the PM were checked.

Histology

Females at 24 h PBM were fixed at 4 °C for 48 h in AFA solution (formaldehyde: ethanol: acetic acid: distilled water, 1.5:12.5:1:10). After washing in phosphate-buffered saline (pH 7.6) and dehydration in 70% to 96% ethanol, the samples were embedded in JB-4 resin following the manufacturer's instructions (Polysciences). Histological sections (2–6 μm thick) were stained with Ehrlich's acid haematoxylin and 0.2% eosin, mounted on glass slides with Euparal Mounting Medium (BioQuipProducts) and observed and photographed. Ten females of each sand fly species were used.

Electron microscopy

Females were dissected at 24 h (*P. argentipes*) or 48 h PBM (*P. orientalis*, *P. papatasi*, *S. schwetzi*), fixed in modified Karnovsky´s fixative [23], and post-fixed with a 2% osmium tetroxide solution (both at 4 °C). The samples were dehydrated in ascending concentration of ethanol (35–100%) then acetone (100%) and embedded in SPURR resin (SPI-chemo). Semithin sections (500 nm)

and ultrathin sections (80–90 nm) were obtained using a Reichert-Jung Ultracut E ultramicrotome. Semithin sections were stained with toluidine blue for light microscopy. Ultrathin sections stained with uranyl acetate and lead citrate [24] were observed and photographed with a Jeol 1011 transmission electron microscope with iTEM 5.1 software (Olympus). Thickness of the PM was measured on representative images using the Image-J software. Together 400 measurements per each sand fly species were obtained (four females per species, 10 images per female and 10 measurements per image).

Assessment of chitinase activity

The exochitinase activity of supernatants from *B. bassiana* culture (preparation described above) or dissected sand fly midguts was quantified using the Chitinase Assay Kit (Sigma, Cat. No. CS0980) by monitoring the hydrolysis of the chitinase substrates (4-Nitrophenyl N, N′-diacetyl-β-D-chitobioside and 4-Nitrophenyl N-acetyl-β-D-glucosaminide). Midguts were dissected at 24, 48 and 72 h PBM and pools of 20 midguts in 40 μL of TRIS-NaCl buffer (20 mM TRIS, 150 mM NaCl, pH 7.6) were used. Specimens were kept on ice during dissections and samples were processed immediately. Midgut pools were homogenized and centrifuged at 12 100 g for 10 min. The assay was performed in triplicates and according to instructions of the manufacturer. Briefly, 2 μL of supernatant were diluted in 98 μL of the substrate solution (1 mg of substrate dissolved in 1 mL of the Assay Buffer, Cat. No. A4855). Samples were incubated for 30 minutes at 37˚C and pH 4.8. The absorption of the released 4-nitrophenol was measured after the addition of the Stop Solution (sodium carbonate solution) colorimetrically at 405 nm on INFINITE M200 spectrophotometer (Schoeller instruments). The exochitinase activity was calculated according to the manufacturer's instructions (one unit release 1 μmole of 4-nitrophenolfrom the appropriate substrate per minute at pH 4.8 and 37˚C).

Effect of chitinases from *S. griseus* and *T. viride* on *Leishmania* growth *in vitro*

L. major promastigotes (at concentration of $1x10^6$/mL) were exposed to chitinases from *S. griseus* and *T. viride* serially diluted in PBS in 96-well flat bottomed microtitre plates. Five μL of each dilution of chitinase were added to 200 μL of the *Leishmania* suspension in culture medium or defibrinated and inactivated rabbit blood. In controls, five μL of PBS were added. Numbers of parasites were counted after 24 h of cultivation at 26 ^{o}C using the Burker cell counter.

Effect of exogenous chitinases from *S. griseus* and culture supernatant from *B. bassiana* on *Leishmania* survival *in vitro*

L. major and *L. donovani* promastigotes at concentration of $1x10^6$/mL in a volume of 100 μL of culture medium were mixed with 100 μL of the supernatant from *B. bassiana* culture (prepared as described above) in 96-well flat-bottomed microtitre plates. The resultant concentration of chitinase was 0.07 U/mL (an identical concentration to that used for experimental infections of sand flies). As a control, 100 μL of the fresh culture medium for *B. bassiana* instead of the supernatant were added. For testing of the effect of *S. griseus* chitinase, 5 μL of chitinase solution in PBS was added to promastigotes at concentration of $1x10^6$/mL in a volume of 200 μL of culture medium to obtain chitinase concentration of 1 U/mL, 0.5 U/mL or 0.07 U/mL. As a control, 5 μL of PBS was added to the culture medium. Numbers of parasites were counted after 24 h of cultivation at 26 ^{o}C using the flow cytometer CytoFLEX S (Beckman Coulter, Inc., Brea, California, USA) equipped with 4 lasers (405 nm, 488 nm, 561 nm, 638 nm) and 13 fluorescence detectors. Dead cells were marked with DAPI (4′, 6-Diamidine-2′-phenylindole

dihydrochloride, 0.005 mg/mL; Thermo Fisher Scientific). The mortality was assessed as a ratio of the number of dead cells showing both green and blue fluorescence (GFP and DAPI) to the number of live cells showing green fluorescence (GFP). In samples where dead cells were completely disintegrated and total numbers of detected cells were significantly lower in comparison with control samples, mortality was counted using the total numbers of parasites in control wells. *Leishmania* promastigotes killed by solution of 1% formaldehyde in PBS and permeabilised with 0.5% Triton X-100 (Sigma) were used as a control for dead cells. GFP was excited using 488 nm laser and its fluorescence emission was detected using 525/40 filter, DAPI was excited by 405 nm laser and detected using 450/50 filter. Analysis of cytometry data was performed using CytExpert software (Beckman Coulter). The experiments were conducted in duplets and repeated 2–3 times.

Detection of midgut O-glycosylated proteins

Midgut lysates from 3–5 days old females of *S. schwetzi* were separated by SDS PAGE (10% gel, reducing conditions) followed by blotting as described previously [26]. Midgut lysates of two *Phlebotomus* species with known glycosylation [26] served as negative (*P. papatasi*) and positive (*P. argentipes*) controls, respectively. The nitrocellulose membrane was blocked overnight at 4˚C in 20 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.6 (TBS-Tw) with 5% bovine serum albumin. Then the membrane was incubated with biotinylated *Helix pomatia* lectin (HPA, 1.25 μg/mL) for 1 h. In the control groups, the HPA was preincubated with the carbohydrate inhibitor N-acetyl-galactosamine (GalNAc, 250 mM) for 30 min. After washing, blots were incubated with streptavidin peroxidase (2.5 μg/mL) for 1 h and developed in 4-chloro-1-naphtol solution.

Statistical analysis

Measurements of parasites and mortality of parasites *in vitro* were compared using Analysis of Variance (ANOVA) including Tukey Post Hoc Test, measurements of the thickness of the PM were tested with Nested ANOVA and Post Hoc Test (t–test with Bonferroni-Holm correction). Differences in defecation between chitinase treated flies and control group were analysed by proportional test with Holm-Bonferroni correction. All the statistical evaluations were performed with statistical software SPSS version 23 or R software (http://cran.r-project.org).

Results

Morphology and ultrastructure of the PM in four sand fly species

Morphology and ultrastructure of the PM of *S. schwetzi* was compared with three species of the genus *Phlebotomus*: *P. orientalis*, *P. argentipes* and *P. papatasi* to ascertain if there are specific morphological traits which may be connected with the long persistence of the PM observed in *S. schwetzi* [18]. Light microscopy and analysis of sections from specimens embedded in JB-4 resin showed that these four sand fly species did not differ substantially in the gross morphology of the PM. The anterior plug, i.e. the most anterior part of the PM secreted by the thoracic midgut, was formed in all the four sand fly species (S1 Fig). The PM was evenly closed on the posterior end, usually forming a short funnel-shaped closed structure called the posterior tail (Fig 1). In all the four species, the PM was originally transparent and then darkened as it became encrusted by heme, this process was apparent beginning 24 h PBM. The only parts of the PM which remained permanently transparent were the AP and the posterior tail.

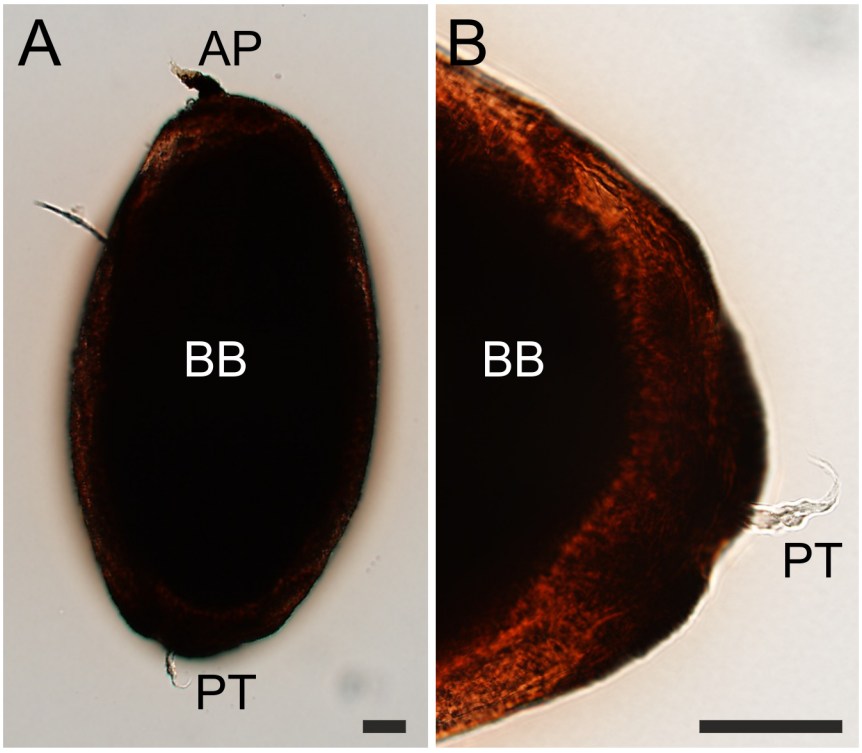

Fig 1. Gross morphology of the *S. schwetzi* PM with detailed image of the posterior tail. The complete PM dissected from the gut of *S. schwetzi* at 24 h PBM (A) and its enlarged posterior end with the transparent posterior tail showed in more detail (B). PT, posterior tail; AP, anterior plug; BB, blood bolus. Scale bars indicate 100 μm.

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Thickness and ultrastructure of the mature PM was assessed using images from electron microscopy. Based on knowledge of the PM kinetics [18], the optimal time for evaluation of the mature PM was 48 h PBM in *P. orientalis*, *P. papatasi* and *S. schwetzi* and 24 h PBM in *P. argentipes* (degradation of the PM proceeds faster in *P. argentipes*, before 48 h PBM [18]). The mature PM of *S. schwetzi* was as thick as those of *P. orientalis* and *P. papatasi*, while the PM of *P. argentipes* was significantly thinner than the PM of these three sand fly species (Table 1). This result corresponds with observations made during dissections of fed females: the PM of *P. argentipes* was extremely fragile and its extraction from the midgut was difficult. The ultrastructure of the mature PM also differed between *P. argentipes* and the remaining three sand fly species. While the mature PM consisted of thin solid outer layer and thick granular inner layer in *S. schwetzi*, *P. orientalis* and *P. papatasi*, the PM of *P. argentipes* appeared fairly

Table 1. Thickness of the PM in four sand fly species measured from TEM images.

Sand fly species	Measurements of the PM thickness				Significance of between-species differences[a]			
	No.	Median	Mean (S.D.) (μm)	Range (μm)	*P. argentipes*	*P. orientalis*	*S. schwetzi*	*P. papatasi*
P. argentipes	400	3.74	3.87 (1.11)	1.54–7.94	-			
P. orientalis	400	7.39	7.22 (1.73)	3.41–13.76	P = 0.007	-		
S. schwetzi	400	6.49	6.68 (1.97)	2.65–12.87	P = 0.016	P = 1.000	-	
P. papatasi	400	6.78	7.16 (2.11)	2.58–15.19	P = 0.007	P = 1.000	P = 1.000	-

[a]Significance of between-species differences was tested by Nested ANOVA and post Hoc Test (t–test with Bonferroni-Holm correction).

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homogeneous throughout the entire cross-section (Fig 2). Thus, the PM of *S. schwetzi* did not show any unique morphological features in comparison with the other two vectors of the genus *Phlebotomus*.

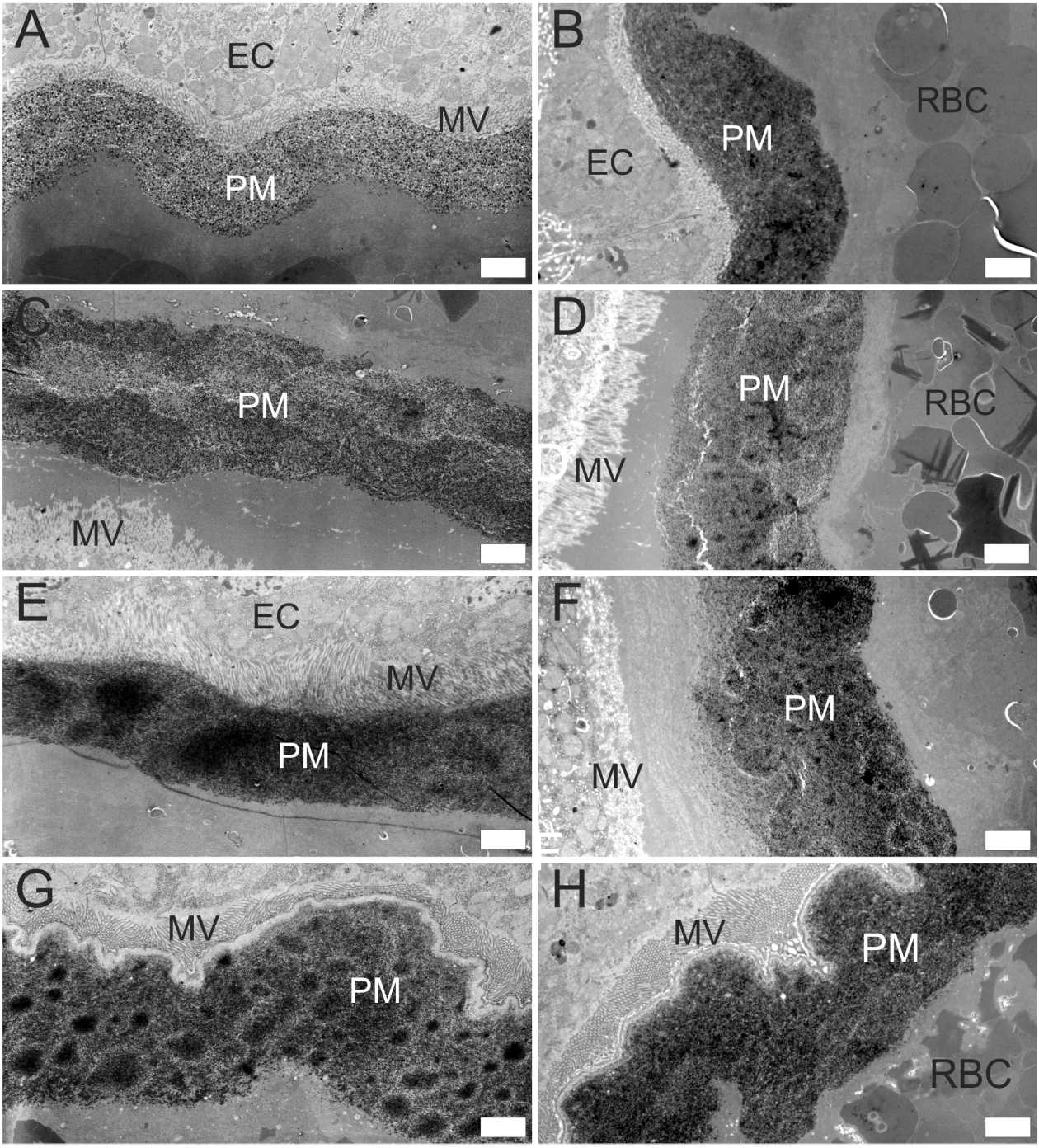

Fig 2. Ultrastructure of the PM in four sand fly species. Electron micrographs of cross sections of the AMG in *P. argentipes* (A, B); *P. orientalis* (C, D); *P. papatasi* (E, F) and *S. schwetzi* (G, H) dissected at 24 h (*P. argentipes*) or 48 h PBM (*P. orientalis*, *P. papatasi*, *S. schwetzi*). PM, peritrophic matrix; MV, microvilli; EC, epithelial cells of the AMG. Scale bars indicate 2 μm.

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Midgut chitinase activity

Next we tested chitinase activity in midguts of the four sand fly species to determine whether the long persistence of the PM in *S. schwetzi* might be caused by lower activity of midgut chitinase in this species. Interestingly, the dynamics and levels of exochitinase activity of *S. schwetzi* were similar to those in *P. papatasi* and *P. orientalis*: activity was detectable at 24 h PBM, peaked at 48 h PBM and then decreased at 72 h PBM (Fig 3). Moreover, the activity levels observed in *S. schwetzi* by 72 h PBM were the highest among all four sand fly species studied. A distinct course of exochitinase activity was observed in *P. argentipes*: the activity peaked at 24 h PBM, decreased by 48 h and declined almost to zero by 72 h PBM. Hence, the long persistence of the PM in *S. schwetzi* cannot be explained by low exochitinase activity in the midgut.

Effect of exogenous chitinases on the survival of *Leishmania* promastigotes *in vitro*

Before feeding exogenous chitinase to *Leishmania*–infected sand flies, we had to prove that chitinase has no lethal effect on the parasites. However, preliminary experiments showed that both commercially available chitinases from *S. griseus* and *T. viride* significantly decreased growth or even killed *Leishmania* promastigotes *in vitro* at all concentrations tested. (S1 Table). Therefore,

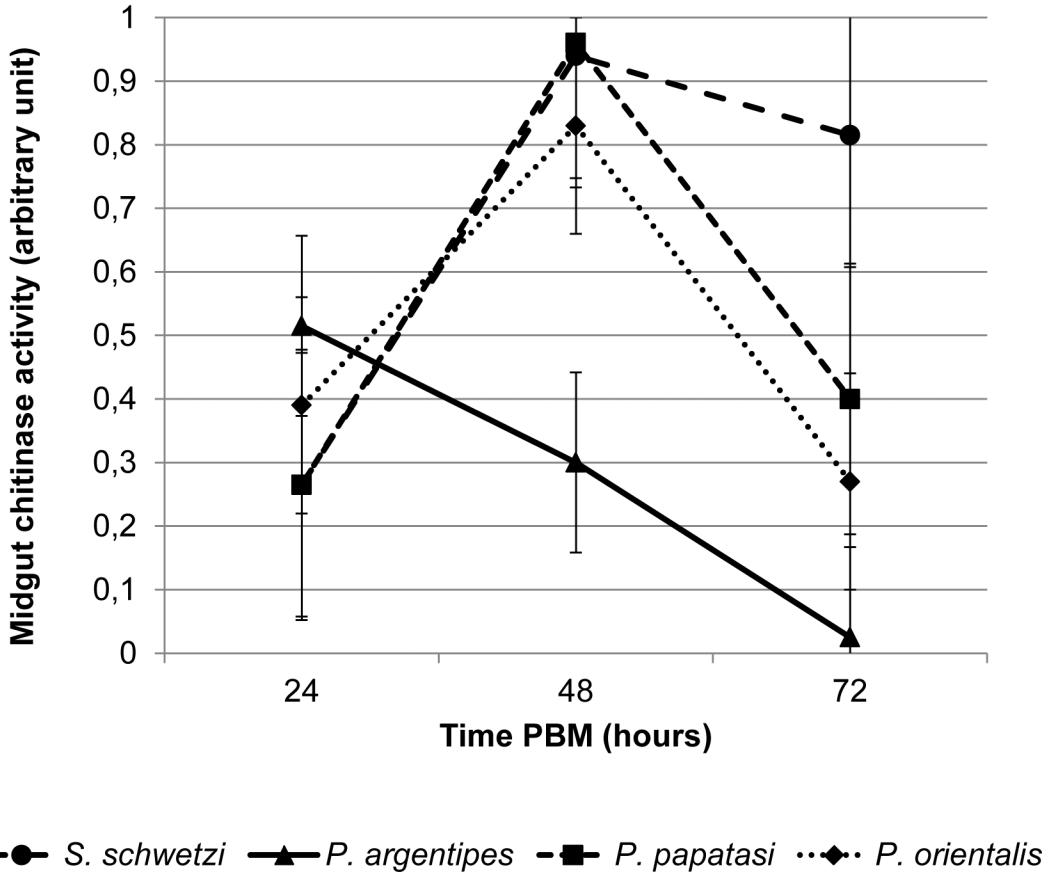

Fig 3. Chitinase activity in midguts of the four sand fly species. The exochitinase activity was quantified using the Chitinase Assay Kit (Sigma) in dissected sand fly midguts at 24, 48 and 72 h PBM. One arbitrary unit of chitinase releases 1 μmole of 4-nitrophenol per minute from the appropriate substrate at pH 4.8 at 37 ˚C. The values are arithmetic means from two experiments.

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we searched for a nontoxic source of chitinase and used the supernatant from the culture of the fungus *B. bassiana*. Flow cytometry was used to study the effect on *Leishmania* parasites *in vitro*. Supernatant from the culture of *B. bassiana* containing chitinase at concentration of 0.07 U/mL had no lethal effect on *L. major* and *L. donovani* promastigotes-mortality was very low and statistically not different from values observed for controls cultures without chitinase (Fig 4, $P = 0.505$ and $P = 0.559$ for *L. major* or *L. donovani*, respectively, ANOVA and Tukey Post Hoc Test). On the other hand, flow cytometry confirmed the lethal effect of chitinase from *S. griseus* on promastigote growth in cultures (S1 Table)-it killed *L. major* and *L. donovani* at all tested concentrations in the same rate as did the negative control (parasites killed by 1% solution of formaldehyde) (Fig 4, $P > 0.05$ for all chitinase concentrations and both *Leishmania* species, ANOVA and Tukey Post Hoc Test).

Effect of culture supernatant from *B. bassiana* on the PM of *S. schwetzi* and development of *Leishmania* parasites *in vivo*

Females of *S. schwetzi* were membrane-fed on a mixture of blood with culture supernatant from *B. bassiana* containing chitinase to disrupt the PM and mimic early disintegration of the

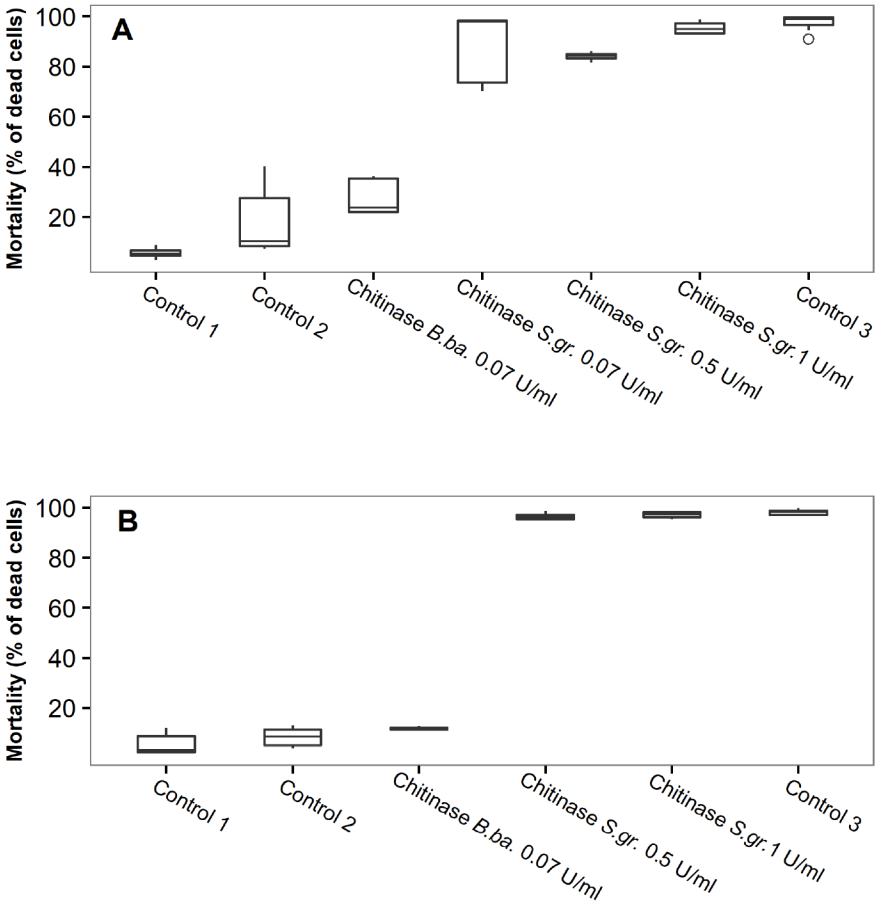

Fig 4. Mortality of *L. donovani* (A) and *L. major* (B) promastigotes exposed *in vitro* to culture supernatant of *B. bassiana* or chitinase from *S. griseus*. The mortality assessed as the ratio of the number of dead cells showing both green and blue fluorescence (GFP and DAPI) to the number of live cells showing green fluorescence (GFP). Control 1, culture medium for *Leishmania* with 0.025 V of PBS; Control 2, culture medium for *Leishmania* mixed at 1:1 with culture medium for *B. bassiana*; Control 3, parasites killed by 1% solution of formaldehyde and permeabilised by 0.5% Triton X-100; *B. ba.*, *Beauveria bassiana*; *S. gr.*, *Streptomyces griseus*.

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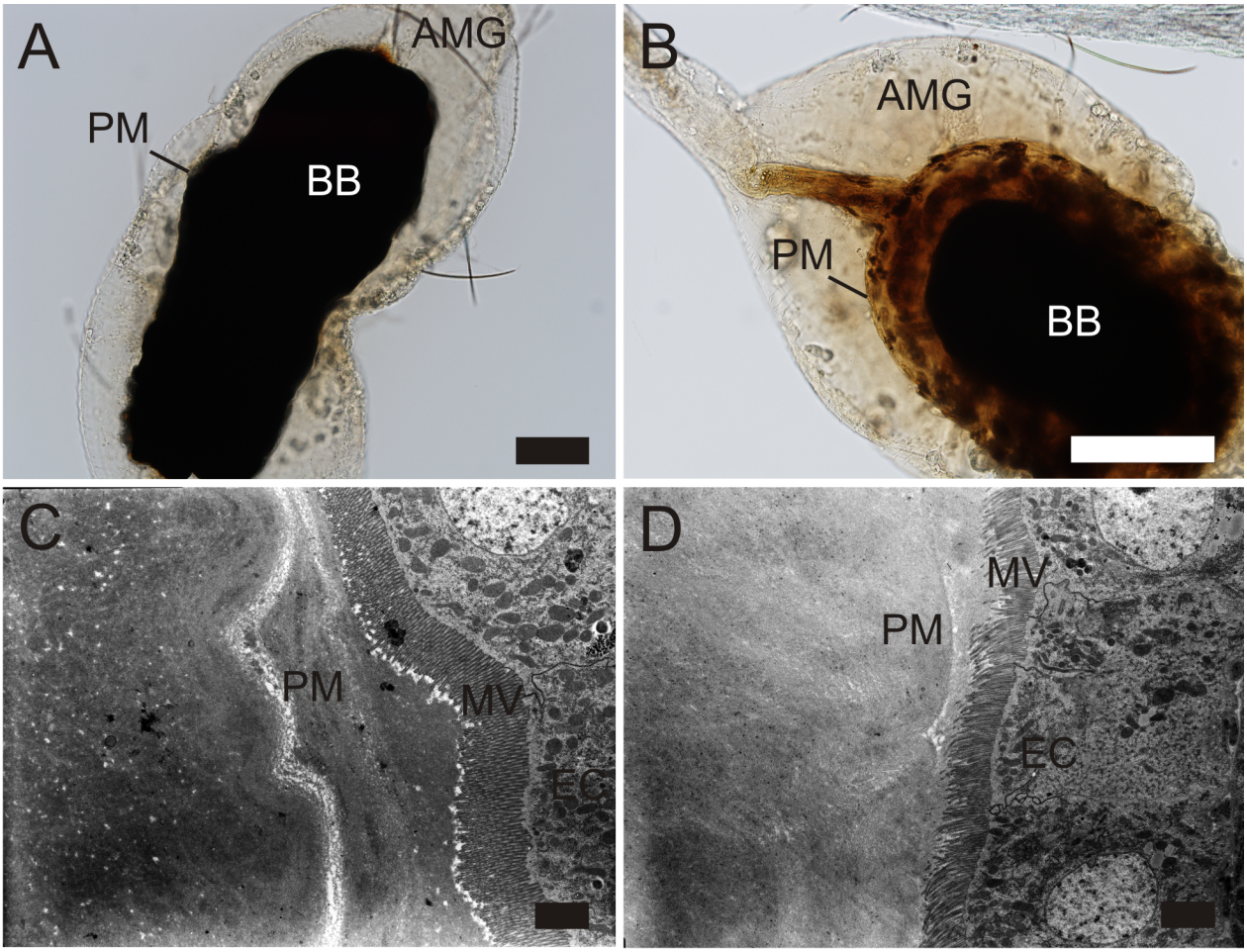

Fig 5. Differences in the PM between *S. schwetzi* females treated with *B. bassiana* culture supernatant and control. A and B, light microscopy; C and D, electron micrographs of cross sections of the abdominal midgut. A, appearance of the PM in a control female on day 3 PBM; B, appearance of the PM in female fed on a mixture of rabbit blood with supernatant of *B. bassiana* containing chitinase on day 3 PBM; C, multilayered thick PM in the control female on day 2 PBM; D, thin PM without apparent inner structure in the female fed on a mixture of rabbit blood with supernatant of *B. bassiana* containing chitinase on day 2 PBM. AMG, abdominal midgut; BB, blood bolus; PM, peritrophic matrix; MV, microvilli; EC, epithelial cells of the AMG. Scale bars indicate 100 μm in A, B and 2 μm in C, D.

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PM occurring in most *Phlebotomus* species. Light microscopy 24 h PBM revealed that the PM was disintegrated in 94% of chitinase-treated females (N = 49) while no disintegration was observed in control females (N = 35). By day 3 PBM the PM in control flies was consistently dark and opaque due to incrustation with heme (Fig 5A) while the PM in chitinase-treated flies remained mostly transparent with patches of dark coloration (Fig 5B). Presence of red blood cells in the ectoperitrophic space was indicative of partial disintegration and increased permeability of the PM in chitinase-treated females.

Electron micrographs of cross sections of the AMG on day 2 PBM confirmed the differences at the structural level. While the PM in control flies was thick and multilayered (Fig 5C), addition of *B. bassiana* culture supernatant to the bloodmeal resulted in thin PMs without apparent inner structure, only scarcely distinguishable from the background (Fig 5D). Surprisingly, although the PM in flies treated with the culture supernatant was weakened and permeable, it maintained its structure in midguts longer than controls (even till day10 PBM) as the defecation in this group was significantly delayed (S2 Fig).

The same culture supernatant from *B. bassiana* with 0.07 U/mL of chitinase was used for experimental infections of *S. schwetzi* with *Leishmania* parasites. Addition of culture supernatants significantly enhanced development of *L. donovani* and *L. major* in *S. schwetzi*. In control females fed on heat-inactivated culture supernatants or fresh culture media mixed with rabbit blood, neither species of *Leishmania* were able to develop mature infections, only very exceptionally survived defecation and then the infections were lost. On the other hand, in groups treated with *B. bassiana* culture supernatants, both *Leishmania* species escaped from the PM (Fig 6A and 6B), developed heavy infections in most females (Fig 7A and 7B) and by day 10 PBM colonized the stomodeal valve in 12% and 36% of females infected with *L. donovani* and *L. major*, respectively (Fig 6C and 6D and Fig 7C and 7D). Moreover, analysis of morphological forms revealed the presence of metacyclic promastigotes in late infections of both *Leishmania* species (Table 2).

Despite colonization of the ectoperitrophic space, the infection rates in females treated with culture supernatants gradually decreased from more than 90% on day 1 PMB to 46% and 47% in *L. donovani* and *L. major*, respectively, on day 10 PBM. In sand flies post defecation, infection rates ranged usually between 20% and 30% in both *Leishmania* species (Fig 7E and 7F) and attachment of parasites to the midgut epithelium was never observed.

Glycosylation of the midgut epithelium

Midgut mucins bearing O-linked glycosylation with terminal N-acetyl-galactosamine have been previously demonstrated to participate in *Leishmania* attachment in so called permissive sand fly vectors [6,26,27]. Therefore, midgut lysate of *S. schwetzi* was subjected to SDS-PAGE followed by blotting with HPA lectin specific for N-acetyl-galactosamine. Midgut lysates of *P. argentipes* and *P. papatasi* served as a positive and negative control, respectively [26]. In *S. schwetzi* midgut lysate, lectin HPA showed specific reactivity with a molecule of apparent molecular mass around 35–40 kDa; however, the reaction was weak in comparison with *P. argentipes*. Control strips with inhibitory sugar added were negative, confirming the specificity of HPA binding (S3 Fig).

Discussion

We demonstrated that the structure and thickness of the PM of *S. schwetzi* is very similar to those of *P. orientalis* and *P. papatasi*. It consists of a thin solid outer layer and an amorphous thick inner layer which correspond with data on the ultrastructure of PMs of several other species of the genera *Phlebotomus* and *Lutzomyia* [11,28–30]. On the other hand, the PM of *P. argentipes* was thin and homogeneous without distinct outer and inner layers which may be related to the exceptionally fast blood digestion described for this sand fly species [18]. The gross morphology of the PM of *S. schwetzi* also resembled other tested sand fly species: all four species had an anterior plug secreted by the thoracic midgut repeatedly detected in sand flies [11,29–31]. The posterior end of the PM was closed, with posterior tail similar to that observed previously in *P. duboscqi* and *P. papatasi* [11,32].

As we did not find significant differences between the PM of *S. schwetzi* and PMs of other sand fly species we tested the hypothesis that the persistence of the PM of *S. schwetzi* could be caused by lower activity of midgut chitinases. Surprisingly, experimental data suggest the opposite; the exochitinase activity in *S. schwetzi* midguts was the highest of the four sand fly species tested. Chitin degradation in insects is catalysed by various chitinolytic enzymes, for example in mosquitoes, chitinolysis is controlled by at least two distinct chitinases (endo- and exochitinase) and β-N-acetylglucosaminidase [33]. Therefore, we cannot exclude the possibility that low activity of enzymes other than exochitinase might be responsible for delayed

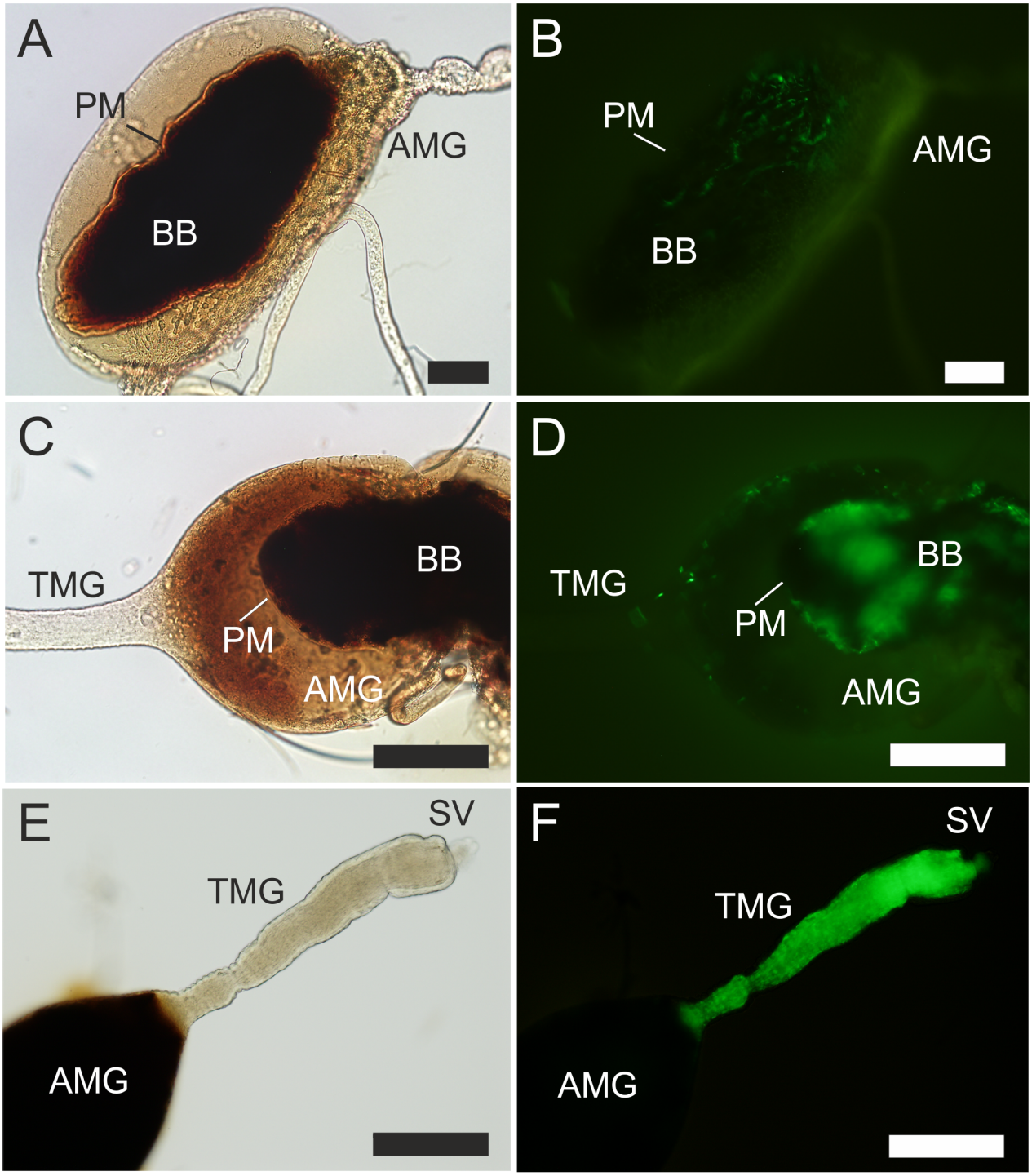

Fig 6. Light and fluorescent microscopy of *S. schwetzi* midguts. A and B, the gut of a control female (infectious meal comprising *L. donovani* promastigotes suspended in a mixture of fresh *B. bassiana* medium and inactivated rabbit blood) on day 3 PBM with *L. donovani* enclosed inside the PM; C and D, the gut of a treated female (infectious meal comprising *L. donovani* promastigotes suspended in a mixture of *B. bassiana* culture supernatant and inactivated rabbit blood) on day 3 PBM showing escape of *L. donovani* from the endoperitrophic space; E and F, the gut of a treated female on day 10 PBM showing colonization of the thoracic midgut and the stomodeal valve by *L. major* promastigotes expressing GFP (green). Images A—B, C—D and E—F are the same guts photographed by light and fluorescent microscopy, respectively. Both *Leishmania* species were marked with GFP protein, the midgut epithelium shows a natural mild autofluorescence. AMG, abdominal midgut; TMG, thoracic midgut; BB, blood bolus; SV, stomodeal valve; PM, peritrophic matrix. Scale bars indicate 100 μm.

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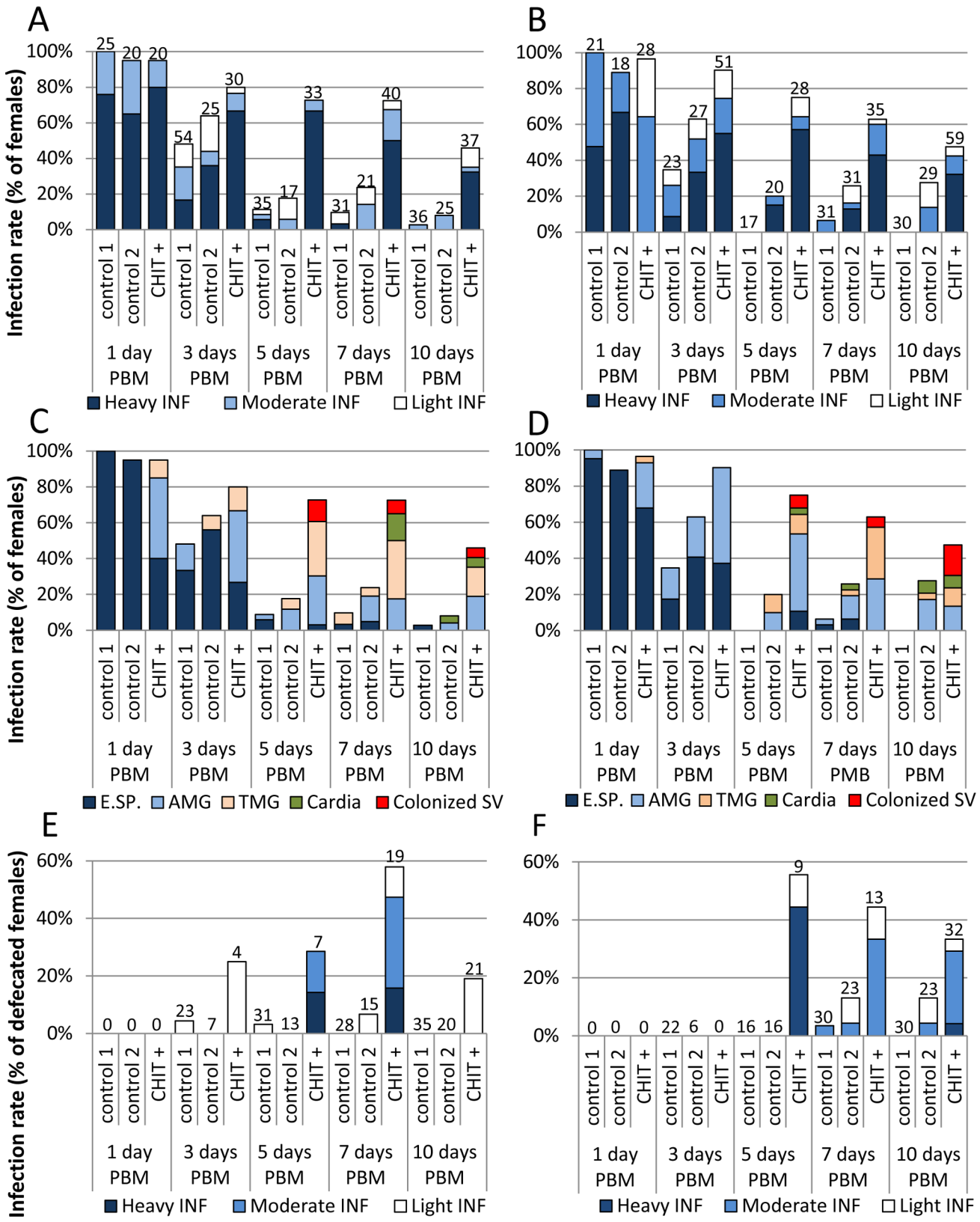

Fig 7. Effect of *B. bassiana* culture supernatant on the development of *L. donovani* and *L. major* in *S. schwetzi*. Experimental infections of *S. schwetzi* with *L. donovani* (A, C, E) and *L. major* (B, D, F) with addition of culture supernatant containing exogenous chitinase (0.07 U/mL) from *B. bassiana*

(CHIT +). In control females, either fresh medium for *B. bassiana* or heat-inactivated supernatant from *B. bassiana* culture was used (control 1 and control 2, respectively). A, B: Rates and intensities of infections. Numbers of dissected females are shown above bars. C, D: Location of *L. donovani* in infected sand flies. E.SP., endoperitrophic space; AMG, abdominal midgut; TMG, thoracic midgut; SV, stomodeal valve. E, F: Rates and intensities of infections in females post defecation. Numbers of dissected females post defecation are shown above bars.

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degradation of PM in *S. schwetzi*. High exochitinase activity in *S. schwetzi* midguts may be connected with the fact that chitinolytic activities of chitinases are related not only to degradation, but are involved also in synthesis and modulation of the PM [33].

Experiments described here and our previous studies [17,18] were performed with *S. schwetzi* from the colony originating in north-western Ethiopia and infected with *L. donovani* parasites isolated in the same region. Previously, *S. schwetzi* originating from Kenya were showed to be refractory to local *L. major* and *L. donovani* [15,16]. The origin of sand flies and parasites is important mainly in context of recent findings from the Mont-Rolland region in Senegal, where *L. infantum* DNA was found in 4.19% of captured *S. schwetzi* females and living parasites were observed in anterior midgut of a single female without a bloodmeal [34]. This fact evokes the question as to what extent is the vector competence of *S. schwetzi* population—specific. This species shows a substantial morphological variability and two morphological forms of *S. schwetzi* ("typical" and "atypical") were found in Senegal [35,36], a large area from Sudan to West Africa [37], Uganda [37] and Kenya [38]. Therefore, revision of the taxonomy of *S. schwetzi* and further experimental studies with sand flies and *Leishmania* parasites originating from different areas would be very interesting. In addition, it is not clear if the prolonged persistence of the PM is the common feature of the genus *Sergentomyia*. Previously the PM was studied in a single member of this genus; *S. arpaklensis* (corresponds to *S. sintoni* based on recent nomenclature). Its PM did not break down with the end of digestion and was defecated intact [39–41], presumably causing refractoriness for the reptile pathogen *L. gymnodactyli* [40].

Importance of the PM in vector competence was supported experimentally by several authors. Addition of a chitinase inhibitor allosamidin to the infective bloodmeal led to entrapment of *L. major* within the PM of *P. papatasi* [10]. Similarly, silencing of the gene for PpChit 1, a sand fly-derived chitinase involved in degradation of the PM [42], reduced *L. major* load in midguts of *P. papatasi* [43] while knockdown of the PpPer1 gene (peritrophin involved in the formation and scaffolding of the PM [42]) led to increase in the parasite load [44].

Here, the addition of the supernatant from *B. bassiana* culture with chitinase activity to the bloodmeals of *S. schwetzi* led to disintegration of the PM which enabled survival of *Leishmania*

Table 2. The relative representation and measurements of three morphological forms of *L. major* and *L. donovani* in guts of *S. schwetzi* by days 7–10 PBM.

Leishmania species	Promastigote form	N (%)	Body length (μm) Mean (S.D.) [Range]	Body width (μm) Mean (S.D.) [Range]	Flagellar length (μm) Mean (S.D.) [Range]
L. major	Elongated nectomonads	161 (80.5)	19.2 (2.9) [14.4–27.2]	1.4 (0.4) [0.7–3.4]	19.2 (3,9) [6.7–33.9]
	Short promastigotes	32 (16.0)	11.7 (1.8) [6.3–13.6]	1.9 (0.7) [1.0–3.7]	14.3 (4.2) [2.7–23.2]
	Metacyclic promastigotes	7 (3.5)	8.0 (1.6) [5.9–10.0]	2.3 (0.7) [1.1–3.0]	17.1 (2.5) [13.0–20.0]
	Total	200 (100)	17.6 (4.3) [5.9–27.2]	1.5 (0.6) [0.7–3.7]	18.3 (4.3) [2.7–33.9]
L. donovani	Elongated nectomonads	117 (58.5)	17.5 (2.4) [14.0–24.6]	1.6 (0.5) [0.7–4.7]	17.8 (3,1) [11.3–26.5]
	Short promastigotes	73 (36.5)	11.2 (1.8) [5.5–13.8]	1.6 (0.5) [0.9–3.3]	14.9 (3.9) [2.9–22.4]
	Metacyclic promastigotes	10 (5.0)	8.4 (2.0) [5.2–10.6]	1.6 (0.3) [1.1–2.1]	18.4 (3.4) [12.9–22.7]
	Total	200 (100)	14.8 (4.0) [5.2–24.6]	1.6 (0.5) [0.7–4.7]	16.8 (3.7) [2.9–26.5]

Sand flies were infected with addition of culture supernatant from *B. bassiana* (chitinase activity 0.07 U/mL).

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parasites in *S. schwetzi*. Both *L. donovani* and *L. major* developed heavy infections in flies treated with culture supernatants containing chitinase, with most important prerequisites for parasite transmission to the next host: presence of metacyclic forms and colonization of the stomodeal valve. The relatively low concentration of chitinase (0.07 U/mL in comparison with 1 U/mL in the experiment of Pimenta et al. (1997)) [10] caused only partial disintegration of the PM. The colour of the PM was more transparent than in control flies which might indicate lower incrustation of the PM with heme. In experiments of Pimenta et al. (1997) [10], the absence of PM caused by addition of exogenous commercial chitinase to the bloodmeal of *P. papatasi* was associated with the total loss or 20% reduction of amastigote- and promastigote-initiated *L. major* infections, respectively. Authors suggested that the lack of the PM exacerbated lethal conditions as a result of proteinase activity in the blood-fed midgut.

Partial degradation of PM due to culture supernatants of *B. bassiana*, together with delayed defecation, enabled *Leishmania* parasites to establish the infection in the midgut of *S. schwetzi*. However, we did not observe the attachment of promastigotes to the midgut epithelium and infection rates decreased with time post bloodmeal. This could be explained by the shortage of ligand molecules in the midgut as O-glycosylated epitopes in *S. schwetzi* midgut were present in very low amounts, significantly lower than in permissive species *P. argentipes* [26]. The presence of O-glycosylated mucins in a permissive vector *P. argentipes* as well as their absence in *P. papatasi*, a specific vector of *L. major*, is in accordance with previously described data [26]. Other factors, like antimicrobial peptides [45, 46] may also negatively influence the establishment of *Leishmania* infections in the gut of *S. schwetzi*.

The results of the present study clearly confirm that the prolonged persistence of the PM till defecation contributes significantly to the refractoriness of Ethiopian *S. schwetzi* to *Leishmania* parasites.

Supporting information

S1 Fig. Gross morphology of the PM in four sand fly species. Guts of *S. schwetzi* (A, B), *P. argentipes* (C, D), *P. papatasi* (E, F) and *P. orientalis* (G, H) were dissected and photographed using the light microscope with DIC at 24 h PBM (A, C, E, G). Sections of sand flies embedded in JB-4 resin were stained with haematoxylin and eosin (B, D, F, and H). Large arrows indicate the anterior plug; small arrows indicate the PM. Scale bars indicate 100 μm.
(TIF)

S2 Fig. Effect of chitinase addition on defecation of *S. schwetzi* females. Defecation of sand flies fed on mixture of inactivated rabbit blood mixed 1:1 with supernatant from the culture of *B. bassiana* containing chitinase (black squares) was compared with defecation on control females fed on the mixture of inactivated rabbit blood mixed 1:1 with medium for *B. bassiana* instead of the supernatant (open circles). Defecation status was assessed under the light microscope. Numbers of females were: 48, 81, 61, 75 and 96 for chitinase-treated group and 46, 77, 51, 62 and 66 for the control group in days 1, 3, 5, 7 and 10 PBM, respectively. The between–groups differences were significant by days 3–10 PBM ($P < 0.05$, tested by proportional test with Holm-Bonferroni correction).
(DOCX)

S3 Fig. Detection of the GalNAc-containing glycoconjugates in three sand fly species. Gut homogenates were fractionated on SDS-PAGE, transferred to nitrocellulose membrane and incubated with biotinylated *Helix pomatia* lectin (HPA) specifically binding GalNAc. PAP, *P. papatasi*; ARG, *P. argentipes*; SER, *S. schwetzi*; +, separated gut homogenate incubated with

lectin HPA; -, incubation of the homogenate with HPA preincubated with specific GalNAc.
(TIF)

S1 Table. Effect of chitinases from *S. griseus* and *T. viride* on *Leishmania* growth *in vitro*.
(DOCX)

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Author Contributions

Conceptualization: Jovana Sadlova, Petr Volf.

Funding acquisition: Jovana Sadlova, Petr Volf.

Investigation: Jovana Sadlova, Miroslav Homola, Jitka Myskova, Magdalena Jancarova.

Methodology: Jovana Sadlova, Miroslav Homola, Petr Volf.

Supervision: Petr Volf.

Writing – original draft: Jovana Sadlova.

Writing – review & editing: Petr Volf.

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An increase of larval rearing temperature does not affect the susceptibility of *Phlebotomus sergenti* to *Leishmania tropica* but effectively eliminates the gregarine *Psychodiella sergenti*

Magdalena Jancarova*, Jana Hlavacova, Jan Votypka and Petr Volf

Abstract

Background: In mosquitoes, it has previously been shown that rearing conditions of immature stages have an effect on the vector competence of adults. Here, we studied the impact of different larval rearing temperatures (27 °C *versus* 32 °C) on the sand fly *Phlebotomus sergenti* Parrot, 1917 and its susceptibility to two parasites: *Leishmania tropica* Wright, 1903, a dixenous trypanosomatid transmissible from sand flies to humans, and *Psychodiella sergenti* Lantova, Volf & Votypka, 2010, a monoxenous sand fly gregarine.

Results: Increased rearing temperature (32 °C) affected the larval developmental times and size of *P. sergenti* adults but had no effect on the susceptibility of *P. sergenti* to *L. tropica*. No differences were found in *Leishmania* infection rates or in the intensities of *Leishmania* infection. Interestingly, increased larval rearing temperature significantly suppressed the development of gregarines. All 117 control sand flies tested were infected with *Ps. sergenti*, and the mean number of gamonts per individual was 29.5. In contrast, only three of 120 sand flies maintained at 32 °C were infected and the mean number of gamonts per individual was just 0.04.

Conclusions: We demonstrated that the increased rearing temperature of *P. sergenti* larvae had no impact on the development of *L. tropica* in adult sand flies but had a profound effect on the gregarine *Ps. sergenti*. We suggest that increasing the larval rearing temperature by 5 °C is a simple and effective way to clean sand fly colonies infected by gregarines.

Keywords: *Phlebotomus sergenti*, *Psychodiella sergenti*, *Leishmania tropica*, Vector competence, Effect of temperature, Gregarines

Background

Phlebotomine sand flies (Diptera: Psychodidae) are blood-sucking insects traditionally divided into three main genera: *Phlebotomus*, *Lutzomyia* and *Sergentomyia*. They occur in a wide variety of habitats from deserts to rainforests. Both sexes feed on natural sugar sources, such as the sap of plants or honeydew, and females also feed on the blood of a wide range of hosts including humans (reviewed by [1, 2]). Eggs are laid on a substrate rich in organic content. Larval development includes four instars, and usually lasts three to four weeks. The pupa stage usually takes from seven to ten days (reviewed by [3]).

Phlebotomine sand flies are known vectors of bacteria (e.g. *Bartonella bacilliformis*), viruses (mainly genus *Phlebovirus*) and *Leishmania* spp., digenetic parasites causing a variety of symptoms ranging from mild cutaneous lesions to mucocutaneous form to fatal visceral disease (reviewed by [2]). *Leishmania* life-cycle involves intracellular amastigotes in the vertebrate host and extracellular promastigotes in the vector.

* Correspondence: magda.jancarova@seznam.cz
Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

Sand flies also harbour their own monoxenous parasites, such as gregarines of the genus *Psychodiella* Votypka, Lantova & Volf 2009 (Apicomplexa: Eugregarinorida), which is found in *Phlebotomus sergenti* (reviewed by [4]). The typical life-cycle of these gregarines starts by the infection of the first-instar larvae by oocysts, which contain sporozoites. In the midgut, the sporozoites escape, attach to the epithelial cells and develop into trophozoites. Later, mature-stage gamonts located either in the gut lumen of the larvae or the haemocoel of adults, undergo sexual development: two complementary gamonts associate in syzygy and form a gametocyst with oocysts inside. In sand fly females, gametocysts stick to the accessory glands and the oocysts are inoculated into the gland lumen to contaminate the surface of eggs (reviewed by [4]). This typical life-cycle is modified in *Psychodiella sergenti*, where sexual development is induced only in blood-fed females [5, 6]. Our previous study showed that coinfection with the gregarine *Ps. sergenti* does not have an apparent effect on the development of *Leishmania tropica* in *P. sergenti* [7].

It has been previously reported that the rearing conditions of larvae have an impact on the maintenance of parasites and their development in mosquito adults. It was shown that the quality of the larval diet changes the vector competence of *Anopheles stephensi* to *Plasmodium yoelii* [8], and that ambient temperature during the larval development of *Aedes albopictus* negatively correlates with the likelihood of adult infection by Chikungunya virus (CHIKV): females developed from larvae kept at 18 °C had higher infection rates than those from larvae kept at 24 and 32 °C [9]. As far as we are aware, similar studies have never been done in sand flies. Therefore, we studied the impact of rearing temperature of immature stages of the Old World sand fly *Phlebotomus sergenti* on the development of *Pychodiella sergenti* and the susceptibility of adult sand flies to *Leishmania tropica*.

Methods

Sand flies and parasites

Two groups of *P. sergenti* (from a colony originating from adults caught in Amnun, Israel) infected with the gregarine *Ps. sergenti* were used in the study: (i) immature stages (eggs, larvae, pupae) maintained at 27 °C; and (ii) immature stages (eggs, larvae, pupae) maintained at 32 °C. Adults of both groups and experimentally infected females were kept at 26 °C. Other parameters of sand fly maintenance were as described by Volf & Volfova [3]. *Leishmania tropica* SU23 (MHOM/TR/98/HM) was maintained at 23 °C on M199 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20 % foetal calf serum (Gibco, Life Technologies, Carlsbad, CA, USA), 1 % BME vitamins (Sigma-Aldrich, St. Louis, MO, USA), 2 % filtered human urine and amikacin (250 μg/ml).

Experimental infections with *Leishmania tropica*

Sand fly females were membrane-fed on suspension of heat-inactivated rabbit blood containing 1×10^6 promastigotes/ml. Blood-fed females were maintained at 26 °C. On days 2 and 7–9 post-blood meal, females were dissected under a stereomicroscope and checked for the intensity and localization of infections using a compound light microscope. Intensities of infection were graded according to Myskova et al. [10] as weak (less than 100 promastigotes/gut), moderate (100–1,000 promastigotes/gut) and heavy (more than 1,000 promastigotes/gut). Data from two independent experiments were pooled and evaluated statistically by means of the Chi-square test using STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA).

Gregarine infection

Different sand fly stages, namely the actively feeding fourth-instar larvae (before defecation of the midgut content) and sugar-fed adults of both sexes (1, 4 and 7 days post-eclosure) were dissected under the stereomicroscope and checked for the presence and number of gregarines. Results were evaluated statistically by the Chi-square and Kruskal-Wallis tests using STATISTICA 12.0.

Morphometric analysis

Measurements of wing length or wing veins are often used to determine the size of adult mosquitoes and sand flies [11, 12]. Therefore, wings of emerged females were dissected from the body and mounted on slides using CMCP-10 mounting medium (Polysciences, Inc., Warrington, PA, USA). Slides were observed under an Olympus BX51 microscope and photographed with Olympus D70 camera software. The effect of temperature was evaluated by measuring the length of the R2, R3, R4, R5, M1 and M2 wing veins, as previously described by Belen et al. [12]. ANOVA was used for statistical evaluation using STATISTICA 12.0.

Results

Morphometric analysis

Larval rearing temperature significantly affected the size of adult sand flies. Measures of all wing veins studied were significantly longer in females originating from larvae developed at 27 °C than those from 32 °C (ANOVA: $F_{(6,55)} = 13.26$, $P < 0.001$). Data from morphometric analysis are shown in Fig. 1. As expected, temperature also affected the rate of development: the average time interval from egg-laying to emergence of adults was 36 days at 27 °C compared to 26 days at 32 °C.

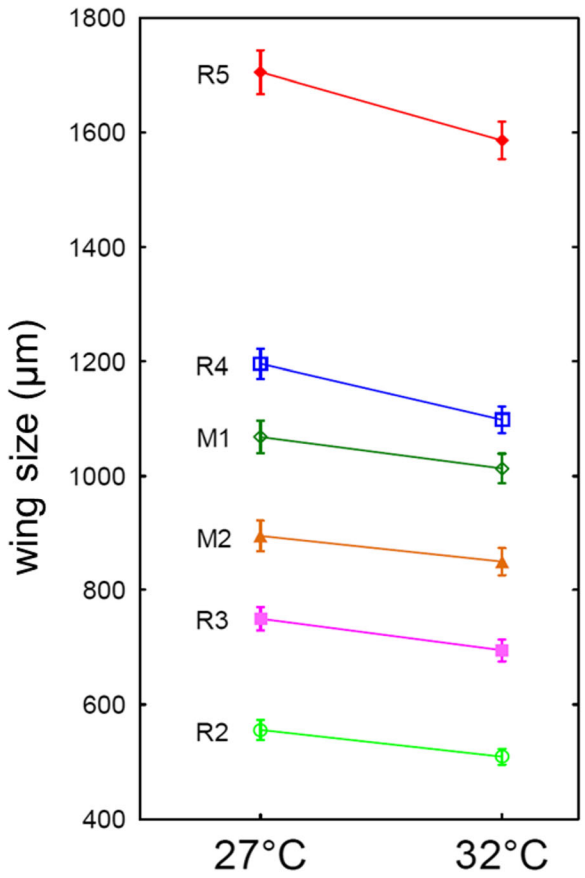

Fig. 1 Wing size of *P. sergenti* females depending on temperature. The association between ambient temperature during immature stage development and the wing size of adult *P. sergenti* females. Females from larvae reared at 27 °C were significantly larger than those from 32 °C. *Abbreviations*: M1, Media 1; M2, Media 2; R2, Radius 2; R3, Radius 3; R4, Radius 4; R5, Radius 5

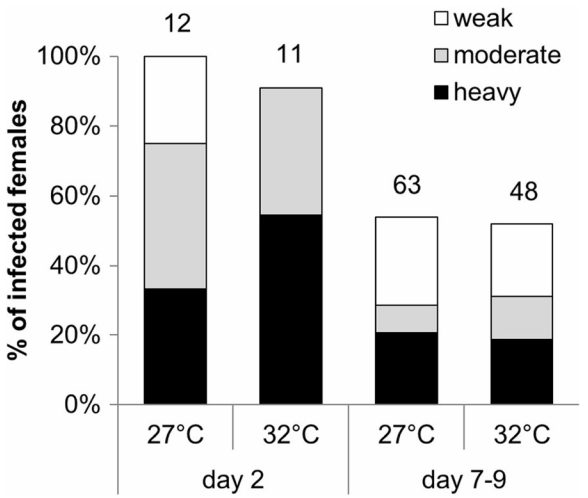

Fig. 2 Development of *L. tropica* in *P. sergenti*. There were no significant differences in infection rates or infection intensity of *L. tropica* in *P. sergenti* females emerged from larvae kept at 27 °C or 32 °C. Numbers of dissected females are shown above the bars

Effects of immature stage rearing temperatures on experimental infections with *Leishmania tropica*

Larval rearing temperature did not affect the infection rates or intensities of *L. tropica* infections in *P. sergenti* females (Fig. 2). No significant differences were found in infection rates (day 2: $\chi^2 = 1.14$, $df = 1$, $P = 0.29$; days 7–9: $\chi^2 = 0.04$, $df = 1$, $P = 0.84$) or in intensities of *Leishmania* infection (day 2: $\chi^2 = 3.36$, $df = 2$, $P = 0.19$; days 7–9: $\chi^2 = 0.85$, $df = 2$, $P = 0.65$).

Effects of immature stage rearing temperatures on gregarine infections

The rearing temperature of immature stages of *P. sergenti* sand flies infected by *Ps. sergenti* had a marked effect on infection rates. All 117 control sand flies tested (60 males and 57 females derived from larvae kept at 27 °C) were infected with *Ps. sergenti* gamonts, with a mean number of 29.5 gamonts per individual. On the other hand, sand flies produced from larvae kept at 32 °C were almost completely gregarine-free: only three of 120 sand flies dissected (60 males and 60 females) were infected by gregarines, with a mean number of 0.04 gamonts per individual. The differences between groups were highly significant ($\chi^2 = 225.29$, $df = 1$, $P < 0.0001$). To investigate in which developmental stage the gregarines disappeared, an additional 32 (kept at 27 °C) and 33 (kept at 32 °C) actively feeding (before defecation of the midgut content) fourth-instar larvae were dissected. Larvae kept at 27 °C were significantly more infected ($\chi^2 = 6.42$, $df = 1$, $P < 0.01$) and the intensity of infection was significantly higher (Kruskal-Wallis test, $F_{(1,41)} = 12.07$, $P < 0.001$).

Discussion

Here we show that the rearing temperature of immature stages did not affect parameters of *Leishmania* infection in adults. Similar studies have been done in mosquitoes, but with contradictory results. There was no observation of a consistent impact of larval rearing temperatures (19, 25 and 31 °C) on the infection, transmission or dissemination of West Nile virus in adults of *Culex tarsalis* [13]. In contrast, *Aedes albopictus* adults obtained from larvae kept at 18 °C had two or six times higher infection rates by Chikungunya virus than adults from larvae kept at 24 and 32 °C, respectively [9]. As expected, larvae kept at higher temperature developed faster and yielded smaller adults. We found that these size differences in *P. sergenti* adults had no effect on the infection rate and intensity of *L. tropica* infections. In mosquitoes, the relationship between vector size and their susceptibility to infection has been studied by various authors, with different outcomes. Larger *Anopheles gambiae* females were significantly more infected by *Plasmodium*

yoelii nigerinesis than smaller ones, but no such effect was found in *Anopheles stephensi* [14]. Similarly, larger *Ae. aegypti* females were more likely to be infected by Dengue virus than smaller ones [11], but the opposite was found by Alto et al. [15]. In two other studies, no effect of mosquito size was described on infection parameters by three different viruses [16, 17]. These findings suggest that there are no general rules across all vectors-parasites/pathogens for a relationship between vector size and infection parameters, but rather results are species specific.

Rearing temperatures of immature stages had major impacts on the intensity of infection and infection rate of gregarines. A significant difference was visible by the fourth-instar larvae: those reared at 27 °C were infected more and had higher intensities of infection compared to those reared at 32 °C. The difference was more pronounced in adults: gregarine gamonts were found in all sand flies developed from larvae reared at 27 °C but in only three adults from larvae reared at 32 °C. This finding seems to confirm a previous hypothesis that the pupal stage is the most limiting part of the *P. sergenti* life-cycle for gregarine survival [18].

We demonstrated that increased temperature eliminated infection by the gregarine *Ps. sergenti* quite efficiently. In our hands, this method was far more effective than the washing of eggs described by Poinar & Thomas [19]. This washing method only reduced the numbers of gregarines *Psychodiella chagasi* and *Ps. sergenti* in colonies of *Lutzomyia longipalpis* and *P. sergenti*, respectively, but had to be repeated again for almost every sand fly generation [18].

We hypothesize that the elimination of gregarines in larvae and pupae reared at 32 °C might be caused by several factors. The increased metabolism of sand flies maintained at higher temperatures [20] leads to a faster reconstruction of larval tissue in pupae, which might be too quick for gregarines. Increased temperatures may also act negatively directly on gregarines, as suggested previously in other gregarine-insect pairs. For example, the effect of temperature was tested on the gametocysts and oocysts of two species of gregarines: *Blabericola migrator* and *B. cubensis*. In both species, no gametocysts completed development or produced oocysts at 10 or 40 °C. Oocyst viability in *B. migrator* was highest at 18 °C (57 %) and 22 °C (77 %), and was markedly decreased at 27 °C (24 %) and 35 °C (2 %), with similar results for *B. cubensis* [21]. The temperature of 32 °C, used in our experiments, might have reduced the number of viable infective oocysts, which would have resulted in lower intensities of larval infections. Furthermore, suboptimal conditions during the pupal stage would result in a further decrease of parasite numbers in adult sand flies.

Another possible explanation of gregarine elimination in *P. sergenti* maintained at 32 °C is the enhanced immune response of the insect. Various beetles maintained at higher temperature display higher phenoloxidase and antibacterial activities [22, 23]. However, the immune response is not straightforwardly correlated with temperature, as individual components of the immune system possess different thermal optima and there is a complex network of interactions between temperature, time and origin of immune challenge. For instance, in *Anopheles stephensi* a maximum expression of nitric oxide synthase was found at 30 °C, while a peak of melanization, phagocytosis and defensin expression was observed at 18 °C [24]. Clearly, further experiments are necessary for an explanation of the mechanism of gregarine elimination in sand flies.

Conclusions

Understanding the biotic and abiotic factors affecting parasite-vector interactions is crucial for predicting the spread of vector-borne diseases and their epidemiology. To our knowledge, this is the first study in sand flies to evaluate the effect of larval rearing temperature on the consequent susceptibility of adults to *Leishmania*. In the natural parasite-vector combination *P. sergenti*/*L. tropica* we did not find any effect. However, increased temperature very efficiently eliminated infection by the gregarine *Ps. sergenti*, and appears to be a novel method for cleaning parasitic gregarines from sand fly colonies. Our results suggest that rearing immature stages at 32 °C is a simple and effective method to obtain gregarine-free colonies.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Authors' contributions

MJ and JH contributed equally to the design of experiments and all experimental procedures. MJ wrote the draft of the manuscript. JV performed statistical analysis and interpretation of data. PV participated in the study design and explanation of results. All authors participated in the revision of the manuscript and approved the final version.

Competing interests

The authors declare that they have no competing of interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Short Communication

The Development of *Leishmania tropica* in Sand Flies (Diptera: Psychodidae): A Comparison of Colonies Differing in Geographical Origin and a Gregarine Coinfection

MAGDALENA JANCAROVA,[1] JANA HLAVACOVA, and PETR VOLF

Department of Parasitology, Faculty of Science, Charles University, Vinicna 7, Prague 2, 128 44, Czech Republic.

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ABSTRACT *Phlebotomus sergenti* Parrot, 1917 is the main vector of *Leishmania tropica*; however, its broad geographical range and molecular heterogeneity suggest possible variability in vector competence. We infected laboratory-reared *P. sergenti* originating from Turkey and Israel to compare their susceptibility to *L. tropica*. In both tested groups, heavy late-stage infections with the presence of metacyclic forms and colonization of the stomodeal valve were observed. The similar development of *Leishmania* in both sand fly colonies indicates that the different geographical origin of *P. sergenti* is not reflected by a different vector competence to *L. tropica*. Additionally, we tested the effect of the gregarine *Psychodiella sergenti* on *L. tropica* coinfections; no apparent differences were found between *P. sergenti* infected or not infected by gregarines.

KEY WORDS *Phlebotomus sergenti*, *Leishmania tropica*, vector competence, coinfection, gregarine

Leishmaniases are vector-borne diseases with a wide range of clinical outcomes. Their causative agents are parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted by the bite of phlebotomine sand flies (Diptera: Psychodidae). *Leishmania tropica* causes cutaneous leishmaniasis in many countries around the Mediterranean basin, the Middle East, Central Asia, and East Africa. The primary specific vector is *Phlebotomus sergenti* Parrot, 1917 (Kamhawi et al. 2000, Volf and Myskova 2007), although other sand fly species have been shown to transmit *L. tropica* in Ethiopia (Gebre-Michael et al. 2004) and northern Israel (Jacobson et al. 2003, Svobodova et al. 2006).

The geographical range of *P. sergenti* is very broad and more widespread than the distribution of *L. tropica*, suggesting some degree of intraspecific variability that may potentially affect the vector competence of different populations of this species (Depaquit et al. 2002). Sequencing of the internal transcribed spacer 2 (ITS2) of 12 populations from 10 countries revealed two principal branches of distinct geographical origin: 1) a more north-east area (Cyprus, Pakistan, Syria, and Turkey) and 2) a more south-west area (Israel, Egypt, Morocco, Sicily; Depaquit et al. 2002). These two branches were confirmed by subsequent studies using random-amplified polymorphic DNA and geometric morphometrics (Dvorak et al. 2006, 2011).

To study the possible consequences of the molecular heterogeneity of *P. sergenti* on the vector competence of *L. tropica*, we established two *P. sergenti* colonies of different geographical origin, one from Turkey (the north-east branch) and the second from Israel (the south-west branch), and experimentally tested their susceptibility to *L. tropica*. As the Turkish colony was naturally infected by the gregarine *Psychodiella sergenti* (Apicomplexa: Eugregarinorida), and the egg-washing procedure by Poinar and Thomas (1984) commonly used to clean gregarines from sand fly colonies is not sufficiently effective in *P. sergenti* (Lantova and Volf 2012), we have now compared the development of *L. tropica* in two groups of Israeli *P. sergenti*, one being infected experimentally by gregarines.

Materials and Methods

Sand Flies and Parasites. Three laboratory colonies of *P. sergenti* were used—1) TU originating from Sanliurfa, Turkey; 2) IS originating from Amnun, Israel; and 3) ISG derived from IS females artificially infected by *Ps. sergenti* as described by Lantova et al. (2010). Sand flies were maintained under standard conditions as previously described by Volf and Volfova (2011). *Leishmania tropica* SU23 (MHOM/TR/98/HM) was maintained at 23°C on M199 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% foetal calf serum (Gibco, Life Technologies, Carlsbad, CA), 1% BME vitamins (Sigma-Aldrich), 2% filtered human urine, amikacin (250 mg/ml), and gentamicin (80 mg/ml).

Experimental Infection. Sand fly females (4–7 d old) were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 1×10^6 promastigotes/ml. This infective dose corresponds to <1,000 parasites per female, as bloodmeal volumes taken by

[1] Corresponding author, e-mail: magda.jancarova@seznam.cz.

various sand fly species ranged from 0.53 to 0.91 µl (Pruzinova et al. 2015). Moreover, promastigote-initiated infections are fully comparable with amastigote-initiated infections (Freitas et al. 2012). Blood-fed females were maintained at 26°C and dissected on days 2 and 7–10 postinfection (p.i.), and their guts were microscopically checked for the presence and localization of *Leishmania* promastigotes. Intensities of infection were graded into three categories according to Myskova et al. (2008)—weak (1–100 promastigotes per gut), moderate (100–1,000 promastigotes per gut), and heavy (>1,000 promastigotes per gut). The experimental infection was repeated two (TU × IS) or three times (IS × ISG). Data were statistically evaluated by means of the χ^2 test using STATISTICA 12.0 software (StatSoft).

Results and Discussion

In the first series of experiments, the development of *L. tropica* was compared in *P. sergenti* TU and IS. Fig. 1A summarizes the data of two independent experiments. Parasites developed well in the females of both colonies tested. In early-stage infections (day 2 p.i.), all dissected females were infected, with a slightly higher proportion of heavy infections found in TU females ($\chi^2 = 6$; df = 2; $P = 0.05$; Fig. 1A). Nevertheless, on days 7–10 p.i., the infection rates and intensities of infection were the same in TU and IS females ($\chi^2 = 0.316$; df = 1; $P = 0.574$ and $\chi^2 = 4.747$; df = 3; $P = 0.191$; respectively). In both tested groups, heavy late-stage infections with anterior migration of *Leishmania* promastigotes, presence of metacyclic forms, and colonization of the stomodeal valve were observed from day 7 (Fig. 1A).

Next, we performed an additional series of experiments comparing IS and ISG females. In mosquitoes, the gregarine *Ascogregarina culicis* has been implicated in maintenance of the chikungunya virus (Mourya et al. 2003). Here, we investigated if the gregarine *Ps. sergenti* has any effect on the development of *L. tropica* in *P. sergenti*. Figure 1B summarizes the data of three independent experiments. No significant differences in infection parameters were detected between IS and ISG females on any day p.i. On day 2, infection rates were 100% and were of similar intensities, with heavy infections observed in a majority of females of both colonies ($\chi^2 = 0.870$; df = 2; $P = 0.647$; Fig 1B). In the late development stage (day 7–10 p.i.), the infection rate was >60% in both *P. sergenti* groups (76% and 64% for IS and ISG, respectively; $\chi^2 = 1.155$; df = 1; $P = 0.283$), intensities of infection were comparable ($\chi^2 = 1.407$; df = 3; $P = 0.704$), and heavy late-stage infections with colonization of the stomodeal valve were observed in ~50% of infected females (Fig. 1B). Thus, we conclude that coinfection by the gregarine *Ps. sergenti* did not have any apparent effect on the development of *L. tropica* in *P. sergenti* in our experimental settings.

In summary, we demonstrate the ability of *L. tropica* to develop equally well in two *P. sergenti* colonies that represent the two different branches previously postulated by Depaquit et al. (2002). Our results support recent findings on the similarity of cytochrome b sequences in specimens from Turkey and Israel (Dvorak et al. 2011) and also correspond with the ability of *P. sergenti* colonies originating from Turkey and Israel to cross breed with no negative effect on their offspring (Dvorak et al. 2006). All these findings question the existence of a *P. sergenti* species complex. It seems that the different geographical origin of

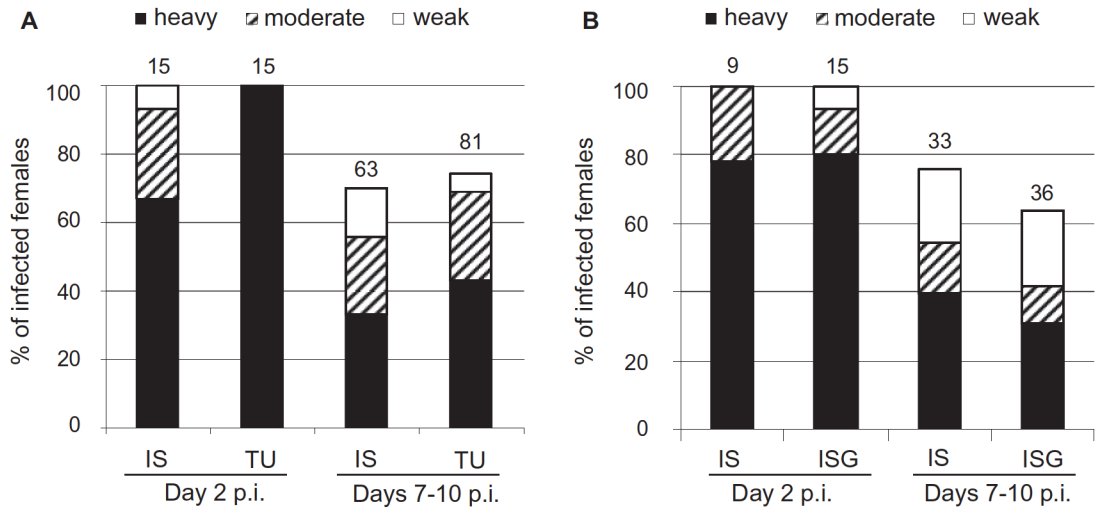

Fig. 1. The development of *L. tropica* in *P. sergenti*: (A) comparison of two *P. sergenti* colonies originating from Turkey (TU) and Israel (IS); (B) comparison of a colony infected by the gregarine *Ps. sergenti* (ISG) and a noninfected control (IS). Intensities of the leishmania infection were estimated as light (<100 promastigotes per gut)—white bar, moderate (100–1,000 promastigotes per gut)—striped bar, and heavy (>1000 promastigotes per gut)—black bar. Numbers above each bar indicate the number of dissected females.

P. sergenti tested here is not reflected by different susceptibility to *L. tropica*. Current results are consistent with the previously described vector competence of various *P. sergenti* populations for *L. tropica* (Svobodova et al. 2006, Kamhawi 2006, Maroli et al. 2013).

This finding on *P. sergenti* corresponds with results on *Leishmania donovani* vectors: two populations of *Phlebotomus orientalis* Parrot, 1936 from endemic and nonendemic areas in Ethiopia were equally susceptible to *L. donovani*, and the authors (Seblova et al. 2013) concluded that factors other than the vector competence of *P. orientalis* play a role in the epidemiology of *L. donovani* in Ethiopia. Similarly, differences in the distribution of *L. tropica* and its main vector *P. sergenti* may be rather attributed to factors other than the different vector competence of various *P. sergenti* populations.

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4 Summary and conclusions

This thesis summarizes results of three publications in peer-reviewed journals and one manuscript. It is focussed on natural barriers and factors which may limit development of viruses, leishmania and gregarines in sand flies.

Firstly, we were interested in the life cycle of sand fly-borne viruses of genus *Phlebovirus*. Phleboviruses are causative agents of human diseases with variety of clinical syndromes but very little is known about their circulation in nature. Their transovarial and sexual transmissions are not efficient enough to keep virus circulation (Tesh and Modi, 1987; Tesh et al., 1992) and no vertebrate reservoirs were found. To study the possible transmission routes of phleboviruses, we chose MASV, non-pathogenic species closely related to TOSV, important human pathogen naturally transmitted by *P. perniciosus* (Charrel et al., 2009).

We proved that infection of first and fourth larval instars by larval food and subsequent transstadial MASV transmission to adults are rather ineffective. Very low infection rate was obtained also by feeding *P. perniciosus* females on blood mixed with virus. Surprisingly, the most efficient way of infection was using the sugar meal: 72% of females and 51% of males of *P. perniciosus* became MASV-positive. Moreover, infected sand flies repeatedly regurgitated the virus particles into the source of sugar which remained infectious at least 24 hours for other naïve individuals: almost 30% of *P. perniciosus* of both genders became positive for MASV after feeding on sugar with spitted virus. Interestingly, MASV infection was found in *P. perniciosus* salivary glands till day 7 post infection but virus particles were expectorated into sugar solution until day 21. These results suggest that virus is not released only through saliva but also by regurgitation from alimentary canal. Maybe, infection through the sugar meal led to different virus development than in sand flies infected through the blood meal and virus do not have to disseminate from midgut or infected salivary glands (Jancarova et al., manuscript).

We suppose that transmission by this type of co-feeding of infected and uninfected sand flies on the same sugar source may serve as an important part of the life cycle of MASV and probably also some other arboviruses (Jancarova et al., manuscript). For example, this might be true for TOSV, where various routes of infection studied (through blood meal, transovarially, venerally) seems to be nonefficient for maintenance of virus in nature (Tesh and Modi, 1984; Tesh and Modi, 1987; Tesh et al., 1992; Maroli et al., 1993). Our results are in agreement with hypothesis that plants and sugar sources, like nectar, could be involved in circulation of other mosquito- and sand fly-borne viruses, namely vesiculoviruses (Johnson et al., 1969; Tesh et al., 1972) and negeviruses (Nunes et al., 2017). Moreover, our results support the theory that vertebrates probably do not have important role in sand fly virus life cycle and rather represent dead-end host (Tesh and Chaniotis, 1975). On the other

hand, our findings oppose the hypothesis that the sand flies could be primary reservoirs of phleboviruses, including MASV (i.e. Alkan et al., 2013).

Sand flies belonging to genera *Phlebotomus* and *Lutzomyia* are proven vectors of human leishmaniasis (rewieved by Maroli et al., 2013) while members of the third main sand fly genus, *Sergentomyia* transmit reptile parasites of genus *Sauroleishmania* and their medical importance is questionable (Dvorak et al., 2018). Vector competence of sand flies is affected by number of physiological and molecular factors. In some sand fly species, particularly in *S. schwetzi*, an important role in *Leishmania* development is also played by PM, the chitin-containing layer secreted by midgut epithelial cells after bloodfeeding (Sadlova et al., 2013). Sadlova and Volf (2009) described positive correlation between the degree of PM disintegration and transformation from short procyclic promastigotes to long nectomonads, morphological stage attaching to midgut to avoid defecation and establish the midgut infection. In *S. schwetzi* the PM remained intact almost until defecation and was proposed to be a key factor responsible for *S. schwetzi* refractoriness to different leishmania species (Sadlova and Volf, 2009; Sadlova et al., 2013).

Our study has confirmed the crucial role of PM in *S. schwetzi*. Addition of *B. bassiana* chitinase in to an infective bloodmeal caused weakened PM which disrupted earlier (24 hours PI: 94% vs 0%), allowing the early escape of *L. major* and *L. donovani* from the endoperitrophic space. Consequently, both *Leishmania* species transformed to metacyclic forms and colonized the stomodeal valve of *S. schwetzi*. On the other hand, in control group, none of the leishmania species established infection in *Sergentomyia* females and all infections were lost during defecation (Sadlova et al., 2018).

We expected that long persistence of PM in *S. schwetzi* could be caused by low midgut chitinase activity and thus we compared *S. schwetzi* with three other sand fly species differing in vector competence to *L. major* and *L. donovani*: *P. argentipes*, *P. papatasi* and *Phlebotomus orientalis*. Surprisingly, the dynamics and levels of exochitinase activity were similar in *S. schwetzi*, *P. papatasi* and *P. orientalis*. Moreover, 72 hours after blood feeding the activity was highest in *Sergentomyia* (Sadlova et al., 2018). Therefore, we concluded that factors other than chitinase activity are responsible for long persistence of PM in *S. schwetzi*.

One of the most important extrinsic factors influencing various aspects of sand fly life is the ambient temperature. As shown in mosquitoes, temperature fluctuation during both larval and adult life affects also the vector competence, however, the results of such studies are contradictory, depending on the vector-pathogen combination. In sand flies the effect of temperature during larval development on vector competence of adults to *Leishmania* was not studied yet. Therefore, we decided to breed *P. sergenti* larvae at different temperature (27°C versus 32°C) and test the susceptibility of adult sand fly females to *L. tropica*, parasite naturally transmitted by this sand fly

vector. Larvae kept at higher temperature developed faster and resulting females were smaller than in larvae maintained at lower temperature. Nevertheless, we did not observe any effect of different larval experimental conditions on the infection rates or intensities of *L. tropica* infections in adult females; both, larval breeding temperature and size of females did not affect these infection parameters (Jancarova et al., 2016).

Interestingly, the larval conditions significantly affected the development of gregarines *Ps. sergenti*, natural pathogen of *P. sergenti*: higher temperature significantly decreased infection rate and intensity of gregarine infection in both, larvae and adults. We hypothesized that this drastic negative effect on gregarines is caused by either: (i) accelerated metabolism of sand flies which provided suboptimal conditions for gregarines development, (ii) direct negative effect of higher temperature on oocysts (as shown in cockroach gregarines by Kolman et al., 2015) and/or (iii) enhanced immune response which may act against gregarines. While the mechanism of gregarine clearance requires further study, the increase of temperature during larval development represents an easy and an effective method how to rid off gregarines from sand fly laboratory colonies (Jancarova et al., 2016).

In nature, *Leishmania* and gregarines may occur together but nothing is known about the possible effect of gregarine presence on sand fly vector competence to *Leishmania*. In mosquitoes it was found that presence of different parasites in the host can modify the impact of pathogens. Thanks to that it might be possible to alter vector competence to another pathogen (Dong et al. 2012; Garza-Hernandéz et al., 2013; Vazeille et al. 2016). We decided to study whether the presence of gregarine *Ps. sergenti* causes any barrier or negative effect for development of *L. tropica* in *P. sergenti*. We did not observe any significant differences in intensity of infection or infection rate of *L. tropica* between females *P. sergenti* infected and non-infected by gregarines (Jancarova et al., 2015).

In conclusion, we brought new information about factors critical for development of various pathogens in sand flies. Particularly we: i) proposed a new model of sand fly-borne viruses circulation in nature, ii) confirmed hypotheses that long persistence of PM is responsible for refractoriness of *Sergentomyia* to *Leishmania* species, iii) described that temperature during larval development and female size of *P. sergenti* did not affect infection parameters of *L. tropica* but effectively reduced gregarines *Ps. sergenti* and last but not least, iv) demonstrated that coinfection of *Ps. sergenti* did not have any visible effect on development of *L. tropica* in *P. sergenti*.

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