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Effect of abiotic and biotic factors on *Leishmania* development in sand fly vectors

Vliv abiotických a biotických faktorů na vývoj leishmanií v přenašečích

Ph.D. thesis / Disertační práce

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Author's declaration / Prohlášení autorky:

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

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Prague, September 29th, 2015

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Supervisor's declaration / Prohlášení školitele:

I declare that Jana Hlaváčová substantially contributed to the experimental work in the three projects presented in her thesis and she had a principal role in writing two of the three publications presented.

Prohlašuji, že se Jana Hlaváčová významně podílela na experimentální práci na třech projektech shrnutých v této disertační práci a že je hlavní autorkou textu dvou publikací zahrnutých do doktorské disertační práce.

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Abstract

The thesis deals with *Leishmania* development in sand flies focusing on factors that could affect the vectorial part of their lifecycle. First, we examined an effect of temperature on *Leishmania* development in sand flies since the global warming was suggested to be one of the most important factors affecting dissemination of leishmaniasis. Development of three *Leishmania* species were studied at two different temperatures (20 and 26°C); *L. infantum* in natural vectors *Lutzomyia longipalpis* and *Phlebotomus perniciosus*, and two closely related *Viannia* species *L. braziliensis* and *L. peruviana* in *Lu. longipalpis*. *Leishmania peruviana* thrived well only at the lower temperature tested. At the higher temperature, most of infections were lost between days 2 and 8 post feeding, i.e. time of blood-meal remnants defecation. This suggests that this Andean species is adapted to slower metabolism of vectors living at lower temperatures. On the other hand, *L. infantum* and *L. braziliensis* successfully developed in sand flies at both temperatures tested, which might be substantial for their further spread to new areas.

Next, an impact of gregarine *Psychodiella sergenti* on *L. tropica* in *Phlebotomus sergenti* was studied. Gregarines are commonly found in sand flies, and in mosquitoes, they can affect development of other parasites. We did not find any significant difference in *L. tropica* development between gregarine infected and non-infected *P. sergenti* females. Parasites developed equally well, heavily infected females and mature infections were observed. Thus, we concluded that co-infection with gregarines did not have any negative impact on *Leishmania* development.

Finally, since the monoxenous trypanosomatid *Leptomonas seymouri* was recently isolated from patients with visceral leishmaniasis and sand flies were discussed as potential vectors of this species, we evaluated the ability of *L. seymouri* to survive and develop in *P. argentipes* and *P. orientalis*, both vectors of *L. donovani*. *Leptomonas seymouri* was capable to persist for several days in both sand fly species tested; nevertheless, infection rates and intensities fell down consequently being almost all lost until the late phase of infection. Neither co-infection with *L. donovani* did not show any difference, although *Leishmania* parasites developed well all days tested. Thus, we assume that a relevance of sand flies in *L. seymouri* transmission is unlikely.

Abstrakt

Práce je věnována vývoji leishmanií v jejich přenašečích flebotomech, se zaměřením na faktory, které mohou tento vývoj ovlivňovat. Za jeden z nejdůležitějších faktorů ovlivňujících šíření leishmaniózy, jsou považovány globální změny klimatu, a proto byla první část práce věnována vlivu teploty na vývoj leishmanií ve flebotomech. Tři druhy leishmanií byly studovány ve dvou rozdílných teplotách (20 a 26 °C); *L. infantum* v přirozených přenašečích *Lutzomyia longipalpis* a *Phlebotomus perniciosus* a dva blízce si příbuzné druhy z podrodu *Viannia*, *L. braziliensis* a *L. peruviana* v *Lu. longipalpis*. *Leishmania peruviana* se vyvíjela dobře pouze v nižší z testovaných teplot, ve vyšší teplotě téměř všechny infekce vymizely mezi dnem 2 a 8 po sání, tj. v době, kdy dochází k defekaci strávené krve. Domníváme se proto, že *L. peruviana*, jakož to druh vyskytující se v Andách, je adaptována na pomalejší metabolismus přenašečů žijících v nižších teplotách. Naopak, druhy *L. infantum* a *L. braziliensis* se úspěšně vyvíjely za obou sledovaných teplot, což by mohlo mít význam pro jejich další šíření do nových oblastí.

Dále jsme studovali vliv gregariny *Psychodiella sergenti* na vývoj *L. tropica* v přenašeči *Phlebotomus sergenti.* Gregariny jsou u flebotomů běžně nacházeny a je navíc známo, že u komárů mohou ovlivnit vývoj jiných parazitů. My jsme však nepozorovali žádný signifikantní rozdíl ve vývoji *L. tropica* v *P. sergenti* s gregarinami a bez gregarin, leishmanie se vyvíjely obdobně a u obou skupin samic byly pozorovány v pozdější fázi vývoje silné infekce. Můžeme tedy shrnout, že koinfekce s gregarinami neměla na vývoj leishmanií žádný vliv.

Vzhledem k tomu, že u pacientů s viscerální leishmaniózou byl nedávno izolován jednohostitelský trypanosomatid *Leptomonas seymouri* a flebotomové jsou zvažováni jako jeho možní přenašeči, v třetí části práce jsme testovali schopnost *L. seymouri* přežívat a vyvíjet se v přenašečích *L. donovani*; *P. argentipes* a *P. orientalis*. *Leptomonas seymouri* byla schopna po několik dní přetrvávat v obou studovaných flebotomech, nicméně procenta nakažených samic a intenzity infekce postupně klesaly, až vymizely. Tento trend nezměnila ani koinfekce s *L. donovani*, ačkoliv se leishmanie po celou dobu sledování vyvíjeli dobře. Role flebotomů v přenosu *L. seymouri* je tedy nepravděpodobná.

Table of contents

1 Introduction

1.1 Leishmaniases with emphasis to the visceral form of the disease and their current spread

Leishmania are digenetic parasitic protozoa (Kinetoplastida: Trypanosomatidae) alternating during their life cycle between vertebrates and sand flies (Diptera: Phlebotominae). They are widespread in tropical and subtropical areas around the world. In human, they cause a severe disease called leishmaniasis, which manifests in a series of forms ranging from simple cutaneous lesions to serious lethal visceral leishmaniasis. Over 20 *Leishmania* species cause leishmaniasis and it is estimated 1, 3 million new cases annually (WHO, 2015). Sand flies from the genus *Phlebotomus* and *Lutzomyia* are the only proven vectors of *Leishmania* pathogenic to humans (reviewed by Maroli et al., 2013).

The spread of leishmaniasis to areas previously regarded non-endemic was recently worldwide recorded (reviewed by Antoniou et al., 2013; Maroli et al., 2013;), and many factors are generally suggested to increase the risk of dispersion of the disease (Shaw 2007; Ready, 2010; Maroli et al., 2013; Bates et al., 2015). Since visceral leishmaniasis is the most widespread and currently emerging disease (Salomón et al., 2008; Harhay et al., 2011; Antoniou et al., 2013; Maroli et al., 2013; Dhimal et al., 2015), we used it as an example to summarize risk factors enabling the spread of the disease.

Visceral leishmaniasis (VL) is a severe and if untreated lethal disease caused by parasites belonging to *L. donovani* complex; *L. infantum* and *L. donovani* (Lukeš et al., 2007). The most affected countries are Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan, where more than 90% of cases occur; however, also other countries of South and Central America, the Mediterranean basin, the Middle East and Central Asia are suffering of the disease. Annually, 300 000 people are estimated to be infected and 20 000 succumb to the disease. The VL manifestation is connected with anemia, weight loss, enlargement of the spleen and liver and irregular fever attack (WHO, 2015). Nevertheless, cutaneous forms of the disease caused by parasites of *L. donovani* complex were recorded as well (BenSaid et al., 2006; Elamin et al., 2008; Svobodová et al., 2009; Badirzadeh et al., 2013; Zhang et al., 2014). Furthermore, post-kala-azar dermal leishmaniasis (PKDL) can appear after the treatment of the visceral form, mainly in patients from East Africa and India (WHO, 2015).

Leishmania infantum is causative agent of visceral leishmaniasis in the Mediterranean basin, central Asia and in the South and Central America. In America it was also called *L. chagasi*. However, it was shown that *L. chagasi* is identical with *L. infantum*, which was brought to Latin America during the colonisation by Europeans (Mauricio et al., 2000). Here, in the new continent, *L. infantum* adapted to the local permissive vector *Lutzomyia longipalpis* (Volf and Myskova, 2007). Twenty *Phlebotomus* species are implicated in its transmission in the Old World and eight *Lutzomyia species* in the New World (reviewed by Maroli 2013). The transmission cycle of *L. infantum* is zoonotic and dogs are considered as the main domestic reservoir hosts. They often suffer from several clinical symptoms as dermal changes, muscular atrophy or lymphadenomegaly, but not all infected dogs have any signs of the disease (Baneth et al., 2008). Also cats are discussed as a potential additional domestic reservoir. Naturally infected cats have been detected worldwide (summarized in Pennisi et al., 2015a) and their infectivity to sand flies from both *Lutzomyia* and *Phlebotomus* were experimentally confirmed (da Silva et al., 2010; Maroli et al., 2007). However, the role of cats in epidemiology of *L. infantum* still remains to be clarified (Maia and Campino, 2011). In wild life several animals such as carnivores (foxes, wolfs, genets, lynxes), rodents (black rats) and opossums were found to be infected and are assumed to serve as additional sylvatic reservoirs of the disease (summarized by Quinnell and Courtenay, 2009; Millán et al., 2014; Pennisi, 2015b). Recently, a role of hares and wild rabits in current outbreak of VL in Madrid was reported (Molina et al., 2012; Jiménez et al., 2014).

Visceral leishmaniasis caused by *L. donovani* occurs predominantly in Indian subcontinent and East Africa (summarized by Ready, 2014); however, it can be also found in Arabian Peninsula and China (reviewed by Maroli et al., 2013, and Salam et al., 2014). In Indian subcontinent, the disease is usually regarded as anthroponosis with *P. argentipes* serving as the vector (Sharma and Singh 2008; Siriwardana et al., 2010; Maroli et al., 2013). On the other hand, in East Africa the epidemiology of VL is less clear. Traditionally, it has been considered as anthroponosis; however, zoonotic transmission has been suggested to play an important role, but mammalian reservoar species has not been fully proven yet (Dereure et al., 2003; Elnaiem, 2011; Kassahun et al., 2015; Rohousova et al., 2015). *Phlebotomus orientalis*, *P. celiae* and/or *P. martini* are associated with the transmission of the disease in Sudan, Ethiopia and Kenya (Elnaiem, 2011; Maroli et al., 2013).

Beside sand flies, also alternative non-vectorial ways such as vertical and sexual transmission (da Silva et al., 2009; Silva et al., 2009; Turchetti et al., 2014), blood transfusion (reviewed in Dey and Singh, 2006, and Mansueto et al., 2014) or sharing of syringes between drugs users (Cruz et al., 2002; Pineda et al., 2002) are suggested to play a role in a local transmission of VL. Importance of non-vectorial transmission of *L. infantum* from dog to dog in non-endemic areas was reported in foxhound hunt clubs and kennels in 18 states in United States and 2 Canadian provinces (Duprey et al., 2006). As *Lu. shannoni* occurs in US and it was shown to be able to get infected from VL dogs (Travi et al., 2002), further studies should be performed on a possibility of establishment of the vectorial transmission in this area (Petersen, 2009). More recently, transmission of VL between five boxers in non-endemic Finland was documented (Karkamo et al., 2014). A probable source of infection was a male dog, which stood in Spain for six months. Authors presume that transmission was more likely via bite wounds and in one case possibly via semen or transplacentally (Karkamo et al., 2014). Consequently, establishment of new VL foci in non-endemic areas in Europe as the result of dog travelling is currently widely discussed (Naucke et al., 2008; Petersen, 2009; Shaw et al., 2009; Menn et al., 2010; Mencke, 2011; Christodoulou et al., 2012; Espejo et al., 2015; Maia and Cardoso, 2015).

In addition to travelling, several other factors such as human migration, deforestation, urbanization, immune status, drug resistance, conflicts, poverty, poor housing or climate changes are generally considered as a risk for the spread of leishmaniasis (summarized in Dujardin et al., 2008; Ready, 2010, and Maroli et al., 2013). Dissemination of VL caused by *L. infantum* to areas previously regarded as non-endemic was recently recorded in Southern Europe (reviewed by Antoniou et al., 2013). New canine leishmaniasis (CanL) foci in pre-Alpine territories in northern Italy with a moderate risk for human VL were described by Maroli et al. (2008), and an outbreak of human VL in Bologna was reported by Varani et al. (2013). Similarly in northern Spain, seropositive dogs and the presence of *L. infantum* vectors (*P. perniciosus* and *P. ariasi*) were found in regions considered to be *Leishmania* free (Ballart et al., 2012; Miró et al., 2012, Ballart et al., 2013). In French Pyrenees, an interesting shift in distribution of seroprevalent positive dogs was seen from valley to hilly villages located in an area of mid-Ariège valley (Dereure et al., 2009). Authors hypothesized that the observed change could be the result of climatic conditions, since an increase in average annual temperature was in hilly areas about 1°C, as well as the usage of protective deltamethrin collars in valley villages. The

evidence about a positive effect of preventive measures (spot-on and/or collars) on a decrease in CanL incidence was also brought in a recently established focus in Calaone, hilly area in north-eastern Italy (Cassini et al., 2013).

An example of a potential risk of introduction of new *Leishmania* species was shown in Greek island Cyprus. Traditionally, dogs infected with *L. infantum* but without human cases have been recorded in the island and only since 2006, autochthonous cases of VL and CL caused by *L. donovani* were reported (Antoniou et al., 2008; Koliou et al., 2014), *Phlebotomus tobbi*, the local vector of *L. infantum*, and *P. galilaeus* were discussed as suspected vectors of the human disease (Mazeris et al., 2010).

In Central Europe, four suspected vectors of *L. infantum* were detected: *P. perniciosus* (Naucke et al., 2008), *P. perfiliewi*, *P. neglectus* (Farkas et al., 2011), and *P. mascitti* (Naucke et al., 2008; Poeppl al., 2013; Melaun et al., 2014; Obwaller et al., 2014). Furthermore, reports of autochthonous cases of visceral leishmaniasis were recorded in horses, dogs and human (Bogdan et al., 2001; Koehler et al., 2002; Müller et al., 2009; Tánczos et al., 2012). Nevertheless, according to data based on climatic modelling (Fisher et al., 2010), establishment of vector transmission of leishmaniasis in Central Europe (Germany) is not probable during the first half of the 21st century.

Urbanization of leishmaniasis in Brazil and Argentina can serve as an example of the increasing risk of VL spread caused by human made environmental changes. In Brazil, originally the rural disease (Deane and Deane, 1962) started urbanizing and has been spreading since 1980s. The first record was obtained from city Teresina (Piauí), followed by cities São Luís (Maranhão) and Montes Claros (Minas Gerais) (reviewed in Harhay et al., 2011). Initially, the majority of cases occurred in Northeast region (Maranhão, Piauí, Caerá, Rio Grande do Norte, Sergipe); but in following years, it was found 20 states in Centralwest, North and Southeast regions (reviewed by Maia-Elkhoury et al., 2008). It is believed that except the environmental changes (e.g. deforestation) also migration of rural populations to urban peripheries, poverty, poor housing, close coexistence of human and animals, and planting of acacias trees may play a role in the expansion (summarized and discussed in Rangel and Vilela, 2008, and Harhay et al., 2011). Although the main *L. infantum* vector *Lutzomyia longipalpis* has a silvatic origin, it was shown to be adaptable to human dwellings and to be an eclectic feeder (reviewed by Lainson and Rangel, 2005). Thus, a change of *Lu. longipalpis* biology and interaction between animals and vectors from urban, peri-urban and sylvatic areas were suggested to be important factors in the

urbanization of the disease (discussed in Lainson and Rangel, 2005; Maia-Elkhoury et al., 2008; Harhay et al., 2011).

In Argentina, the first VL urban focus was detected in 2006 in Posadas, Misiones (Salomón et al., 2008); *Lutzomyia longipalpis* and CanL were found associated with the new VL focus (Cruz et al., 2010; Santiny et al., 2010). More recently, cases of CanL were described for the first time also in another city of Misiones, Puerto Iguazú (Acosta et al., 2015). Furthermore, spread of *Lutzomyia longipalpis* was recently recorded in northeast region in provinces Formosa, Corrientes and Chaco (Salomón et al., 2009a; Salomón et al., 2009b; Salomón et al., 2011), and in Salta province in northwest (Bravo et al., 2013). Dog movement and incidence of CanL were suggested to be a risk for VL transmission in these areas (Salomón et al., 2009a; Salomón et al., 2009b; Bravo et al., 2013).

An example of the complexity of various factors affecting VL expansion was described in Nepal (Dhimal et al., 2015), where the northward spread and autochthonous VL cases were newly discovered outside the endemic area, in a hilly western region (Pandey et al., 2011; Schwartz et al., 2011; Pun et al., 2013).

The spread and increase in the number of cases of leishmaniasis can be also affected by HIV-*Leishmania* co-infection. It was suggested that AIDS increases the risk of VL 100-1000 times in VL endemic countries (reviewed in Desjeux and Alvar, 2003). Higher tissue parasite loads, more frequent parasithemia in peripheral blood and higher rates of post-kala-azar dermal leishmaniasis in HIV-VL patients led to the assumption that these patients could be more infectious for sand flies and therefore serve as a potential reservoir hosts. Furthermore, lower cure rates, higher mortality, relapses and drug toxicity rates were observed in HIV-positive VL patients, and conversely VL seems to help in progress of HIV disease (summarized in Alvar et al., 2008; Diro et al., 2014; Monge-Maillo et al., 2014; van Griensven, 2014).

An interesting situation was observed in Europe, where the co-infection changed the age of VL patients from the mostly children disease to HIV positive adults, mainly intravenous drug users as the population with the highest risk (Desjeux and Alvar, 2003; Alvar et al., 2008). However, usage of HAART (the highly active antiretroviral therapy) positively affected the incidence of co-infected patients in Europe (del Guidice et al., 2002; de la Rosa et al., 2002). Unfortunately, an access to proper treatment in developing countries such as East Africa region, which are highly epidemic for both VL and HIV, is complicated and remains challenging (Diro et al., 2014).

1.2 *Leishmania* development in sand flies

In sand flies, *Leishmania* development is restricted to the digestive tract (Fig. 1). During the feeding on an infected host, *Leishmania* amastigotes are taken up with the ingested blood meal (Fig. 1a). These are small rounded cells without flagella, which are adapted to live in mammalian hosts (Walters, 1993; Rogers et al., 2002). The blood feeding is a signal for epithelial cells of the midgut to start with a synthesis of a peritrophic matrix (PM), which is a semipermeable envelope composed mainly of proteins, glycoproteins and chitin. Its main function is a protection of cells of the gut from damage by digestive enzymes, heme detoxification, mechanical damage, and it is also a barrier for pathogens, which can be taken with the blood meal (summarised in Lehane, 1997 and Terra, 2001; Pascoa et al., 2002).

In the intraperitrophic space, amastigotes transform to short cells with short flagella called procyclic promastigotes (Fig. 1b). These intensively divide, and after the PM is broken by sand fly chitinases (Ramalho-Ortigão and Traub-Csekö, 2003; Ramalho-Ortigão et al., 2005) procyclics transform to long nectomonads (Sádlová and Volf, 2009) (Fig. 1c); highly motile promastigotes forms, which later transform to short nectomonads (Walters, 1993); named also leptomonads by Rogers et al. (2002) (Fig. 1d). Short nectomonads then undergo the second intensive division (Rogers et al., 2002; Gossage et al., 2003). In the ectoperitrophic space, parasites attach to the gut, whereby they avoid their expulsion from the vector's digestive tract within blood meal remnants. In the subgenus *Leishmania*, nectomonads move anteriorly and attach to the midgut inserting their flagella between microvilli of epithelial cells (Walters et al., 1989a; Walters, 1993; Sacks and Kamhawi, 2001).

According to a specificity of vector-*Leishmania* interaction, sand flies are divided into two groups: specific (restrictive) and permissive vectors (reviewed by Kamhawi, 2006, and Volf and Myskova, 2007). Specific vectors transmit only a single *Leishmania* species and have midgut receptors for species-specific leishmanial terminal carbohydrate lipophosphoglycan (LPG). For example, *Phlebotomus papatasi* possesses galectin-PpGalec specific for terminal galactose residues on LPG of *Leishmania major* (Kamhawi et al., 2004). Furthermore, a flagellar protein FLAG1/SMP1 was recently suggested to play a role in an initial interaction of *L. majo*r with the midgut of its restrictive vector *P. papatasi* (Di-Blasi et al., 2015). On the other hand, permissive vectors allow the development to a wide spectrum of *Leishmania* species and another biding system different

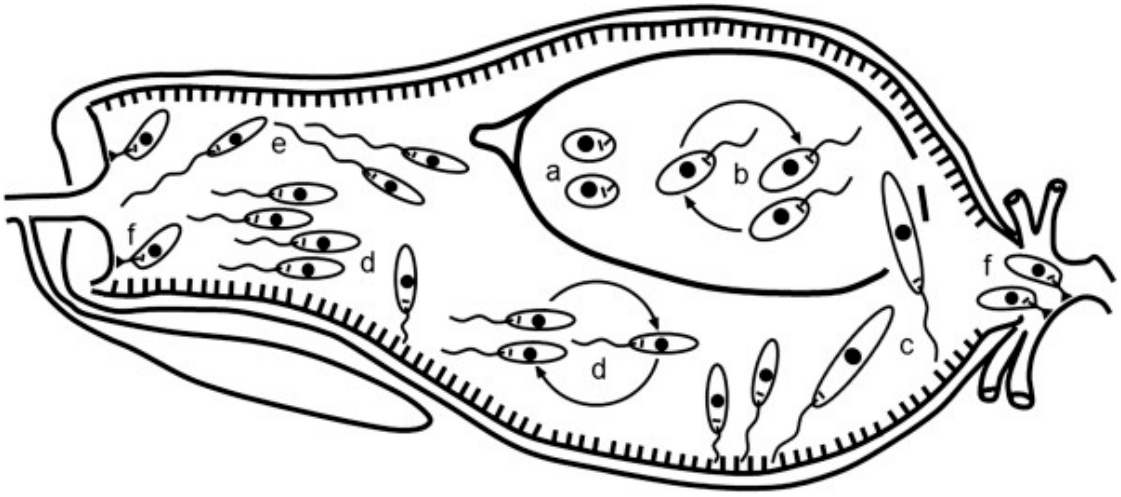

Fig. 1: *Leishmania* development in sand fly (adapted from Dostálová and Volf, 2012). A. amastigotes in the blood-meal; b. dividing procyclic promastigotes inside the peritrophic matrix; c. long nectomonads escaping from the peritrophic matrix and attaching to the midgut; d. short nectomonads (leptomonads) dividing, attaching to the midgut and migrating forward to the stomodeal valve; e. metacyclic promastigotes; f. haptomonads attaching to the chitin lawyer of the stomodeal valve and pylorus.

from the interaction with LPG is involved in the attachment of the parasites. It is hypothesised that an interaction between O-glycosylated proteins with N-acetylgalactosamin (GalNAc) on midguts of permissive vectors with lectins on the surface of promastigotes could play a role in the binding (Myskova et al., 2007).

In the New World subgenus *Viannia*, nectomonads move also posteriorly and attach to the chitin layer of the hindgut (Fig. 1f) (Lainson et al., 1977; Walters et al., 1989b; Walters, 1993). The role of the midgut attachment in *Viannia* is still a matter of controversy and remains to be elucidated (Johnson and Hertig, 1970; Azevedo-Pereira et al., 2007; Bates, 2007; Soares et al., 2010; Wilson et al., 2010).

In the late stage infection, there is a characteristic forward movement of nectomonads to the stomodeal valve and their transformation to metacyclic promastigotes (Fig. 1e). The metacyclics are small, highly motile forms possessing long flagella (Walters, 1993; Rogers et al., 2002; Gossage et al., 2003) and different type of LPG (McConville et al., 1992; Mahoney et al., 1999; Soares et al., 2002; Soares et al., 2005). These forms are infectious for mammalian hosts and are transmitted during the blood feeding (reviewed in Bates, 2007). Two principal mechanisms of transmission were described. The first is based on a damage of cuticular lining of the stomodeal valve by haptomonad promastigotes attached by their flagellar tips, which are transformed and form a hemidesmosomal type of

junction (Fig. 1f). Demage is caused by parasite's chitinases and lead to a poor function of the valve and a reflux of the parasites into a host skin (Schlein et al., 1992, Volf et al., 2004; Rogers et al., 2008). The second mechanism is based on a mechanical block of an anterior part of the gut by a plug from parasites enclosed in filamentous proteophosphoglycan, which can cause difficulties during the feeding of sand fly (Rogers et al., 2002).

In conclusion, *Leishmania* have to overcome several critical moments to complete their development in the sand fly. Firstly, they must survive an effect of digestive proteolytic enzymes during the digestion of a blood meal (Dillon and Lane, 1993; Secundino et al., 2010; Telleira et al., 2010; Dostálová et al., 2011; Santos et al., 2014; Pruzinova et al., 2015). Subsequently, they must avoid their expulsion together with the digested blood by escaping from the peritrophic matrix (Sádlová and Volf, 2009; Pruzinova et al., 2015), which is followed by an attachment to the gut (Walters, 1993; Sacks and Kamhawi, 2001; Myskova et al., 2007). Last but not least, they have to cope with a complex reaction of an immune system of the sand fly (Boulanger et al., 2004; Diaz-Albiter et al., 2012; Telleria et al., 2013).

1.3 Effects of temperature on *Leishmania* development in sand flies

The global warming is assumed to be an important risk factor for leishmaniasis; and therefore, knowledge on the effect of temperature on sand flies and *Leishmania* could help us to better understand the process of its dissemination and predict its behaviour in the future.

It was previously shown, that temperature has a significant effect on the development of sand flies (Theodor, 1936; Endris et al., 1984; Guzmán and Tesh, 2000; Kasap and Alten, 2005; Kasap and Alten, 2006; Benkova and Volf, 2007; Chelbi and Zhioua, 2007). Temperature affects all developmental stages; eggs, larvae incubation and its further development, pupation and adult emergence. Many authors recorded that time of the development, i.e. oviposition time, incubation period or mean generation time negatively correlate with the rising temperature (Endris et al., 1984; Guzmán and Tesh, 2000; Kasap and Alten, 2005; Kasap and Alten, 2006; Chelbi nad Zhioua, 2007).

Experiments provided by Benkova and Volf (2007) showed, that not only the speed of the development but also metabolic processes such as digestion and defecation are

significantly affected by different mean temperatures. At a lower temperature (23°C) authors observer slower defecation and oviposition compared to a higher temperature (28°C). Furthermore, blood-fed females lived longer at the lower temperature. On the contrary, no differences in mortality of sugar fed females were observed between two temperatures tested (Benkova and Volf, 2007).

Various thermal sensitivities in different developmental stages of *P. papatasi* (colony originating from Turkey) were demonstrated by Kasap and Alten (2005). Sand flies were examined in the range from 15 to 32°C. The lowest temperature for eggs was 11,6°C, larval stages required minimally 19,8°C, developmental zero value for pupae was 17,6°C and temperature at least 20,3°C was needed to complete the development from egg to adult (Kasap and Alten, 2005). The optimum temperature for this *P. papatasi* colony was suggested to be 28°C (Kasap and Alten, 2006).

Importantly, it has been found that temperature tolerance differs between species. For example, *P. papatasi* and *P. perniciosus*, i.e. species from temperate Europe, are less sensitive to lower temperatures compared to tropical *Lu. longipalpis* (Guzmán and Tesh, 2000). In contrast at height temperature (32°C), *Lu. anthophora* development was not negatively affected (Endris et al., 1984), while *P. papatasi* showed higher mortality of eggs and therefore lower emergence of adults compared to temperatures ranged between 20 and 28°C (Kasap et al., 2005).

Leishmania promastigotes develop in the sand fly gut, and therefore it is obvious to suppose that they may be affected by changes caused by altered ambient temperatures during life of their vectors. Positive correlation of rising temperature (10-25°C) with a proportion of infected females of *P. ariasi* and speed of *L. infantum* multiplication was detected by Rioux et al. (1985). Authors mentioned that from 20°C rising temperature encouraged promastigotes to colonise the stomodeal valve. Furthermore, they concluded that the optimal temperature for *L. infantum* development in *P. ariasi* is 25°C and discussed this fact with the absence of *L. infantum* but the presence of the vector in Cévennes region, above 600 m a.s.l.. A negative effect of faster digestion and defecation on *L. amazonensis* development at higher temperature (28°C) observed Leaney (1977). He presumed that parasites do not have enough time to escape from the endoperitrophic space and establish infection in *Lu. longipalpis* due to the faster digestion at higher temperature. Conversely, Sadlova et al. (2013) did not find any significant difference in late stage infections of *L. donovani* in *Lu. longipalpis* between lower (21°C) and usual (26°C) temperature, even that degradation of peritrophic matrix was faster at the higher

temperature. The importance of digestion, especially timing of defecation on *Leishmania* development in sand flies, was recently demonstrated by Pruzinova et al. (2015). Authors hypothesised that prolonged time between break-down of peritrophic matrix and defecation of sand fly may provide promastigotes more time to escape from the endoperitrophic space, establish infection in the midgut and thereby prevent of their expulsion with blood meal remnants (Pruzinova et al., 2015).

The importance of ambient temperature on vector competence was repeatedly shown in mosquitoes. It affected not only their survivorship, length of development and gonotrophic cycles (Bayoh and Lindsay, 2003; Aytekin et al., 2009; Delatte et al., 2009; Christiansen-Jucht et al., 2014), but also their immune system (Murdock et al., 2012) and vectorial susceptibility to *Plasmodium* parasites (reviewed by Lefévre et al., 2013). It was shown that increasing temperature decreases an extrinsic incubation period (the time *Plasmodium* need to complete the development in mosquitoes) of *Plasmodium yoelii* in *Anopheles stephensi* (Paaijmans et al., 2012) and also a gonotrophic cycle of the mosquito (Paaijmans et al., 2013). Getting together, the synchronisation of the length of vector's gonotrophic cycle and the extrinsic incubation period is assumed to might have an impact on the transmission. At 22°C, mosquitoes are prepared to feed again when the high proportion of them is infectious (harbouring sporozoites in salivary glands). On the other hand at 26°C, mosquitoes are ready to feed before the extrinsic incubation period is completed and parasites are, therefore, supposed to wait until the next feeding (Paaijmans et al., 2013). In line with previous studies, Eling et al. (2001) observed a decreasing extrinsic incubation period of *P. falciparum* in increasing temperature, however, no oocysts were found in *An. stephensi* reared at 30°C. Nevertheless, it was shown that only first 30 hours after infection, when ingested gametocytes are in the blood meal and ookinets migrate across the wall of the midgut, are critical for the exposure to 30°C. Later development was not affected with the elevated temperature. Notably, faster digestion at higher temperatures was suggested to lower chance of ookinets to migrate across the midgut wall and possibility of their digestion together with the ingested blood was proposed (Eling et al., 2001).

Moreover, nitric oxide synthase expression, which was shown to be an immune response to the early development of *Plasmodium* (Luckhart et al., 1998), is temperature-dependant and is highest at 30-34°C compared to lower temperatures tested (Murdock et al., 2013). Additionally, also other components of the mosquito immune system such as humoral melanisation, phagocytosis, defensin and cecropin expression can be affected by

temperature, diurnal temperature fluctuation and time of day of infection (Murdock et al., 2012; Murdock et al., 2013).

Data obtained on the development of *Plasmodium* in mosquito vectors at different ambient temperatures showed strong evidence of complex interaction affecting the parasite as well as the vector. Given that both sand flies and mosquitoes are ectothermic insects strongly affected by ambient temperature, further studies on *Leishmania* development in sand flies at different temperatures await investigation.

1.4 Effects of microbiota on *Leishmania* development in sand flies

During their life cycle, sand flies live in different environments and feed on different meal. Larvae develop in soil rich in organic material, for example in animal shelters and burrows, caves, or termite hills. Adults of both sexes feed on a sugar meal and females also on a blood meal (Feliciangeli, 2004; Maroli et al., 2013). All these feedings are an opportunity to ingest bacteria, fungi and other parasites, which may colonize the gut of sand flies. This chapter is, therefore, focused on the effect of microbiota on *Leishmania* development in sand flies with emphasis on gregarine co-infection.

Wide range of bacteria colonizing guts of wild-caught or laboratory-reared sand flies was detected by several authors (Schlein et al., 1985; Volf et al., 2002; Mukhopadhyay et al., 2012; Sant'Anna et al., 2012; Maleki-Ravasan et al., 2014). As a blood meal is usually sterile, adults obtain microbiota mainly during the feeding on plants and a possibility of transtadial passage from larvae to adults was also observed (Volf et al., 2002). Interestingly, differences in bacterial flora between populations of sand flies were repeatedly detected (Schlein et al., 1985; Gouveia et al., 2008; Hillesland et al., 2008), and subsequently the association between environment and microbiota composition was discussed (Gouveia et al., 2008). It has been suggested that bacterial and fungi microflora of sand flies could be an important factor in leishmaniasis epidemiology (Schlein et al., 1985; Volf et al., 2002; Sant'Anna et al., 2012; Maleki-Ravasan et al., 2014).

In mosquitoes, it was previously demonstrated that microbiota have an effect on vector competence (Dong et al., 2009; Moreira et al., 2009; Cirimotich et al., 2011; Tchioffo et al., 2013; Ramirez et al., 2014). Recently, Dennison et al. (2014) summarized three possible ways, which may play a role in microbiota-parasite interaction:

i) competition for resources; ii) activation of immune system by microbiota; and iii) direct effect by secondary metabolites produced by microbiota. For instance, infection by Wolbachia in *Aedes aegypti* was shown to limit infection of dengue virus, Chikungunya virus and *Plasmodium gallinaceum*. As *Wolbachia* is an intracellular insect endosymbiont, competition for resources, mainly for cholesterol and lipids, with both viruses and *Plasmodium,* and also stimulation of vector's immune system were suggested to play a role in the parasites limitation (Moreira et al., 2009). Pan et al. (2012) described that *Wolbachia* infection in *Ae. aegypti* induces production of reactive oxygen species molecules (ROS), which subsequently activate Toll immune pathway, which results in production of antimicrobial peptides (AMPs) defensin and cecropin. These are finally involved in inhibition of proliferation of the dengue virus (Pan et al., 2012). A negative effect of ROS radicals on *Plasmodium falciparum* infection in *Anopheles stephensi* induced by *Wolbachia* was further observed by Bian et al. (2013). However, the effect of *Wolbachia* on *Plasmodium* development may be temperature-dependant as was shown in a study of Murdock et al. (2014). Another example of the bacterial immune priming is a contribution of *Actinobacter* sp. at modulation of the immune response against *P. falciparum*, which was suggested to be regulated by the immune deficiency (IMD) insect immune signalling pathway (Bahia et al., 2014). An example of direct effect of secondary metabolites was shown in the study by Cirimotich et al. (2011). It describes an inhibitory effect of *Enterobacter* bacterium (isolated from *An. arabiensis*) on *P. falciparum* and *P. berghei* mediated by ROS produced by the bacteria.

Besides to bacteria and viruses, microsporidian *Vavraia culicis* was shown to have an effect on *Plasmodium* infection and immune response of mosquitoes (Bargielowski and Koella, 2009). *Anopheles gambiae* infected as larvae by microsporidian parasite possessed more effective melanisation and less profitable *P. berghei* infection compared to non-infected individuals (Bargielowski and Koella, 2009).

In sand flies, the interaction between *Leishmania* and microbiota remains less investigated; however, published studies indicate that fungi and bacteria can significantly affect *Leishmania* development. Potential of fungi to inhibit *Leishmania major* development was described in experimentally infected *P. papatasi* by Schlein et al. (1985). Significantly reduced intensities of *L. mexicana* infection were observed in *Lu. longipalpis* females prior fed on yeast bacteria *Asaia* and *Ochrobactrum intermedium*, and a combination of *Asaia* sp. and yeast *Pseudozyma*. *Ochrobactrum intermedium* and co-infection of *Asaia* and *Pseudozyma* also significantly reduced percentages of infected sand

flies (Sant´Anna et al., 2014). Additionally, authors demonstrated a positive effect of *L. mexicana* on longevity of sand flies, when they were subsequently infected with entomopathogenic bacteria *Serratia marcescens* (56% with vs. 11% without *Leishmania* infection). This led to a hypothesis that there may exist mutually beneficial interaction between *Leishmania* and sand flies, which prolong the life of sand flies and thereby increase the probability of *Leishmania* transmission. Interestingly, *Serratia marcescens* suppressed the growth of *L. mexicana* (Sant´Anna et al., 2014) and lysed *L. chagasi*/*infantum* (Moraes et al., 2008) *in vitro*. Further studies on *Leishmania*-microbiota interaction in sand flies should be performed since it was described that gut bacteria can activate immune system of sand flies (Boulanger et al., 2004; Diaz-Albiter et al., 2012; Telleria et al., 2013). In addition, some sand fly colonies as well as wild caught sand flies were found infected by *Wolbachia* (Kassem and Osman, 2007; Parvizi et al., 2013; Bordbar et al., 2014). These intracellular bacteria are known to have a negative effect on *Plasmodium* and viruses in mosquitoes (Moreira et al., 2009; Pan et al., 2012; Bian et al., 2013).

Apart from bacteria and fungi, sand flies and mosquitoes are often found to be infected with gregarines (Apicomplexa: Eugregarinorida) of the genera *Psychodiella* and *Ascogregarina*, respectively. In mosquitoes they were studied extensively, even in co-infection with other parasites. On the other hand, relatively little is known about sand fly gregarines of genus *Psychodiella* and no experimental co-infections with other microbes were studied yet. Therefore, we decided to study effects of gregarines on *Leishmania* development in sand flies.

The life cycle of *Psychodiella chagasi*, gregarine of *Lutzomyia longipalpis*, is shown in the Figure 2. Briefly, infection starts by ingesting oocysts by first instar larvae. In the midgut, sporozoites hatch from oocysts, attach to epithelial cells (Fig. 2a) and transform into trophozoites. In later infection, sexual development can be detected in the larval gut lumen or the body cavity of adults (Fig. 2b). Gamonts form syzygy (Fig. 2c) and transform to gametocysts containing oocysts (Fig. 2d). In adults, gametocysts attach to the accessory glands of females and oocysts are then injected into the lumen of glands. Finally, during ovipositing of females, oocysts (together with contents of the glands) attach to the chorion of eggs and are thus a source of infection for the next generation of sand flies (Fig. 2e) (summarized by Lantova and Volf, 2014). Importantly, sexual development of *Ps. sergenti* takes place only in blood-fed females, contrary to other species, which sexual development was observed also in males and unfed females (Lantova and Volf, 2012).

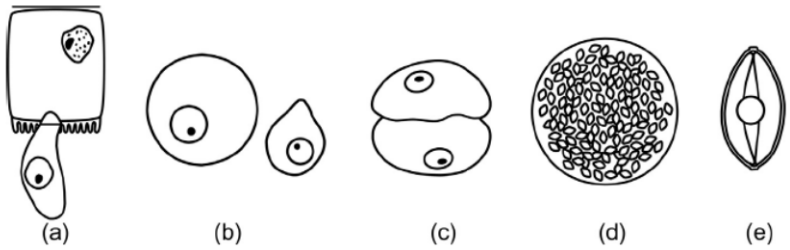

Fig. 2: The developmental cycle of *Psychodiella chagasi* (adapted from Lantova and Volf, 2014). A. sporozoit attaching to the epithelial cell; b. gamonts; c. gamonts in syzygy; d. gametocyst with oocysts; e. sporulated oocyst.

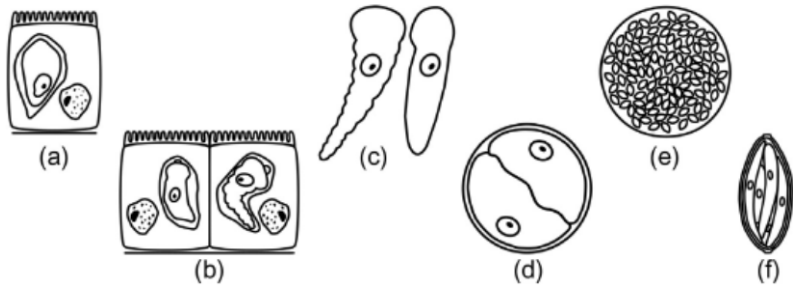

Fig. 3: The developmental cycle of *Ascogregarina culicis* (adapted from Lantova and Volf, 2014). A. sporozoit in the epithelial cell; b. trophozoites in epithelial cells; c. gamonts; d. young gametocyst; e. mature gametocyst with oocysts; f. oocyst with sporozoites.

The different life cycle was observed in gregarines of mosquitoes. *Ascogregarina culicis* development is shown as an example in Figure 3. Larvae ingest oocysts with sporozoites, which after their release penetrate into epithelial cells of the gut (Fig. 3a) and transform to trophozoites (Fig. 3b), which escape to the lumen following a rupture of the cells. Here they attach to the gut but afterwards during pupation, they migrate to the Malphigian tubules. In adults, gamonts form syzygy (Fig. 3c,d) and develop into gametocysts containing oocysts (Fig. 3e). The oocysts are defecated into water, where they serve as an infectious stadium for other larvae (Fig. 3f) (reviewed in Lantova and Volf, 2014).

Only two works studied an impact of gregarines on sand flies. Wu and Tesh (1989) observed significantly higher mortalities in adults of *Lutzomyia longipalpis* infected with gregarine *Psychodiella chagasi*, when compared to the non-infected group. Lantova et al. (2011) observed not only higher mortality of adults of *P. sergenti*, but also increased mortality of immature stages. The higher mortality was more obvious, when larvae were

reared under more dense conditions. As *Ps. sergenti* does not live intracellularly and higher mortality was observed in crowded conditions, authors speculated that competition for nutrients could be one of the main reasons that caused higher mortality of larvae. Interestingly, no significant effect on sand fly fecundity was observed neither by Lantova et al. (2011) nor by Wu and Tesh (1989).

In mosquitoes, results on the effect of gregarines on larvae, pupae and adults are contradictory. Some authors observed their negative effect on larvae (Barrett, 1968; Sulaiman, 1992; Copeland and Craig, 1992; Garcia et al., 1994) and pupae (Barrett, 1968; Siegel et al, 1992). For example, *Ascogregarina culicis* increased larval mortality of its natural host *Aedes aegypti* (Barrett, 1968; Sulaiman, 1992). Larvae and pupae were stunted and Malpighian tubules of pupae and adults were heavily damaged. This damage was a result of gametocysts development and a degree of damage was positively correlated with an infection rate (Barrett, 1968). Correspondingly, pathological processes in Malphigian tubules such as tubule distension, liquefaction of tubule content or parasite melanisation were observed in *Ae. albopictus* infected with *As. taiwanensis* by Comiskey et al. (1999a). Similarly, *As. culicis* caused enlarged and deformed nuclei in infected midgut cells of *Ae. geniculatus* larvae (Kramar, 1952). On the other hand, McCray et al. (1970) did not find any negative effect of *As. culicis* on *Ae. aegypti* and Tseng (2004) did not find any effect on larval survival of *Ae. albopictus* infected with *As. taiwanensis*. In adults, no significant effect was observed in *Ae. aegypti* infected with *As. culicis* (McCray et al., 1970) while an effect of gregarines on body size or size of wings was observed by Siegel et al. (1992) and Tseng (2004). Furthermore, the parasite manifestation can be additionally affected by several other factors such as unnatural mosquito-gregarine combination (Copeland and Craig, 1992; Garcia et al., 1994), number of infesting parasites and/or deficient nutrition (Walker et al., 1987; Comiskey et al., 1999 a, b).

Interesting results came out from co-infection experiments of mosquitoes with gregarines and other parasites. Fellous and Koella (2010) showed that co-infection of *As. culicis* and the microsporidium *Vavraia culicis* delayed pupation of *Ae. aegypti*, when an infection dose of both parasites was high and mosquitoes were reared in low nutrition conditions. Additionally, *Ascogregarina* decreased production of *Vavraia* spores and conversely, infection with *Vavraia* reduced a number of *Ascogregarina* oocysts under a low food diet of mosquitoes (Fellous and Koella, 2009). Authors summarised that the hosts of the parasites are affected not only by interaction between two parasite species, but also by the availability of food resources. Importantly, the host‘s immune system was

considered as a factor, which could play a role in the interaction of parasites (Fellous and Koella 2009; Fellous and Koella, 2010).

Ability of *As. taiwanensis* to enhance development of helminth *Dirofilaria immitis* (Nematoda: Onchocercidae) in *Ae. albopictus* was demonstrated by Comiskey et al. (1999b). They also showed that co-infection and high nutrients resulted in lower mortality post blood meal. This is opposite to what was observed in females infected only with *Dirofilaria*, despite the highest level of damage of Malpighian tubules and melanisation in co-infected mosquitoes. Authors assumed that the impact of *Ascogregarina* infection on host physiology and immune system may be favourable for both, the host and *Dirofilaria*. However, in *Ae. triseriatus*, Beier (1983) did not find any effect of *As. barreti* on the development and a number of invective *Dirofilaria immitis* larvae.

The role of *Ascogregarina* on maintenance of vector born viruses was studied by Mourya et al. (2003) and Miller and DeFoliart (1979). Mourya et al. (2003) showed that *As. culicis* can play a role in vertical transmission of Chikungunya virus to *Ae. aegypti* trough gregarine oocysts. However, the same virus was not vertically transmitted by *As. taiwanensis* to *Ae. albopictus* and also transmission of dengue-2 virus was not seen neither by *As. culicis* nor by *As. taiwanensis* (Mourya et al., 2003). Similarly, *As. barreti* did not enhance infection of the La Crosse virus in *Ae. triseriatus* as its oocysts did not contain the virus, it seems that it does not serve as the mechanism for the distribution of the virus (Miller and DeFoliart, 1979).

In conclusion, interaction between gregarines and their insect hosts is a complex multifactorial process that may have a significant impact not only on the development of vectors but also on a course of development of other co-circulating pathogens, which should be kept in mind during evaluation of parasite-vector experiments.

1.5 Monoxenous trypanosomatids in immunocompromised humans

Monoxenous trypanosomatids circulate usually within their insect hosts, however it was suggested that some of them may sometimes switch to dixeny and it is supposed that organisms of immunosuppressed patients can allow them to survive and explore new niches (Lukeš et al., 2014). The ability to survive at elevated temperatures occurring in warm-blooded vertebrates and infect and survive inside macrophages and fibroblasts

(McGhee and Cosgrove, 1980; Matteoli et al., 2009; Pereira et al., 2010a) was previously observed in several monoxenous trypanosomatids.

Unusual "leishmaniasis like" infections caused by insect trypanosomatids were found in immunodeficient HIV patients (Dedet and Pratlong, 2000; Pacheco et al., 1998; Morio et al., 2008). A case with clinical features of visceral leishmaniasis caused by trypanosomatid probably related to *Blechomonas pulexsimulantis* (a trypanosomatid found in a dog's flea) was diagnosed in HIV positive men form Rio de Janeiro (Pacheco et al., 1998). Another report was brought by Morio et al. (2008). It describes infection by a monoxenous flagellates from the genus *Herpetomonas* in HIV-positive man, which manifested as a continual frontal headache and a high fever. More recently, several cases of co-infection of *Leishmania donovani* and monoxenous *Leptomonas seymouri* were observed in VL patients in India (Srivastava et al., 2010; Ghosh et al., 2012; Singh et al., 2013). *Leptomonas seymouri* belongs to the subfamily Leishmaniinae (Jirků et al., 2012), and the species was originally isolated from a cotton stainer *Dysdercus suturellus* (Hemiptera: Pyrrhocoridae) (Wallace, 1977). Similarly to HIV infection, visceral leishmaniasis caused by *L. donovani* is assumed to have an immunosuppressive effect on patients, which may prepare good conditions enabling to *L. seymouri* infect human hosts (Ghosh et al., 2012). Interestingly, around 95% of sequencing reads were found belonging to *L. seymouri* by the whole genome analysis of isolates from VL patients in India (Singh et al. 2013). Here should be noted that *L. seymouri* replicate faster than *L. donovani* in cultures and would easily overgrow *Leishmania* after isolation (Srivastava et al., 2010).

A possible transmission route to human patients remains to be yet elucidated, however, it was discussed that an infection per os, contaminatively by contact with a parasite or transmission by a vector could occur (Chicharro and Alvar 2003; Morio et al., 2008; Srivastava et al., 2010). In India, *P. argentipes*, a vector of *L. donovani*, has been hypothesized as a potential vector of *L. seymouri* (Srivastava et al., 2010; Singh et al., 2013). It was shown that, similarly as *Leishmania* in sand flies, also monoxenous trypanosomatids are able to bind to guts of medically important mosquito *Aedes aegypti in vitro* and/or *in vivo* (Fampa et al., 2003; Corrêa-da-Silva et al., 2006; d'Avila-Levy et al., 2008; Pereira et al., 2010b). Fampa et al. (2003) detected that *Blastocrithidia culicis* binds *in vitro* to the gut of *Ae. aegypti* by their body and by inserting their flagella between microvilli, and moreover, it is able to divide and persist in *Ae. aegypti in vivo*. Furthermore, *in vivo* attachment of *B. culicis* by the cell body and by inserting flagella between midgut microvilli, degradation of epithelial cells, and parasites located in

haemocoel were seen in *Ae. aegypti* (Corrêa-da-Silva et al., 2006). Except to the gut, *B. culicis* is also capable to adhere to outer surface of salivary glands of *Ae. aegypti* after injection to haemocoel *in vivo* (Nascimento et al., 2010). *In vitro*, attachment by the anterior flagellum was observed. Further, parasites were seen in the space between basal lamina and the plasma membrane of glandular cells, in the cytoplasm of salivary gland lobules, and inside glandular cell (Nascimento et al., 2010). Authors suggested that an invasion of salivary glands may be a part of *B. culicis* life cycle in mosquitoes.

Interestingly, the research group of Fampa et al. (2003) demonstrated that *Blastocrithidia culicis* and *Angomonas deanei* attach to the gut of sand fly *Lutzomyia longipalpis in vitro*. Validity of this finding is, however, questionable. As was shown by Wilson et al. (2010), *in vitro* attachment does not mimic the situation *in vivo*. For example, *Leishmania braziliensis* does attach to midgut of *Phlebotomus papatasi in vitro*, despite it is not able to develop in *P. papatasi in vivo*. Thus, other barriers must be overcome by parasite to successfully complete the development in the sand fly vector (Wilson et al. 2010).

Homolog of leishmanial surface metallopeptidase (gp63) was found to play a role in flagellates midgut binding and macrophage interaction (Santos et al., 2006; Matteoli et al., 2009; Pereira et al., 2010a,b). In *Leishmania*, gp63 takes part in pathogenesis in mammalian hosts such as facilitation of macrophage phagocytosis, evasion of complement-mediated lysis, extracellular matrix interaction, resistance to antimicrobial peptides or interaction with extracellular matrix (summarised in Yao, 2010). In sand flies, the role of gp63 is less clear. Hajmová et al. (2004) suggested that it enhances early stage development of *L. amazonensis* in *Lu. longipalpis*. *In vitro* binding essay provided by Jecna et al. (2013) showed significantly lower midgut binding of *L. amazonensis* gp63 down regulated strain compared to the wild type. On the other hand, Joshi et al. (2002) did not see any effect of gp63 on *L. major* development in *P. duboscqi*. Moreover, in both *Leishmania* and monoxenous trypanosomatids, gp63 has a nutrient function in the gut of insect (reviewed in d'Avila-Levy et al., 2014).

2 Objectives

Leishmania are parasites of the gut of ectothermic sand fly vectors living in a changing environment, which can have an effect on vector's metabolism or its immune system. In addition, these sand flies may host other parasites/microbes which may, directly or indirectly, affect *Leishmania* development in sand fly midgut. Thus, the thesis focuses on abiotic and biotic factors, namely temperature and other parasites and their effect on *Leishmania* in vectors.

The main objectives of the study were:

1. to evaluate effects of temperature on the development of *Leishmania* in sand flies naturally occurring in different habitats; *Leishmania infantum* in *Phlebotomus perniciosus* and *Lutzomyia longipalpis*, and *Leishmania peruviana* and *Leishmania braziliensis* in *Lutzomyia longipalpis*.

2. to elucidate effects of gregarine *Psychodiella sergenti* on *Leishmania tropica* development in *Phlebotomus sergenti*.

3. to test the capability of *Leptomonas seymouri* to develop in *Phlebotomus orientalis* and *Phlebotomus argentipes* in sugar- and blood-initiated infection, and compare its development with *Leishmania donovani* during co-infection.

3 List of publications

1. **Hlavacova, J.**, Votypka, J., & Volf, P. (2013). The effect of temperature on *Leishmania* (Kinetoplastida: Trypanosomatidae) development in sand flies. *Journal of Medical Entomology*, *50* (5), 955-958.

2. Jancarova, M., **Hlavacova, J.**, & Volf, P. (2015). The development of *Leishmania tropica* in sand flies: a comparison of colonies differing in geographical origin and a gregarine co-infection. *Journal of Medical Entomology*, doi: 10.1093/jme/tjv135.

3. Kraeva, N., Butenko, A., **Hlaváčová, J.**, Kostygov, A., Myškova, J., Grybchuk, D., Leštinová, T., Votýpka, J., Volf, P., Opperdoes, F., Flegontov, P., Lukeš, J., & Yurchenko, V. (2015). *Leptomonas seymouri*: Adaptations to the dixenous life cycle analyzed by genome sequencing, transcriptome profiling and co-infection with *Leishmania donovani*. *PLoS Pathogens*, *11* (8): e1005127.

4 Summary and conclusions

The thesis summarises results of three publications, which are based on three projects I was involved in during my PhD study. We studied different factors which might affect *Leishmania* development in sand flies, particularly ambient temperature and presence of other protozoan parasites.

Firstly, we were interested in the effect of temperature on *Leishmania* development in sand flies. Temperature was suggested to influence *Leishmania* transmission by a direct impact on the parasite development and indirectly by the presence of the vector (Ready, 2008). Particularly in Europe, climate change was assumed as one of the main factors affecting the spread of the disease (Ready, 2010), recently detected in several areas (Antoniou et al., 2013). Therefore we primarily examined the development of *Leishmania infantum* in its vectors at two different temperatures (20 and 26°C). *Lutzomyia longipalpis* and *Phlebotomus perniciosus* were used in experiments since they are natural vectors of this parasite in Latin America and Europe, respectively.

We have shown that even though intensities of infection were lower at the lower temperature on day 2 post blood-meal (PBM), on day 8 PBM *L. infantum* developed comparably well with heavy late stage infections and colonization of the stomodeal valve frequently observed in all combinations tested. The lower intensities of infection on day 2 PBM indicate that multiplication of promastigotes was slower at the lower temperature; however it did not have any negative impact on parasite establishment and further development in the vectors. In the line with slower multiplication of parasites, defecation of sand flies was also slower at the lower temperature in both species tested. While more than 80% of *Lu. longipalpis* females digested and defecated blood meal remains by day 3 PBM, this process took approximately 6-7 days at 20°C. Digestion and defecation of *P. perniciosus* was significantly slower; at 26°C, almost 90% of females were defecated on day 4 PBM and only two thirds of defecated females were observed by day 7 PBM at 20°C. The observation corresponds with a study previously provided by Volf and Killick-Kendrick (1996), who showed remarkable difference in dynamics of digestion in various sand fly species.

Importance of timing of digestion, defecation and peritrophic matrix disintegration was recently shown by Sadlova et al. (2013) and Pruzinova et al. (2015). They revealed that a time frame between break-down of peritrophic matrix and defecation is essential for

further parasite development and establishment of the infection. Sádlová and Volf (2009) demonstrated that the break-down of peritrophic matrix enables the transformation of procyclic promastigotes to long nectomonads (Sádlová and Volf, 2009) and saliva of sand flies are supposed to have an effect on promastigote differentiation (Charlab and Ribeiro, 1993). Taking together, these suggest that in sand flies possessing a short time between the break-down of the peritrophic matrix and defecation, *Leishmania* does not have enough time to transform to stadia responsible for the establishment in the ectoperitrophic space of the midgut and they are defecated (Pruzinova et al., 2015). The typical example of such refractory species is *Sergentomyia schwetzi* (Lawyer et al., 1990; Sadlova et al., 2013; Pruzinova et al., 2015).

In addition to *L. infantum*, we compared the development of two *Viannia* species *L. braziliensis* and *L. peruviana* in permissive vector *Lu. longipalpis*. Even though both species are closely related, they occur in different biotopes and cause a different manifestation of the disease. *Leishmania braziliensis* is a lowland species in Latin America causing cutaneous disease but often progress to mucocutaneous lesions. Conversely, *L. peruviana* inhabits Andean mountains and the disease manifests as simple cutaneous lesions (reviewed by Lainson, 2010). Experimental infections comparing their development at 20 and 26°C brought interesting results. Whereas *L. braziliensis* developed well in both temperatures tested causing heavy late stage infections, *L. peruviana* did well only at 20°C, while at 26°C nearly all infections were lost between days 2 and 8, i.e. the period of blood meal defecation. We suppose that this mountain species is adapted to sand flies living at lower ambient temperatures and that delayed defecation gives promastigotes more time for establishment in the vector's digestive tract. Furthermore, considering the ability of *L. infantum* and *L. braziliensis* to develop well also in lower temperatures, we highlighted that researches and medical doctors should be aware of further spread of the disease to higher latitudes and altitudes.

Secondly, we tested the effect of gregarines on *Leishmania* infection in sand flies. It was previously shown that gregarines have an impact on parasites in mosquitoes, (Comiskey et al., 1999b; Mourya et al., 2003; Fellous and Koella 2009; Fellous and Koella 2010). In our study we compared *L. tropica* development in two colonies of *Phlebotomus sergenti* originated from Israel, one infected and the other non-infected by gregarine *Psychodiella sergenti*. We did not find any significant difference in *L. tropica* development neither in percentages of infected females nor in intensities of infection in any day tested between gregarine infected and non-infected groups of females. Heavy late stage infections

and stomodeal valve colonisation were observed from day 7 in both groups tested. Therefore, we have assumed that there is no apparent effect of gregarines on *L. tropica* development in its specific vector *P. sergenti* tested in our experimental setting.

Finally, the third project was aimed to investigate a potential of *Leptomonas seymouri*, the monoxenous trypanosomatid from the subfamily Leishmaniinae, to infect warm-blooded vertebrates and its ability to successfully colonize sand fly vectors. Several cases of *L. seymouri* and *Leishmania donovani* co-infections were recently recorded in patients suffering from visceral leishmaniasis in India (Srivastava et al., 2010; Ghosh et al., 2012; Singh et al., 2013) and, among others possible routs of infection, sand flies were discussed to might play a role in its transmission (Srivastava et al., 2010; Singh et al., 2013). Therefore, we tested the development of *L. seymouri* in *Phlebotomus argentipes* and *P. orientalis*, two natural vectors of *L. donovani* in India and East Africa, respectively. As a way of infection of sand flies is not known, two principle possibilities were tested; an infection via a sugar meal and via a blood meal.

In sugar-fed females, parasite numbers as well as rates of infection were gradually decreasing in time. On day 2 post sugar meal, 100% of *P. orientalis* and 59% of *P. argentipes* females were infected; however, until day 9 only a third of females remained infected, usually with just a few persisted parasites. Results obtained in blood meal experiments were even less positive. On day two, low numbers of freely moving parasites, localised in the ingested blood, were observed and less than 50% of females were infected. On day 9, the infection persisted in only a few females and in very low densities. No parasite's binding to the gut was observed, conversely to what was found in some monoxenous trypanosomatids in mosquitoes (Fampa et al., 2003; Corrêa-da-Silva et al., 2006).

Furthermore, co-infection experiments with *L. donovani* were performed to test if it might enhance *L. seymouri* development in sand flies. Female sand flies were membrane-fed on blood meal containing mixture of mCherry-expressing *L. seymouri* and GFP-expressing *L. donovani*. Results were similar to the previous experiments, only few females were found infected with low numbers of *L. seymouri* on day 5 post feeding. On the other hand, *L. donovani* developed well and thriving heavy late-stage infections were observed.

Our study showed that *L. seymouri* is capable to persist for some time in the digestive tract of the natural vectors of *L. donovani*. However, the infection falls gradually down in all experimental settings tested and it appears plausible that parasites may be

defecated with meal remnants. Thus, a role of sand flies in *L. seymouri* transmission to human seems to be unlikely, and other possible routs of infection should be further investigated.

In conclusion, the results included in the thesis bring novel information about the effect of temperature and other parasites on *Leishmania* in sand flies and their vector competence. We demonstrated that temperature can be a limiting factor for *Leishmania* development in sand flies. Further, we detected that gregarine *Psychodiella sergenti* does not have any negative effect on *P. sergenti* competence to *L. tropica*. Finally, we showed that monoxenous trypanosomatid *Leptomonas seymouri* can for some time persist in the digestive tract of two vectors of *L. donovani*, either alone or in co-infection with *L. donovani*, but it is not capable to produce late-stage infection. Thus, the association of monoxenous trypanosomatids with "leishmaniasis like" diseases in immunodeficient and visceral leishmaniasis patients raise a question about their clinical relevance, which remains to be learned about in the future.

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Hlavacova, J., Votypka, J., & Volf, P. (2013). The effect of temperature on *Leishmania* (Kinetoplastida: Trypanosomatidae) development in sand flies. *Journal of Medical Entomology*, *50*(5), 955-958.

The Effect of Temperature on *Leishmania* (Kinetoplastida: Trypanosomatidae) Development in Sand Flies

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ABSTRACT The spread of leishmaniasis to areas where it was previously considered nonendemic has been recently found in the New and Old Worlds, and climate changes are suspected as a crucial factor responsible for this spread. Ambient temperature is known to significantly affect the metabolism of sand flies and their developmental times, but little is known about the effect of temperature on the *Leishmania* life cycle in vectors. This study assesses the effect of temperature on the development of two closely related New World *Viannia* species, *Leishmania braziliensis* and *Leishmania peruviana*, in the permissive vector *Lutzomyia longipalpis*, and on the development of New and Old World *Leishmania infantum* in its natural vectors *Lu. longipalpis* and *Phlebotomus perniciosus*, respectively. The mountain species *L. peruviana* developed well in sand fly females kept at 20°C, whereas at 26°C, most infections were lost during the defecation of bloodmeal remains; this suggests an adaptation to the slower metabolism of sand flies living at lower ambient temperature. On the contrary, *L. infantum* and *L. braziliensis* developed well at both temperatures tested; heavy late-stage infections were observed in a majority of sand fly females maintained at 20°C as well 26°C. Frequent fully developed infections of *L. infantum* and *L. braziliensis* at 20°C suggest a certain risk of the spread of these two *Leishmania* species to higher latitudes and altitudes.

KEY WORDS sand fly, leishmaniasis, *Viannia*, climate change

Leishmania (Kinetoplastida: Trypanosomatidae) are digenetic parasites causing leishmaniasis; their only proven vectors are phlebotomine sand flies (Diptera: Phlebotominae). *Leishmania* infecting mammals belong to two subgenera, *Leishmania* and *Viannia*, which differ not only in their distribution but also in their development in the sand fly vector (reviewed by Lainson 2010).

The two *Viannia* species chosen for this study, *Leishmania braziliensis* and *Leishmania peruviana*, are phylogenetically closely related but differ in the clinical outcome of the disease and occur in very distinct biotopes. Whereas *L. peruviana* causes only cutaneous leishmaniasis and its distribution is restricted to the Andean mountains, *L. braziliensis* can progress to mucocutaneous lesions and is widely distributed in the lowlands of Latin America (Lainson 2010). The third parasite species tested, *Leishmania infantum*, is the causative agent of visceral and cutaneous leishmaniasis in many countries of the Old World, as well as the New World, where it is known as *Leishmania chagasi.* In the Old World, *L. infantum* is transmitted by *Phlebotomus* sand flies, including *Phlebotomus perniciosus* (reviewed by Killick–Kendrick 1999). Because of European colonists and their dogs, it was introduced to Latin America (Mauricio et al. 2000), where it adapted to the local permissive sand fly *Lutzomyia longipalpis* (Volf and Myskova 2007).

Recently, *L. infantum* has spread to new areas and higher latitudes, where it was previously considered nonendemic. For example, a new focus of human visceral leishmaniasis was detected in the city of Posadas (northeast Argentina) (Salomon et al. 2008). In Europe, the northward spread of *L. infantum* has been recorded in northern Italy (Maroli et al. 2008) and Catalonia, Spain (Ballart et al. 2012). A progressive increase in *L. infantum* seroprevalence in dogs has been observed in the foothills of the French Pyrenees (Dereure et al. 2009) and in the Alpujarras region in Spain (Martín–Sánchez et al. 2009).

The spread of leishmaniasis may be enhanced by several factors, including human-made and environmental changes, immune status, and drug resistance (reviewed by Dujardin 2006). In Europe, climate changes are considered a crucial factor responsible for the spread of the disease. Ready (2008) stressed that changes of climate and ambient temperature can affect the distribution of leishmaniases via sand fly abundance or via the effect of temperature on parasite development in the vector. Ambient temperature significantly affects the digestion, metabolic processes, and developmental times of sand flies (Benkova and Volf 2007), but there is only one publication about the effect of temperature on the *Leishmania* life cycle in vectors: Rioux et al. (1985) demonstrated that *L. infantum* develops in the digestive tract of *Phlebotomus*

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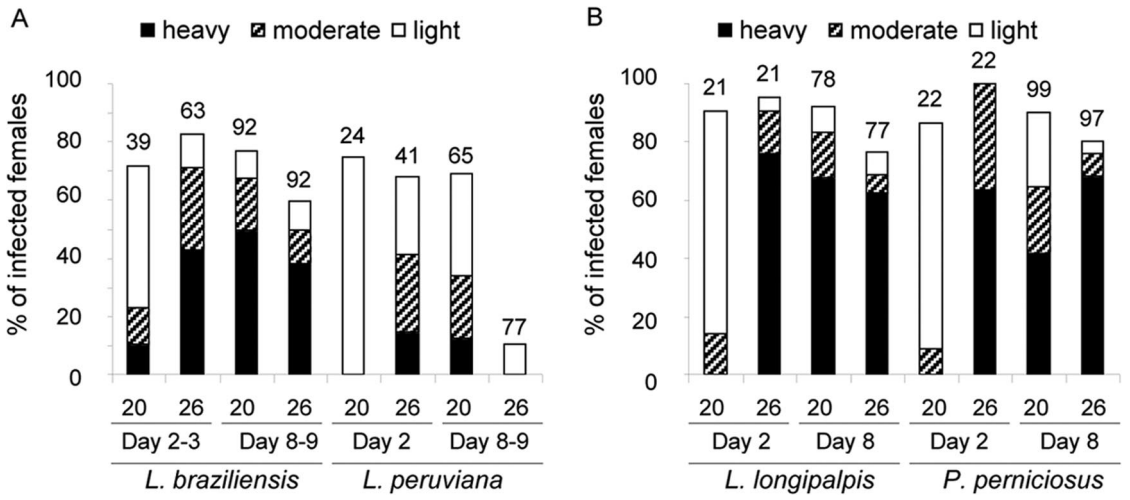

Fig. 1. Development of *Leishmania* strains in sand flies maintained at 20°C and 26°C. (A) *Leishmania braziliensis* and *Leishmania peruviana* in *Lutzomyia longipalpis*. (B) *Leishmania infantum* in *Lu. longipalpis* and *Phlebotomus perniciosus*. Intensities of infection were estimated as: light (<100 promastigotes/gut), white bar; moderate (100–1,000 promastigotes/gut), striped bar; and heavy (>1,000 promastigotes/gut), black bar. Numbers above each bar indicate the number of dissected females. Numbers on the "x" axis: 20 = 20°C; 26 = 26°C.

ariasi better at higher temperatures compared with the lower temperatures tested.

Therefore, the current study is focused on the effect of temperature on the development of three different *Leishmania* species; *L. infantum* was tested in its two natural vectors, *P. perniciosus* and *Lu. longipalpis*, whereas the development of two *Viannia* species, *L. braziliensis* and *L. peruviana*, was compared in their unnatural vector *Lu. longipalpis*, a permissive sand fly species frequently used as a laboratory model.

Materials and Methods

Sand Flies and Parasites. Laboratory colonies of *P. perniciosus* (Murcia, Spain) and *Lu. longipalpis* (Jacobina, Brazil) were reared at 25–26°C under standard conditions. Three *Leishmania* species were used: *L. infantum* (MCAN/PT/2005/IMT373), *L. braziliensis* (MHOM/PE/1993/LC2177), and *L. peruviana* (MHOM/PE/1990/HB86).

Experimental Infections. Sand fly females were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 1×10^6 (for *L. infantum*) or 5×10^6 (for *L. braziliensis* or *L. peruviana*) promastigotes per milliliter of blood. Blood-fed females were maintained at 20 or 26°C, and on days 2–3 and 8–9 post-bloodmeal (PBM), either examined under a light microscope or used for DNA isolation. Microscopically, parasite loads were graded into three categories as described by Myskova et al. (2008). Infection rates (percentage of infected females) and intensities of infection were compared by the χ^2 test (S-PLUS 2000).

Real-Time Polymerase Chain Reaction. Extraction of total DNA from infected females was performed using a High Pure PCR Template Preparation Kit (Roche, Czech Republic) according to the manufacturer's instructions. DNA was eluted in 100 μl elution buffer and stored at −20°C. Quantitative polymerase chain reaction was performed by the SYBER Green detection method (iQSYBER Green Supermix, Bio-Rad, Hercules, CA). DNA of *L. braziliensis* and *L. peruviana* was amplified according to method by Castilho et al. (2008) and *L. infantum* according to the method by Mary et al. (2004). Statistical evaluation was performed by the Kruskal–Wallis test and Mann–Whitney *U* test (STATISTICA 6.1, StatSoft).

Sand Fly Defecation. *P. perniciosus* and *Lu. longipalpis* females were fed through a chick-skin membrane on heat-inactivated rabbit blood, and the method described by Benkova and Volf (2007) was used to compare their defecation times at different temperatures. Briefly, fully blood-fed females were individually placed in small glass vials, maintained at 20 or 26°C and checked twice a day under a binocular microscope for defecation. Data were evaluated by the Mann–Whitney *U* test (STATISTICA 6.1).

Results and Discussion

Experimental infections of *L. braziliensis* and *L. peruviana* were studied in *Lu. longipalpis* to compare the development of these two *Viannia* species at different ambient temperatures (20 vs. 26°C). On days 2 and 3 PBM, the infection rate was comparable for all four parasite–temperature combinations studied. However, at 26°C, both *Viannia* species multiplied faster, resulting in more numerous heavy infections than at 20°C (*L. braziliensis:* χ^2 test $P < 0.00002$; *L. peruviana:* χ^2 test $P < 0.0005$) (Fig. 1A).

On days 8 and 9 PBM, *L. braziliensis* developed well in both temperatures tested. Despite the fact that the infection rate was significantly higher at 20°C (χ^2 test $P < 0.02$), the intensity of *L. braziliensis* infections

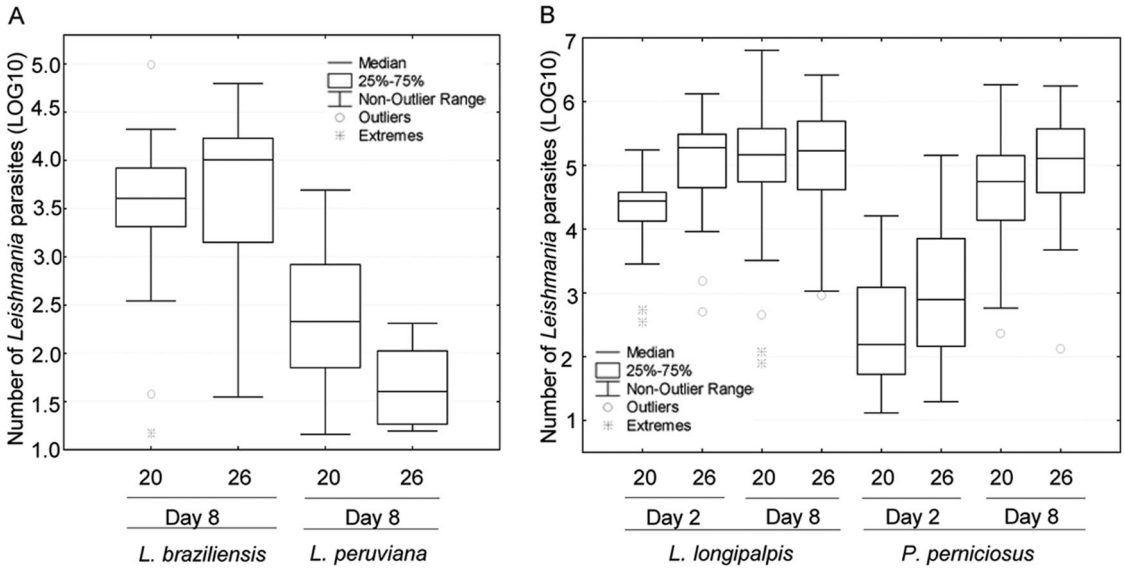

Fig. 2. Analysis of *Leishmania* parasites loads in infected sand flies maintained at 20 and 26°C by quantitative polymerase chain reaction. (A) *L. braziliensis* and *L. peruviana* in *Lu. longipalpis*. (B) *L. infantum* in *Lu. longipalpis* and *P. perniciosus*. Numbers on the "x" axis: 20 = 20°C; 26 = 26°C.

measured by quantitative polymerase chain reaction (parasite loads) did not significantly differ between the two temperatures tested (Figs. 1A and 2A). In contrast, *L. peruviana* developed well only in *Lu. longipalpis* females maintained at 20°C, whereas at 26°C, its infection rates and parasite loads were extremely low on days 8–9 PBM (Figs. 1A and 2A).

These experiments revealed that early stage infections of *L. peruviana* thrive at 26°C, but that almost 100% of infections are then lost between days 2 and 8. This is the time when sand fly females defecate and parasites might be expelled with bloodmeal remnants. We studied the timing of *Lu. longipalpis* defecation and found that it is affected by the ambient temperature. The lower temperature tested delayed defecation for ≈3 d. Whereas at 26°C, >80% females defecated by day 3 PBM (72 h), at 20°C, it took ≈6–7 d PBM (160–168 h) for a similar percentage of females to defecate (Fig. 3). Delayed defecation at 20°C provided more time to parasites to become established in the sand fly gut. Because of the fact that *L. peruviana* is a geographically restricted mountain species, we suppose that it is adapted to the slower metabolism of sand flies living in lower ambient temperatures.

The development of *L. infantum* was compared in its two natural vectors, *Lu. longipalpis* and *P. perniciosus*, and at two ambient temperatures, 20 and 26°C. In all vector–temperature combinations tested, *L. infantum* developed well, producing high infection rates and heavy late-stage infections on day 8 PBM (Figs. 1B and 2B). In early stage infections (day 2 PBM), however, the temperature affected the intensity of infections, with lower parasite loads observed at 20°C in both vectors (Figs. 1B and 2B). This suggests that the lower ambient temperature resulted in slower parasite growth within the bloodmeal, but did not have any negative effect on the establishment of *L. infantum* within the midgut. Fully developed heavy late-stage infections were frequently observed at both temper-

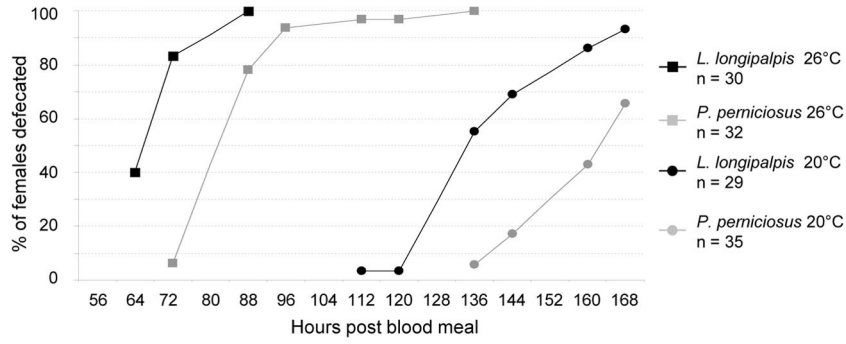

Fig. 3. Defecation of blood-fed *Lu. longipalpis* and *P. perniciosus* females maintained at 20 and 26°C.

atures tested and both vectors, *Lu. longipalpis* and *P. perniciosus* (Figs. 1B and 2B).

Similarly to *Lu. longipalpis*, lower ambient temperature also delayed defecation in *P. perniciosus* (Fig. 3). Whereas at 26°C, almost 90% of females defecated by day 4 PBM (96 h), at 20°C, the defecation was delayed for >3 d and only two-thirds of *P. perniciosus* females were defecated by day 7 PBM (168 h). Comparison of two vectors revealed that at both temperatures tested, *Lu. longipalpis* digest the bloodmeal faster and defecate significantly earlier than *P. perniciosus* (T = 20°C: Mann–Whitney *U* test $P < 0.0001$; T = 26°C: Mann–Whitney *U* test $P < 0.0001$) (Fig. 3). This finding corresponds with the previous observations of Volf and Killick–Kendrick (1996), who showed that at 25°C, *Lu. longipalpis* digest the bloodmeal more quickly than *P. perniciosus* and other four sand fly species tested.

Recent findings of new leishmaniases foci in Latin America and Europe urge for more data on the development of *Leishmania* in their vectors. Here we demonstrate the ability of *L. infantum* and *L. braziliensis* to develop heavy late-stage infections in sand flies even at 20°C. This could be an important factor enabling the spread of leishmaniases into new areas. Therefore, the risk of spread of these two *Leishmania* species to higher latitudes and altitudes should not be neglected.

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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

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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

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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

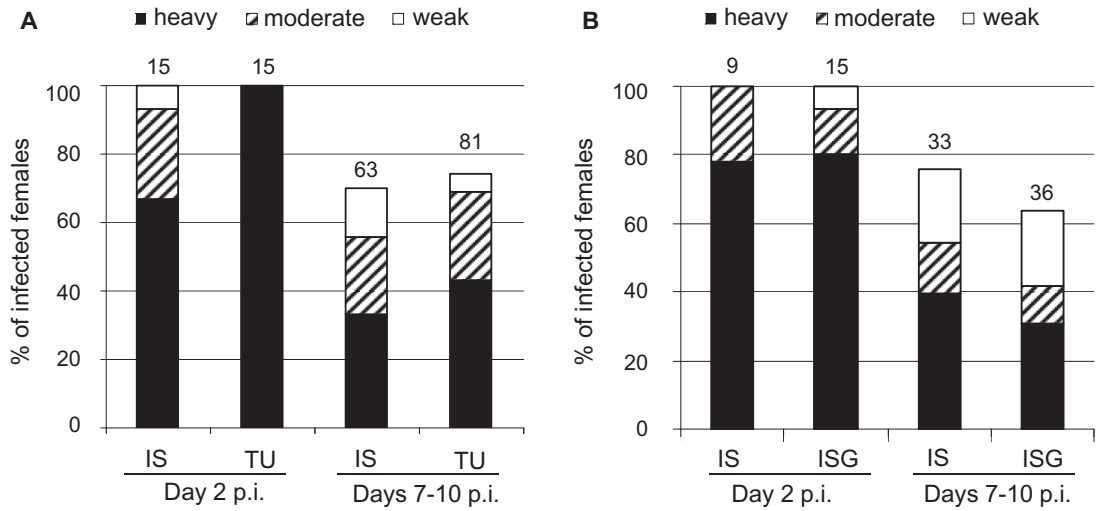

Fig. 1. The development of *L. tropica* in *P. sergenti*: (A) comparison of two *P. sergenti* colonies originating from Turkey (TU) and Israel (IS); (B) comparison of a colony infected by the gregarine *Ps. sergenti* (ISG) and a noninfected control (IS). Intensities of the leishmania infection were estimated 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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RESEARCH ARTICLE

Leptomonas seymouri: Adaptations to the Dixenous Life Cycle Analyzed by Genome Sequencing, Transcriptome Profiling and Co-infection with *Leishmania donovani*

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Abstract

The co-infection cases involving dixenous *Leishmania* spp. (mostly of the *L*. *donovani* complex) and presumably monoxenous trypanosomatids in immunocompromised mammalian hosts including humans are well documented. The main opportunistic parasite has been identified as *Leptomonas seymouri* of the sub-family Leishmaniinae. The molecular mechanisms allowing a parasite of insects to withstand elevated temperature and substantially different conditions of vertebrate tissues are not understood. Here we demonstrate that *L*. *seymouri* is well adapted for the environment of the warm-blooded host. We sequenced the genome and compared the whole transcriptome profiles of this species cultivated at low and high temperatures (mimicking the vector and the vertebrate host, respectively) and identified genes and pathways differentially expressed under these experimental conditions. Moreover, *Leptomonas seymouri* was found to persist for several days in two species of *Phlebotomus* spp. implicated in *Leishmania donovani* transmission. Despite of all these adaptations, *L*. *seymouri* remains a predominantly monoxenous species not capable of infecting vertebrate cells under normal conditions.

Author Summary

In this work we performed a comprehensive evaluation of the infective potential of *Leptomonas seymouri*, repeatedly isolated from kala-azar patients infected by *Leishmania donovani* in India and neighboring countries, and have tested the capacity of this monoxenous

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trypanosomatid to utilize the sand fly vectors permissive for *Leishmania donovani*. We concluded that despite several genetic adaptations it has developed, *Leptomonas seymouri* remains a predominantly monoxenous species not able to infect mammalian macrophages either alone or in co-infection with *Leishmania*. Under certain circumstances it is able to infect mammals, but probably only when the host is immunocompromised by infection with another pathogen, such as *Leishmania donovani* or HIV.

Introduction

Flagellates of the family Trypanosomatidae are single-celled obligatory parasites. They can be either dixenous (i.e. those with two hosts in their life cycle—*Trypanosoma*, *Leishmania*, and *Phytomonas* spp.) or monoxenous (i.e. those having only one host). For decades, monoxenous trypanosomatids of insects were effectively neglected. However, this situation is rapidly changing, as a remarkable diversity of these flagellates is being revealed within insects—a group which is known to be extraordinarily species rich [1,2]. In addition, the study of these parasites is expected to shed light on the origin of the dixenous life cycle (alternation of an insect vector and a vertebrate or plant host). It is generally accepted that the dixenous species have evolved from their monoxenous kins and that this transition has happened independently at least three times during the evolution of Trypanosomatidae, as the dixenous genera *Trypanosoma*, *Leishmania*, and *Phytomonas* are interspersed by the monoxenous genera *Angomonas*, *Blastocrithidia*, *Blechomonas*, *Crithidia*, *Herpetomonas*, *Kentomonas*, *Leptomonas*, *Paratrypanosoma*, *Sergeia*, *Strigomonas*, and *Wallacemonas* (S1 Fig) [3,4]. This suggests that some (presumably) monoxenous species may occasionally try switching to dixeny. Indeed, the presence of the monoxenous trypanosomatids in vertebrates has been noted already about 100 years ago [5]. More recently, several monoxenous flagellates belonging to the genera *Herpetomonas*, *Crithidia*, *Leptomonas*, and *Blechomonas* have been identified from human clinical isolates [6–8]. Importantly, most of them involved immuno-compromised individuals, leading to a hypothesis that these usually non-infectious species may explore new ecological niches in vertebrates that have their immune system suppressed [9,10]. Within this paradigm, about two dozen cases of monoxenous trypanosomatids co-infecting humans along with various *Leishmania* spp. have been reported almost exclusively from the Indian subcontinent. Most of them implicated causative agents of visceral leishmaniasis (kala-azar) of the *L. donovani* complex [11]. It was also demonstrated that both dixenous and monoxenous flagellates may be transmitted by the same *Phlebotomus* vector, yet the evidence is not very strong [12,13]. The cytochrome b and 18S rRNA-based PCR analyses were confined to the isolates from a small geographical area and the identity of non-*Leishmania* parasites could not be elucidated to the species level.

The species most often recovered from co-infections in leishmaniasis patients is *Leptomonas seymouri* Wallace, 1959 [14]. Together with all *Leishmania* spp. it belongs to the subfamily Leishmaniinae (S1 Fig) [15] and was originally isolated from a cotton stainer *Dysdercus suturellus* (Hemiptera: Pyrrhocoridae) [16]. Nonetheless, when a broad-scale survey of trypanosomatids parasitizing pyrrhocorids throughout the world was undertaken, none of the samples proved to contain *L. seymouri* [17]. So the question remains whether the original isolate was obtained from a specific host (e.g. species that is evolutionary adapted for parasite's life cycle). *L. seymouri* can even multiply in plants under experimental conditions [18] proving it to be non-fastidious and able to adapt to quite different environments.

Recent whole-genome analysis of kala-azar clinical isolates from splenic aspirates demonstrated heavy "contamination" with unidentified *Leptomonas* sp. [19]. This result is not so

surprising provided that both parasites are almost indistinguishable by morphology and that *Leptomonas* outgrows *Leishmania* in culture [20].

We speculate that several species of monoxenous trypanosomatids are capable of surviving in the hostile environment of the vertebrate body. Molecular details of such adaptation are not worked out, yet it is clear that some monoxenous trypanosomatids must be able to tolerate heat shock up to the temperatures they might experience in warm-blooded vertebrates. Indeed, a number of representatives of the genera *Crithidia* and *Herpetomonas* can withstand elevated temperature reaching 37°C [21–23].

In this study we addressed the issue of *Leishmania–Leptomonas* co-infection from the point of view of the monoxenous partner. To understand molecular mechanisms and biochemical pathways responsible for survival within warm-blooded vertebrates, we have demonstrated that *Leptomonas seymouri* can withstand elevated temperatures *in vitro*, sequenced its genome, and assessed transcriptional profiles of cells cultivated in different conditions. Furthermore, we tested *L. seymouri* ability to survive in *Phlebotomus argentipes* and *P. orientalis*, two sand fly species implicated in *Leishmania donovani* transmission.

Results

Identification of *Leptomonas seymouri* in clinical kala-azar isolates

Whole genome sequencing of two clinical Indian kala-azar field isolates, a strain resistant to sodium antimony gluconate therapy (Ld 39, May 2000, Muzaffarpur, Bihar) and a strain sensitive to treatment (Ld 2001, February 2000, Balia, Uttar Pradesh), revealed numerous (over 95%) sequences apparently derived from *Leptomonas* sp. in addition to those of *L. donovani* [19]. These isolates were cultivated from splenic aspirates in frame of a large screen aimed to understand molecular differences between confirmed kala-azar cases. For precise identification of the co-infecting species we applied an arsenal of molecular tools developed over the years [24–27]. Three genetic loci, namely 18S rRNA, glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), and ITS regions were amplified, sequenced and compared with other representatives of the subfamily Leishmaniinae [15]. 18S rRNA sequences of the isolates Ld 39 and Ld 2001 (GenBank accession numbers KP717894 and KP717895, respectively) were identical and indistinguishable from the corresponding sequence of *L. seymouri* (GenBank accession number AF153040). gGAPDH sequences (GenBank accession numbers KP717896 and KP717897 for isolates Ld 39 and Ld 2001, respectively) were nearly identical with only 1 nt substitution in the coding sequence. They both were very similar (except for the degenerative primer sequences) to the gGAPDH sequence of *L. seymouri* (GenBank accession number AF047495). 18S rRNA and gGAPDH sequences are informative for higher level taxonomy, and are usually adequate for the genus (and up) level ranking [4,28]. For proper species identification we used other well-established markers, ITS1 and ITS2 [14,20,29]. Their sequences were identical with the exception of a 2 nt-long indel (GenBank accession numbers KP717898 and KP717899 for isolates Ld 39 and Ld 2001, respectively). BLAST search revealed 100% identity with the ITS1-5.8S rRNA region of *L. seymouri* (GenBank accession number JN848802).

The data presented above allowed us to conclude that the monoxenous co-infectant of the clinical kala-azar isolates Ld 39 and Ld 2001 is *L. seymouri*. We also would like to note that the cases of co-infections of *Leishmania* and *Leptomonas* are likely underreported in the literature, as several sequences attributed to *L. donovani* in GenBank do in fact belong to *L. seymouri*. Our analysis of the ITS-containing region, SL, gGAPDH, HSP70, HSP83, RNA polymerase II, α-tubulin and some mitochondrial genes (A6, cytb, COI, COII, COIII, NADH) revealed that 38 out of 170 (22%) and 3 out of 217 (1.4%) ITS sequences of *L. seymouri* were misidentified as

Leishmania donovani and *L. tropica*, respectively (see S1 Table for GenBank accession numbers).

Leptomonas seymouri withstands elevated temperature typically associated with vertebrate infection

The presence of monoxenous *L. seymouri* in co-infections with dixenous *L. donovani* implies several adaptations to the environment of the human body. One of the important factors to be considered is temperature. Typical monoxenous trypanosomatids of the insect gut are temperature-sensitive and cannot withstand conditions of the warm-blooded vertebrates [6]. In order to investigate temperature resistance of several trypanosomatid species *in vitro*, we compared growth kinetics of two different *Leptomonas* species, *L. seymouri* ATCC 30220 (hereafter used as a proxy of filed isolated Ld 39 and Ld 2001, which were not available) and *L. pyrrhocoris* H10, under different experimental conditions. Parasites were incubated at temperatures 23°C, 29°C, and 35°C for up to 7 days. The highest temperature (35°C) approximately corresponds to that faced by the flagellates upon transfer from a sand fly into a vertebrate. To imitate the conditions of insect gut and vertebrate blood, SDM and two-phased blood-agar were used, respectively. No considerable difference was observed in growth kinetics of two trypanosomatid species incubated at 23°C in both media. Interestingly, increasing the cultivation temperature to 29°C and 35°C inhibited growth of *L. pyrrhocoris*, while growth of *L. seymouri* was not significantly affected (Fig 1). We concluded that *L. seymouri* is capable of withstanding the elevated temperature reaching that of the human body. In contrast, *L. pyrrhocoris* is temperature-sensitive and halts its cell division in non-optimal conditions. In all cases, cultivation on blood-agar medium resulted in higher cells density.

Light microscopy of Giemsa stained smears of *L. seymouri* cultivated under different experimental conditions revealed statistically significant morphological changes (Fig 2). The most noticeable one was shortening of the free portion of the flagellum observed in cells cultivated at high temperature. This phenomenon was observed for both media used but it was more

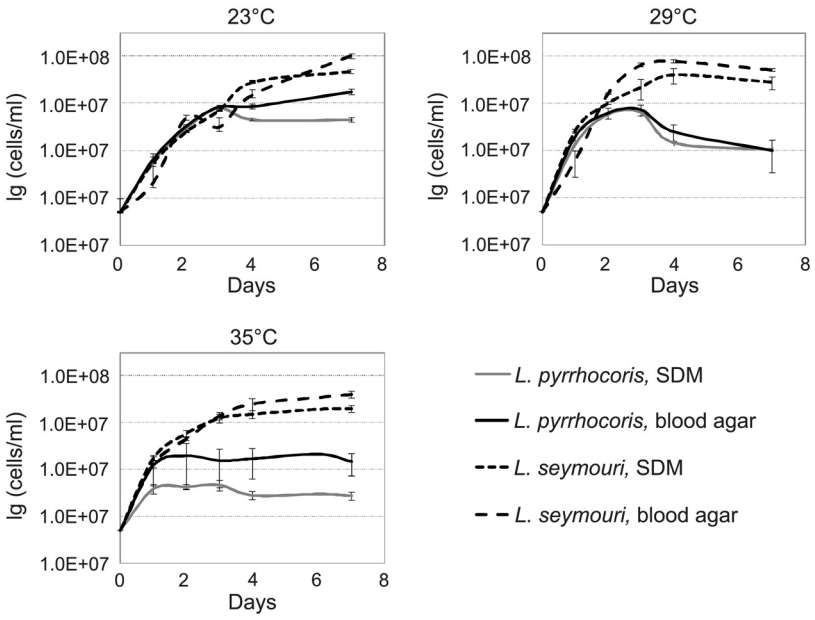

Fig 1. Growth kinetics of *Leptomonas pyrrhocoris* and *L. seymouri* at 23°C, 29°C, and 35°C in SDM and blood-agar media. Data from 3 independent biological replicates are presented.

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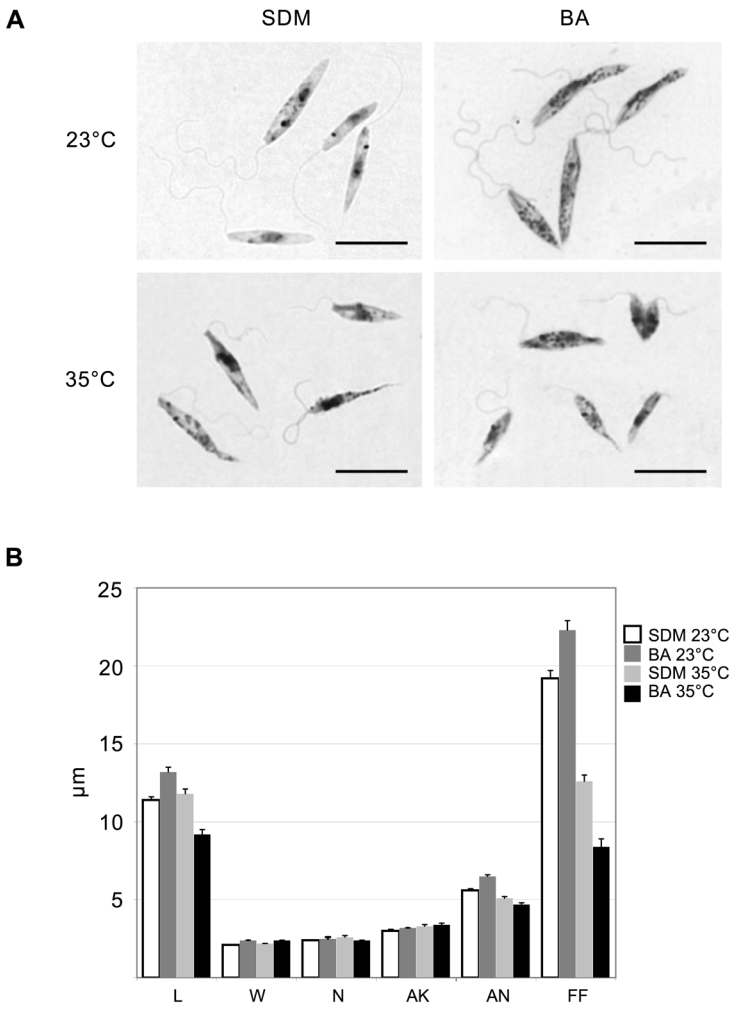

Fig 2. Morphology of *Leptomonas seymouri* cells cultivated *in vitro* in SDM and blood-agar media at low (23°C) and high (35°C) temperature. A, Giemsa-stained slides, scale bar is 10 µm. **B**, ANOVA statistical analysis of 100 cells, average and standard error are presented in micrometers (µm). L–length, W–width, N–length of the nucleus, AK–distance between the kinetoplast and anterior end of the cell, AN–distance between the nucleus and anterior end of the cell, FF–free flagellum length.

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pronounced in blood-agar. Also elevated temperature resulted in more diverse body sizes and shapes with the most conspicuous feature being elongated and tapered posterior end of some cells.

Genome of *Leptomonas seymouri*

The genome of *L. seymouri* ATCC 30220 was assembled into 1,222 scaffolds (maximum length 326,845 bp) with N50 of 70,646 bp and a total assembly length of approximately 27.3 Mbp. This is a substantial improvement over the previously reported assembly of the unidentified *Leptomonas* sp. (14,518 contigs with maximum length of 26,366 and N50 of 3,370 bp) [19]. Both assemblies had almost the same total genome length (27.3 and 27.4 Mb). Importantly, over 85% of the reads could be cross-mapped (length fraction = 0.9; similarity fraction = 0.9) confirming identity of the *L. seymouri* isolates. The number of annotated protein-coding genes, 8,488, was also within the range of previously reported genomes (6,451 for *Phytomonas* sp.

HART1; 8,309 for *Leishmania major*; 10,109 for *Trypanosoma brucei*) [30–32]. Consistent with other trypanosomatids, the protein-coding genes lack conventional introns. The only exceptions reported so far in *Trypanosoma* spp. and *Leishmania* spp. are poly(A) polymerase and DEAD/H RNA helicase [32,33]. Indeed, their *L. seymouri* orthologs also contain introns and thus require *cis*-splicing for proper expression.

A typical aspect of the *L. seymouri* genome is that it contains a relatively small number of genes that have undergone tandemly linked duplications. Using a cutoff value of 10^{-50}, the number of genes present in two or more homologous copies has been estimated at about 9.9% in *L. seymouri*. Same numbers for *Phytomonas* sp., *L. major*, *T. brucei*, and *C. fasciculata* are 9.6%, 18.3%, 26.0%, and 40.2%, respectively. This is one of the major components determining differences in genome size among these species.

Metabolic pathways in *L. seymouri*

Genomic information was used to predict the metabolic pathways in *L. pyrrhocoris* and *L. seymouri*, two phylogenetic kins with different sensitivity to temperature and ability to co-infect vertebrate hosts (S2 Fig). In essence, the metabolism in these two species is very similar, with important features and differences highlighted below. A classical glycolytic pathway, partly inside glycosomes (as inferred from the presence of peroxisome targeting signals), is responsible for the metabolism of various exogenous sugars (S2 Table). Carbohydrate metabolism is characterized by an incomplete aerobic oxidation because one of the classical mitochondrial tricarboxylic acids (TCA) cycle enzymes (NAD-linked isocitrate dehydrogenase) is absent. However, the other TCA cycle enzymes can be used for the inter-conversion of metabolic building blocks required for gluconeogenesis and other biosynthetic purposes (S3 Table). While both *L. pyrrhocoris* and *L. seymouri* are able to synthesize their own pyrimidines, they depend on a supply of external purines. They lack the capacity to oxidize aromatic amino acids and require an external supply of most of the essential amino acids, cofactors and vitamins for growth (S4 Table). Both *Leptomonas* spp. have a fully developed mitochondrion with 9 of the 10 TCA cycle enzymes present, a complete respiratory chain with the respiratory complexes I—IV, and a fully functional mitochondrial F_1-ATPase (S5 Table).

Although lacking the alternative oxidase found in many other trypanosomatids, *L. seymouri* possesses an alternative NADH dehydrogenase gene. Our analysis predicts that it is able to feed on a large variety of polysaccharides, carbohydrates, both hexoses and pentoses, with the anticipated end products of carbohydrate metabolism being acetate, succinate, carbon dioxide, ethanol, alanine, and D-lactate. *L. seymouri* has a complete set of β-oxidation enzymes, which are associated with the mitochondrion. A few additional lipid-metabolizing enzymes are present in the glycosomes. It appears that the analyzed flagellate does not possess a type-I system of fatty acid synthesis, but makes its fatty acids in the cytosol by the action of a series of elongases (S6 Table). It is able to oxidize 16 of the 20 amino acids, but the necessary enzymes for the metabolism of lysine and the three aromatic amino acids (phenylalanine, tyrosine and tryptophan) are lacking. The urea cycle is not functional since two mitochondrial enzymes of the cycle are missing (S7 Table). The remaining three cytosolic enzymes have all been acquired by lateral gene transfer and allow arginine to be utilized in polyamine biosynthesis. Surface proteins, previously identified in *Trypanosoma*, *Leishmania* and *Crithidia* spp., have also been found in *Leptomonas* (S8 Table). Homologues of GP63, amastin, 3'-nucleotidase, integral membrane protein, prohibitin, membrane-bound acid phosphatases MBPA1 and MBPA2 and tartrate-sensitive acid phosphatase, but not oligosaccharyl transferase, are present. Protection against oxidative stress in monoxenous trypanosomatids differs from their dixenous kins. In addition to the trypanothione system and the presence of many homologues of tryparedoxins and

peroxiredoxins, all monoxenous species analyzed thus far have a bacterial-type catalase acquired by lateral gene transfer (S9 Table).

Enzymes of the RNA interference pathway, namely the homologs of the Argonaute (AGO1) and the two dicer proteins (DCL1 and DCL2) were not detected in *L. seymouri* (S10 Table). Importantly, they were found in the genome of *L. pyrrhocoris* arguing that these two closely related species differ in their ability to regulate gene expression by RNA interference.

Lateral gene transfer

In the evolution of Trypanosomatidae many events of lateral gene transfer (LGT) have taken place, since genes of bacterial origin are frequently encountered in all trypanosomatid lineages [34]. This suggests that an ancestral flagellate had already acquired such genes, which include a number of enzymes of glycolysis, pentose-phosphate shunt and pyrimidine biosynthesis, as well as trypanothione reductase and pterin transporters [35–37]. Some LGT events including genes involved in sucrose and pentose sugar metabolism, haem synthesis and urea cycle seem to be more recent and specific to the Leishmaniinae clade that comprises *Leishmania*, *Crithidia* and *Leptomonas* spp. [38–40]. Even more recent acquisitions, shared only among *Crithidia* spp. and *Leptomonas* spp. include catalase, the diaminopimelate-metabolizing enzymes and those of β-glucosidase, nitroalkane oxidase, phenolic acid dehydrogenase and glycerol dehydrogenase families (S11 Table). In total, 70 out of 586, or 12% of all the metabolic genes analyzed, have resulted from the events of lateral transfer.

Gene family analysis using the OrthoMCL approach

For this analysis full proteomes for 23 trypanosomatid species were downloaded from TriTrypDB v. 7.0 and combined with newly annotated proteins from *L. seymouri*, *L. pyrrhocoris*, *B. ayalai* and *Paratrypanosoma confusum* (S12 Table). Comprehensive characterization of *L. seymouri* gene family repertoire and its comparison to that of other trypanosomatids may help to shed light on possible adaptations of this species to the dixenous lifestyle. Recently, a comparative genomics approach was used to define a "gene kit" implicated in cell invasion and intracellular parasitism in *Leishmania* spp. and *Trypanosoma cruzi* [41]. Authors have found that despite substantial differences in mechanisms of host cell invasion and survival within the host cell, 3,340 orthologous gene clusters are exclusively shared between intracellular parasites when compared to extracellular *T. brucei*. Many proteins within these clusters were already proven to play a pivotal role in *Leishmania* and *Trypanosoma* virulence (e.g. GP63, amastin, ascorbate peroxidase), while functions of other proteins require further detailed investigation.

In our study we were aiming to identify candidate proteins in *L. seymouri* that may define its ability to occasionally infect warm-blooded organisms. For that purpose Orthologous Groups (OG) presence/absence patterns in *L. seymouri* were analyzed and compared to those of other trypanosomatids. In the reference dataset for OrthoMCL analysis several *Leishmania* spp. (medically and veterinary important dixenous species), along with *C. fasciculata* and *L. pyrrhocoris* (both never encountered in vertebrates) are of primary interest for comparison with *L. seymouri*. According to a widely accepted view of trypanosomatid phylogeny, *Leptomonas* spp. are most closely related to *Crithidia* spp., and together they form a clade that clusters as a sister group to the genus *Leishmania* [2,15] (S2 Fig). Firstly, OG content was compared in *L. seymouri*, *C. fasciculata*, and *L. pyrrhocoris* in order to exclude from the analysis OGs that are present in typical monoxenous trypanosomatids. *Leptomonas pyrrhocoris* has a typical promastigote morphology and dwells in insect species of the family Pyrrhocoridae [17,42], while *C. fasciculata* uses various culicids as hosts [43–45]. Notably, some representatives of the genus *Crithidia* (*C. hutneri*, *C. luciliae thermophila*) can survive at temperatures of the mammalian

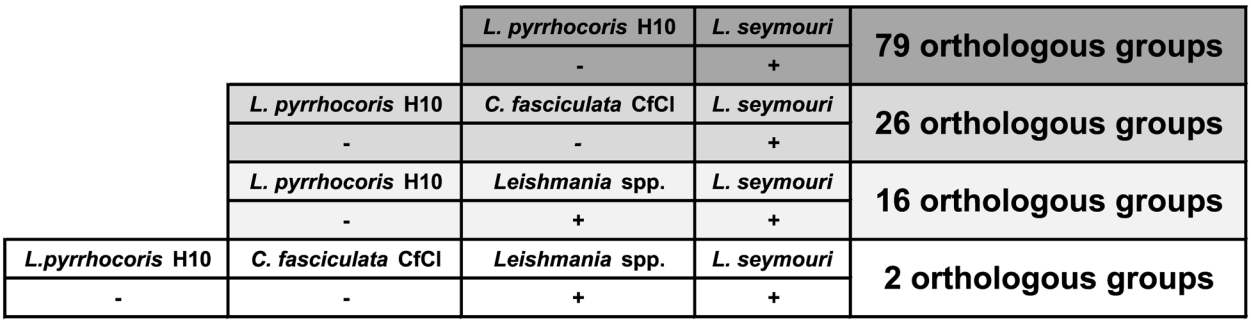

Fig 3. Orthologous group presence (denoted by "+")/absence (denoted by "-") patterns for *Leptomonas seymouri*, *Leptomonas pyrrhocoris*, *Crithidia fasciculata*, and several *Leishmania* species. The number of present OGs is indicated on the right.

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and avian bodies [21,22]. Therefore *C. fasciculata* may possess genes involved in survival at elevated temperatures, and in order to exclude possible biases caused by the presence of *C. fasciculata* genes in our OrthoMCL analysis, OG repertoire comparisons were performed twice: with and without *C. fasciculata* in the datasets being compared.

Out of 7,935 *L. seymouri* OGs, 79 OGs were absent in *L. pyrrhocoris*, and 26 OGs were absent from both *L. pyrrhocoris* and *C. fasciculata* (S13 Table and Fig 3). Our assumption is that among the genes belonging to the above-mentioned groups there are at least several that predispose *L. seymouri* metabolism to dixeny. Fifty five out of 79 OGs absent in *L. pyrrhocoris* do not have any functional annotation assigned and thus represent a broad field for further studies (S13 Table). Nevertheless, several genes identified by comparative genomics approach in our study were already proven to play a pivotal role in parasite survival and virulence (see below).

In order to further narrow down the set of such genes we introduced one more condition into the comparison: gene family present in *L. seymouri* must be also present in all *Leishmania* species considered in the analysis (*L. braziliensis* MHOM/BR/75/M2903, *L. braziliensis* MHOM/BR/75/M2904, *L. donovani* BPK282A1, *L. infantum* MCAN/ES/98/JPCM5, *L. major* MHOM/IL/80/Friedlin, and *L. mexicana* MHOM/GT/2001/U1103). A reptile parasite *L. tarentolae* ParrotTarII was not included in the analysis due to its inability to infect warm-blooded organisms [46]. Additional BLASTP search (E-value $\leq 10^{-10}$) for proteins belonging to OGs and meeting the criteria stated above was performed in order to determine whether these OGs have related OGs with homologous proteins clustered separately by the sensitive OrthoMCL algorithm. Cases when related OGs have a presence/absence pattern which violates the above-mentioned criteria are not discussed here since unambiguous conclusion cannot be made concerning the role of such proteins in *L. seymouri* thermotolerance.

Sixteen OGs absent from *L. pyrrhocoris* are shared by *L. seymouri* and *Leishmania* spp. Importantly, only 2 of them are absent from both *L. pyrrhocoris* and *C. fasciculata* (Fig 3). These two OGs represent a kinase-like protein and a ubiquinol-cytochrome *c* reductase-like protein. According to the results of additional BLASTP search, the latter protein OG does not have any related OGs and all of its orthologs in the TriTrypDB are annotated as ubiquinol-cytochrome *c* reductase-like proteins. Aiming to identify homologs of this protein in other species beyond the TriTrypDB, we conducted a BLAST search against the NCBI nr database and found a close homolog only in *Strigomonas culicis* (ubiquinol-cytochrome *c* reductase subunit 6, E-value $\leq 10^{-30}$, protein accession number: EPY16273.1). The kinase-like protein mentioned above has weak hits with E-value over 10^{-30} to several other OGs containing protein kinases. Due to the relatively high E-values of the BLAST hits and quite unspecific annotations

of kinases within related OGs, this protein was not excluded from our analysis (although several related OGs have absence/presence patterns that differ from the required ones), and its possible role in *L. seymouri* thermotolerance cannot be ruled out.

Having excluded the requirement of OG being absent from *C. fasciculata*, the overlap mentioned above extends to 16 OGs (Fig 3), which include one more group with putative protein kinases as well as putative anaphase-promoting complex subunit, putative epsin and several hypothetical proteins with unknown functions. In order to obtain a global picture of corresponding OG distribution for subunits of the anaphase-promoting complex and putative epsin, we extended analysis of OG presence/absence patterns to the whole dataset of 27 trypanosomatid species. As expected, OGs containing these proteins have shown nearly omnipresent distribution (being absent from *L. pyrrhocoris* as required in our analysis and additionally missing in several *Trypanosoma* spp.). Additional BLAST search (with more relaxed parameters) for these proteins against proteins belonging to other OGs also did not return any hits. Such results can be explained assuming considerable sequence diversity in these proteins families. For epsins, a group of eukaryotic proteins broadly implicated in clathrin-mediated endocytosis, there is evidence for substantial sequence dissimilarities and lineage-specific protein architecture [47]. Anaphase-promoting complex is a multi-subunit E3 ubiquitin ligase that is necessary for proteolytic degradation of crucial cell cycle regulators, which causes segregation of sister chromatids [48]. Taking into account a universal role of the proteins mentioned above and their phyletic patterns (especially their presence in several monoxenous species) we conclude that they are unlikely to be involved in *L. seymouri* thermotolerance. Interestingly, 3 OGs containing hypothetical proteins (OG_09193, OG_10013, and OG_10042) within the group of 16 OGs fully satisfy the conditions applied in the study, including the absence of closely related OGs. Moreover, these groups of homologous proteins do not occur in any *Trypanosoma* spp. and in monoxenous trypanosomatids for which genome sequences are available (except for *C. fasciculata*). Proteins within these groups represent primary targets for additional studies aiming to reveal mechanisms contributing to *L. seymouri* thermotolerance.

Leptomonas seymouri harbors dsRNA viruses

Prompted by our observation that *L. seymouri* lacks RNAi machinery (see above) and by patterns of RNAi retention in Trypanosomatidae [49], we also examined *L. seymouri* for the presence of dsRNA viruses. Two complementary methods, the nuclease digestion assay and immunofluorescence microscopy, were used [50]. Indeed, the anti-dsRNA antibodies detected small sharp dots, which are reminiscent of those found in the virus-positive isolate of *Leishmania guyanensis* [51]. Importantly, these putative viral particles did not co-localize with the mitochondrion (Fig 4A). The nuclease digestion assay of *L. seymouri* RNA was performed in parallel with the virus-free *Blechomonas pulexsimulantis* used as a negative control [52]. It detected dsRNA bands resistant to DNase I and S1 nuclease, which were present in RNA preparations from *L. seymouri* (Fig 4B). Interestingly, this dsRNAs differ in size from that of the previously characterized LRV1 virus of *Leishmania guyanensis* (1.5 + 2.9 kb versus 5.3 kb, respectively) [53,54]. It remains to be investigated whether this reflects critical differences in genomic organization of viruses, such as segmented versus whole dsRNA genomes.

Whole transcriptome profiles of *L. seymouri* cultivated at different temperatures

To identify genes and/or pathways responsible for thermoresistance of *L. seymouri*, we profiled whole transcriptomes of the parasites cultivated at low (23°C) and high (35°C) temperature. We presumed that in addition to genetic factors (e.g. chromosome ploidy) regulation of gene

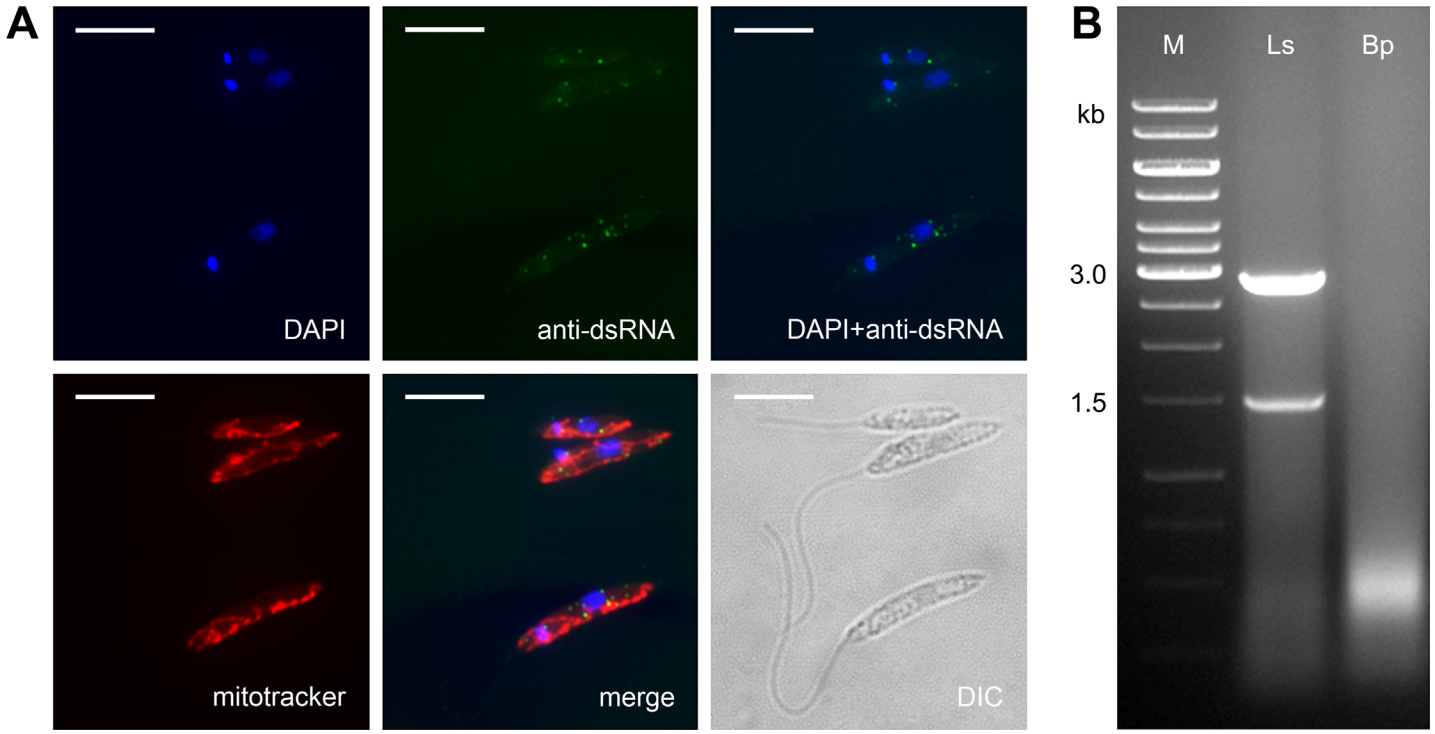

Fig 4. *Leptomonas seymouri* harbors dsRNA viruses. A, Cells were stained with DAPI, mitotracker Red and anti-dsRNA antibody for viral detection. Scale bar is 10 μm. **B**, Total RNA samples isolated from *Leptomonas seymouri* (Ls) and *Blechomonas pulexsimulantis* (Bp) were treated with DNase I and S1 nuclease and separated by gel electrophoresis. M– 1 kb ladder (Thermo Fisher Scientific).

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expression may also be involved in adaptation to dixeny. Reads passing the filtering step (61.4; 52.5; 39.1 million reads for replicates at 23°C and 61.1; 58.9; 37.9 million reads for replicates at 35°C) were used in subsequent analyses. Out of 8,488 genes identified in the *L. seymouri* genome 8,482 genes were recovered in our analysis. Results of the FDR test are shown in S3 Fig. In total, 340 genes (4% of the total number) were shown to be differentially expressed at the elevated temperature (S14 Table). Expression of 139 genes (1.6% of the total number) was found to be down-regulated at 35°C, whilst 201 genes (2.4% of the total number) were upregulated at least 1.5 fold (p-value ≤ 0.05). Several interesting cases are discussed in detail below.

Synthesis of sterols. Sterols and related compounds are important membrane components of the living cells that define cell membrane's fluidity. They act as bidirectional regulators by stabilizing the membrane and raising its melting point at high temperature, and by preventing phospholipids from clustering together and stiffening at low temperature [55]. Sterol biosynthesis is a fairly conserved biochemical pathway in eukaryotes responsible for the production of cholesterol in animals and several C24-alkyl sterols (ergostane-based sterols) in fungi, plants, and trypanosomatids. In *L. major*, genetic ablation of C14α-demethylase (C14DM) results in a complete loss of ergostane-based sterols and accumulation of C14-methylated sterols. Genetically modified (c14dm$^{-/-}$) parasites were viable but exhibited some remarkable defects including increased membrane fluidity, and hypersensitivity to heat stress [56]. In *T. brucei* the decrease in the levels of squalene synthase and squalene monooxidase led to the depletion of cellular sterol intermediates and end products, impaired cell growth and aberrant morphologies, DNA fragmentation and profound modification of mitochondrial structure and function [57].

In *L. seymouri* numerous enzymes implicated in biosynthesis of C24-alkyl sterols were down-regulated at elevated temperature. This list includes sterol C14DM, squalene monooxygenase, lanosterol synthase, C-5 sterol desaturase, and lathosterol oxidase.

Oxidative stress protection. As mentioned above, the protection against oxidative stress in monoxenous trypanosomatids is unique since in addition to the trypanothione/ tryparedoxins/peroxiredoxins systems, they heavily rely on a bacterial-type catalase. In *L. seymouri*, expression of the trypanothione reductase goes down upon cultivation at 35°C, but this decrease is compensated by overexpression of two other enzymes, namely catalase and ascorbate-dependent peroxidase. The expression of an enzyme responsible for superoxide anions detoxification (superoxide dismutase) is also upregulated at elevated temperature.

Carbohydrate and fatty acids metabolism. Several glycosomal components of the carbohydrate metabolism are significantly down-regulated in *L. seymouri* at elevated temperature. These are glucose-6-phosphate isomerase, ATP-dependent 6-phospho-1-fructokinase, glycosomal phosphoglycerate kinase, fumarate hydratase, fumarate reductase, and malate dehydrogenases. Conversely, expression of some genes, such as pyruvate phosphate dikinase and cytosolic fumarase, is up-regulated at 35°C.

Consistent with the above-mentioned observations, *L. seymouri* catabolism of fatty acids by β-oxidation is enhanced at high temperature. Several enzymes implicated in this reaction, namely enoyl-CoA hydratase, elongase 4, and several desaturases (delta-6 fatty acid desaturase, delta-5 fatty acid desaturase, stearic acid desaturase), are all upregulated at 35°C. Enhanced catabolism is accompanied by diminished *de novo* synthesis, as is evidenced by the inhibition of three consequent elongases (1 to 3) responsible for the synthesis of saturated fatty acids.

Experimental infection of *Phlebotomus* spp. with *Leptomonas seymouri*

Experimental infections of the two proven vectors of *Leishmania donovani*, *Phlebotomus orientalis* and *P. argentipes*, were compared side-by-side. Insects were fed on either blood or sugar meals to mimic the range of conditions which may favor infection (Fig 5A and 5B). On day 2 after infective sugar meal all females of *P. orientalis* were infected, while the infection rate of *P. argentipes* females was lower (59%) (Fig 5A). Intensity of infection was generally weak in both species tested. On day 6 p. i. percentages of infected sand flies decreased to 77% and 46% for *P. orientalis* and *P. argentipes* females, respectively. On day 9 every third female remained infected, yet most of them harbored only few flagellates.

Infection *via* the blood meal was less efficient when compared to the sugar meal. On day 2 less than half of blood-fed females of *P. orientalis* and *P. argentipes* were infected (47% and 32%, respectively). Freely moving promastigotes were found enclosed in the ingested blood. On days 6 and 9 *L. seymouri* promastigotes persisted only in a few females (Fig 5B).

Co-infection of sand flies with *Leptomonas seymouri* and *Leishmania donovani*

The experimental co-infection of sand fly females of *P. argentipes* were performed by blood meals containing either mCherry- (*L. seymouri*, ATCC-30220) and/or GFP-expressing (*L. donovani*, strain GR-374) flagellates. In the control dissection of five sand flies performed just a few hours p. i., both mCherry- and GFP-labeled cells were encountered at about 100 cells of each species *per* sand fly gut. On day 2 p. i. the infection rate of *L. seymouri* was lower than that of *L. donovani* (82.6% versus 95.7%, respectively), and also the intensity of infection with the former species was significantly weaker (Fig 5C). The differences between both parasite species became even more pronounced on day 5 p. i., when the percentage of infected sand flies remained unaltered for *L. donovani* (86.7%), while it markedly dropped for *L. seymouri*

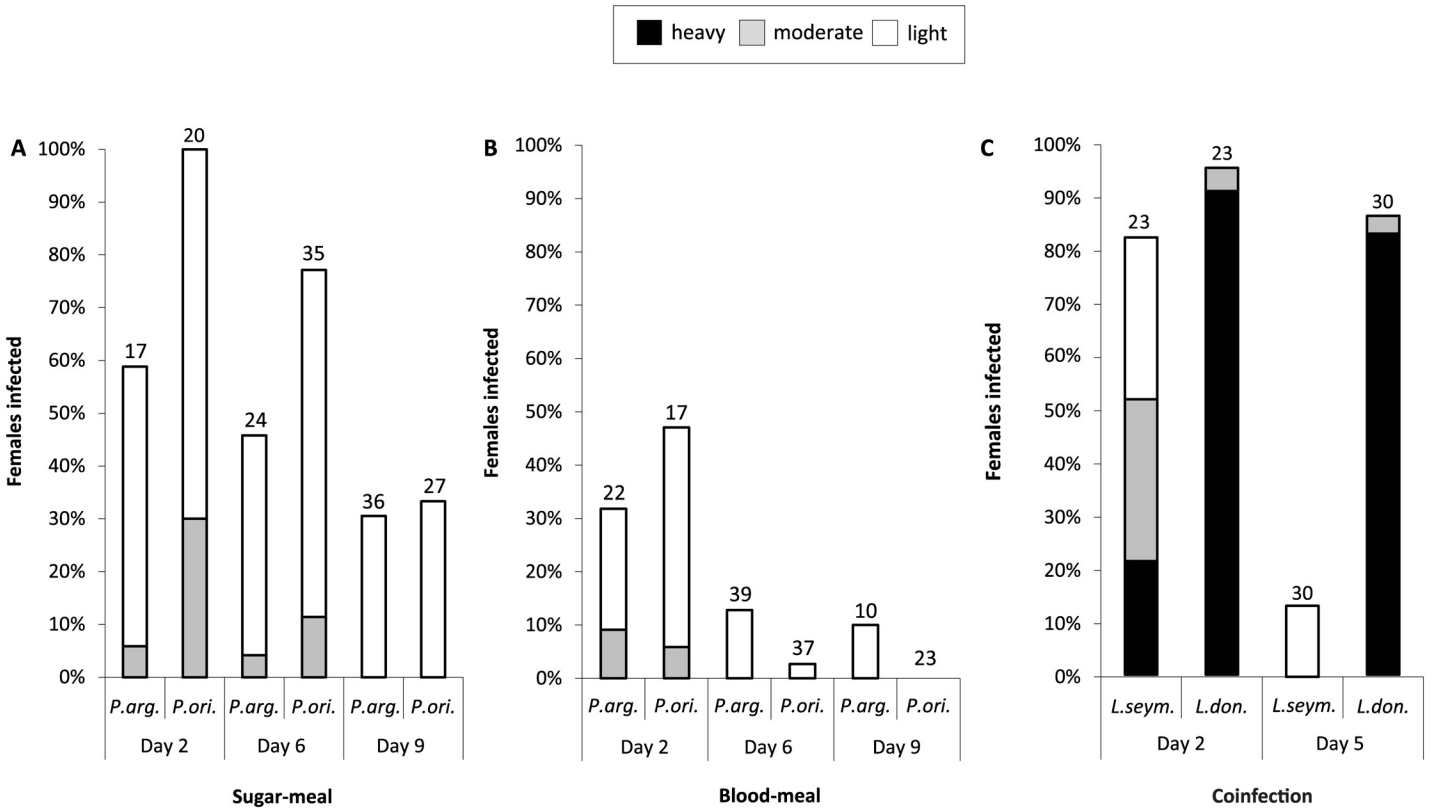

Fig 5. Experimental infection and co-infection of *Phlebotomus* spp. *Phlebotomus argentipes* and *P. orientalis* were infected with *Leptomonas. seymouri* using sugar- (A) or blood- (B) meal method. Intensity of infection was assayed on days 2, 6, and 9 post infection and defined as light (less than 100 promastigotes, white bar), moderate (100–1,000 promastigotes, grey bar), or heavy (over 1,000 promastigotes, black bar) depending on the number of parasites per gut. **C**, Experimental co-infection of *P. argentipes* with mCherry *Leptomonas seymouri* and GFP *Leishmania donovani* GR-374. Intensity of infection was assayed on days 2 and 5 post infection with blood meal and defined as light, moderate, or heavy as above. Numbers above each bar indicate the number of dissected females.

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(13.3%). Moreover, the intensity of infection with *L. donovani* was high, whereas the few insects still infected with *L. seymouri* harbored only negligible number of free swimming mCherry-expressing cells (Fig 5C).

Infection of macrophages

Survival of parasites inside mammalian host cells J774 or BMMϕ was evaluated 3, 4, 5 and 6 days p. i. No viable *L. seymouri* cells were found in macrophages by fluorescent microscopy or in Giemsa-stained smears. In contrast, the control represented by *L. donovani* survived inside both J774 and BMMϕ cells. Similar results were obtained using peritoneal macrophages from BALB/c mice. The transformation assay has confirmed microscopic observations, as no *L. seymouri* cells were found after the lysis of macrophages. On the contrary, *L. donovani* propagated very well under the same conditions. Similar results were obtained when either J774 or BMMϕ macrophages were simultaneously co-infected with both parasites.

Discussion

Here we performed a multifarious evaluation of the infective potential of *L. seymouri*, repeatedly isolated from kala-azar patients infected by *L. donovani* in India and neighboring countries, and have tested the capacity of this monoxenous trypanosomatid to utilize the sand fly

vectors permissive for *L. donovani*. Moreover, we attempted to find genetic and corresponding metabolic adaptations responsible for its survival at 35°C.

Firstly, we have sequenced the whole genome of *L. seymouri* and compared it with *L. pyrrhocoris* and *C. fasciculata*, the only monoxenous species for which high-quality assemblies are available. Twenty six OGs carried by the thermotolerant *L. seymouri* and absent in these closely related thermosensitive flagellates may potentially be associated with this adaptation. Including dixenous species into the comparative analysis narrowed down our search to just two OGs shared by *L. seymouri* and five *Leishmania* spp. and absent from *L. pyrrhocoris* and *C. fasciculata*, namely a kinase-like protein and a ubiquinol-cytochrome *c* reductase-like protein. It was shown previously that protein kinases are involved in amastigote differentiation in *Leishmania* spp. [58], a process in which temperature switch plays a decisive role [59]. Moreover, our search has identified a number of proteins with specific distribution among trypanosomatid lineages (e.g. absent in *Trypanosoma* spp. and/or *Leishmania* spp. but present in monoxenous flagellates) that are prime targets for functional analysis. In any case, the fact that the vast majority of genes within OGs with this phyletic distribution are annotated as hypothetical proteins with unknown function indicates our scarce knowledge of trypanosomatid metabolism.

A number of metabolic changes observed in *L. seymouri* exposed to elevated temperature are evocative of those in *Leishmania* amastigotes or *T. brucei* bloodstream forms in glucose-poor environment [60,61]. For example, inhibition of the *de novo* synthesis of sterols in *L. seymouri* resembles *Leishmania* amastigotes in which the relative abundance of C24-alkyl sterols was significantly decreased upon their differentiation from procyclics [62,63]. Similarly to their dixenous cousins, *L. seymouri* cells at high temperature reduce the uptake of glucose and shift their acetyl-CoA production in mitochondria from mainly pyruvate-based to the fatty acids-derived [64–66].

The detection of double-stranded viruses in *L. seymouri* is particularly relevant in the light of recent findings that their presence in *Leishmania guyanensis* correlates with its virulence and metastatic potential [51,67]. While molecular mechanisms of this phenomenon are just becoming to be understood, it is already clear that the host immune response is rewired [68,69]. We and others have detected dsRNA-containing viruses in several other monoxenous trypanosomatids parasitizing dipteran and heteropteran insects [27,51,70,71], but their relationships to the characterized viruses of *Leishmania* still remain a mystery. Two analyzed *Leptomonas* spp. differ in their acceptability for dsRNA viruses. This indicates fundamentally different mechanisms they may utilize to regulate their gene expression.

In summary, we conclude that although *L. seymouri* has developed several adaptations that allow it to grow well at 35°C, it remains a predominantly monoxenous species not able to infect mammalian macrophages either alone or in co-infection with *Leishmania*. This agrees with a recent report on selective elimination of *Leptomonas* from co-cultures with *Leishmania* [72]. Under certain circumstances it is able to infect mammals, but probably only when the host is immunocompromised by infection with another pathogen, such as *L. donovani* or HIV [14,73]. However, it is quite likely that such co-infections are much more frequent than the available literature suggests. This conclusion is further supported by our finding that *L. seymouri* can survive up to 9 days in the same sand fly species that is responsible for the transmission of pernicious *Leishmania* spp. Therefore, it will be important to analyze samples from patients suffering from visceral and other leishmaniases with primers specific for *L. seymouri* and related (presumably) monoxenous trypanosomatids to address the possibility that we see only the tip of the iceberg. In addition to the capacity to withstand elevated temperature, other factors, such as its ability to escape the host immune response, may likely play an important role in establishment of the *Leptomonas* infection in mammals. We cannot exclude the

possibility that some isolates of *L. seymouri* may be exclusively transmitted by sandflies and spend part of their life cycle in vertebrates similar to their *Leishmania* spp. relatives.

Materials and Methods

Ethics statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act Number 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number 24/773/08-10001) and were performed under the Certificate of Competency (Registration Number CZU945/05 ext. CZ02573) and the Permission Number 31114/2013-MSMT-13 ext. 24115/2014-MZE-17214 of the Ministry of the Environment of the Czech Republic.

Origins of strains

Leptomonas seymouri isolate ATCC 30220 was obtained from the American Type Culture Collection (ATCC, Manassas, USA). It was isolated from the cotton stainer *Dysdercus suturellus* in the United States in 1959. *Leptomonas pyrrhocoris* isolate H10 [17], *Blechomonas ayalai* isolate B08-376 [52] and *Leishmania donovani* isolate MHOM/ET/2010/GR374 have originated from the research collections at Charles University in Prague, Institute of Parasitology in České Budějovice, and Life Science Research Centre in Ostrava.

In vitro cultivation and morphological analysis

Cultures of the monoxenous trypanosomatids were routinely maintained in the Schneider's Drosophila medium (SDM) (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific), 50 units/ml of penicillin, 50 μg/ml of streptomycin (both from Sigma-Aldrich, St. Louis, USA), and 10 μg/ml of hemin (Jena Bioscience GmbH, Jena, Germany) at 23°C. All isolates used in this work can also be cultivated in the Brain Heart Infusion (BHI) medium (Sigma-Aldrich) supplemented with 10% FBS and antibiotics as above, or in the two-phased blood-agar medium [74].

To estimate the dynamics of growth, 5 x 10^4 parasites were seeded into the SDM or the blood-agar medium. Cultures were incubated at 23°C, 29°C, and 35°C for 7 days. Cell numbers were counted using a hemocytometer and plotted in log scale. Morphology of the cells cultivated at low (23°C) and high (35°C) temperature, either in SDM or blood-agar media, was analyzed at day 4 (exponential phase) after staining cells with Giemsa as described previously [75,76]. One hundred cells per sample were measured and analyzed using ANOVA statistical models [77].

Leishmania donovani (MHOM/ET/2010/GR374) transfected with Green Fluorescent Protein (GFP) was cultured in M199 medium (Sigma) containing 20% heat-inactivated FBS (Thermo Fisher Scientific) supplemented with 1% BME vitamins (Sigma), 2% sterile urine, 50 units/ml penicillin, 250μg/ml amikacin (Bristol-Myers Squibb, New York, USA), and 150 μg/ml of geneticin, G418 (Sigma).

PCR amplification, cloning and sequencing

The internal transcribed spacer, ITS region of the rRNA locus was amplified using primers IAMWE and Tc5.8-rev and conditions described elsewhere [78]. Total genomic DNA samples

of clinical Indian kala-azar field isolates Ld_39 and Ld_2001 were used as templates [19]. The 18S rRNA and gGAPDH genes were PCR-amplified, cloned into the pCR2.1 vector system (Thermo Fisher Scientific), sequenced and analyzed as described previously [79,80]. The obtained sequences were deposited to GenBank with the following accession numbers: KP717894, KP717895 (18S rRNA); KP717896, KP717897 (gGAPDH); KP717898, KP717899 (ITS1 + ITS2 regions).

Genome assembly and annotation

The *Leptomonas seymouri* ATCC 30220 genome was sequenced with 100 nt paired-end reads using the Illumina HiSeq 2000 platform (Macrogen, Seoul, South Korea). Prior to assembly, reads were subjected to trimming and filtering using CLC Genomics Workbench v. 7.0 (CLC Inc, Aarhus, Denmark): regions with Phred quality < 20 were trimmed, no more than one N was allowed in the remaining sequence, then TruSeq adapter trimming and a minimum length threshold of 75 nt were applied.

Draft genome of *L. seymouri* was assembled with the CLC Genomics Workbench v. 7.0 employing a De Bruijn graph-based algorithm with the average coverage of 180 x. Augustus v. 2.5.5 was used to annotate the draft genome of *L. seymouri* [81]. Prediction accuracy of Augustus was improved by retraining using a training set of *L. seymouri* conserved proteins. In brief, *de novo* assembled contigs were searched against proteins in the TriTrypDB v. 7.0 database [82] (BlastX E-value $\leq 10^{-5}$) and best BLAST hits were chosen based on the following criteria: a) E-value $\leq 10^{-30}$, b) hit length longer than 80 amino acids (aa), c) percent identity higher than 40. Subsequently, a non-redundant training set of 727 high-confidence gene models with unambiguous start site positions was created based on best BLAST hits to annotated proteins from TriTrypDB and RNA-seq coverage data. Non-redundancy of the training set was achieved by excluding genes with more than 70% identity at the amino acid level. Further analysis of the Augustus annotation included manual curation of predicted genes based on transcriptome sequencing data, e.g. removing start sites predicted in regions with no transcriptomic coverage and adding transcribed ORFs >200 aa in length not predicted by Augustus. For tRNA gene prediction tRNAscan-SE Search Server [83] was used with default parameters. For annotating other non-coding RNAs BlastN algorithm (E-value $\leq 10^{-10}$) was employed with subsequent manual inspection of BLAST results. As a result, 8,488 genes were annotated in the *L. seymouri* genome, which has been submitted to the NCBI (BioProject accession number PRJNA285179) and the TriTryp database, a part of the EuPathDB [84].

Gene family analysis using the OrthoMCL approach

Orthologous groups are the set of genes descended from a single common ancestral gene, containing both paralogs and orthologs. OGs for *L. seymouri* proteins were inferred using the OrthoMCL v.2.0 software [85]. Full proteomes for 23 trypanosomatid species were downloaded from the TriTrypDB v. 7.0 and combined with newly annotated proteins from *L. seymouri* and 3 other trypanosomatid species (*Leptomonas pyrrhocoris*, *Blechomonas ayalai* and *Paratrypanosoma confusum*). The reference protein dataset was subjected to removal of poor quality proteins (based on sequence length and percent of in-frame stop codons), all vs. all BLAST (E-value 10^{-10}) and a clustering procedure implemented in the OrthoMCL algorithm. This resulted in 19,866 OGs, 7,935 of which contained proteins of *L. seymouri*.

Whole transcriptome data processing and analysis

L. seymouri was cultivated at 23°C and 35°C for 75 hrs. Total RNA was isolated from 2.5 x 10^7 cells using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the

manufacturer's instruction. The mRNA-derived libraries were sequenced with 100 nt paired-end reads on the Illumina HiSeq 2000 platform (Macrogen). Total of 3 independent biological replicates were analyzed. The whole transcriptome data from this study have been submitted to TriTrypDB database [82].

Differential gene expression analysis was done using the RNA-Seq tool in CLC Genomics Workbench. Raw reads were subjected to quality-based trimming (regions with Phred quality < 20 were trimmed, no more than one N was allowed in the remaining sequence), adapter trimming, and a minimum length threshold of 30 bp. Processed reads were then mapped to the annotated *L. seymouri* genome with the following parameters: maximum number of mismatches, 2; minimum fraction of read length mapped, 0.8; minimum identity within the mapped sequence, 0.8; maximum number of best-scoring hits for a read, 30. All libraries were mapped as paired-end, and expression values (RPKM) for each gene were calculated. To identify gene sets that are differentially expressed between the two conditions, the FDR test was employed [86]. Genes with expression fold change ≥ 1.5 and FDR p-value ≤ 0.05 were chosen for further analyses.

Gene ontology (GO) terms for genes up- and down-regulated at high temperature were generated using the Blast2GO plugin in CLC Genomics Workbench [87]. Initially, BlastP search against the NCBI nr database was performed, GO terms associated with all the hits were retrieved, and most appropriate GO terms were selected according to the standard Blast2GO procedure. GO term enrichment was assessed using Fisher's exact test.

Detection of dsRNA viruses

For detection of dsRNA viruses, two complementary protocols were used. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI), mitotracker Red CMXRos (both from Thermo Fisher Scientific) and mouse monoclonal anti-dsRNA antibody (Scicons, Szirák, Hungary), followed by goat anti-mouse IgG–Alexa Fluor 488 (Thermo Fisher Scientific) antibody as described previously [50]. In addition, 50 μg of total RNA isolated using TRI reagent (Sigma-Aldrich) was treated with 1 unit of DNase I (New England Biolabs, Ipswich, USA) at 37°C for 1 hr, followed by digestion with 35 units of S1 nuclease (Sigma-Aldrich) for 45 min at the same temperature. Samples were analyzed on 0.8% native agarose in 1xTAE buffer [88].

Establishing a fluorescent strain of *L. seymouri*

A fragment encoding mCherry fluorescent protein was amplified with primers 5′-TTAT CCATGGTTAGTAAAGGAGAA-3′ and 5′-TGTTAGCGGCCGCTTATGCGGTACCAG AACC-3′ using plasmid p2686 as a template [89]. The resulting 745 bp fragment was cloned into the pF4T7polNLS1.4sat vector digested with *Nco*I and *Not*I replacing the T7 polymerase ORF [90]. Log-phase *L. seymouri* cells (4×10^7) were transfected with 15 μg of *Swa*I-linearized pF4mCherry1.4sat as described before [91]. Recombinant clones were selected on agar—BHI growth medium supplemented with 10% FBS, 40mM HEPES, pH 7.4 and nourseothricin (Jena Bioscience) at final concentration of 250 μg/ml. Expression of mCherry was confirmed by fluorescence microscopy.

L. seymouri development in sandflies

Colonies of two sand fly species, *Phlebotomus orientalis* and *P. argentipes*, both representing major proven vectors for *L. donovani*, were maintained under standard conditions as described elsewhere [92]. Females of both colonies were fed either through a chick-skin membrane on suspension of heat-inactivated rabbit blood containing exponentially growing 1×10^7 promastigotes per ml of blood or on 20% sucrose solution containing 5×10^7 promastigotes per ml. In

order to recognize sugar-fed females, the sucrose solution was stained by indigo carmin. Blood- and sugar-fed females were kept at 26°C with free access to 50% sucrose solution by day 1 post infection (p. i.).

Sand fly females were dissected at different intervals p. i. (1–2, 5–6 and 7–9 days). Numbers and location of flagellates in the sand fly gut were checked microscopically. Parasite loads were graded as previously described, i.e.: light (< 100 parasites/gut), moderate (100–1,000 parasites/gut) and heavy (> 1,000 parasites/gut) [93].

Leptomonas and *Leishmania* co-infection and development in sand flies

Females of *P. argentipes* were fed through a chick-skin membrane on suspension of heat-inactivated rabbit blood containing 1 x 10^6 per ml promastigotes of *Leptomonas seymouri* mCherry (passage 4) and 1 x 10^6 per ml promastigotes of *Leishmania donovani* (MHOM/ET/2010/GR374) GFP (passage 10) originating from exponentially growing cultures. Assorted blood-fed females were kept at 26°C with free access to 20% sucrose solution. Sand fly females were dissected on days 2 and 5 p. i., and the presence of parasites as well as other characteristics were analyzed as described previously [93].

In vitro infection of mouse macrophages with *Leptomonas seymouri* and *Leishmania donovani*

Macrophage cell line J774 was cultured in complete RPMI-1640 medium (Sigma) containing 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2mM of L-glutamine, and 0.05 mM of β-mercapto-ethanol (all from Sigma) at 37°C with 5% CO_2. Bone marrow was obtained by flushing of tibias and femurs of BALB/c mice and flagellates were cultured in complete RPMI-1640 medium (Sigma) supplemented as above along with 20% of L929 fibroblast cell culture supernatant serving as a source of macrophage colony-stimulating factor at 37°C with 5% CO_2. The differentiation from bone marrow precursor cells to bone marrow-derived macrophages proceeded for 7 to 8 days in sterile polystyrene Petri dishes. The bone marrow derived macrophages (BMMϕ) were washed and seeded into plates at density of 5 x 10^5 cells per ml. Consequently, stationary cultures of *Leishmania donovani* (GFP), *Leptomonas seymouri* (mCherry), alone or in combination were added in ratio of 8:1 (parasites: BMMϕ). Three days p. i. BMMϕ were extensively washed with pre-warmed RPMI-1640 to remove excess of parasites and the viability of trypanosomatids was monitored by fluorescence microscope Olympus CX-31 (Olympus, Tokyo, Japan) up to 6 day p. i. In addition, Giemsa staining was used to analyze intracellular forms in macrophages by light microscopy. All experiments were performed in two independent biological replicates.

To analyze survival of parasites, the transformation growth assay was used [94]. In brief, macrophages infected with *Leishmania donovani*, *Leptomonas seymouri*, alone or in combination for 96 hrs were extensively washed with RPMI-1640 and lysed with 0.016% SDS in RPMI-1640 for 7 min at room temperature to release their intracellular forms. The lysis reactions were neutralized by RPMI-1640 supplemented with 17% heat-inactivated FBS. Parasites were spun down at 3,200 rpm for 10 min at 4°C, washed in RPMI-1640, and re-suspended in a relevant promastigote medium (BHI or M199) supplemented with an appropriate selective antibiotic at 23°C. For macrophages co-infected with both parasites, two types of media and antibiotics were assessed. The status of viable parasites was checked for 6 consecutive days.

Supporting Information

S1 Fig. Evolutionary relationships among trypanosomatids based on SSU rRNA sequences. Green color depicts bodonids species used as an outgroup. Yellow and red colors represent

monoxenous and dixenous parasites, respectively. Clade Leishmaniinae is marked. Modified from [28].
(EPS)

S2 Fig. Cladogram showing phylogenetic relationships among the members of sub-family Leishmaniinae.
(EPS)

S3 Fig. Volcano plot of the *L. seymouri* genes differentially expressed at low (23°C) and high (35°C) temperature. Positive and negative Δlog2RPKM values indicate genes up- and downregulated, respectively. Statistically significant (FDR p-value ≤ 0.05) up- and downregulated (with a 1.5-fold threshold) genes are boxed.
(EPS)

S1 Table. GenBank accession numbers of *L. seymouri* sequences misidentified as *Leishmania* spp.
(XLSX)

S2 Table. List of genes involved in glycolysis.
(XLSX)

S3 Table. List of genes involved in carbohydrate metabolism.
(XLSX)

S4 Table. List of genes involved in amino acid metabolism.
(XLSX)

S5 Table. List of genes of mitochondrial origin.
(XLSX)

S6 Table. List of genes involved in fatty acid biogenesis.
(XLSX)

S7 Table. List of genes involved in urea cycle.
(XLSX)

S8 Table. List of genes encoding surface proteins.
(XLSX)

S9 Table. List of genes involved in oxidative stress response.
(XLSX)

S10 Table. List of genes involved in RNA interference.
(XLSX)

S11 Table. List of genes acquired by lateral transfer.
(XLSX)

S12 Table. Proteome datasets used in the OrthoMCl analysis.
(XLSX)

S13 Table. List of all 79 OGs analyzed in the study.
(XLSX)

S14 Table. List of genes differentially expressed at low (23°C) and high (35°C) temperature.
(XLSX)

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Author Contributions

Conceived and designed the experiments: VY JL PV. Performed the experiments: NK JH AK JM DG TL JV. Analyzed the data: AB FO PF AK. Contributed reagents/materials/analysis tools: PV. Wrote the paper: VY PF JL JV NK.

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