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Sauroleishmania-sand fly interactions

Interakce sauroleishmanií s flebotomy

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Ph.D. thesis / Disertační práce

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AUTHOR'S DECLARATION

I hereby declare that I have elaborated this Ph.D. thesis independently. The data presented herein are the results of my own work or collaboration with co-authors of the attached manuscripts. I also declare that I have cited properly all scientific literature sources used and that this thesis or any substantial part of it has not been submitted for another of the same academic degree.

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Prohlašuji, že jsem tuto disertační práci vypracovala samostastně. Data v ní prezentovaná jsou výsledky mé vlastní práce nebo spolupráce se spoluautory přiložených rukopisů. Taktéž prohlašuji, že jsem uvedla všechny použité literární zdroje a že tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

In Prague, 9th February 2024

V Praze, dne 9. února 2024

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Mgr. Lucie Tichá

SUPERVISOR'S DECLARATION

I declare that Lucie Tichá substantially contributed to the experimental work in the project presented in this Ph.D. thesis and that she had a principal role in the writing of three publications presented.

PROHLÁŠENÍ ŠKOLITELE

Prohlašuji, že se Lucie Tichá významně podílela na experimentální práci v rámci projektu prezentovaného v této disertační práci a je hlavní autorkou textu tří publikací.

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ABSTRACT

Sauroleishmania is a group of less studied parasites that belong to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). They circulate between reptile hosts and sand fly vectors (Diptera: Psychodidae). Due to the non-pathogenic character of its species, little is known about their development in reptiles and sand flies. The main objective of this project was to elucidate some missing aspects of *Sauroleishmania* life cycle. A major part of this thesis aimed to test the susceptibility of various sand fly species to different *Sauroleishmania* isolates and describe their development in the sand fly intestinal tract. A minor part was devoted to the study of infection in reptiles.

First, we investigated the development of *Leishmania* (*Sauroleishmania*) *tarentolae* in three *Phlebotomus* species. Sand flies were infected through membrane on promastigote suspension and dissected at various time intervals post infection. *Leishmania* (*S.*) *tarentolae* developed in all three species tested and underwent peripylarian type of the development. Moreover, heavy parasite loads were frequently found in Malpighian tubules, which is a unique localization among *Leishmania* parasites. To summarize the current knowledge on *L.* (*S.*) *tarentolae*, we have also written a review describing the origin, life cycle and application of this *Sauroleishmania* species.

Next, we described the development of two selected *Sauroleishmania* species, *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali*, in various sand flies of the genera *Sergentomyia* and *Phlebotomus*. Herein, we observed that the same *Sauroleishmania* species can undergo both, peripylarion or hypopylarian development, and that this is influenced by the sand fly vector. The susceptibility of *Phlebotomus* spp. to *Sauroleishmania* infection has been clearly demonstrated and we propose that *Phlebotomus* sand flies may play a role as alternative vectors of *Sauroleishmania*.

In addition, we studied the host feeding preferences of *Sergentomyia minuta*, a natural vector of *Sauroleishmania*, and compared it with *Phlebotomus papatasi*. *Sergentomyia minuta* refused to feed on mice and rabbits but was attracted to and fed on a human volunteer. Thus, the anthropophilic behaviour of this species has been experimentally demonstrated, further highlighting its potential involvement in the transmission of human pathogens. Contrarily, *P. papatasi* fed on *Tarentola mauritanica* geckos, supporting the role of this species as alternative vector of *Sauroleishmania*.

Second part of the project focused on *Sauroleishmania* development in reptiles. *Hemidactylus turcicus* geckos have been experimentally infected with *L.* (*S.*) *adleri* or *L.* (*S.*) *hoogstraali* using sand-fly derived parasites and examined using xenodiagnosis. The presence of parasites was not confirmed in any of the infected geckos. Possible explanations might be a wrongly chosen route of infection or loss of infectivity of the *Sauroleishmania* isolates used. Therefore, we studied the infection of wild-caught reptiles in Italy. Blood of various reptiles was examined by PCR and *L.* (*S.*) *tarentolae* DNA was found in *T. mauritanica* geckos. Interestingly, DNA of the human pathogenic *Leishmania* (*Leishmania*) *infantum* and amastigote forms were found in the bone marrow of *T. mauritanica* geckos. The sympatric occurrence of *L.* (*S.*) *tarentolae* and *L.* (*L.*) *infantum* was observed in sand flies and geckos but also in sheltered dogs, which were found serologically positive for both species.

ABSTRAKT

Sauroleishmanie jsou méně zkoumanou skupinou parazitů, kteří náleží do rodu *Leishmania* (Kinetoplastida: Trypanosomatidae). Jejich životní cyklus zahrnuje plazí hostitele a flebotomy (Diptera: Psychodidae). Sauroleishmanie nejsou patogenní pro člověka, a tak je o jejich vývoji ve flebotomech a gekonech známo velmi málo. Hlavním cílem této práce tedy bylo otestovat vnímavost různých druhů flebotomů k sauroleishmaniové infekci a popsat vývoj těchto parazitů ve střevě přenašeče; menší část pak byla věnována výzkumu infekcí v plazech.

Nejprve jsme se zabývali vývojem *Leishmania* (*Sauroleishmania*) *tarentolae* ve třech druzích flebotomů rodu *Phlebotomus*. Flebotomové byli infikováni kulturou promastigotů sáním přes membránu a jejich střeva byla vyšetřována v různých časových intervalech po infekci. *Leishmania* (*S.*) *tarentolae* se vyvíjela ve všech třech zkoumaných druzích, typ vývoje byl peripylární. Kromě toho se parazité v hojném počtu vyskytovali v Malpigických trubicích, což je pro parazity rodu *Leishmania* unikátní lokalizace. Dosavadní poznatky o *L.* (*S.*) *tarentolae* jsme také shrnuli do přehledového článku popisujícího původ, životní cyklus a využití tohoto druhu sauroleishmanie.

Dále jsme popsali vývoj dvou vybraných druhů sauroleishmanií, *L.* (*S.*) *adleri* a *L.* (*S.*) *hoogstraali*, v různých druzích flebotomů rodů *Sergentomyia* a *Phlebotomus*. Ukázalo se, že stejný druh sauroleishmanie může procházet jak peripylárním, tak hypopylárním typem vývoje, a že způsob vývoje je ovlivněn právě druhem přenašeče. Vnímavost zástupců rodu *Phlebotomus* k sauroleishmaniím byla experimentálně prokázána, proto se domníváme, že tito flebotomové tak mohou sloužit jako alternativní přenašeči sauroleishmanií.

Studovali jsme take hostitelské preference flebotomů *Sergentomyia minuta*, kteří jsou přirozenými přenašeči sauroleishmanií, a porovnali je s preferencemi *Phlebotomus papatasi*. *Sergentomyia minuta* odmítla sát na myších a králicích, ale sála na lidském dobrovolníkovi. Antropofilní chování prokázané experimentálně tak svědčí o možném zapojení *S. minuta* do přenosu lidských patogenů. Naproti tomu *P. papatasi* sál na gekonech *Tarentola mauritanica*, což podporuje hypotézu o jeho úloze jako alternativního přenašeče sauroleishmanií.

Druhá část projektu se pak zabývala vývojem sauroleishmanií v plazech. Gekoni *Hemidactylus turcicus* byli experimentálně infikováni promastigoty *L.* (*S.*) *adleri* nebo *L.* (*S.*) *hoogstraali* izolovanými z infikovaných flebotomů, a byli opakovaně vyšetřováni pomocí xenodiagnostiky. Přítomnost parazitů nebyla potvrzena u žádného z nakažených gekonů. Možným vysvětlením může být nevhodně zvolený způsob infekce nebo ztráta infektivity použitých kmenů sauroleishmanií. Proto jsme dále studovali infekce volně žijících plazů v Itálii; krev různých druhů plazů byla vyšetřována pomocí PCR. DNA *L.* (*S.*) *tarentolae* byla zachycena u gekonů *T. mauritanica*. V těchto gekonech však byla navíc nalezena také DNA patogenní *Leishmania* (*L.*) *infantum* a rovněž byly pozorovány amastigotní formy v kostní dřeni gekonů. Sympatrický výskyt *L.* (*S.*) *tarentolae* a *L.* (*L.*) *infantum* byl popsán u flebotomů, gekonů a také u psů, kteří byli sérologicky pozitivní pro oba tyto druhy.

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
dqPCR	Duplex real-time Polymerase Chain Reaction
H.	*Hemidactylus*
IFAT	Immunofluorescence antibody test
L.	*Leishmania*
La.	*Laudakia*
LPG	Lipophosphoglycan
MTs	Malpighian tubules
P.	*Phlebotomus*
PCR	Polymerase Chain Reaction
Po.	*Podarcis*
qPCR	Real-time Polymerase Chain Reaction
S.	*Sauroleishmania*
Se.	*Sergentomyia*
T.	*Tarentola*
TOSV	Toscana virus
Tr.	*Trypanosoma*
WHO	World Health Organisation

INTRODUCTION

1. *Leishmania* and its life cycle

Leishmaniases are a group of neglected tropical diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). The disease itself has several manifestations, ranging from less severe cutaneous leishmaniasis to mucocutaneus or visceral form; the last one may be life-threatening in untreated cases. Over one billion people live in endemic areas and more than one million new cases occur annually, research on leishmaniasis is therefore of great veterinary and medical importance (WHO, 2023).

Leishmania spp. have a digenetic life cycle, circulating between a wide range of vertebrate hosts and blood-feeding insects. Leishmaniases are primarily zoonoses, and humans are thus rather incidental hosts, although purely anthropological cycles have also been described for *Leishmania* (*Leishmania*) *donovani* and *Leishmania* (*Leishmania*) *tropica* in certain areas (reviewed by Bates, 2007). The genus consists of about 50 described species, of which 20 are associated with human diseases. It is currently divided into four subgenera, of which *Leishmania*, *Viannia* and *Mundinia* include species infecting mammals or humans, whilst the subgenus *Sauroleishmania* is traditionally thought to be exclusively associated with reptiles (Killick-Kendrick et al., 1986; reviewed by Akhoundi et al., 2016; Espinosa et al., 2018).

Phlebotomine sand flies (Diptera: Psychodidae) are main vectors of *Leishmania* and among more than 900 described sand fly species, approximately 100 are proven or suspected vectors of *Leishmania* spp. pathogenic to humans (reviewed by Maroli et al., 2013). Medically important are mainly the members of the genera *Phlebotomus* and *Lutzomyia*, which are associated with the transmission of human leishmaniases in the Old World and New World, respectively. However, biting midges (Diptera: Ceratopogonidae) have recently been confirmed to be also involved in the circulation of these parasites, specifically species of the subgenus *Mundinia* (Becvar et al., 2021).

The development of *Leishmania* in sand flies is initiated by ingestion of blood containing intracellular amastigote forms. Shortly after feeding, the ingested blood meal is surrounded by a peritrophic matrix produced by midgut epithelial cells. In the endoperitrophic space, amastigotes transform into extracellular flagellated promastigotes and multiply rapidly. After the disintegration of the peritrophic matrix, promastigotes are released and attach to the surface of the sand fly intestinal tract, particularly to the microvilli of the midgut. Attachment of promastigotes is a specific process involving surface molecules of both, the parasite and the vector. Midgut attachment is a crucial stage in the life cycle of *Leishmania* in vectors, as it prevents the parasites from being expelled from the gut along with blood meal remnants. The detachment of promastigotes is accompanied by their further proliferation and migration into various parts of the sand fly gut (reviewed by Bates, 2007; Dostalova and Volf, 2012)

In mammal-infecting species, promastigotes migrate anteriorly into thoracic part of the midgut and colonize the stomodeal valve. They attach to the chitin lining of the stomodeal valve and disrupt it by chitinase. Damage of the stomodeal valve along with the production of metacyclic stages are essential for successful infection of the vertebrate host. After blood-feeding of infected sand fly female, the metacyclic promastigotes are released into feeding lesion and invade the host blood cells, where they transform into the amastigotes and further replicate (reviewed by Dostalova and Volf, 2012).

The intravectorial development is species-specific process, and three groups of leishmania parasites have been distinguished based on its differences (Lainson and Shaw, 1987). The Suprapylaria (subgenus *Leishmania*) includes mammal-infecting species of the Old

World, whose development takes place in the anterior parts of sand fly intestinal tract. Promastigotes do not colonize the hindgut and develop exclusively in the midgut (**Figure 1**).

The second group Peripylaria consists of mammal-infecting species of the New World (subgenus *Viannia*). After blood meal digestion, during late-stage infections, promastigotes of *Viannia* species develop first in the hindgut. Attachment to the chitin lining of the hindgut is followed by anterior migration in the sand fly gut (midgut and stomodeal valve) (**Figure 1**). In both groups, Suprapylaria and Peripylaria, infection of the vertebrate host occurs during blood-feeding of infected sand fly.

The third group includes reptilian parasites of the subgenus *Sauroleishmania;* they are known as Hypopylaria and their late-stage development is restricted to the sand fly hindgut only (**Figure 1**). Therefore, contaminative mode of infection or ingestion of infected sand flies have been considered as possible modes of *Sauroleishmania* transmission to reptiles.

The subgenus *Mundinia* has been established quite recently and thus its development in natural vectors has not been described in detail yet. However, it seems that it is similar to Suprapylaria as parasites were found exclusively in the midgut of sand flies and *Culicoides* midges (Becvar et al., 2021).

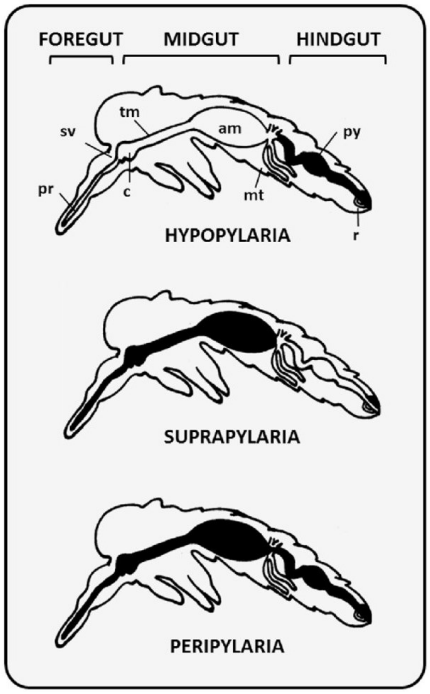

Figure 1. The three *Leishmania* sections Hypopylaria, Suprapylaria and Peripylaria proposed by Lainson and Shaw (1987). The distribution of parasites in the sand fly intestinal tract is shown in black. (pr) proboscis, (sv) stomodeal valve, (c) cardia, (tm) thoracic midgut, (am) abdominal midgut, (mt) Malpighian tubules, (py) pylorus, (r) rectum (adapted from Kaufer et al., 2017).

2. *Sauroleishmania*

2.1 Origin and taxonomic position

First reports of reptiles being carriers of these trypanosomatids occurred already in the early 20th century. Based on their morphological characters, flagellates isolated from reptiles were usually assigned to the genera *Leptomonas* or *Herpetomonas* (Telford, 2009). Amastigote-like cells were observed in the blood of the common wall gecko (*Tarentola mauritanica*) for the first time by Chatton and Blanc (1914); later it was shown that cultures of "leptomonas" can be obtained by cultivation of the blood of these geckos (Chatton and Blanc, 1918). While investigating protozoans of Egyptian lizards, Wenyon (1920) observed new parasite species from the common chameleon (*Chamaeleo chamaeleon*; syn. *Chamaeleon vulgaris*) and described it as *Leishmania chamaeleonis*. The same author also stated that leptomonad forms seen earlier by Chatton and Blanc were in fact *Leishmania* and proposed the name *Leishmania tarentolae* for this species.

In 1973, the term *Sauroleishmania* was first used by Ranque in his Ph.D. thesis (Ranque, 1973). The same name was also proposed few years later by Saf'janova (1982), who suggested to include all reptile-infecting species of *Leishmania* into a separate group. The group itself was first described as a separate genus and the taxonomic position of *Sauroleishmania* has undergone several changes over the years (Killick-Kendrick et al., 1986).

It is generally accepted that dixenous parasites of the genus *Leishmania* emerge from the monoxenous parasites of insects and monophylly of this genus is currently well supported (Harkins et al., 2016; reviewed by Akhoundi et al., 2016; Espinosa et al., 2018). An important milestone in *Leishmania* classification was the system presented by Lainson and Shaw in 1987, who divided these parasites into three groups based on their development in vectors: Suprapylaria (subgenus *Leishmania*), Peripylaria (subgenus *Viannia*) and Hypopylaria (subgenus *Sauroleishmania*) (Lainson and Shaw, 1987), for more details see above. This system was subsequently supported by molecular data and *Sauroleishmania* was placed into genus *Leishmania* as one of its subgenera (Croan et al., 1997; Noyes et al., 1997).

More recently, two major phylogenetic lineages were distinguished: sections Euleishmania and Paraleishmania (Cupolillo et al., 2000), see **Figure 2**. The latter mentioned consists of genera *Endotrypanum* and *Porcisia*, while the section Euleishmania (the true *Leishmania*) comprises of the genus *Leishmania* and its subgenera, i.e. *Leishmania*, *Sauroleishmania*, *Viannia* and *Mundinia* (formerly *Leishmania enriettii* complex) (Harkins et al., 2016; Espinosa et al., 2018; reviewed by Kostygov and Yurchenko, 2017). Although relatively recently described, the subgenus *Mundinia* forms an ancient group taking place at the base of a phylogenetic tree of the genus *Leishmania* (Espinosa et al., 2018). The subgenus *Viannia* subsequently diverges from *Leishmania* and *Sauroleishmania*, which are sister groups (Fraga et al., 2010; Schönian et al., 2018).

The phylogenetic placement of *Sauroleishmania* among the other mammal-infecting subgenera suggests that this group arose through adaptation of these species to reptiles (reviewed by Akhoundi et al., 2016; Schönian et al., 2018). It is relatively species-rich subgenus and currently consists of 21 described species, from which two are still unnamed (reviewed by Akhoundi et al., 2016). Among these, *Leishmania* (*Sauroleishmania*) *tarentolae* was described as a type species (Wenyon et al., 1920; Killick-Kendrick et al., 1986).

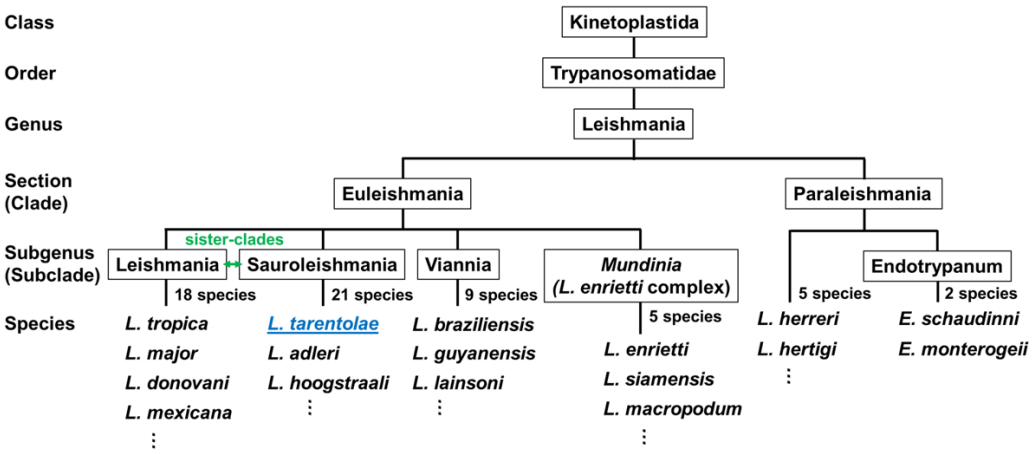

Figure 2. The phylogenetic classification of the genus *Leishmania* (adapted from Klatt et al., 2019).

The distribution of *Sauroleishmania* is restricted to the Old World as it is closely connected with its insect vectors. The group was defined by Killick-Kendrick et al. in 1986 as trypanosomatids of the blood or intestinal tract of reptiles occurring as amastigotes and/or promastigotes. The insect vectors are sand flies of the genus *Sergentomyia*, in which parasites develop as promastigotes. Mechanism of transmission is unclear but assumed to be by bite and/or ingestion of infected sand fly (Killick-Kendrick et al., 1986).

2.2 Development in reptile hosts

Among all *Leishmania* subgenera, *Sauroleishmania* forms a neglected group as they are thought to be associated exclusively with reptiles, being non-pathogenic to mammals, including humans and domestic animals. So far, *Sauroleishmania* spp. have been isolated from various reptilian hosts, mainly lizards and geckos of the families Agamidae, Gekkonidae, Lacertidae, Scincidae and Varanidae (Belova, 1971; reviewed by Wilson and Southgate, 1979). *Sauroleishmania* is confined only to the Old World reptiles and its distribution in the New World has not been reported yet. Various species of lizards from North and Central America, Venezuela and Brazil have been consistently tested negative on blood cultures (Dollahon and Janovy, 1974). The only exception is *Leishmania* (*S.*) *henrici*, which was detected in *Anolis* sp. in Martinique (Garnham, 1971).

There are very few studies describing the development of *Sauroleishmania* in reptiles. It was shown that *Sauroleishmania* spp. can be observed in different tissues within the body of reptilian hosts. The development of *Leishmania* (*S.*) *chamaeleonis* was found to be restricted to the intestine and cloaca of chameleons where parasites occur as promastigotes. *Leishmania* (*S.*) *henrici* develops in the intestine and cloaca of iguanas in promastigote stage but was also found to invade blood stream in small numbers. However, the vast majority of *Sauroleishmania* isolates have been obtained by blood or internal organ cultures (reviewed by Wilson and Southgate, 1979). Infection in reptiles is usually cryptic and until the onset of molecular methods it was hard to detect by means other than cultivation. The detection of parasites on blood smears or tissue impressions has repeatedly been shown to be very difficult and rare (Belova, 1971; Wallbanks, 1982). Intracellular amastigote stages of *Sauroleishmania* are therefore detected scarcely but, unlike mammalian *Leishmania* spp., amastigotes were reported in various types of blood cells, including leukocytes (Elwasila, 1988), monocytes (David, 1929;

Rioux et al., 1969; Edeson and Himo, 1973), erythrocytes (Paperna et al., 2001) and even thrombocytes (Telford, 1979).

At the beginning of the 20th century, amastigotes were observed in the blood of the common wall geckos (*T. mauritanica*) in south Tunisia (Chatton and Blanc, 1914). In France, groups of 3-10 amastigotes were found in monocytes of *T. mauritanica* geckos and the species was identified as *Leishmania* (*S.*) *tarentolae* (Rioux et al., 1969). The same *Sauroleishmania* species was reported in white-spotted wall geckos (*Tarentola annularis*) in Sudan; almost 20 % of reptiles was positive for *Sauroleishmania* and amastigote forms were found in leukocytes in groups of 3-9 (Elwasila, 1988). A single infected monocyte with amastigotes was reported in blood smears of starred agama (*Laudakia stellio*; syn. *Agama stellio*) in Palestine with the new species described as *Leishmania* (*S.*) *agamae* (David, 1929). Similar observations were later confirmed by Edeson and Himo (1973) in Lebanon. The authors noted that although some *La. stellio* specimens were identified as negative on smears, promastigote stages appeared in cultured blood in nearly 30 % of these lizards over time. This further confirms that the blood or internal organ cultivation is the most effective diagnostic method (Belova, 1971; Edeson and Himo, 1973).

In addition to white blood cells, *Sauroleishmania* amastigotes have also been observed in other types of blood cells. *Leishmania* (*S.*) *zuckermani* was found to invade circulating erythrocytes of the Turner's thick-toed geckos (*Chondrodactylus turneri*; syn. *Pachydactylus* turneri) in South Africa. Amastigotes were found singly or in groups of 3-7 within parasitophorus vacuoles (Paperna et al., 2001). Telford (1979) investigated the infection of two *Sauroleishmania* species in the brilliant ground agama (*Trapelus agilis*; syn. *Agama agilis*) and the common wonder geckos (*Teratoscincus scincus*) in Pakistan. In both species, amastigote stages were seen in thrombocytes on peripheral blood smears (Telford, 1979).

Data from a large survey between 1963-1966 monitoring *Sauroleishmania* prevalence in lizards in the Turkmenistan shows that infection rate of reptiles is variable. The highest values were observed in the Caspian bent-toed geckos (*Tenuidactylus caspius*; syn. *Gymnodactylus caspius*) (20-30 %) and steppe agama (*Trapelus sanguinolentus*; syn. *Agama sanguinolenta*) (14,5 %), while it was relatively low (approximately 1 %) in other reptile species tested positive for *Sauroleishmania* (Belova, 1971). The same variability has also been reported by other authors. Relatively high infection rate (19 %) was seen in *T. annularis* geckos in Sudan (Elwasila, 1988), similarly in *T. mauritanica* geckos in Algeria (15,7 %) (Sergent et al., 1914), while only 8 % of *La. stellio* scored positive for *L.* (*S.*) *agamae* in Lebanon (Edeson and Himo, 1973).

It is believed that reptiles may remain infected for their whole life and that infection in lizards is not affected by seasonality. Naturally infected specimens were found in winter and early spring, before the first generation of sand flies has emerged. These findings suggest that reptiles positive prior to sand fly season starts must have been infected before winter or even earlier, showing that minimum duration of parasite survival is at least 5-6 months. Moreover, 20 % of geckos displayed the presence of *Sauroleishmania* spp. in April, which was comparable to the previous year's infection rate at the peak of the season. The hypothesis was also supported by long-term persistence of infections in lizards kept in laboratory conditions, although reptiles were repeatedly tested after relatively short time (i.e., 2 months) (Belova, 1971).

Similarity of *Sauroleishmania* with mammal-infecting *Leishmania* spp. led the first researches in the field to idea that reptiles may serve as a reservoir of the human leishmaniases. Sergent et al. (1914) investigated a zoonotic disease called Biskra boil caused by *Leishmania* spp. in Algeria; the flagellates isolated from *T. mauritanica* geckos were similar to *Leishmania* (*L.*) *tropica* and geckos were therefore considered as natural reservoirs of cutaneous leishmaniasis. Since then, several isolates have been obtained from different reptile species, but

the role of reptiles in the epidemiology of leishmaniasis has not been confirmed (Belova, 1971) and this research area has long been completely overlooked. A significant milestone was the molecular detection of pathogenic *Leishmania* spp. in lizards and snakes from the Northwest China (Chen et al., 2019; Zhang et al., 2019). In addition to reptilian parasites, several mammalian species such as *Leishmania* (*L.*) *turanica*, *L.* (*L.*) *tropica* and *L.* (*L.*) *donovani* complex have been identified among the infected reptiles. Moreover, similar findings were reported in the Mediterranean basin, where *L.* (*S.*) *tarentolae* and *L.* (*L.*) *tropica* occur in sympatry (Mendoza-Roldan et al., 2022a). These findings re-open the debate regarding the role of reptiles in the transmission of mammalian leishmaniases.

2.3 Sand fly vectors

Parasite of the genus *Leishmania* have digenetic life cycle and most species are transmitted to vertebrate hosts by phlebotomine sand flies (Diptera: Psychodidae). In *Sauroleishmania*, the transmission is closely associated with sand flies of the genus *Sergentomyia*, which feed preferentially on cold-blooded vertebrates (Killick-Kendrick et al., 1986). *Sergentomyia* sand flies are widely distributed throughout the Old World, most of them inhabiting tropical areas where species of the genus *Phlebotomus* are relatively scarce. *Sergentomyia* spp. are commonly found in the Afrotropical and Oriental regions, but also in the Mediterranean basin of the Palearctic, and their overall distribution extends across Asia to the Australasian region (reviewed by Akhoundi et al., 2016). The taxonomy of the genus *Sergentomyia* (França and Parrot, 1920) and the morphological identification of species is complicated. The genus currently consists of more than 300 described species, which are divided into ten subgenera (reviewed by Akhoundi et al., 2016).

It is known that *Sergentomyia* spp. can also harbour parasites other than *Sauroleishmania*, mainly trypanosomes of reptiles (Adler and Theodor, 1935; Ashford et al., 1973; Gramiccia et al., 1989). In addition, their role in the transmission of human pathogenic *Leishmania* spp. (reviewed by Maia and Depaquit, 2016) and other human pathogens, like *Toscana phlebovirus* (Charrel et al., 2006), has also been discussed. With the advent of molecular methods, DNA detections of mammal-infecting *Leishmania* spp. in *Sergentomyia* spp. have become increasingly frequent. For instance, *Leishmania* (*L.*) *major* DNA was found in *Se. minuta* in Portugal (Campino et al., 2013) or in *Se. clydei* and *Se. sintoni* in Iran (Parvizi and Amirkhani, 2008; Ayari et al., 2016). *Leishmania* (*L.*) *infantum* DNA was detected in *Se. minuta* in Portugal (Pereira et al., 2017) and Italy (Pombi et al., 2020), while DNA of *Leishmania* (*Leishmania*) *donovani* was reported in *Se. minuta* in Sicily (Abbate et al., 2020) and *Se. squamipleuris* in Kenya (Owino et al., 2021).

Although referred as mostly herpetophilic, it was shown that some members of the genus *Sergentomyia* have broader host range and occasionally feed on various mammals, including humans (Quate, 1964; reviewed by Maia and Depaquit, 2016). Blood meal identification studies have repeatedly described the detection of mammalian blood in many species of this genus. For example, mammalian blood has been detected in *Se. minuta* (Pombi et al., 2020; González et al., 2020; Abbate et al., 2020), *Se. schwetzi* (Quate, 1964; Polanska et al., 2020) *Se. gemmea*, *Se. iyengari* (Sirapattanapipong et al., 2018), *Se. bedfordi* and *Se. adleri* (Quate, 1964). The feeding of *Sergentomyia* spp. on mammals further supports the potential involvement of these sand flies in the transmission of mammalian leishmaniases. However, molecular detection alone is not sufficient to incriminate a sand fly vector and more evidence is needed to better address this issue (reviewed by Maia and Depaquit, 2016).

It should be noted that sand fly feeding is not only dependent on host preferences but is influenced by many environmental, physiological, and ecological factors and thus is not surprising that most sand fly species are considered as opportunists (Quate, 1964; Svobodova et al., 2003; González et al., 2021). This is also confirmed by the fact that although members of the genus *Phlebotomus* feed mainly on mammals, some species have been reported to feed occasionally on reptiles. This behaviour was most studied in *Phlebotomus papatasi* (Adler and Theodor, 1935; Quate, 1964), but has also been observed in other *Phlebotomus* species such as *P. caucasicus*, *P. sergenti* (Adler and Theodor, 1929; Belova, 1971), *P. orientalis* (Quate, 1964), *P. martini* and *P. rodhaini* (Mutinga and Ngoka, 1981). Nevertheless, the potential role of *Phlebotomus* sand flies in the life cycle of *Sauroleishmania* has not been further studied.

The life cycle of *Sauroleishmania* in sand fly vectors has not been elucidated yet (**Figure 3**). The parasites are ingested while blood-feeding of sand fly females and although not described, it probably occurs *via* a pool feeding mechanism, similarly as in mammalian *Leishmania* spp. (reviewed by Bates, 2007). Once ingested into the abdominal midgut and enclosed within peritrophic matrix, amastigotes transform to promastigotes and proliferation of promastigote stages begins. It was reported that *Sergentomyia* spp. produce relatively thick peritrophic matrix compared to sand fly vectors of the genera *Phlebotomus* and *Lutzomyia* (Lawyer et al., 1990; Shatova et al., 1991). Therefore, it has been suggested that the peritrophic matrix prevents the promastigotes from escaping into the ectoperitrophic space, and these are thus passed out into the sand fly hindgut, where they attach to its chitinous lining (reviewed by Bates, 2007). It has also been shown that in *Sergentomyia schwetzi* the time frame between degradation of peritrophic matrix and defecation of the blood meal remnants is relatively short, which may prevent promastigotes from attaching to the midgut microvilli (Sadlova et al., 2018). This type of the *Sauroleishmania* development in sand fly hindgut was described as hypopylarian (Lainson and Shaw,1987).

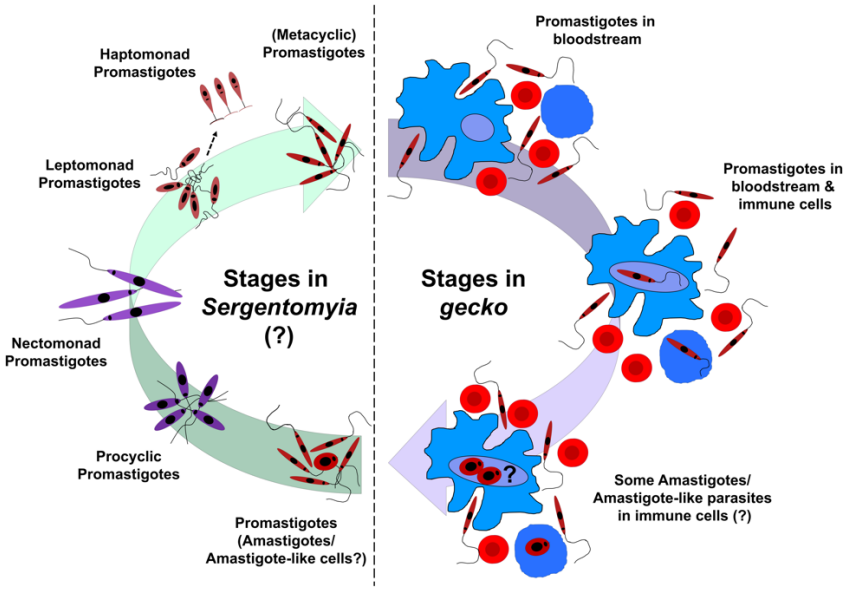

Figure 3. Simplified life cycle of *Sauroleishmania* in sand flies and geckos (adapted from Klatt et al., 2019).

There are very few records of *Sauroleishmania* infections in their natural vectors. Heisch (1958) described a new parasite species from *Latastia* sp. in Kenya named *L*. (*S*.) *adleri* and assumed that *Sergentomyia clydei* (originally described as *Phlebotomus clydei*) is its natural vector. Infections in *Se*. *clydei* took anterior position in the gut, flagellates were present in the midgut and resembled those observed in culture cultivated from lizard blood. Author reported that sand fly females were collected in Kenya, in the same area as infected lizard, and some engorged specimens contained nucleated blood cells. A few decades later, *Leishmania* (*S*.) *adleri*-like DNA was detected in *Se. clydei* in Iran (Oshaghi et al., 2009). A role of *Se. clydei* in the transmission of *L*. (*S*.) *adleri* is supported by finding of DNA of this parasite species in *Se. clydei* females in Mali and Niger (Krüger et al., 2023).

Killick-Kendrick (1979) described natural infections in *Sergentomyia minuta* females collected by prof. J. A. Rioux in France, in the same area where *T. mauritanica* geckos were found to be positive for *L*. (*S*.) *tarentolae* few years earlier (Rioux et al., 1969). Parasite development varied among infected flies and promastigotes were found in various parts of the gut (both midgut and hindgut). Moreover, heavy parasite loads were present also in Malpighian tubules. Killick-Kendrick (1979) stated that although not typical for *Leishmania* parasite, presence of promastigotes in Malpighian tubules may be a part of life cycle of some *Sauroleishmania* species. Another possible explanation of this non-typical development was the confusion of *Sauroleishmania* with monoxenous trypanosomatids such as *Leptomonas*. More recently, *Leishmania* (*S*.) *tarentolae* was isolated from the same sand fly species in Italy, but the development of the parasite has not been further specified (Maroli et al., 1988).

An unspecified promastigote infection was found in *Sergentomyia sintoni* in the northeast of Iran (Nadim et al., 1968), where *Leishmania* (*S*.) *gymnodactyli* was later isolated from the same sand fly species and characterized by isoenzyme method. Almost 15 % of the dissected *Se*. *sintoni* displayed promastigote infection in the midgut (Rashti and Mohebali, 1994). Another unspecified promastigote infections have also been reported in *Se. sintoni* and *Sergentomyia dentata* in the northwest of Iran (Rassi et al., 1997), *Se. sintoni* and *Se. clydei* in the southeast of Iran (Kassiri and Jahanifard, 2012), and in *Sergentomyia sinkiangensis* in China (Zhang and Leng, 1997).

A more detailed description of the development of *Sauroleishmania* in sand flies were provided by studies using xenodiagnosis of infected reptiles. Adler and Theodor (1929) described development of *Leishmania* (*S*.) *ceramodactyli* in colonized females of *P. papatasi* fed on infected gecko *Stenodactylus doriae* (syn. *Ceramodactylus doriae*). Promastigote infection was found in 35 % of females, in most of them heavy parasite loads were observed. Attached forms were found in the hindgut but flagellates were also present in the anterior parts of the gut and reached up to the cardia. These data were then compared with experimental membrane feeding of *P. papatasi* on cultured promastigotes: no significant differences in development were observed, out of 115 sand fly females, 109 became positive (95 %). The parasites mostly occupied the hindgut, where they attached to its chitinous intima. However, *Leishmania* (*S*.) *ceramodactyli* also tended to migrate anteriorly in the gut and occurred in the cardia when infections were heavy (Adler and Theodor, 1929).

In the same study, development of *L*. (*S*.) *tarentolae* in *P. papatasi* was described and compared with *L*. (*S*.) *ceramodactyli*. Both tested *L*. (*S*.) *tarentolae* strains occupied an anterior position in the gut. Promastigotes were present in the midgut and cardia, but no parasites were localized in the hindgut (Adler and Theodor, 1929). Development of *L*. (*S*.) *tarentolae* was also studied in *Se. minuta* (originally described as *Phlebotomus parroti*) fed on *Tarentola mauritanica* gecko co-infected with *Sauroleishmania* and *Trypanosoma platydactyli*. Authors reported that promastigotes of *Sauroleishmania* were found in the midgut and cardia, but it

should be noted that mixed infection was present in all three dissected sand flies (Adler and Theodor, 1935).

Although susceptible to some *Sauroleishmania* species, *Phlebotomus papatasi* did not support the development of *Leishmania* (*S.*) *hoogstraali* described by McMillan (1965) from the Mediterranean house geckos (*Hemidactylus turcicus*). All sand fly females fed on infected gecko were found negative after defecation. Lainson et al. (1977) tested the susceptibility of New World sand fly species, *Lutzomyia longipalpis*, to *L.* (*S.*) *hoogstraali*. Sand flies were fed on suspensions of promastigotes through a membrane, but only a single female (6 %) became positive with just a few apparently damaged flagellates in the midgut and pylorus. Similar results were obtained studying the development of *L.* (*S.*) *gymnodactyli* in experimentally infected females of *Sergentomyia arpaklensis*. Promastigotes multiplied abundantly in the engorged blood meal, but infection was lost after defecation (Shatova et al., 1991).

2.4 The mechanism of *Sauroleishmania* transmission

Formerly, even transovarial transmission in reptiles was considered, but this hypothesis was quickly disproven as the offspring of naturally infected *Te. caspius* female bred in laboratory were all tested negative (Belova, 1971). Currently, it is known that *Sauroleishmania* life cycle is closely connected with sand flies, but the exact mode of transmission from vectors to reptile hosts has never been demonstrated in laboratory conditions and thus is still unclear. It was described that *Sauroleishmania* are transmitted either *via* sand fly bite and/or by ingestion of infected sand fly, depending on the *Sauroleishmania* development in the vectors (Killick-Kendrick et al., 1986).

Some *Sauroleishmania* spp. develop late-stage infections in the sand fly hindgut and thus ingestion of infected sand fly or contaminative way of transmission have been discussed (Killick-Kendrick et al., 1986; reviewed by Bates, 2007). Contrarily, other authors reported anterior migration of some *Sauroleishmania* spp. in the sand fly gut, which suggests that the parasites are transmitted while blood-feeding of infected sand fly female, similarly to mammal-infecting *Leishmania* spp. (Adler and Theodor, 1929; Adler and Theodor, 1935; Rashti and Mohebali, 1994). It is known that *Sauroleishmania* spp. develop in sand flies as promastigotes, but further morphological forms have not been distinguished. Stages infectious to reptiles have not been revealed and it is not clear whether metacyclogenesis and metacyclic stages are present in *Sauroleishmania*, although analogy similar to mammal-infecting *Leishmania* spp. can be assumed (reviewed by Bates, 2007).

Indeed, some authors have succeeded in experimentally infecting reptiles with cultures of *Sauroleishmania* promastigotes. McMillan (1965) infected the Mediterranean house geckos (*H. turcicus*) with *Leishmania* (*S.*) *adleri* by inoculating promastigote culture intraperitoneally into geckos previously tested negative for *Sauroleishmania*. However, authors do not mention any further information on the course of the infection.

Dollahon and Janovy (1974) inoculated intracardially several New World reptile species by promastigote cultures of *L.* (*S.*) *adleri*, *L.* (*S.*) *agamae* or *L.* (*S.*) *tarentolae*. In the brown basilisk (*Basiliscus vittatus*) and the four-lined ameiva (*Holcosus quadrilineatus*; syn. *Ameiva quadrilineata*), *L.* (*S.*) *adleri* was detectable for up to 56 days and 10 days, respectively. *Leishmania* (*S.*) *agamae* survived in the six-lined racerunner (*Aspidoscelis sexlineatus*; syn. *Cnemidophorus sexlineatus*) for up to 10 days and *L.* (*S.*) *tarentolae* could not be established in any of the tested reptiles. Although the parasites survived in the host for several weeks or months, it should be noted that these modes of infection are very far from a natural mechanism.

In addition, relatively large volumes of parasitic suspension have been used which may explain the partial persistence of parasites and their detectability over time.

Nevertheless, most attempts to infect reptiles with *Sauroleishmania* spp. in laboratories have failed. The European green lizards (*Lacerta viridis*) were infected either intraperitoneally, subcutaneously, or orally by suspension of *L*. (*S*.) *agamae* promastigotes. All experimental infections failed, and parasite antigens were found in various tissues examined by an immunoenzyme method (Ingram and Molyneux, 1984a). Similar findings were shown in experiment with the Elmenteita rock agama (*Agama caudospinosa*) conducted by the same authors (Ingram and Molyneux, 1984b).

In conclusion, the above findings may suggest certain host-species specificity of *Sauroleishmania*, but also point to the need to better address this topic.

OBJECTIVES

Although described more than century ago, subgenus *Sauroleishmania* still forms a neglected group of parasites, mainly due to the non-pathogenic character of its species. Little is known about their development in sand fly vectors or reptilian hosts. A reliable laboratory model has not been established and relatively outdated publications on *Sauroleishmania* research often show contradictory results.

This Ph.D. thesis is the result of a long-term research project that builds on my master thesis and aims to fill a gap in knowledge about parasites of the subgenus *Sauroleishmania*. We tested the susceptibility of various sand fly colonies to different *Sauroleishmania* species and described their development in these sand flies. We were particularly interested in the differences between sand flies of the genus *Sergentomyia* and *Phlebotomus* with the focus on localization of the *Sauroleishmania* parasites in the sand fly intestinal tract. The project also marginally dealt with experimental infections of reptiles.

The main objectives of this thesis were:

1. Test the susceptibility of various sand fly species to different *Sauroleishmania* spp. and investigate their potential role in the life cycle of *Sauroleishmania*.

   Laboratory bred sand flies were experimentally infected through a membrane and parasite survival into the late stage of infection has been recorded. Members of the genera *Sergentomyia* and *Phlebotomus* were compared and their ability to feed on reptiles was examined.

2. Describe the development of *Sauroleishmania* spp. in sand flies.

   Sand fly females (*Sergentomyia schwetzi*, *Phlebotomus papatasi*, *P. duboscqi*, *P. sergenti*, *P. orientalis*, *P. argentipes* and *P. perniciosus*) were experimentally infected by cultured promastigotes and dissected at multiple time intervals post blood meal. Development of various *Sauroleishmania* isolates was studied with the focus on localization of parasites in the sand fly intestinal tract and their morphological forms.

3. Describe the development of *Sauroleishmania* spp. in reptiles.

   Geckos were experimentally infected by selected *Sauroleishmania* spp. and their infectiousness to sand flies was tested using xenodiagnosis. Detection of parasites in various tissues was examined. We aimed to describe the development of *Sauroleishmania* in geckos and establish laboratory model for further studies.

LIST OF PUBLICATIONS

Ticha, L., Kykalova, B., Sadlova, J., Gramiccia, M., Gradoni, L. & Volf, P. (2021). Development of various *Leishmania* (*Sauroleishmania*) *tarentolae* strains in three *Phlebotomus* species. *Microorganisms*, *9*(11), 2256.

Mendoza-Roldan, J. A., Votypka, J., Bandi, C., Epis, S., Modry, D., **Ticha, L.**, Volf, P. & Otranto, D. (2022b). *Leishmania tarentolae*: A new frontier in the epidemiology and control of the leishmaniases. *Transboundary and Emerging Diseases*, *69*(5), e1326-e1337.

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Development of various *Leishmania* (*Sauroleishmania*) *tarentolae* strains in three *Phlebotomus* species.

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Article

Development of Various *Leishmania* (*Sauroleishmania*) *tarentolae* Strains in Three *Phlebotomus* Species

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Abstract: *Leishmania* (*Sauroleishmania*) *tarentolae* is transmitted by reptile-biting sand flies of the genus *Sergentomyia*, but the role of *Phlebotomus* sand flies in circulation of this parasite is unknown. Here, we compared the development of *L.* (*S.*) *tarentolae* strains in three *Phlebotomus* species: *P. papatasi*, *P. sergenti*, and *P. perniciosus*. Laboratory-bred sand flies were membrane-fed on blood with parasite suspension and dissected on days 1 and 7 post blood meal. Parasites were measured on Giemsa-stained gut smears and five morphological forms were distinguished. In all parasite-vector combinations, promastigotes were found in Malpighian tubules, often in high numbers, which suggests that this tissue is a typical location for *L.* (*S.*) *tarentolae* development in sand flies. All three studied strains colonized the hindgut, but also migrated anteriorly to both parts of the midgut and colonized the stomodeal valve. Significant differences were demonstrated between sand fly species: highest infection rates, high parasite loads, and the most frequent anterior migration with colonization of the stomodeal valve were found in *P. perniciosus*, while all these parameters were lowest in *P. sergenti*. In conclusion, the peripylarian type of development was demonstrated for three *L.* (*S.*) *tarentolae* strains in three *Phlebotomus* sand flies. We suggest paying more attention to *Phlebotomus* species, particularly *P. perniciosus* and *P. papatasi*, as potential secondary vectors of *Sauroleishmania*.

Keywords: *Sauroleishmania*; *Leishmania tarentolae*; sand flies; *Phlebotomus*; experimental infections

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1. Introduction

Protozoans of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are causative agents of leishmaniases, neglected tropical diseases that affect millions of people worldwide. *Leishmania* parasites have digenetic life cycle, circulating between a wide range of reservoir hosts and phlebotomine sand flies (Diptera: Psychodidae). Members of this genus were recently divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania*, and *Mundinia* [1]. While the subgenera of *Leishmania* and *Viannia* have been intensively studied, little is known about *Sauroleishmania* and their life cycle.

Sauroleishmania was first described as a separate genus in 1973 [2], later the same subgeneric name was used to separate all reptiles-infecting species from those that infect mammals [3]. Subsequently, other species were placed into this genus and its definition was better constituted [4]. The phylogenetic position of *Sauroleishmania* was unclear for a long time, based on recent molecular data, it is now generally accepted that *Sauroleishmania* form a monophyletic group that belongs within the genus *Leishmania* [5]. The subgenera *Leishmania* and *Sauroleishmania* are sister-groups that divided relatively lately, and some studies suggest the hypothesis of the origin of *Sauroleishmania* parasites due to the adaptation of mammalian species to the reptile hosts [5–7]. Currently, 21 species belonging to the subgenus *Sauroleishmania* are described, including two unnamed species [5].

Sauroleishmania has been a neglected group of parasites so far as it was considered as non-pathogenic for mammals. They are regarded as parasites of reptiles and they have been repeatedly isolated from many different reptile species, mainly lizards of the families Agamidae, Gekkonidae, Iguanidae, Lacertidae, and Scincidae [8,9]. However, some members of this subgenus were able to infect, at least transiently, mammals or mammalian cells [10–13]. Recently, DNA of these parasites was detected in asymptomatic rodent [14], canine blood [15], or human blood [16]. These findings are raising questions about the current biosafety level of these parasites [7].

The mechanism of *Sauroleishmania* transmission remains unclear; it is considered that reptiles are infected by sand fly bite and/or by its ingestion [4,17]. Proven natural vectors are reptile-biting sand flies of the genus *Sergentomyia* [4,18]. On the other hand, the role of *Phlebotomus* sand flies in *Sauroleishmania* circulation remains unclear. Some *Phlebotomus* species occasionally feed on reptiles [8,19,20] and old studies reported that some *Sauroleishmania* parasites can develop late-stage infections in *Phlebotomus* sand flies [8,17,21]. Nevertheless, further research in this area is needed. *Sauroleishmania* development in sand flies is localized in the hindgut and thus is described as hypopylarian [22]. However, there are some older records of the anterior migration of these parasites in the sand fly gut [23–25], which indicates that some *Sauroleishmania* parasites might be transmitted by bite via the mechanism known for mammalian species [26].

Leishmania (*S.*) *tarentolae* is the most studied *Sauroleishmania* species. It was first isolated from a common wall gecko, *Tarentola mauritanica* [27–29]. In these geckos, amastigotes were found inside the monocytes [30]. The infection of *L.* (*S.*) *tarentolae* was also discovered in some other gecko species, namely *Cyrtodactylus kotschyi* [31] and *Tarentola annularis*, in which amastigotes were observed inside leucocytes [32]. However, DNA of *L.* (*S.*) *tarentolae* was recently detected in lacertid lizards, *Podarcis siculus* [15], suggesting that geckos might not be exclusive hosts for this *Sauroleishmania* species. In Italy, promastigotes typed as *Leishmania* (*S.*) *tarentolae* were isolated from *Sergentomyia minuta* in Calabria [18] and Rome provinces [33]; in both localities, the infection rates of *Se. minuta* females were relatively high (7.1% and 2.3%, respectively). In addition to *Se. minuta*, two other sand fly species were reported as potential vectors of *L.* (*S.*) *tarentolae*: *Sergentomyia antennata* and *Phlebotomus papatasi* [17,21,23]. Nevertheless, a detailed description of the development in sand flies is absent.

In this study, we compared the development of three *L.* (*S.*) *tarentolae* strains of different origin in three sand fly species of the genus *Phlebotomus*, with the focus on the localization of parasites in the sand fly gut and description of various morphological stages. The potential role of these sand fly species in the transmission of *Sauroleishmania* is also discussed.

2. Materials and Methods

2.1. Parasites and Sand Flies

Three *Leishmania* (*S.*) *tarentolae* strains of different origin were used for the experiments. RTAR/IT/1981/ISS21-G6c isolated from *Tarentola mauritanica* in Apulia and typed by Multilocus Enzyme Electrophoresis (MLEE) [29], RCYR/IT/1981/ISS24-CK3 isolated from *Cyrtodactylus kotschyi* also in Apulia and typed by MLEE [31] and IMIN/IT/2017/ISS3200-RM-5 isolated from *Sergentomyia minuta* in Latium and typed by ribosomal ITS1-PCR RFLP [33,34]. Originally, all strains were isolated in Evans' modified Tobie's medium, quickly cryopreserved after a few subinoculations, and maintained thereafter at the *Leishmania* cryobank of the Istituto Superiore di Sanità, Rome. For this study, recently thawed parasites were cultivated at 23 °C in SNB-9 blood agar [35] with Medium 199 (Sigma-Aldrich, Prague, Czech Republic) as an overlay, supplemented with 20% fetal calf serum (Gibco, Prague, Czech Republic), 1% Basal Medium Eagle vitamins (Sigma-Aldrich, Prague, Czech Republic), 2% sterile urine, and 250 µg/mL amikacin (Amikin, Bristol-Myers Squibb, Prague, Czech Republic). For experimental infections of sand flies, low passage parasites were used, they were washed by centrifugation (2400× *g* for 5 min) and resuspended in sterile saline solution.

Development of different *Leishmania* (*S.*) *tarentolae* strains was studied in three sand fly species occurring in the Mediterranean area. We used laboratory-reared colonies of *Phlebotomus perniciosus* (originating from Spain), *Phlebotomus papatasi*, and *Phlebotomus sergenti* (both originating from Turkey). Sand flies were maintained at 26 °C with a 14 h light/10 h dark photoperiod and fed on 50% sucrose. For a detailed description, see [36].

2.2. Sand Fly Infections

Sand fly females (5–9 days old) were infected by feeding through a chick-skin membrane on heat-inactivated sheep blood (LabMediaServis, Jaromer, Czech Republic). Based on preliminary experiments, the infectious dose was set to 5×10^6 promastigotes per 1 mL. Engorged females were separated and maintained under the same conditions as the colonies. Dissections were performed at two time-intervals post blood meal (PBM): on day 1 PBM (before defecation, an early stage of infection) to confirm the experimental blood feeding was successful, and on day 7 PBM (several days after defecation, a late stage of infection). The abundance of parasites and their localization in the sand fly gut were examined under the light microscope. The infections were graded as light (<100 parasites per gut), moderate (100–1000 parasites per gut) and heavy (>1000 parasites per gut), as described previously [37]. All experiments were repeated at least twice for each *Sauroleishmania* strain–sand fly combination. Gut smears of infected sand flies were prepared for morphological forms determination. Differences in intensities of infections were tested by Chi-square test using the software SPSS version 23.

2.3. Morphometry of Parasites

Smears of sand fly guts infected by different *Leishmania* (*S.*) *tarentolae* strains were prepared on day 7 post blood meal. Morphometry of promastigotes on day 1 PBM was not performed as the numbers of parasites were too low. Gut smears were fixed with methanol, stained with Giemsa, and examined under the light microscope with an oil-immersion objective. Parasites were photographed with an Olympus D70 camera and measured using ImageJ software. Body length, body width, and flagellar length of 150 randomly selected promastigotes from at least three different sand flies were recorded (for each *Sauroleishmania* strain–sand fly combination). Criteria for morphological forms published by previous authors were modified [21,38,39] and following morphological stages were determined: (i) long nectomonads: body length ≥14 µm; (ii) short nectomonads: body length <14 µm and flagellar length <2 times body length; (iii) metacyclic promastigotes: body length <14 µm and flagellar length ≥2 times body length; (iv) rounded paramastigotes; and (v) haptomonads.

3. Results

Development of three *L.* (*S.*) *tarentolae* strains was followed from day 1 to 7 post blood meal (PBM) and all strains showed similar results. On the other hand, *Leishmania* development significantly differed between sand fly species on day 7 PBM (for detailed statistics, see Table S1).

3.1. Development of L. (S.) tarentolae in P. papatasi

In total, 208 *P. papatasi* females were dissected. On day 1 PBM, the infection rates ranged from 70–100% for individual *Sauroleishmania* strains. Parasites grew slowly at the beginning and only light or moderate infections were recorded (Figure 1). Promastigotes were present in the endoperitrophic space, inside ingested blood meal surrounded by peritrophic matrix.

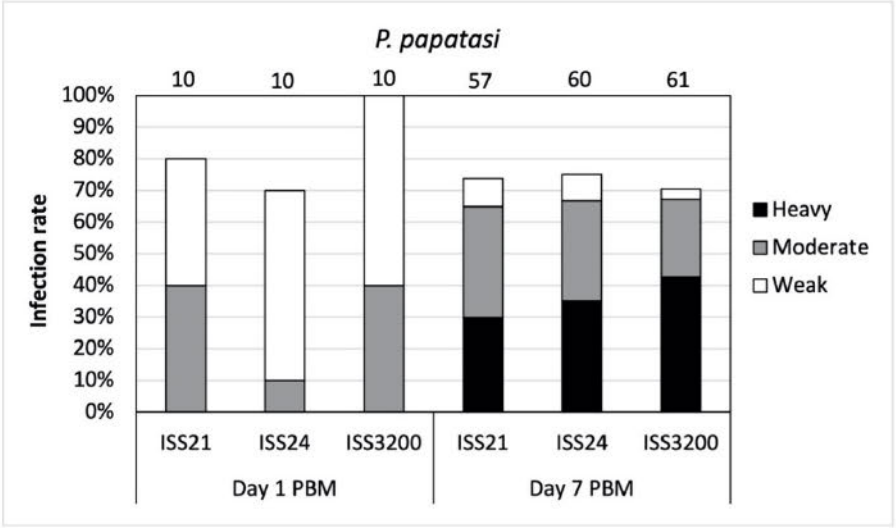


Figure 1. Infection rates and intensities of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus papatasi* on days 1 and 7 post blood meal (PBM). Intensities of infections were classified into three categories: light (<100 parasites/gut), moderate (100–1000 parasites/gut), and heavy (>1000 parasites/gut). Numbers of dissected sand fly females are given above the columns.

On day 7 PBM, fully developed late-stage infections were observed in all three *L.* (*S.*) *tarentolae* strains. Infection rates reached about 70% for each strain and the majority of infections were either heavy or moderate (Figure 1). Statistical differences in the intensities of infections among strains were non-significant ($X^2 = 4.389$, df = 6, $p = 0.624$).

In the majority (55%) of infected *Phlebotomus papatasi* females, *Sauroleishmania* underwent peripylarian development; promastigotes colonized the hindgut and spread anteriorly to the midgut (Figure 2). Tendency towards an anterior position in the midgut was quite frequent and the stomodeal valve was successfully colonized in 8% of infected females. In the hindgut, both attached (haptomonads) and free-swimming forms (nectomonads) were observed along the entire length of the hindgut. Promastigotes were frequently found also in Malpighian tubules (98% of infected females, see Figure 2) and often were present there in high numbers.

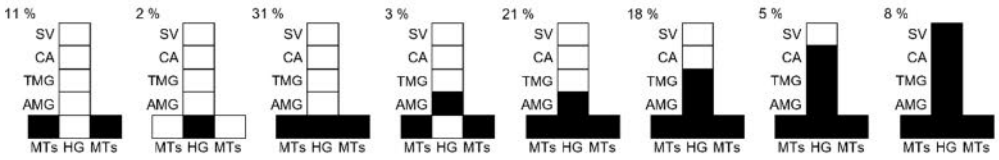


Figure 2. Localization of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus papatasi* on day 7 post blood meal. HG, hindgut; MTs, Malpighian tubules; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. Percent distribution of localization patterns among the infected females is shown in the top left of each stylized diagram. Only localizations present in more than 1% of females are depicted; for more details about each *Sauroleishmania* strain, see Figure S1.

3.2. Development of L. (S.) tarentolae in P. sergenti

In total, 205 *P. sergenti* females were examined for *Sauroleishmania* infections. On day 1 PBM, high infection rates (80–90%) were observed in all three *Sauroleishmania* strains (Figure 3). Nevertheless, the majority of infections were low or moderate, similarly to *Phlebotomus papatasi*. All parasites were localized within the endoperitrophic space.

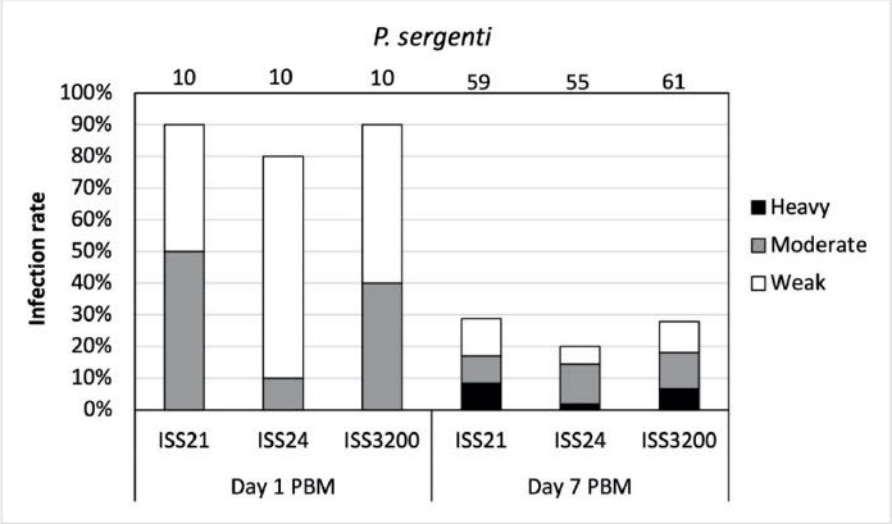


Figure 3. Infection rates and intensities of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus sergenti* on day 1 and day 7 post blood meal (PBM). Intensities of infections were classified into three categories: light (<100 parasites/gut), moderate (100–1000 parasites/gut), and heavy (>1000 parasites/gut). Numbers of dissected sand fly females are given above the columns.

The development of *L.* (*S.*) *tarentolae* in *P. sergenti* was less successful after defecation as infection rates were less than 30% on day 7 PBM. The intensities of infections were mostly light or moderate (Figure 3) and differences among the *Sauroleishmania* strains were not significant ($X^2 = 5.822$, df = 6, $p = 0.443$).

In *P. sergenti* females, most parasites were localized in the Malpighian tubules and the hindgut: 81% of infected females had parasites limited to these two tissues (hypopylarian type of development). Both attached and free forms occurred along the hindgut. In contrast to *P. papatasi*, tendency to anterior migration was lower and the peripylarian type of development was recorded only in 18% of infected females: flagellates were observed in abdominal and thoracic parts of the midgut, but never colonized the cardia or the stomodeal valve (Figure 4).

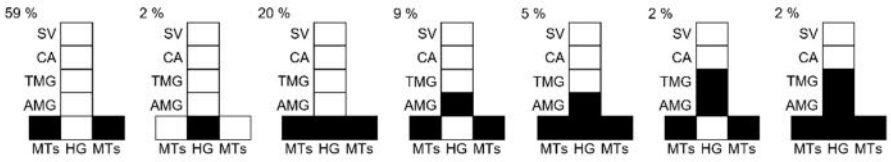


Figure 4. Summarized localization of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus sergenti* on day 7 post blood meal. HG, hindgut; MTs, Malpighian tubules; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. Percent distribution of localization patterns among the infected females is shown in the top left of each stylized diagram. Only localizations present in more than 1% of females are depicted; for more details about each *Sauroleishmania* strain, see Figure S2.

3.3. Development of L. (S.) tarentolae in P. perniciosus

In total, 203 *P. perniciosus* females were dissected and examined for *Sauroleishmania* infection. On day 1 PBM, the percentage of infected sand flies was relatively high (70–90%) in all *Sauroleishmania* strains. Majority of the infections were light (Figure 5) with all parasites presented in bloodmeal enclosed by the peritrophic matrix.

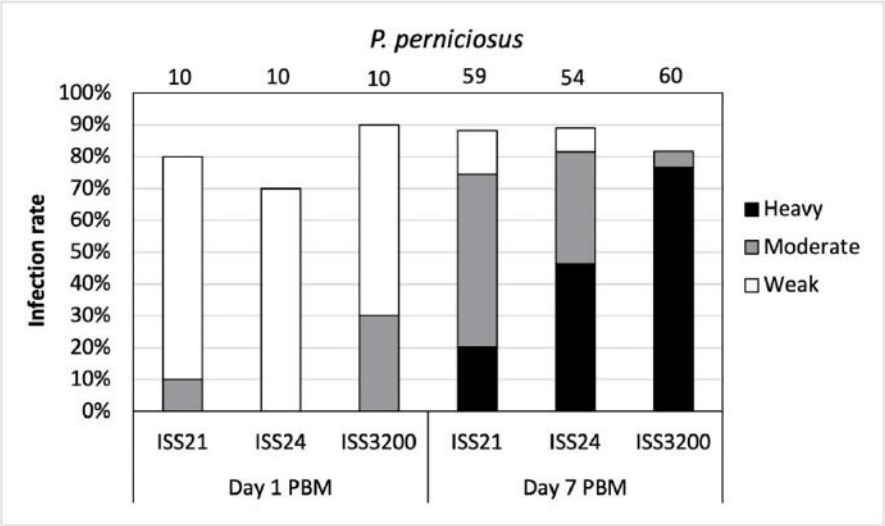


Figure 5. Infection rates and intensities of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus perniciosus* on day 1 and day 7 post blood meal (PBM). Intensities of infections were classified into three categories: light (<100 parasites/gut), moderate (100–1000 parasites/gut), and heavy (>1000 parasites/gut). Number of dissected sand fly females are given above the columns.

On day 7 PBM, late-stage infections developed in 80–90% of *P. perniciosus*, most of them were moderate or heavy (in all three *Sauroleishmania* strains). Differences in intensities of infections among strains were significant ($X^2 = 52.459$, df = 6, $p = 0.000$). In *L.* (*S.*) *tarentolae* strain ISS3200, almost 80% of heavy infections was recorded (Figure 5).

In *P. perniciosus*, promastigotes often occupied hindgut, Malpighian tubules, and midgut; their anterior migration was more frequent than in the other two sand fly species tested. Peripylarian type of development clearly prevailed in *P. perniciosus*: in two thirds of infected females, parasites were localized in the midgut, and colonization of the stomodeal valve was observed in 19% of females (Figures 6 and 7a). Malpighian tubules were colonized in all infected sand flies (Figure 6) and mostly harbored heavy parasite loads (Figure 7b). In the hindgut, we distinguished both attached and free forms (similarly to *P. papatasi* and *P. sergenti*).

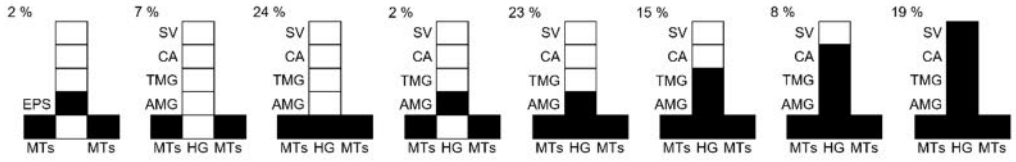
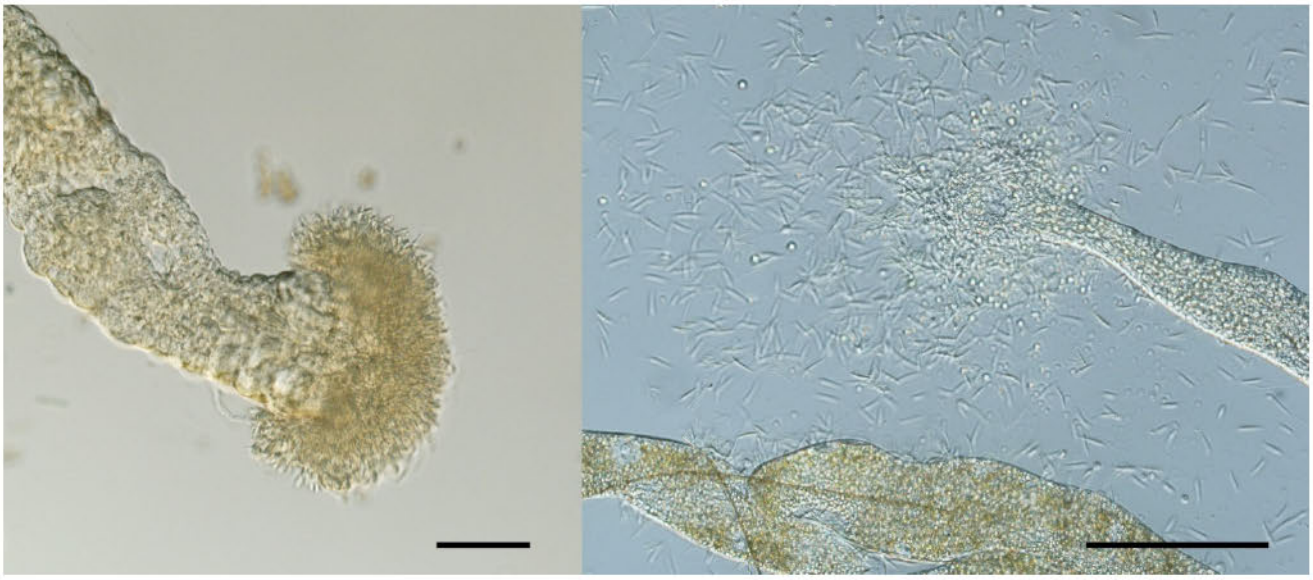


Figure 6. Summarized localization of three *Leishmania* (*S.*) *tarentolae* strains in *Phlebotomus perniciosus* on day 7 post blood meal. EPS, endoperitrophic space; HG, hindgut; MTs, Malpighian tubules; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. Percent distribution of localization patterns among the infected females is shown in the top left of each stylized diagram. Only localizations present in more than 1% of females are depicted; for more details about each *Sauroleishmania* strain, see Figure S3.



(**a**) (**b**)

Figure 7. *Phlebotomus perniciosus* infected with *Leishmania* (*S.*) *tarentolae* strain ISS3200 on day 7 post blood meal: (**a**) colonization of stomodeal valve (Nomarski interference contrast, 100× magnification); (**b**) presence of flagellates in Malpighian tubules (Nomarski interference contrast, 200× magnification). Scale bar = 100 µm.

3.4. Morphometry of Promastigotes from Gut Smears

Sauroleishmania morphological forms were studied on day 7 post blood meal in all three sand fly species and compared with those from the culture. In total, 1350 promastigotes from sand fly guts and 450 promastigotes from cultures were photographed, measured, and five morphological forms were distinguished: elongated nectomonads, short nectomonads, metacyclic promastigotes, rounded paramastigotes, and haptomonads. All these forms were seen moving in native preparations from sand fly guts or the cultures and therefore we considered them as typical *Sauroleishmania* developmental stages. For detailed morphometry of individual forms, see the Supplementary Materials (Table S2). The spectrum of morphological forms produced by *L.* (*S.*) *tarentolae* in *Phlebotomus* sand flies is shown in Figure 8.

In the cultures (seven days old, stationary phase of growth), only three types of flagellates were present: elongated nectomonads (16%), short nectomonads, which represented the prevailing forms (64%), and metacyclic promastigotes (20%).

In the sand fly gut, all five types of promastigotes were found. Most prevailing forms were short nectomonads (59%) and elongated nectomonads (26%). Both of these forms were also observed in a variation with significantly shortened flagella, whose average length was around 4.6 µm. The body length of elongated nectomonads was variable and in some parasite cells reached up to 29 µm. The metacyclic promastigotes (14%) were observed in all *Sauroleishmania* strain–sand fly combinations (Table 1) and they appeared in two types, with the short thin body or as rounded metacyclics. Paramastigotes were another morphological form present in the sand fly gut. These forms have small rounded body (~5 µm by 4.5 µm) and very short flagella (~1.2 µm) with the kinetoplast beside the nucleus. Paramastigotes were found in all three sand fly species, but in very low numbers (less than 2% for each). Haptomonads were the least abundant forms found (less than 1%) as they are strongly attached to the cuticular lining of the gut and thus it is hard to detect them on gut smears.

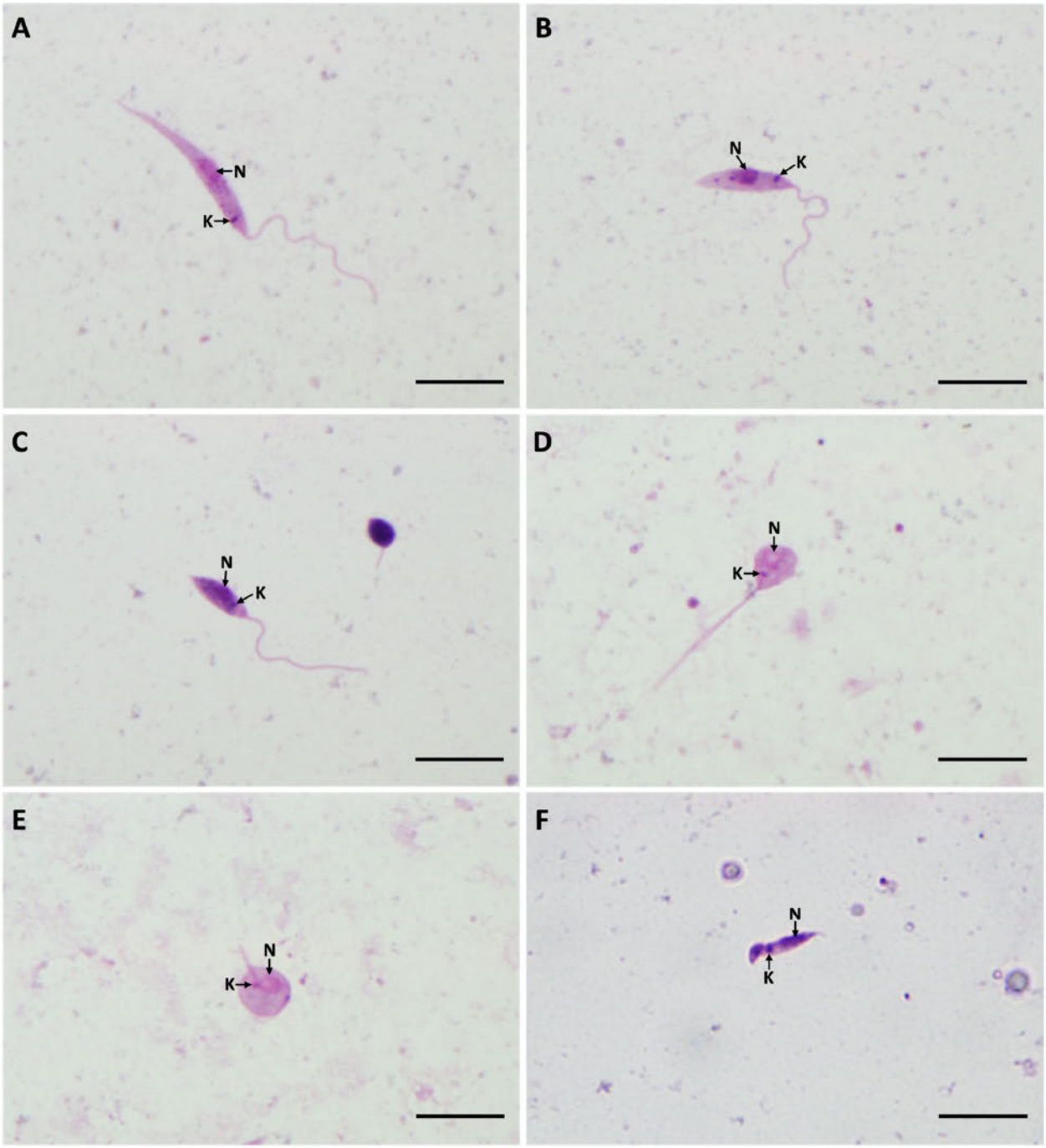


Figure 8. *Leishmania* (*S.*) *tarentolae* morphological forms in *Phlebotomus* sand flies: (**A**) elongated nectomonad; (**B**) short nectomonad; (**C**) metacyclic promastigote; (**D**) rounded metacyclic promastigote; (**E**) rounded paramastigote; (**F**) haptomonad. N, nucleus; K, kinetoplast (stained by Giemsa, 1000× magnification, scale bar = 10 µm).

Table 1. Proportion of metacyclic forms developing in culture and in sand flies.

Sauroleishmania Strain	Culture	*P. papatasi*	*P. sergenti*	*P. perniciosus*
ISS21	18%	15%	15%	16%
ISS24	21%	12%	12%	12%
ISS3200	22%	11%	15%	16%

4. Discussion

Over the last decades, *Leishmania* (*Sauroleishmania*) *tarentolae* has been commonly used as a model organism due to its biosafety level and easy cultivation of laboratory-adopted strains. This parasite species has made a significant contribution to the study of kinetoplast DNA (kDNA) and RNA editing [40–42], it has been used to express human recombinant proteins, and it is also considered for application in the immunotherapy of mammalian leishmaniases [7,11,12,43,44]. In contrast, basic aspects of its development in geckos and sand flies still remain unclear. In this study, we compared the development of various *L.* (*S.*) *tarentolae* isolates in three sand fly species of the genus *Phlebotomus* and demonstrated significant differences in susceptibility of these sand fly species. We are aware that for a better understanding of the *Sauroleishmania* life cycle, it would be important to study the development of *L.* (*S.*) *tarentolae* in its natural vector, *Sergentomyia minuta* [18,33]. A colony of *Se. minuta* has recently been established in our laboratory in Prague. Unfortunately, this species is not willing to feed experimentally through any type of membrane (both chicken and gecko skins were tested) and we assume that feeding on infected geckos might be the only possibility how to infect these sand flies.

Phlebotomus (*Phlebotomus*) *papatasi* is a common species occurring in the Mediterranean area. It is a specific vector of *Leishmania major* and *Leishmania turanica* [45,46], but is also considered to be potential vector of some *Sauroleishmania* species [17]. Our experiments revealed that all three *L.* (*S.*) *tarentolae* strains were able to develop late-stage infections in this sand fly species. The infection rates were relatively high (about 70% for each strain) with the majority of heavy or moderate parasite loads. *Sauroleishmania* had peripylarian development in the majority of infected females and the stomodeal valve was colonized in 8% of females. These findings are in the agreement with the old study comparing the development of two *L.* (*S.*) *tarentolae* strains in *P. papatasi*: in both strains tested, parasites were seen in anterior midgut on days 3 to 5 post infection [21]. Although *P. papatasi* feeds primarily on mammals, several studies on host preferences have reported this species being an opportunistic feeder [47,48]. In addition, the ability of *P. papatasi* to feed on cold-blooded vertebrates has been repeatedly demonstrated [8,21,49]. Thus, we confirm that these sand flies may play a role in *Sauroleishmania* transmission.

Phlebotomus (*Paraphlebotomus*) *sergenti* is a specific vector of *Leishmania tropica* [50] in the Middle East and Maghreb area. According to our study, the development of *L.* (*S.*) *tarentolae* was less successful in this sand fly species, as most of the infections were lost after defecation. In all *Sauroleishmania* strains, less than 30% of dissected females were positive on day 7 post blood meal (PBM) with the majority of weak and moderate infections. In some *P. sergenti* females, *Sauroleishmania* underwent a peripylarian type of development, but in majority they were limited to Malpighian tubules and hindgut. The low tendency of anterior migration in *P. sergenti* might be due to the inability of parasites to attach to *P. sergenti* midgut epithelium as this species is known to be a specific vector of *L. tropica* [50]. These findings suggest that *P. sergenti* is not a very suitable host for our *L.* (*S.*) *tarentolae* strains. Although *P. sergenti* is considered to be an opportunistic species [47,51] and its ability to feed on geckos has been also reported [21], our results suggest that this sand fly species is unlikely to serve as a *L.* (*S.*) *tarentolae* vector.

Phlebotomus (*Larroussius*) *perniciosus* is an abundant sand fly species in the western part of the Mediterranean area, a major vector of *Leishmania infantum* [52], and under laboratory conditions it is permissive for several *Leishmania* species [53]. In *P. perniciosus*, high infection rates (80–90%) were observed on day 7 PBM in all three *L.* (*S.*) *tarentolae* strains. Most infections were of moderate or heavy intensity, parasites colonized the midgut in two thirds of infected females and 19% of them colonized the stomodeal valve. Our results showed that *P. perniciosus* was highly susceptible for all three *L.* (*S.*) *tarentolae* strains studied. Although several studies have reported the broad host range of *P. perniciosus* [54–56], its willingness to feed on geckos has not been recorded yet. *Leishmania* promastigotes isolated from *P. perniciosus*, even in females collected in Mediterranean sites where *Se. minuta* and *L.* (*S.*) *tarentolae* are sympatric, were repeatedly typed as *L. infantum*, but

never as *L.* (*S.*) *tarentolae* [18,52,55,57,58]. However, DNA of *L.* (*S.*) *tarentolae* has recently been detected in *P. perniciosus* [15,59] and in another member of subgenus *Larroussius*, *Phlebotomus perfiliewi* [16]. This suggests that more attention should be given to *Larroussius* species as potential secondary vectors of *L.* (*S.*) *tarentolae*.

Members of the subgenus *Sauroleishmania* are traditionally classified as Hypopylaria and their development is localized in the hindgut [22]. Our results showed that in all three *Phlebotomus* species studied, *L.* (*S.*) *tarentolae* occupied both posterior and anterior parts of the midgut and thus had a peripylarian type of development. Peripylarian development prevailed in *P. papatasi* and *P. perniciosus* while it was less frequent in *P. sergenti*. The anterior migration of this *Sauroleishmania* species was also recorded in females of *Sergentomyia minuta* experimentally infected by feeding on gecko *Tarentola mauritanica*, which was positive for mixed infections of *L.* (*S.*) *tarentolae* and *Trypanosoma platydactyli* [17,23].

The occurrence of *L.* (*S.*) *tarentolae* in Malpighian tubules was surprising, as this behavior is more typical for *Endotrypanum* and monoxenous trypanosomatids [60]. In our study, Malpighian tubules (MTs) were colonized in all parasite-vector combinations and often by heavy parasite loads: promastigotes were densely packed in the lumen of MTs. Simultaneous presence of flagellates in blood meal and MTs in few partially defecated females on day 7 PBM suggests that *L.* (*S.*) *tarentolae* enters MTs immediately after the peritrophic matrix is broken. Thus, we suppose that MTs represent the main location for these parasites. Previously, the infection of promastigotes in MTs was recorded only in five specimens of *Se. minuta* in the south of France [25]. It was assumed that these sand flies were infected by *L.* (*S.*) *tarentolae*, as this parasite was isolated from geckos *T. mauritanica* in the same area [30] and infected *Se. minuta* females were collected nearby drainage holes in stone walls where the geckos lived [25]. Our results suggest that the colonization of MTs is a regular part of the life cycle for at least some *Sauroleishmania* species.

There are only few records of *Sauroleishmania* morphological forms in the available literature [21,24,49]. Within this study, we distinguished five morphological forms. On day 7 PBM, the most prevailing forms were elongated nectomonads and short nectomonads. Furthermore, we also observed stages that were morphologically determined as metacyclic promastigotes. In mammalian species, these stages are highly infective for the vertebrate hosts, and they could be distinguished both morphologically and biochemically, as the surface lipophosphoglycan (LPG) is highly modified during the process of metacyclogenesis [61]. However, it should be noted that metacyclogenesis has never been described in *Sauroleishmania* and it is not clear if these forms are infectious for the reptiles. Other less abundant forms we observed were haptomonads and small rounded cells with very short flagella classified as paramastigotes. These rounded forms were previously seen in *P. papatasi* experimentally infected with *L.* (*S.*) *ceramodactyli* [21], and therefore we considered them as typical stages of *Sauroleishmania* life cycle in their vectors. As *Sauroleishmania* developmental stages have not been described previously, there is no evidence of the individual forms' infectivity and further research in this area is needed [26].

Some authors have also described another morphological form typical for *Sauroleishmania*, so-called "fisherman's floats" [24,49]. These stages have an expanded body in the nucleus area and noticeably attenuated posterior ends. However, "fisherman's floats" have not been defined in more detail and judging by the authors' drawings, they are very variable. In our morphological analysis, we sometimes noticed the forms that were quite similar to the "fisherman's floats". Nevertheless, we decided not to record them as a specific category as these cells were quite variable and often damaged. Moreover, we did not observe any "fisherman's floats" in the native preparations and thus we cannot exclude the possibility that forms described on smears by previous authors [24,49] were artifacts resulting from gut smear preparations.

5. Conclusions

- We demonstrated the ability of *L.* (*S.*) *tarentolae* to develop in the midgut of three *Phlebotomus* sand flies. The most permissive to parasite development was *P. perniciosus* and we suggest that this species, together with *P. papatasi*, could be involved in *L.* (*S.*) *tarentolae* circulation and should be considered as potential secondary vectors of this parasite.
- *L.* (*S.*) *tarentolae* was frequently found in anterior midgut and stomodeal valve of infected sand fly females, which challenges previous definition of its hypopylarian development (limited to hindgut only). Interestingly, heavy parasite loads were frequently found in Malpighian tubules, which suggests that this localization, found previously in monoxenous *Crithidia* parasites, is unique among *Leishmania* but typical for *L.* (*S.*) *tarentolae* development in *Phlebotomus* sand flies.
- For better understanding of *Sauroleishmania* life cycle, its morphological forms, and localization in the sand fly gut, it would be important to study the development of *L.* (*S.*) *tarentolae* in its proven natural vector *Sergentomyia minuta*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9112256/s1, Table S1: Comparison of intensities of late-stage infections between three sand fly species. Figure S1: Detailed localization of three *Leishmania* (*S.*) *tarentolae* strains (ISS21, ISS24, and ISS3200) in *Phlebotomus papatasi* on day 7 post blood meal. Figure S2: Detailed localization of three *Leishmania* (*S.*) *tarentolae* strains (ISS21, ISS24, and ISS3200) in *Phlebotomus sergenti* on day 7 post blood meal. Figure S3: Detailed localization of three *Leishmania* (*S.*) *tarentolae* strains (ISS21, ISS24, and ISS3200) in *Phlebotomus perniciosus* on day 7 post blood meal. Table S2: Detailed morphometry in microns of individual forms of *Leishmania* (*S.*) *tarentolae*.

Author Contributions: Conceptualization, P.V. and L.T.; Methodology, P.V., L.T., M.G. and L.G.; Formal analysis, J.S.; Investigation, L.T. and B.K.; Writing—original draft preparation, L.T. and P.V.; Writing—review and editing, P.V., J.S., M.G. and L.G.; Supervision, P.V.; Funding acquisition, L.T. and P.V. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Leishmania tarentolae: A new frontier in the epidemiology and control of the leishmaniases.

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REVIEW

Leishmania tarentolae: A new frontier in the epidemiology and control of the leishmaniases

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Abstract

Leishmaniasis (or the leishmaniases), classified as a neglected tropical parasitic disease, is found in parts of the tropics, subtropics and southern Europe. *Leishmania* parasites are transmitted by the bite of phlebotomine sand flies and million cases of human infection occur annually. *Leishmania tarentolae* has been historically considered a non-pathogenic protozoan of reptiles, which has been studied mainly for its potential biotechnological applications. However, some strains of *L. tarentolae* appear to be transiently infective to mammals. In areas where leishmaniasis is endemic, recent molecular diagnostics and serological positivity to *L. tarentolae* in humans and dogs have spurred interest in the interactions between these mammalian hosts, reptiles and *Leishmania infantum*, the main aetiologic agent of human and canine leishmaniasis. In this review, we discuss the systematics and biology of *L. tarentolae* in the insect vectors and the vertebrate hosts and address questions about evolution of reptilian leishmaniae. Furthermore, we discuss the possible usefulness of *L. tarentolae* for new vaccination strategies.

KEYWORDS
leishmaniasis/leishmaniases, *Leishmania infantum*, *Leishmania tarentolae*, *Sauroleishmania*, *Sergentomyia*, vaccine

1 | THE STORY OF TWO SISTERS: REPTILIAN AND MAMMALIAN LEISHMANIAS

Trypanosomatids of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) are responsible for a significant health burden to mammals, including humans, in many tropical, subtropical and temperate regions, with 20 *Leishmania* spp. associated with human diseases (Okwor & Uzonna, 2016; Otranto & Dantas-Torres, 2013). For example, zoonotic visceral leishmaniasis caused by *Leishmania infantum* is a neglected disease of medical and veterinary importance worldwide, with the agent being transmitted by sand flies of the genera *Phlebotomus* in the Old World (Maroli et al., 2013) and *Lutzomyia* in the New World (Dantas-Torres et al., 2012).

A group of 21 less-studied leishmaniae, belonging to *Sauroleishmania*, is usually associated with sand flies of the genus *Sergentomyia*, which have long been considered to feed primarily on cold-blooded vertebrates (Akhoundi et al., 2016). Among them, *Leishmania* (subgenus *Sauroleishmania*) *tarentolae* was described from the gecko *Tarentola mauritanica* in Europe, North Africa and the Middle East (Telford, 2009). Although it has long been considered non-pathogenic and specific to its reptilian hosts, some strains of *L. tarentolae* (e.g., the strain LEM-125) were shown under laboratory conditions to cause transient infections in mammalian cells, differentiating into the amastigote stage, but not efficiently replicating within mammalian macrophages (Adler, 1962; Breton et al., 2005; Novo et al., 2015; Taylor et al., 2010). However, the unexpected detection of *L. tarentolae* in a mummy (Novo et al., 2015) and in human blood (Iatta et al., 2021; Pombi et al., 2020) triggered further investigations of the role of this trypanosomatid in the context of the leishmaniases and their control. Other members of the subgenus *Sauroleishmania*, such as *Leishmania adleri*, have also been associated with cutaneous leishmaniasis in humans (Coughlan et al., 2017; Manson-Bahr & Heisch, 1961), reflecting the understudied status of *Sauroleishmania*. Moreover, understanding the biology of *L. tarentolae* is highly relevant, given the myriad of applications in biotechnology due to (i) apparent absence of pathogenicity for humans and other mammals, (ii) easy and inexpensive cultivation and (iii) robustness as a platform for the production of recombinant proteins (Klatt et al., 2019; Niimi, 2012). For example, *L. tarentolae* exhibits mammalian-like post-translational modifications, which makes it a useful source for expressing functional mammalian antibody fragments and human glycoproteins (Jørgensen et al., 2014; Klatt & Konthur, 2012), such as N-glycans erythropoietin (Cantacessi et al., 2015) and amyloid precursor protein alpha (Klatt et al., 2013). Importantly, the finding of *L. tarentolae* in dogs, reptiles (i.e., both geckos and lizards), sand flies and humans in the same area where *L. infantum* is endemic (2021aIatta et al., 2021; Mendoza-Roldan et al., 2021, 2022; Pombi et al., 2020) opens many questions about the interactions between both trypanosomatid flagellates, potentially offering new opportunities for vaccines and/or immune-protection strategies to control canine and human leishmaniases. This review provides a comprehensive account of the main features of *L. tarentolae* systematics, phylogenetics and evolution, along with its biology in the insect vectors and the vertebrate hosts.

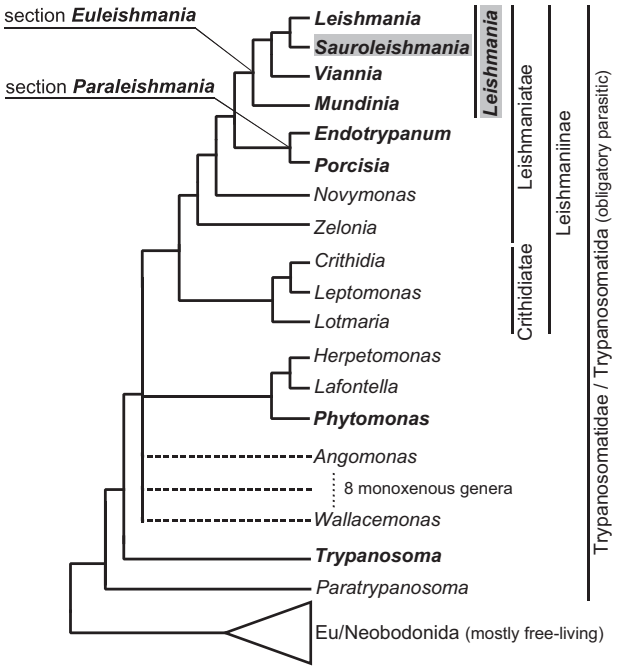

FIGURE 1 A schematized tree summarizing multiple phylogenetic reconstructions, mostly 18S rRNA gene-based and showing relationships been monoxenous and heteroxenous (in bold) trypanosomatids and between *Leishmania* four subgenera

2 | ORIGIN, EVOLUTION AND SYSTEMATICS OF SAURIAN-ASSOCIATED *LEISHMANIA*

The genus *Trypanosoma* has long been considered the most basal trypanosomatid branch, supporting the dixenous origin of this family. However, the branching of the recently described monoxenous flagellate *Paratrypanosoma confusum* between free-living bodonids and parasitic trypanosomatids (Flegontov et al., 2013), favours the insect-first scenario, in which the ancestral flagellate first invaded insects, and then only subsequently colonized vertebrate hosts, probably through blood feeding (Lukeš et al., 2018). The derived dixenous lifestyle evolved from the monoxenous one several times independently, initially in *Trypanosoma* and later on in the two-host genera *Leishmania* and *Phytomonas*, which are phylogenetically nested within the monoxenous trypanosomatids (Lukeš et al., 2014; Lukeš et al., 2014, 2018; Maslov et al., 2013).

Despite the fact that *Leishmania* spp. have been intensively studied, there are many open questions regarding their taxonomy and phylogeny. Both concepts recently underwent substantial changes described below (Cupolillo et al., 2000; Espinosa et al., 2018; Harkins et al., 2016; Klatt et al., 2019; Kostygov & Yurchenko, 2017; Kostygov, et al., 2021). All *Leishmania* spp. belong to the subfamily Leishmaniinae within the family Trypanosomatidae in the order Trypanosomatida (Figure 1). On closer examination, leishmaniae are grouped together with the newly described monoxenous genera *Novymonas*, *Borovskyia* and *Zelonia* in the infrafamily Leishmaniatae, while two established and

species-rich monoxenous genera, *Leptomonas* and *Crithidia*, together with *Lotmaria* form the infrafamily Crithidiatae (Figure 1). With monophyly well supported, all dixenous leishmaniae form two major sister lineages informal designated as sections or divisions: section *Paraleishmania* brings together the genera *Endotrypanum* and *Porcisia* (formerly *Paraleishmania*; see Kostygov & Yurchenko, 2017), while the genus *Leishmania* belongs to the section *Euleishmania* (the true *Leishmania*). Members of this genus are further divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia* (formerly the *Leishmania enriettii* complex) (Figure 1). The subgenus *Sauroleishmania* was established half a century ago, although its type species *L. tarentolae* was described much earlier (Wenyon, 1920), and includes more than 20 species, which are restricted to the Old World (Akhoundi et al., 2016). *Sauroleishmania* spp. are known as reptilian parasites that have been consistently detected in various reptiles belonging to the saurian families Agamidae, Gekkonidae, Lacertidae, Scincidae and Varanidae originating from Mediterranean Europe, North Africa and the Middle East (Telford, 2009; Wilson and Southgate, 1979), yet there are some interesting exceptions. Unlike most *Sauroleishmania* spp., *L. adleri* is capable of infecting mammals (Coughlan et al., 2017) and causes transient skin symptoms in humans (Manson-Bahr & Heisch, 1961) and asymptomatic infections in hamsters and mice (Adler, 1962). An undescribed species of *Sauroleishmania* (different from *L. adleri* and *L. tarentolae*) was found to cause visceral leishmaniasis in humans and dogs in China (Chen et al., 2019; Yang et al., 2013). Moreover, *L. tarentolae* promastigotes are capable of invading mammalian (including human) dendritic cells (DC) and macrophages, where they differentiate into an amastigote-like form, yet there is no unambiguous evidence of their replication (Breton et al., 2007; Taylor et al., 2010).

There are three mutually exclusive hypotheses postulating the origins of the genus *Leishmania* from the Palearctic or the Neotropics, or from the supercontinent before its split into present continents (Akhoundi et al., 2016; Harkins et al., 2016; Klatt et al., 2019; Lukeš et al., 2007; Schönian et al., 2018). The oldest fossil record of a protist parasite is represented by *Paleoleishmania proterus* found in the midgut lumen of a blood-filled female of the sand fly *Palaemyia burmitis* entrapped in mid-Cretaceous amber (~100 MYA) in Myanmar (Poinar, 2004; Poinar & Poinar, 2004a). Promastigotes were mixed with nucleated reptilian blood cells, likely representing the ancestor of the genus *Sauroleishmania* (Poinar & Poinar, 2004a, 2004b). This finding implies that *Sauroleishmania* forms a sister clade to all other *Leishmania* species. However, the phylogenetic position of *Sauroleishmania* between the mammal-infecting subgenera *Leishmania* and *Viannia* suggests that this species-rich subgenus switched from mammals to reptiles (Klatt et al., 2019; Schönian et al., 2018). Although the available fossil record supports reptiles as early hosts of *Leishmania*-like parasites, the reptile-infecting subgenus *Sauroleishmania* must have arisen later, after the adaptation of *Leishmania* to mammals. While subsets of data can be used to support each of these hypotheses, the prevailing view places the origin of *Leishmania* in the Mesozoic, prior to the breakup of Gondwana.

3 | GUT FEELING: *LEISHMANIA TARENTOLAE* DEVELOPMENT IN A SAND FLY GUT

Sauroleishmania spp. are generally transmitted by reptile-biting sand flies of the genus *Sergentomyia*, with many species found infected by various *Sauroleishmania* species (Karimi et al., 2014; Killick-Kendrick et al., 1986; Maroli et al., 1988; Rashti & Mohebali, 1994). Although *Sergentomyia* spp. feed primarily on reptiles, some species have been reported to bite mammals, including humans, raising a question about the role of these vectors in the transmission of mammal-infecting *Leishmania* species, particularly *L. infantum* (Maia & Depaquit, 2016). Nevertheless, involvement of other sand fly genera in *Sauroleishmania* transmission should also be considered. Indeed, *L. tarentolae* DNA was recently detected in *Phlebotomus perfiliewi* (Pombi et al., 2020), *Phlebotomus perniciosus* (Latrofa et al., 2021; Mendoza-Roldan et al., 2021) and heavy late-stage infections were demonstrated experimentally in *Phlebotomus papatasi* (Adler & Theodor, 1929), *P. perniciosus* and *Phlebotomus sergenti* (Ticha et al., 2021). This may be due to the fact that many *Phlebotomus* species are opportunistic feeders, and their host-seeking behaviour may vary depending on the location, season and host availability (Quate, 1964). Their willingness to feed on cold-blooded animals has been repeatedly documented, with a prominent case of *P. papatasi* (Adler & Theodor, 1929; Belova, 1971; Quate, 1964), which is susceptible to *Sauroleishmania* spp. infection (Adler & Theodor, 1929; Ticha et al., 2021). Collectively, these data suggest that sand flies of the genus *Phlebotomus* may play a role as alternative vectors in the circulation of *L. tarentolae*, and therefore in its transmission to non-reptilian hosts (Ticha et al., 2021). As *Sergentomyia* is a genus exclusively present in the Old World, the transmission cycle of *L. tarentolae* in Brazil must be due to other vectors, possibly by *Lutzomyia* spp., as *L. tarentolae* was shown to develop in *Lutzomyia longipalpis* under laboratory conditions (Diaz-Albiter et al., 2018).

Based on their development in vectors, Lainson and Shaw (1987) classified parasites of the genus *Leishmania* into three groups (Figure 2(a)). The Suprapylaria (subgenus *Leishmania*) includes mammal-infecting species of the Old World (e.g., *L. infantum*), whose development is restricted to the midgut. The Peripylaria (subgenus *Viannia*) encompasses mammal-infecting species of the New World (e.g., *Leishmania braziliensis*), which develop in the hindgut and then migrate to anterior midgut. The third group consisting of reptile-infecting species (subgenus *Sauroleishmania*, including *L. tarentolae*) was named as Hypopylaria (Figure 2(a)). The development of these parasites was believed to be limited to the hindgut, suggesting that transmission to reptiles occurs when infected sand fly is ingested. In contrast, species with suprapylarian or peripylarian type of development are transmitted to mammals by sand fly bites (Bates, 2007).

However, some *Sauroleishmania* species are capable of an anterior migration in the sand fly gut, associated with colonization of the anterior midgut (Figure 2(b)) (Adler & Theodor, 1929, 1935; Ticha et al., 2021) and it is assumed that the hypopylarian type of development occurs only in some *Sauroleishmania*-sand fly combinations. The

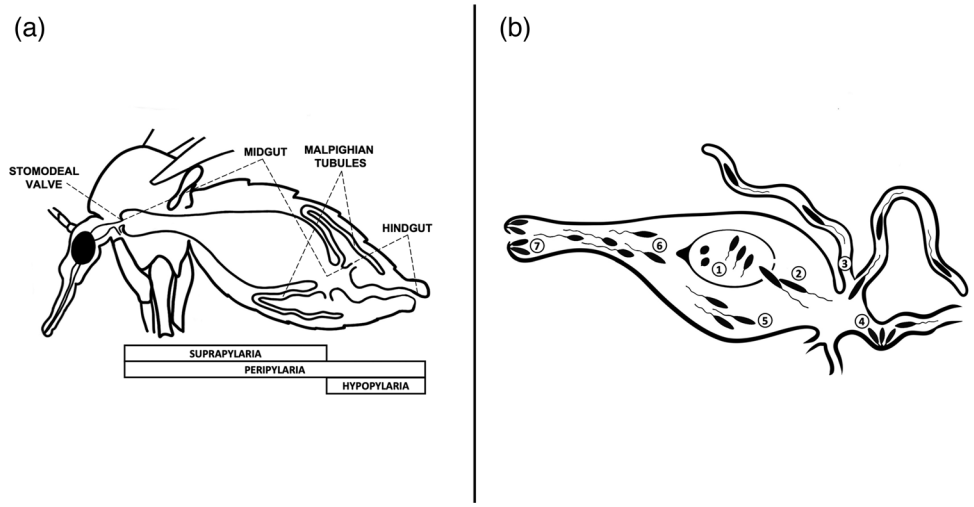

FIGURE 2 Sand fly digestive tract and summary Suprapylaria/Peripylaria/Hypopylaria (a) and development of *Leishmania tarentolae* (b)

development seems to be influenced by the insect, since *L. tarentolae* undergoes the peripylarian type of development in *P. papatasi* and *P. perniciosus*, yet the hypopylarian development prevails in *P. sergenti* (Ticha et al., 2021). Such variability in the vector-parasite interaction may be due to different behaviour of *Sauroleishmania* species to escape from the blood meal surrounded by the peritrophic matrix and by their capacity to attach to different parts of the sand fly gut.

It has also been proposed that the hypopylarian type of development occurs when promastigotes cannot cross the peritrophic matrix and are passed into the hindgut (Bates, 2007). Indeed, the role of peritrophic matrix in parasite life cycle is important. For example, its delayed degradation in *Sergentomyia schwetzi* is known to cause the refractoriness of this vector to mammal-infecting *Leishmania* species (Sádlová et al., 2018). However, further studies on *Sauroleishmania*-sand fly interactions are necessary to confirm these hypotheses.

Although *S. minuta*, the proven natural vector of *L. tarentolae*, is one of the most abundant sand flies in the Mediterranean (Maroli et al., 1988), only two studies described the development of *L. tarentolae* in this sand fly species (Adler & Theodor, 1935; Telford, 2009). Females of *S. minuta* (erroneously referred to as *Phlebotomus parroti* in the original description (Telford, 2009) were experimentally infected by feeding on gecko *T. mauritanica* carrying a mixed infection of *L. tarentolae* and *Trypanosoma platydactyli*. Both parasites acquired an anterior position in the sand fly gut, with *Sauroleishmania* promastigotes found in the midgut and cardia, but not in the hindgut (Adler & Theodor, 1935). Recently, the development of *L. tarentolae* in Malpighian tubules of three *Phlebotomus* species was experimentally demonstrated (Ticha et al., 2021). The localization in Malpighian tubules is rather unique for the genus *Leishmania*, with only two other reports of unidentified promastigotes in *Sergentomyia garnhami*, *Sergentomyia antennata* (Kaddu, 1986) and in *S. minuta* (Killick-Kendrick et al., 1979). An examination of laboratory bred *S. minuta* females that were allowed to feed on naturally infected geckos revealed that *L. tarentolae* is able to colonize the Malpighian tubules of both *Sergentomyia* spp. and *Phlebotomus* spp. (Ticha et al., unpublished). Though there are only few records of *Sauroleishmania* morphological forms in vectors (Adler & Theodor, 1929, 1935), they do not differ from those known for *Leishmania* in mammals, but the infectious stages for reptiles are not known (Bates, 2007). So far, a successful experimental transmission of *Sauroleishmania* from sand flies to reptilian hosts has not been demonstrated.

Two possible modes of transmission may be considered. The hypopylarian type of development of some *Sauroleishmania* species suggests that reptiles become infected by ingestion of a sand fly. On the contrary, species with the peripylarian type might be transmitted by bite, via the pool-blood feeding mechanism, similarly to mammal-infecting *Leishmania* (Bates, 2007). Colonization of the stomodeal valve and disruption of its surface are essential for effective transmission of *Leishmania* to its mammalian hosts, as it facilitates the regurgitation of parasites from the midgut (Dostálová & Volf, 2012). The presence of *L. tarentolae* promastigotes in the cardia and colonization of the stomodeal valve in *Phlebotomus* spp. (Adler & Theodor, 1929; 1935) support the idea of transmission by bite (Figure 3). However, the localization of *L. tarentolae* in Malpighian tubules raises a third possible scenario, namely the transmission by prediuresis. When feeding on a host, sand fly females regularly excrete urine to concentrate proteins in bloodmeal and restore weight and water balance (Sádlová et al., 2013). Viable *L. major* promastigotes, including the metacyclic form, were found in urine droplets discharged by infected *P. papatasi* and *Phlebotomus duboscqi* females, during feeding (Sádlová & Volf, 1999). *Leishmania* promastigotes in urine droplets may enter bite wounds or mucosal membranes. As urine is secreted from Malpighian tubules and passes the hindgut (both tissues being the typical location of *L. tarentolae* promastigotes), the role of prediuresis in *Sauroleishmania* transmission should be considered plausible and therefore further studied.

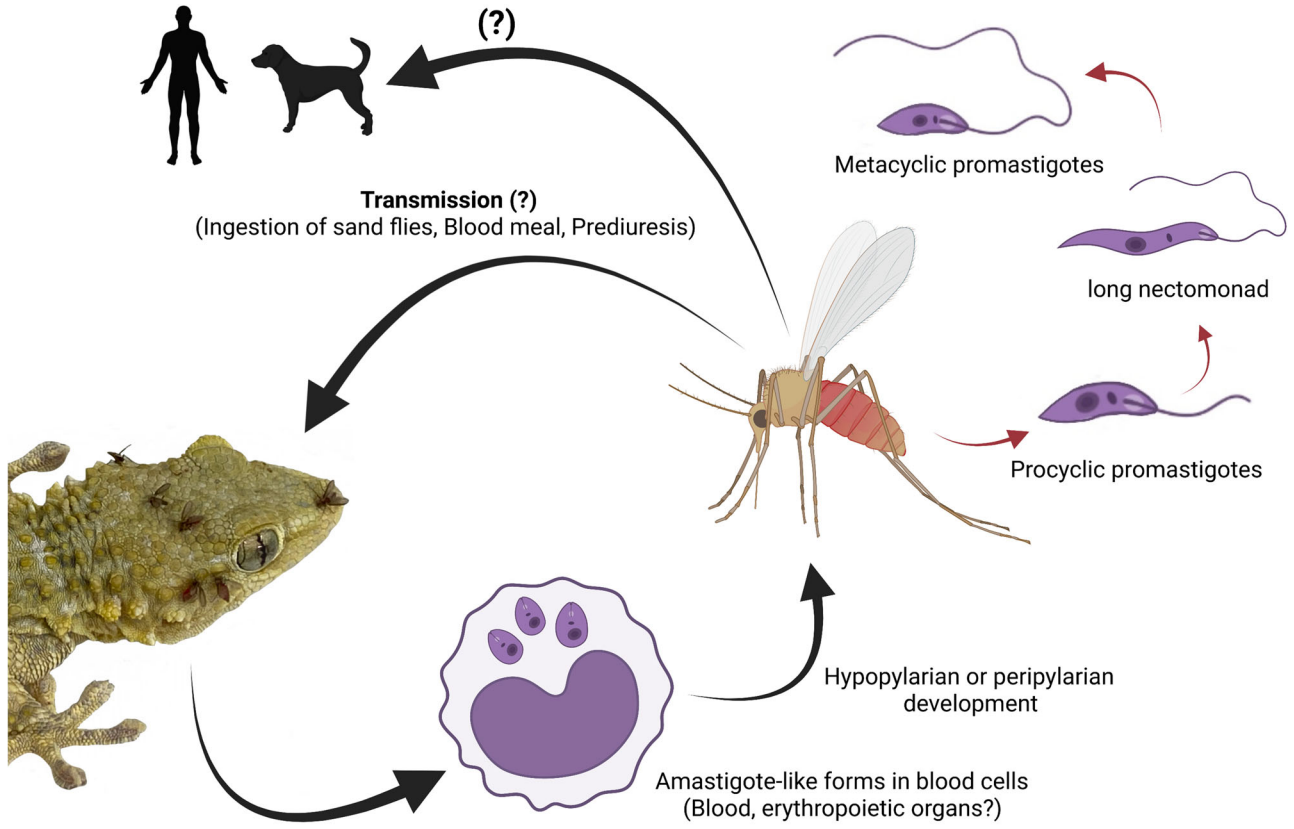

FIGURE 3 Life cycle of *Leishmania tarentolae* in vectors and hosts. In reptiles, amastigote-like forms develop in blood cells, and parasite DNA has been detected in blood and erythropoietic organs. Sand flies ingest infected blood cells and parasites differentiate into promastigote forms and undergo hypopylarian or peripylarian type of development. Possible transmission routes to vertebrate hosts are via sand fly bite, oral ingestion of the fly or contaminative way by prediuresis. Transmission and development in mammals are not known

4 | MAMMALIAN EXPOSURE TO *LEISHMANIA TARENTOLAE* AND THE ROLE OF REPTILES IN THE LEISHMANIASES

In the early years, *L. tarentolae* was classified as *Leptomonas* while researchers investigated *T. mauritanica* gecko as a possible reservoir of a zoonotic disease called Biskra boil and caused by *Leishmania* spp. (Sergent et al., 1914). Soon after, while describing different types of reptilian flagellates from Egypt, Wenyon (Wenyon, 1920) mentioned that the species isolated by Sergent (Sergent et al., 1914) was in fact a *Leishmania*, later becoming *L. tarentolae*. At the moment of the first isolation of *L. tarentolae*, some authors hypothesized that geckos could be reservoirs of cutaneous leishmaniasis caused by *Leishmania tropica* and/or *Leishmania major* (Chatton & Blanc, 1914; McMillan, 1966; Sergent et al., 1914; Wenyon, 1920). Also, other *Sauroleishmania* species were suspected to be causative agents of cutaneous leishmaniasis or oriental sore. For example, *L. adleri* was isolated from the blood of *Latastia longicaudata* lizards in Kenya (Heisch, 1958), and was believed to be a strain of *Leishmania donovani*. Unlike *L. tarentolae*, more studies confirmed the pathogenic effect of *L. adleri* as the causative agent of cutaneous leishmaniasis in rodents and humans (Coughlan et al., 2017; Manson-Bahr & Heisch, 1961). It was hypothesized that interactions between mammalian and reptilian leishmania (i.e., *L. tarentolae* in mammals and *L. donovani* in reptiles) could ultimately result in partial dilution of species, thus immunization and protection, within the two sister clades (Mutinga & Ngoka, 1981).

Furthermore, additional attempts were made to identify and isolate *Sauroleishmania* from endemic areas of human and canine leishmaniasis. Axenic cultures of *L. tarentolae* were obtained from France (Gao et al., 2001) and Italy (Mendoza-Roldan et al., 2022; Pozio, et al., 1983) with reports of *L. tarentolae* in different species of reptiles (Klatt et al., 2013; Klatt et al., 2019; Mendoza-Roldan et al., 2022), sand flies (Mendoza-Roldan et al., 2021) and mammals (Iatta et al., 2021) (Figure 4; Novo et al., 2015; Pombi et al., 2020; Annex 1). In particular, *L. tarentolae* is widely distributed and can infect saurian reptiles from the Gekkonidae (i.e., *Mediodactylus kotschyi*, *Tarentola annularis*, *T. mauritanica*) and the Lacertidae (i.e., *Podarcis filfolensis*, *Podarcis siculus*) families in the Mediterranean context (Figure 4; Annex 1) (Elwasila, 1988; Klatt et al., 2013; Mendoza-Roldan et al., 2022; Pozio et al., 1983).

While studying the molecular prevalence of *L. infantum* in human donors, sand flies and dogs from central Italy, *L. tarentolae* was detected by nested-PCR in humans and sand flies (i.e., *Phlebotomus* and *Sergentomyia*) (Pombi et al., 2020). This finding was most likely related to the *Sergentomyia* spp. transmitting *L. tarentolae* while feeding on humans (Mendoza-Roldan et al., 2021; Pombi et al., 2020). Moreover, the substantial reduction in anti-*L. infantum* antibody titres of more

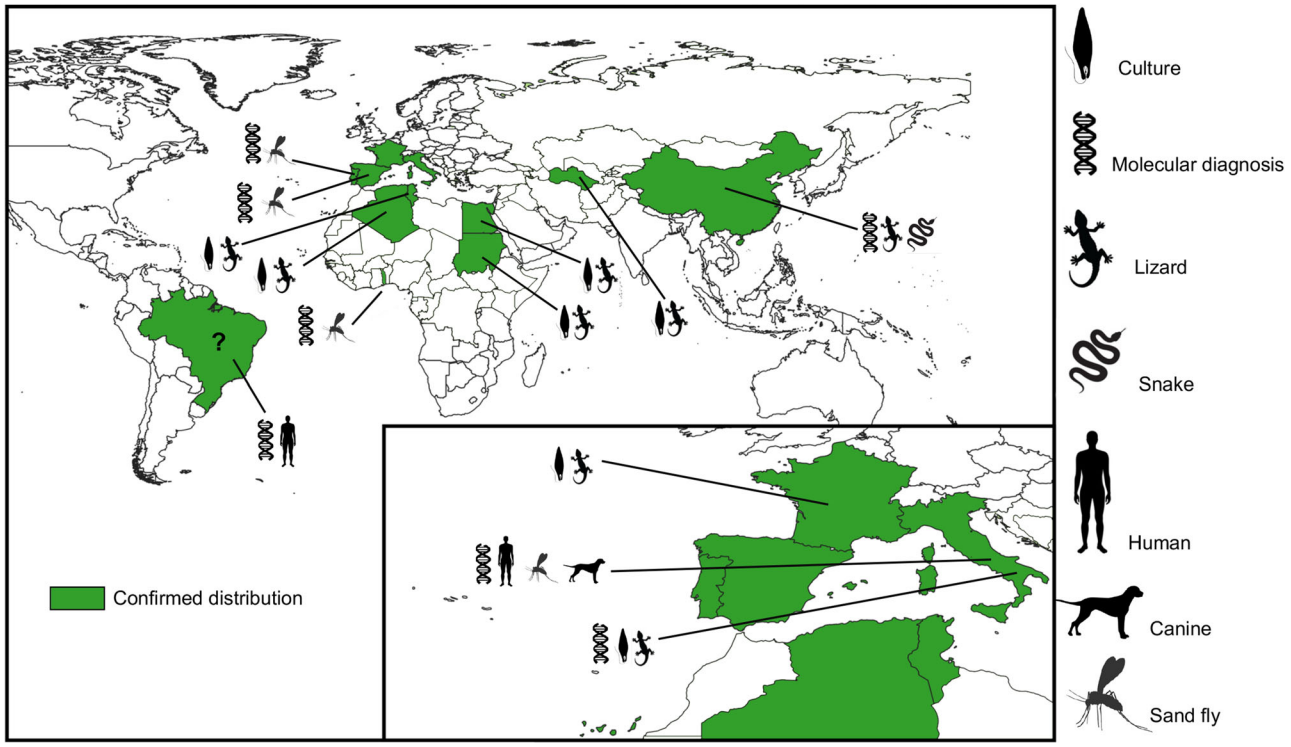

FIGURE 4 Distribution map of *Leishmania tarentolae* based on isolates and molecular detection in reptiles, sand flies and mammals (Annex 1). Dark green represents confirmed distribution; question mark refers a controversial finding concerns parasites detected in bone marrow and intestinal tissue samples from a 300-year-old Brazilian mummy based on a kDNA amplicon matching to *L. tarentolae* (Novo et al. 2015), which, however, does not agree with the geographical distribution of the subgenus *Sauroleishmania*

than half of the population of *L. infantum*-seropositive and clinically healthy sheltered dogs, sampled throughout the year (that is, during the transmission and non-transmission season), raised questions about the possibility of dogs being exposed to *L. tarentolae* (Cavalera et al., 2021). In fact, circumstantial evidence suggested by the seasonal variation in antibody levels depending on the sand fly activity and sympatric occurrence of *L. tarentolae* and *L. infantum* could possibly indicate a protective effect of the exposure to *L. tarentolae* in areas endemic to canine leishmaniasis, reducing the clinical manifestation of leishmaniasis in dogs. The likelihood of infection by *L. tarentolae* in mammals was further confirmed serologically and molecularly in southern Italy, both in humans (Iatta et al., 2021) and in sheltered dogs (Mendoza-Roldan et al., 2021). Moreover, the finding of *S. minuta* as the most abundant species in canine leishmaniasis endemic areas (Mendoza-Roldan et al., 2021; Pombi et al., 2020), further suggested the possibility of mammalian exposure to *L. tarentolae*, also considering the feeding behaviour of this sand fly species on humans and dogs.

Capability of pathogenic mammalian-associated *Leishmania* to infect reptiles was studied in the late 1960s and 1970s and was ultimately disregarded, mainly given the physiological differences between mammals and reptiles (e.g., reptiles being ectotherms and mammals endotherms) (Belova, 1971; McMillan, 1966). Nevertheless, Belova (1971) described experimental infections of reptiles with mammalian-associated *Leishmania* spp., and this was later confirmed by molecular detection of various *Leishmania* spp. (i.e., *L. donovani*, *L. tropica*, *L. turanica*) in saurians and snakes in China (Chen et al., 2019; Zhang et al., 2019). Furthermore, *L. infantum* was molecularly detected in lizards in areas of canine leishmaniasis in southern Italy, in sympatric occurrence with *L. tarentolae* (Mendoza-Roldan et al., 2022). The infection of *L. infantum* in reptiles was further corroborated through the retrieval of amastigote forms in the bone marrow of geckoes (Mendoza-Roldan et al., 2022). These molecular findings suggest the interaction between both *Leishmania* species and ultimately raise the question who was infected first – reptiles by *Leishmania* or mammals by *Sauroleishmania*?

5 | *LEISHMANIA TARENTOLAE* AND THE CELLULAR MODEL

Leishmania tarentolae is broadly used for a range of biotechnological applications, from protein production to its exploitation as a model for drug discovery (Klatt et al., 2019). In the area of bio-molecular studies, *L. tarentolae* was firstly exploited to investigate gene amplification (Ouellette et al., 1991; White et al., 1988) and RNA editing in the mitochondrion (Blum et al., 1990). In parallel, *L. tarentolae* was developed as a platform for recombinant protein production (Cantacessi et al., 2015), and then commercialized by Jena Bioscience (Jena, Germany) under the name LEXSY. The LEXSY system allows the expression of target proteins either in a constitutive or inducible form, as intracellular or secretory molecules (https://www.jenabioscience.com/). The

strain P10, on which the LEXSY system is based, was likely derived from the TARII/UC strain of the parasite, isolated by Parrot from an Algerian gecko (*T. mauritanica*) (Klatt et al., 2019; Parrot, 1949). Among the variety of microbial and cellular platforms to produce recombinant proteins (e.g., prokaryotes, yeasts, mammalian cells, insect cells), *L. tarentolae* found its niche thanks to some specific characteristics. First, the maintenance and growth of *L. tarentolae* is accomplished at a low cost: promastigotes are easily cultured in aerobic conditions as continuous suspension culture at 26°C, in different synthetic media (Cantacessi et al., 2015; Kushnir et al., 2005). Second, growth characteristics are suitable to scale the production to industrial levels, by growing parasites in bioreactors, with the potential of harvesting high yields of recombinant proteins from engineered strains (Niimi, 2012). Third, *L. tarentolae* presents a protein glycosylation pattern that is very likely to overlap that of pathogenic Trypanosomatidae (Murphy et al., 2020), but is also similar to that of mammals (Cantacessi et al., 2015). Based on the above characteristics, *L. tarentolae* is an interesting system for protein studies (e.g., X-ray Crystallography) and for the production of protein antigens, for example for sero-diagnostic applications and vaccine development. To date, the use of this protist to produce antigens for diagnostic application has been limited to experimental studies on antigens from pathogenic species of *Leishmania* or *Trypanosoma* (Rezaei et al., 2019; Rooney et al., 2015) and from viruses (Baechlein et al., 2013; Varotto-Boccazzi et al., 2021). In this context, a recent paper showed that a recombinant protein produced in *L. tarentolae* allows reliable serological diagnosis of SARS-CoV-2 infection (Varotto-Boccazzi et al., 2021). However, while in the presence of biantennary glycosylation structures, N-glycosylation in *L. tarentolae* is not completely overlapping that of mammals (Cantacessi et al., 2015). Therefore, the capability of a given viral antigen produced in *L. tarentolae* to match the diagnostic patterns should always be carefully compared with the same antigen expressed from mammalian cells.

In view of its safety and easy culturing, *L. tarentolae* has been investigated as a surrogate pathogen in candidate vaccines, aimed at protecting against human pathogenic *Leishmania* species. In a first seminal paper, Breton et al. (2005) showed that *L. tarentolae* promastigotes are engulfed by DCs in vitro, inducing proper maturation of these cells, with expression of major histocompatibility complex class II (MHCII) and costimulatory molecules. More importantly, this study showed that intraperitoneal administration of live *L. tarentolae* in BALB/c mice determined polarization of the immune response toward Th1 pathway, with significant protection against challenge with *L. donovani*. In successive pre-clinical studies, live *L. tarentolae* promastigotes were assayed as candidate vaccines in association with adjuvants, with cross-protective immunity against *L. major* (Haghdoust et al., 2022; Keshavarzian et al., 2020). While the above studies had been performed using non-engineered strains of *L. tarentolae*, thus exploiting some form of cross-immunity with human pathogenic species, other studies employed genetically modified strains of *L. tarentolae*, engineered for expression of antigens from human pathogenic leishmanias (Salari et al., 2020; Saljoughian et al., 2013) and/or of immune-modulating molecules (Montakhab-Yeganeh et al., 2017), such as proteins from the sand fly saliva (Katebi et al., 2015). These studies generally showed that whole live promastigotes from engineered strains of *L. tarentolae* determined protection in animal models against pathogenic species, including *L. infantum* and *L. major*.

In parallel with the above studies on anti-*Leishmania* vaccines, *L. tarentolae* was investigated for its potential as a platform to generate anti-viral vaccines. Targeted viruses include human immunodeficiency virus 1 (Breton et al., 2007), human papillomavirus (Bolhassani et al., 2015) and hepatitis C virus (Ansari et al., 2019). The engineered strains of *L. tarentolae* have so far been assayed only in animal models, either as living vehicles for the antigens (Ansari et al., 2019; Breton et al., 2007), or just as biofactories for antigen production (Bolhassani et al., 2015). The first approach is obviously based on the assumption that the targeting of *L. tarentolae* to DCs should facilitate the delivery of viral antigens to secondary lymphoid organs, ensuring their presentation to CD4+T cells (Breton et al., 2005; Breton et al., 2007). In the second approach, the antigen is administered after purification. Overall, studies above led to encouraging results in animal models, in terms of the generation of virus-neutralizing antibodies.

6 | CONCLUDING REMARKS

Leishmania tarentolae is a promising protist for its biotechnological applications, of which very little is known regarding its biological cycle, transmission pathways and overall biology. However, the interaction that *L. tarentolae* may have, in endemic areas of canine leishmaniasis, with *L. infantum* and its implications on the pathogenicity and epidemiological cycles of canine and human leishmaniasis are subjects that require further research to better understand natural scenarios. This may open new opportunities for the development of vaccines and/or immune-protection strategies to control leishmaniases. Yet, this knowledge may be translocated to other areas where *Leishmania* and *Sauroleishmania* occur in sympatry.

Furthermore, recent efforts and studies regarding *L. tarentolae* transmission have demonstrated that this species could have a peripylarian type of development and may colonize the stomodeal valve in *Phlebotomus* spp., supporting transmission via pool-blood feeding, as seen in mammal-infecting species of *Leishmania*. Additionally, sand fly prediuresis and consequent contaminative transmission, as well as hosts feeding on infected sand flies, could be another mechanism to infect vertebrates. However, the transmission and development in reptilian hosts and *Sergentomyia* sand flies have yet to be unravelled. Finally, although *L. tarentolae* has historically been considered non-pathogenic and unlikely to infect mammals, some cultured strains have been shown to be transiently infectious to mammals. The fact that reference laboratory strains are probably non-infectious even for reptiles, spurs the need for new isolates to fully understand the natural development of *L. tarentolae* in reptiles and in mammals. In addition, this species has been studied as a model for anti-*Leishmania* vaccines and a platform to generate antiviral vaccines with overall encouraging results in animal models, in terms of the generation of virus-neutralizing antibodies. The overall picture presented in this review is useful in understanding the implications of the interactions of these sister clades *Leishmania*,

which may be applied knowledge to improve diagnostic tools, efficient control and treatment of a neglected disease that is a high burden to our society.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

AUTHOR CONTRIBUTION

All authors contributed equally to the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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APPENDIX

Annex 1. Reference list of the distribution of *Leishmania tarentolae*

Continent	Country	Host	Method of identification	Reference
America	Brazil	Human	Molecular	Novo et al. (2015)
Europe	Portugal	Sand fly	Molecular	Maia et al. (2015)
Europe	Spain	Sand fly	Molecular	Bravo-Barriga et al. (2016)
Europe	France	Gecko	Culture	Rioux et al. (1969)
Europe	Italy	Gecko	Culture	Pozio et al. (1983)
Europe	Italy	Reptiles	Molecular	Mendoza-Roldan et al. (2021)
Europe	Italy	Dog	Molecular	Mendoza-Roldan et al. (2022)
Europe	Italy	Human	Molecular	Pombi et al. (2019); Iatta et al. (2021)
Europe	Italy	Sand fly	Molecular	Latrofa et al. (2018)
Africa	Algeria	Gecko	Culture	Telford (2009)
Africa	Tunisia	Gecko	Culture	Telford (2009)
Africa	Egypt	Gecko	Culture	Wenyon (1921)
Africa	Sudan	Gecko	Culture	Telford (2009)
Africa	Togo	Sand fly	Molecular	Ferlet et al. (2021)
Asia	China	Lizard	Molecular	Zhang et al. (2019)
Asia	China	Snake	Molecular	Chen et al. (2019)
Asia	Turkmenistan	Gecko	Culture	Garnham (1971)

Novo, S. P., Leles, D., Bianucci, R., & Araujo, A. (2015). *Leishmania tarentolae* molecular signatures in a 300 hundred-years-old human Brazilian mummy. *Parasites & vectors*, *8*, 72. https://doi.org/10.1186/s13071-015-0666-z

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Leishmania (*Sauroleishmania*) *tarentolae* isolation and sympatric occurrence with *Leishmania* (*Leishmania*) *infantum* in geckoes, dogs and sand flies.

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Leishmania (*Sauroleishmania*) *tarentolae* isolation and sympatric occurrence with *Leishmania* (*Leishmania*) *infantum* in geckoes, dogs and sand flies

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Abstract

The trypanosomatid protist *Leishmania tarentolae* is a saurian-associated parasite vectored by the *Sergentomyia minuta* sand fly. This study aimed to confirm the circulation of *L. infantum* and *L. tarentolae* in sand flies, reptiles and dogs and to isolate new strains of these protists. Reptilian and sheltered dog blood samples were collected, and sand flies were captured. Samples were tested for *Leishmania* spp. using duplex real-time PCR (dqPCR) and real-time PCR (qPCR); the origin of blood meal was identified in engorged sand flies by conventional PCR. The reptilian blood and intestinal content of sand fly females were cultured. Dog sera were tested by IFAT using both *Leishmania* species. Four *Tarentola mauritanica* geckoes were molecularly positive for *L. infantum* or *L. tarentolae*, with no co-infections; moreover, amastigote-like forms of *L. infantum* were observed in the bone marrow. 24/294 sand flies scored positive for *Leishmania* spp. by dqPCR, 21 *S. minuta* and two *Phlebotomus perniciosus* were positive for *L. tarentolae*, while only a single *Ph. perniciosus* was positive for *L. infantum*. Blood meal analysis confirmed reptile and dog in *S. minuta*, dog and human in *Ph. perniciosus* and dog in *Phlebotomus neglectus*. Two axenic strains of *L. tarentolae* were obtained. Twelve of 19 dogs scored positive for *L. infantum* and *L. tarentolae* by IFAT and three of them also for *L. infantum* by dqPCR, and six by qPCR. These data confirm the sympatric circulation of *L. infantum* and *L. tarentolae* in geckoes, sand flies, and dogs, and suggest that geckoes may be infected with *L. infantum*.

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

Author summary

Leishmania tarentolae is a saurian-associated parasite recently reported in geographical areas where *Leishmania infantum* is endemic. To confirm the circulation of both protists in sand flies, reptiles and dogs and to isolate new *Leishmania* spp. strains, reptilian and sheltered dog blood samples were collected and sand flies captured. Samples were molecularly tested for *Leishmania* spp. and sandflies for origin of blood meal. Dog sera were tested by IFAT for both *Leishmania* species and reptilian blood and intestine of sand flies were cultured. Four *Tarentola mauritanica* geckoes were positive for *L. infantum* or *L. tarentolae*; moreover, amastigote-like forms of *L. infantum* were observed in the bone marrow. 24/294 sand flies scored PCR positive for *Leishmania* spp. Reptile and dog blood were found in *S. minuta*, dog and human in *Ph. perniciosus* and dog in *Phlebotomus neglectus*. Two axenic strains of *L. tarentolae* were obtained. Twelve of 19 dogs scored positive for *L. infantum* and *L. tarentolae* by IFAT and three of them also for *L. infantum* by PCR, and six by qPCR. Data confirm the sympatric circulation of *L. infantum* and *L. tarentolae* in geckoes, sand flies, and dogs, and suggest that geckoes may be infected with *L. infantum*.

Introduction

Leishmaniases are important diseases affecting mammals, including humans, in tropical, subtropical, and temperate regions, with more than 350 million people infected worldwide [1]. The genus *Leishmania* (Kinetoplastea, Trypanosomatidae), transmitted predominantly by phlebotomine sand flies (Diptera, Psychodidae), includes more than fifty species which infect mammals and reptiles. From those, about twenty parasitize humans, causing cutaneous, mucocutaneous and visceral leishmaniasis [2,3]. In the Mediterranean basin, *Leishmania infantum* is the main species, causing zoonotic cutaneous and visceral leishmaniasis in humans, and infects more than 2.5 million dogs [4]. In the same area, other pathogenic species of the subgenus *Leishmania* occur (e.g., *Leishmania donovani* in Cyprus and *Leishmania tropica* in Greece), while species typical of reptiles, belonging to the subgenus *Sauroleishmania*, (e.g., *Leishmania chameleonis* and *Leishmania tarentolae*) were found in Algeria, France, and Italy [3,5–7]. Indeed, ecological and anthropic drivers (i.e., climate changes, animal translocation, wildlife movements, or globalization) have amplified the risk of alien *Leishmania* spp. introduction [8], and the spreading of sand fly populations in new localities results in the northward shift of leishmaniasis [9].

The abovementioned factors pose new challenges to medical and veterinary practitioners, given the possibility of diagnostic inaccuracies or cross-reactivities, such as in the case of the simultaneous occurrence of *L. infantum* and *L. tropica* in dogs in Israel [10] and of *L. infantum* and *L. tarentolae* in dogs in Italy [7].

In particular, saurian-associated *L. tarentolae* has been detected in geckoes *Tarentola mauritanica* and *Mediodactylus kotschyi* from Italy [11] and *Tarentola annularis* from Sudan [12]. Recently, this species was molecularly detected in lizards *Podarcis filfolensis* and *Podarcis siculus* [7,13]. The species was molecularly detected also in human blood in central Italy [14], in islanders of the Pelagie archipelago [15] and in sheltered dogs in Italy [7]. Mammalian species *L. infantum* was molecularly detected in lizards (i.e., *P. siculus*) and geckoes (i.e., *T. mauritanica*, *Hemidactylus turcicus*) inhabiting dog shelters in southern Italy [13]. The high abundance of the natural vector *Sergentomyia minuta* and the detection of human and dog blood in engorged females [14,16] suggest the possibility of mammalian exposure to *L. tarentolae*. Indeed, both *L. infantum* and *L. tarentolae* may infect the same sand fly species where their

distribution overlaps. In Italy, this is the case of *Phlebotomus perfiliewi* and *Phlebotomus perniciosus* that were molecularly positive for *L. tarentolae* [7,14] and *S. minuta* for *L. infantum* [14,17–19]. In addition, experimental infections demonstrated that *L. tarentolae* develops in *Ph. perniciosus* and *Phlebotomus papatasi* [20], the main vectors of *L. infantum* and *Leishmania major*, respectively. Although scientific interest on *L. tarentolae* is continually growing, its life cycle, pathogenicity, tropism and overall biology remain largely unknown, despite many isolations and molecular characterization efforts [11,20–23]. The obtained strains have been of great importance for the development of biotechnological tools, given by the production of cultured promastigotes at the industrial level [2,24]. In addition, two isolates are available commercially in culture, namely, TarII (ATCC: 30267) (Algeria, 1939; [21]) and LEM125 (France, 1981; [25]) strains, both obtained from *T. mauritanica* geckoes. Importantly, LEM125 strains can be transiently infectious to mammals, hence posing a biosafety risk not yet assessed [26–28]. Conversely, TarII strains are considered nonpathogenic and have suffered considerable modifications in their kinetoplast DNA (kDNA) due to the constant culture passages through the many years since the first isolation, losing proteins not essential for their life cycle in culture medium [24]. However, the whole genome sequence is available only for TarII strain [29,30]. Therefore, new information on the epidemiology and biology of *L. tarentolae*, as well as the isolation of new strains are needed to better understand the possible infection in mammals. Overall, this study aimed to evaluate the circulation of *L. tarentolae* and *L. infantum* in reptiles, sand flies, and dogs in an endemic area of canine leishmaniasis (CanL) and to obtain new strains of both *Leishmania* species in axenic cultures.

Methods

Ethics statement

Protocols for the collection of reptiles were approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 12/20), and authorized by the Ministry for Environment, Land and Sea Protection of Italy (Approval Number 0073267/2019), the Societas Herpetologica Italica and the Istituto Superiore per la Protezione e la Ricerca Ambientale (Approval Number 71216).

Methods and samples are summarized in a flowchart (Fig 1).

Study area

Geckoes, lizards, and sand flies were collected from May to November 2021 in two locations endemic for CanL, near Valenzano municipality (with 4.2 km of distance between each other), Apulia region, Italy [9]. These locations were Campus of Veterinary Medicine, University of Bari "Aldo Moro" (site 1; 41˚1'31.584"N, 16˚54'3.6288"E) and a private owned dog shelter (site 2; 41˚03'04.3"N, 16˚53'39.7"E), where dog blood samples were also collected and screened (Fig 2). Site 1 had a Mediterranean environment characterized by olive trees, the presence of typical "*muretti a secco*" (stone walls) where reptiles and sand flies thrive (Fig 3A), while site 2 was a high-walled shelter, with few olive trees where dogs and pigeons were kept (Fig 3B).

Sample collection

Reptile capturing and processing. Adult reptiles (except for gravid females) were captured by lassoing or by hand, or in the case of snakes using herpetological hooks and sampled before release in the original home range. A small amount of blood (~200μl) obtained via cardiocentesis (in snakes from the ventral coccygeal vein) was used for smears (stained with Diff-Quik), cultivation (~50μl) in a modified Tobie-Evans (TE) medium [31], and the rest was

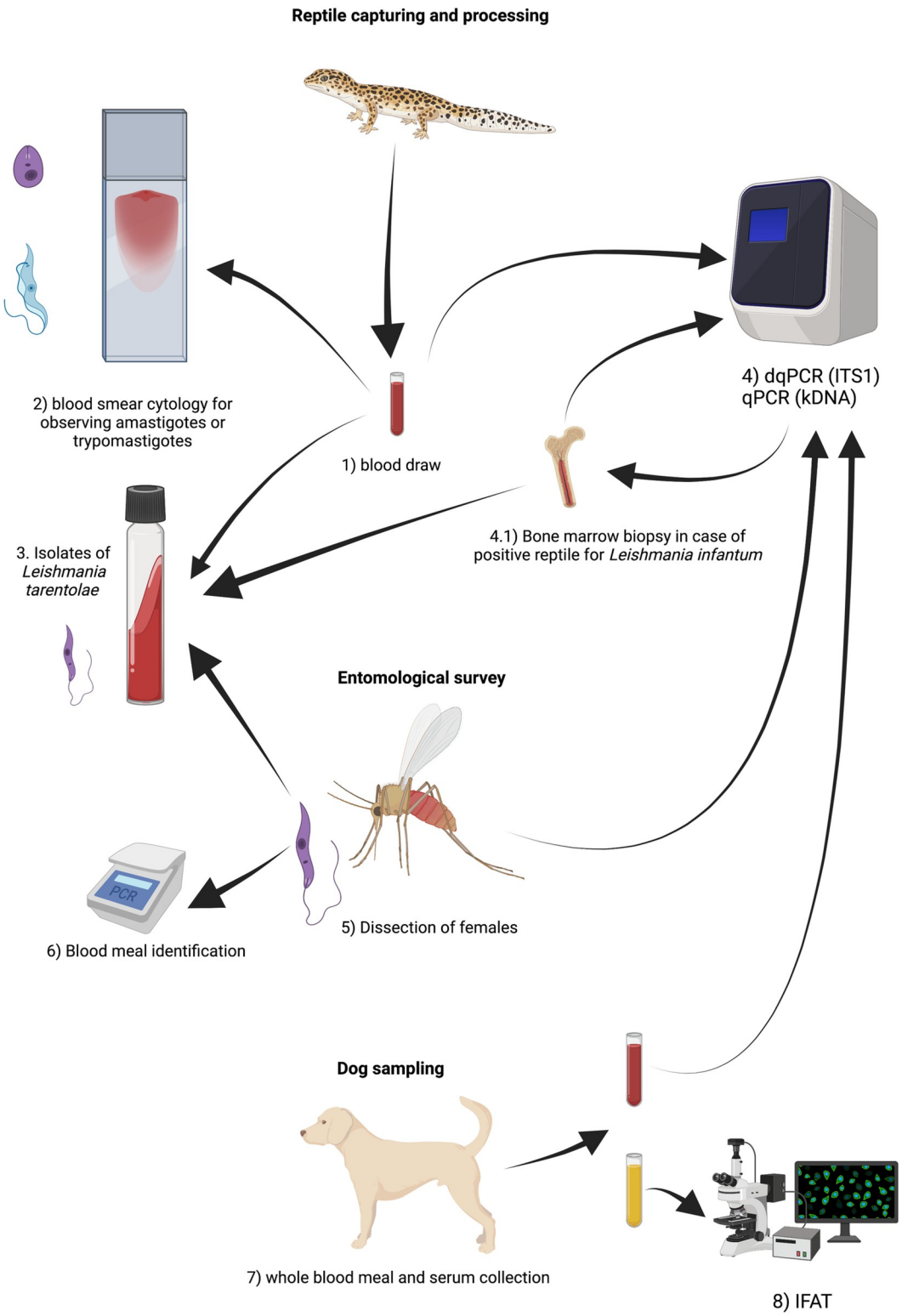

Fig 1. Flowchart of methods and samples used, divided by sections: reptile capturing and processing, entomological survey and dog sampling. Created in: https://biorender.com/.

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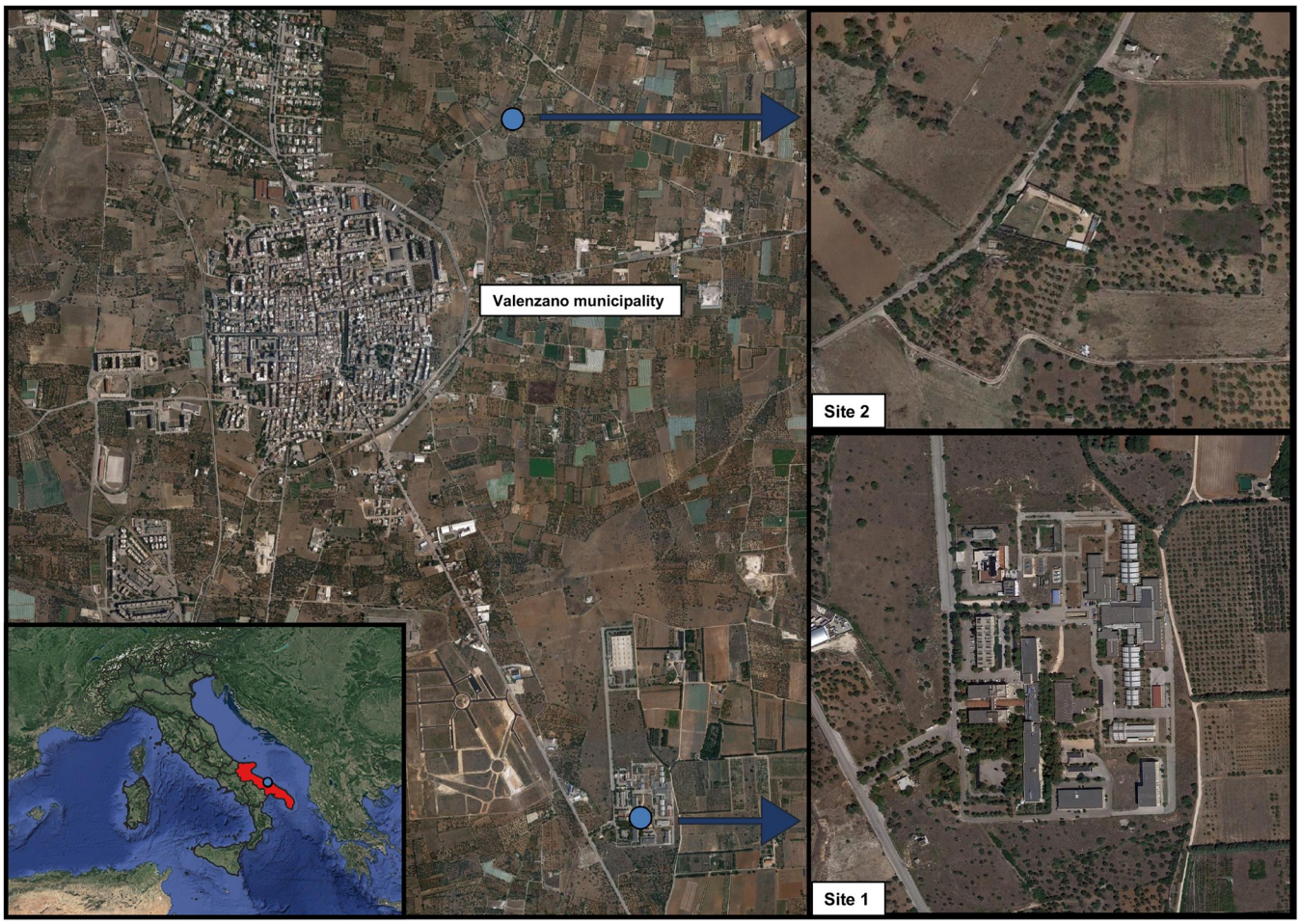

Fig 2. Geographic location of reptile and dog blood sample collection sites and sand fly capturing, in the surroundings of Valenzano municipality, Apulia, Region. (Publicly available satellite shapefiles from http://mt0.google.com/vt/lyrs=s&hl=en&x={x}&y={y}&z={z}; in QGIS 3.4).

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stored at −20˚C until molecularly processed. Geckoes, positive for *L*. *infantum* in blood, were further assessed by bone-marrow biopsy of the femur, according to protocols described elsewhere [32,33]. Briefly, a regular hypodermic needle of adequate size and length (30G X 5/16") was used to collect the sample. The area was prepared aseptically, and the needle was inserted into the bone with continuous and steady pressure and a slight rotational movement. The bone marrow was collected by aspirating the syringe with a slight but steady negative pressure. The obtained material was used for smears in films, cultivation, and DNA extraction.

Entomological survey. During the study period, sand flies were collected twice a week using a sticky trap area of 1 m^2 (32 papers; 21.0 × 29.7 cm) and 4 CDC light traps set from 5:00 p.m. to 8:00 a.m. Collections were carried out during the sand fly activity season [13] until the total absence of sand flies (i.e., three consecutive negative captures). Specimens were stored in 70% ethanol and morphologically identified using taxonomic keys [34]. Alive females collected by CDC traps were dissected with a drop of saline solution and the gut was observed under a microscope to determine the presence of flagellates [20]. The gut content of positive females was cultivated in a modified Tobie-Evans (TE) medium [31].

Dog sampling. Nineteen dogs housed in site 2 were sampled in May and November 2021. A complete physical examination was performed by a veterinarian to assess the health status of

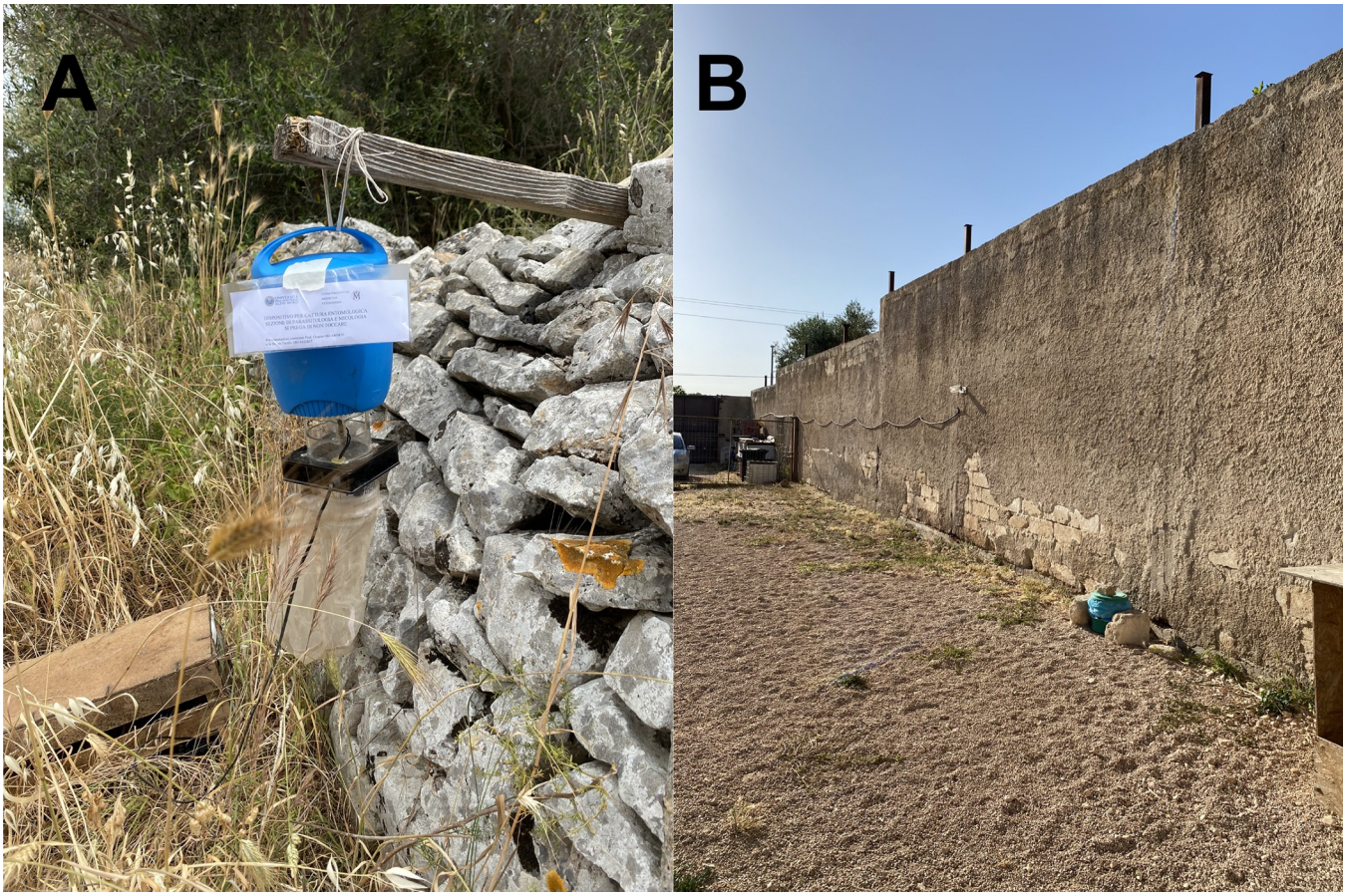

Fig 3. Environmental characteristics of both collection sites. A) "muretto a secco" where the CDC light traps were placed in the campus of Veterinary Medicine, University of Bari "Aldo Moro". B) a high-walled private-owned dog shelter.

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the animals. From each dog, whole blood was collected in vacuum containers with K3 EDTA (2.5 ml) and serum collection tubes with clot activator (5 ml).

Serological testing

Dog serum samples were tested by IFAT for the detection of IgG anti-*L. infantum* as described previously [35]. To evaluate exposure to *L. tarentolae*, IFAT was performed using promastigotes of *L. tarentolae* (strain RTAR/IT/81/ISS21-G.6c/LEM124) following the same procedure as for *L. infantum* IFAT. For both IFAT, serum samples of a dog positive for *L. infantum* by cytological and molecular analyses, and a healthy dog negative for *L. infantum*, were used as positive and negative controls, respectively. The samples were scored as positive when they produced clear cytoplasmic and membrane fluorescence of promastigotes from a cut-off dilution of 1:80 [36]. Positive sera were titrated by serial dilutions until negative results were obtained.

Molecular biology

Genomic DNA (gDNA) was extracted from reptile and dog blood samples and cultures, using the GenUPBlood DNA commercial kit (Biotechrabbit GmbH, Hennigsdorf, Germany), according to the manufacturer's instructions. DNA was extracted from bone marrow films

using the QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). gDNA was extracted from the thorax and abdomen (heads and last abdominal segments were removed for morphological identification) of each sand fly female ($n = 294$) using an in-house method as previously described [37]. All samples (i.e., blood, bone marrow, and sand flies) were tested by duplex real-time PCR (dqPCR) for the detection of *L. infantum* and *L. tarentolae* and were considered positive with Ct values up to 38.0 and 38.6, respectively, as described previously [38]. Blood samples were also tested for *L. infantum* kDNA minicircle (120 bp) by real-time PCR (qPCR), using the protocol described elsewhere [39]. gDNA from *L. infantum* promastigotes cultured in Tobie-Evans medium from an infected dog living in Italy (zymodeme MON-1) and from *L. tarentolae* (strain RTAR/IT/81/ISS21-G.6c/LEM124) were used as controls. gDNA extracted from a blood sample from a lizard and a dog tested negative for *Leishmania* spp. was used as a negative control.

For sequence analyses, culture isolates were amplified by conventional PCR (cPCR) using primers L5.8S/LITSR targeting a partial region of the internal transcribed spacer 1 (ITS1, ~300bp) and ran the PCR protocol as described elsewhere [40]. A fragment of the Heat-shock protein 70 gene (*hsp*70, 1,245 pb) was also amplified using specific primers and the PCR protocol was run as described elsewhere [41].

Engorged sand flies ($n = 10$) were tested for blood-meal identification by cPCR using primers targeting the vertebrate 16S rRNA gene (600 bp), and the PCR protocol was run as previously described [14]. All PCR reactions consisted of 4 μl of gDNA and 46 μl of PCR mix containing 3 mM MgCl2, 10 mM Tris–HCl (pH 8.3) and 50 mM KCl, 125 μM of each dNTP, 1 pmol/μl of each primer, and 2 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). The amplified products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a Gel Logic 100 gel documentation system (Kodak, NY, USA). The amplicons were purified and sequenced in both directions using the same primers as for PCR, employing Big Dye Terminator v.3.1 chemistry in an automated sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). All sequences were aligned using Geneious prime software and compared to those available in GenBank using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/blast.cgi).

The genetic relationship of *Leishmania* species was evaluated using representative *hsp*70 sequences obtained from culture isolates, and those of reference laboratory strains of *L. tarentolae* and *L. infantum*, along with relevant trypanosomatids available in the GenBank database. The phylogenetic tree was inferred using the maximum likelihood (ML) method based on the Kimura 3-parameter model [42], selected by best-fit model analysis, and based on the lowest score obtained by Bayesian Information Criterion (BCI), using MEGA6 software (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences between sites (4 categories (+G, parameter = 0.1772)). Evolutionary analyses were conducted with 5000 bootstrap replicates using MEGA6 software [43]. The corresponding *hsp*70 sequence of *Trypanosoma brucei gambiense (*Accession number: KP208736.1) was used as an outgroup.

Results

Reptile capturing and processing

During the seven-month study period, 37 reptiles of three species (two *Hierophis carbonarius* snakes, seven *Podarcis siculus* lizards, and 28 *T. mauritanica* geckoes) were captured and sampled. In particular, two *H. carbonarius*, seven *P. siculus*, 13 *T. mauritanica*) were collected at site 1, while 15 *T. mauritanica* were collected at site 2. *Leishmania tarentolae* was isolated from a *T. mauritanica* gecko from site 2 (Table 1). On cytological blood smear examination,

Table 1. Trypanosomatid detection in *Tarentola mauritanica* geckoes using blood cytology, isolation (in TE medium), dqPCR and qPCR; values of the threshold cycle (Ct) are reported.

Gecko identification number	Blood cytology	Isolation	dqPCR (Ct)	qPCR (Ct)
3	*Leishmania* spp.*	-	*Leishmania infantum* (37.50–38.6*)	*Leishmania infantum* (29.07*)
5	*Trypanosoma* sp. *Leishmania* spp.*	-	-	*Leishmania infantum* (36.81)
6	*Trypanosoma* sp.	*Leishmania tarentolae*	*Leishmania tarentolae* (26.95)	-
14	*Trypanosoma* sp.	-	-	-
15	-	-	*Leishmania tarentolae* (25.09)	-

*from bone marrow.

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trypomastigotes, most likely of *Trypanosoma* cf. *platydactyli*, (Fig 4A) were observed in three *T. mauritanica* geckoes from site 2, whereas amastigote forms of *Leishmania* spp. were not found in blood. Of the 37 reptile blood samples examined by dqPCR and qPCR, four (10.81%) *T. mauritanica* geckoes were positive at site 2. Specifically, two geckoes were positive for *L. tarentolae*, one being the source of the *L. tarentolae* strain RTAR/IT/21/Ct-25.09, while two other geckoes were positive for *L. infantum* (one scored positive by dqPCR and one by qPCR; Table 1). Bone marrow films from the two *L. infantum* PCR positive geckoes revealed amastigote forms within monocytes and macrophages (Fig 4B; Table 1). Furthermore, DNA extracted from bone marrow films was positive in one gecko (dqPCR Ct-38.6, qPCR Ct-29.07), confirming *L. infantum* infection (Table 1).

Entomological survey

A total of 716 phlebotomine sand flies (i.e., 474 *S. minuta*, 206 *Ph. perniciosus*, and 36 *Ph. neglectus*) were collected, of which 294 were females (231 *S. minuta*, 52 *Ph. perniciosus*, and 11

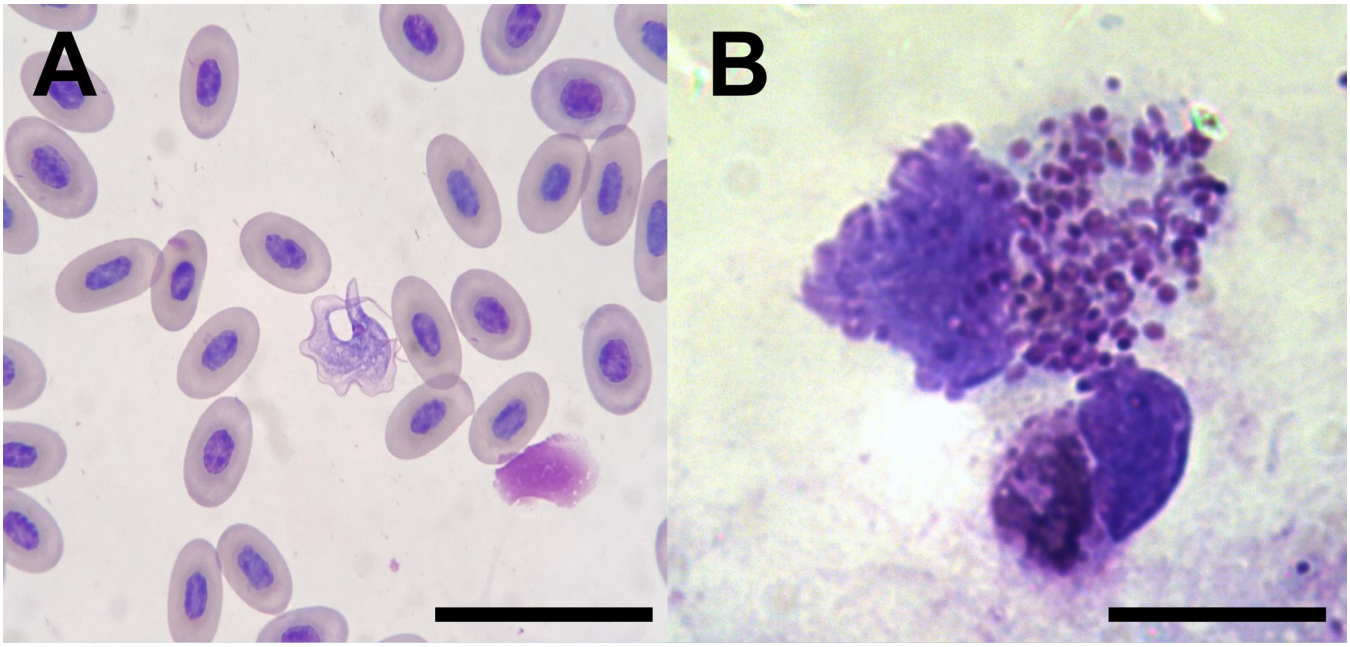

Fig 4. Trypanosomatidae from geckoes. A) *Trypanosoma* sp. trypomastigotes in blood of *Tarentola mauritanica* gecko. B) molecularly identified *Leishmania infantum* amastigote-like forms in leukocyte of *Tarentola mauritanica* gecko. Scale bars A) 50 μm; B) 10 μm.

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Table 2. Sand fly females tested for *Leishmania infantum* and/or *Leishmania tarentolae* by duplex quantitative PCR. The mean (M), minimum (Min), maximum (Max) and standard deviation (sd) values of the threshold cycle (Ct) are reported.

Sand flies	*Leishmania tarentolae*				*Leishmania infantum*				
	P/T (%)	Ct			P/T (%)	Ct			P/T (%)
		M	Min-max	sd		M	Min-max	sd	
S. minuta	21/231 (9.1)	33.35	19.3–37.73	6.3	0/231	-	-	-	21/231 (9.1)
Ph. perniciosus	2/52 (3.8)	37.58	36.54–38	1	1/52 (2)	29.69	-	-	3/52 (5.7)
Ph. neglectus	0/11	-	-	-	0/11	-	-	-	0/11
P/T (%)	23/294 (7.8)				1/294 (0.3)				24/294 (8.1)

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Ph. neglectus). Of the sand fly females, 24 scored positive for *Leishmania* spp. (8.1%) by dqPCR (Table 2). Among them, 21 (87.5%) *S. minuta* and two (8.3%) *Ph. perniciosus* were positive for *L. tarentolae*, while one (4.2%) *Ph. perniciosus* for *L. infantum*. Out of the 44 females collected using CDC light traps, four *S. minuta* were microscopically positive for promastigotes; *L. tarentolae* was confirmed by dqPCR for three of them only and one axenic culture was established after 30 days. Furthermore, host blood meal sequences were obtained for five out of ten engorged females, with high nucleotide identity (99.8–100%) of *T. mauritanica* (1×; AN JQ425060) and dog (1×; AN MN699634) in *S. minuta*, of humans (2×; AN OL521838 and MK617278) in *Ph. perniciosus*, and dog (1×; AN MN699634) in *Ph. neglectus*.

Dog sampling

Of 19 dogs serologically examined, 12 (63.2%) scored positive against promastigotes of *L. infantum* and *L. tarentolae* by IFAT in May and 11 (58.8%) in November 2021 (Table 3). Dog blood samples tested by dqPCR and qPCR only yielded positive results for *L. infantum* in November 2021. Specifically, three dog samples were positive by dqPCR and six samples by qPCR, being three animals positive for both methods (Table 3).

Isolation and sequence analyses

Overall, new strains of *L. tarentolae* were isolated from *T. mauritanica* (strain RTAR/IT/21/RI325) (Fig 5A) and from *S. minuta* (ISER/IT/21/SF178) (Fig 5B) from the same locality (site 2). Sequence analyses of the ITS1 region and *hsp*70 gene (Fig 6) confirmed the *L. tarentolae* identification of the isolates (Accession Numbers: RTAR/IT/21/RI325 –OM831140; ISER/IT/21/SF178 –OM831137).

Discussion

Data demonstrate the sympatric circulation of *L. infantum* and *L. tarentolae* in geckoes, sand flies, and dogs in an area endemic for CanL. Synanthropic geckoes were found to be PCR positive for *L. infantum* and *Ph. perniciosus* for *L. tarentolae*. Importantly, the finding of *L. infantum* amastigotes in the bone marrow of geckoes suggests that these animals are not only exposed, but also infected with this *Leishmania* species.

Both reptile species (i.e., *P. siculus*, *T. mauritanica*) sampled in this study, have previously been recorded PCR positive to *L. infantum* and *L. tarentolae* in the same geographical area, though the prevalence for both *L. tarentolae* (12.5%) and *L. infantum* (4.1%) was higher [13]. In the study presented herein, trypanosomatids were only detected in *T. mauritanica* geckoes probably because it was the main species of reptiles collected. Importantly, sympatric occurrence of these protists (with no co-infection of both *Leishmania* spp.) was observed in geckoes collected from site 2 (a dog shelter), with a prevalence of 13.3% for both *Leishmania* spp. and

Table 3. Antibody titers against *Leishmania infantum* and *Leishmania tarentolae* promastigotes detected by indirect fluorescent antibody test (IFAT) according to sampling time (May and November 2021) and serum dilution (1:80 to 1:2560). Values of the threshold cycle (Ct) are reported for dqPCR and qPCR that were only positive for *Leishmania infantum*.

Dog No.	May 2021		November 2021			
	IFAT		IFAT		dqPCR (Ct)	qPCR (Ct)
	Leishmania infantum	*Leishmania tarentolae*	*Leishmania infantum*	*Leishmania tarentolae*	*Leishmania infantum*	
1	1:2560	1:2560	1:2560	1:1280	33.80	26.20
2	1:320	1:320	1:640	1:640	35.23	29.18
3	1:80	1:80	Died		-	-
4	1:320	1:640	1:640	1:640	-	-
5	1:320	1:320	1:640	1:160	-	-
6	1:80	1:80	1:160	-	-	-
7	1:640	1:640	1:2560	1:1280	-	27.93
8	1:640	1:320	1:640	1:320	-	-
9	-	-	1:80	1:80	-	29.06
10	1:2560	1:2560	1:2560	1:640	-	-
11	1:1280	1:1280	1:2560	1:2560	38.34	28.05
12	1:640	1:1280	1:1280	1:640	-	32.16
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	1:80	1:80	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-

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26.6% for trypomastigotes. *Trypanosoma* sp. was observed in this study co-infecting three hosts with *L. tarentolae*, in blood cytology, with different combinations of diagnostic techniques. Though not identified at species level, *Trypanosoma* cf. *platydactyli* was previously

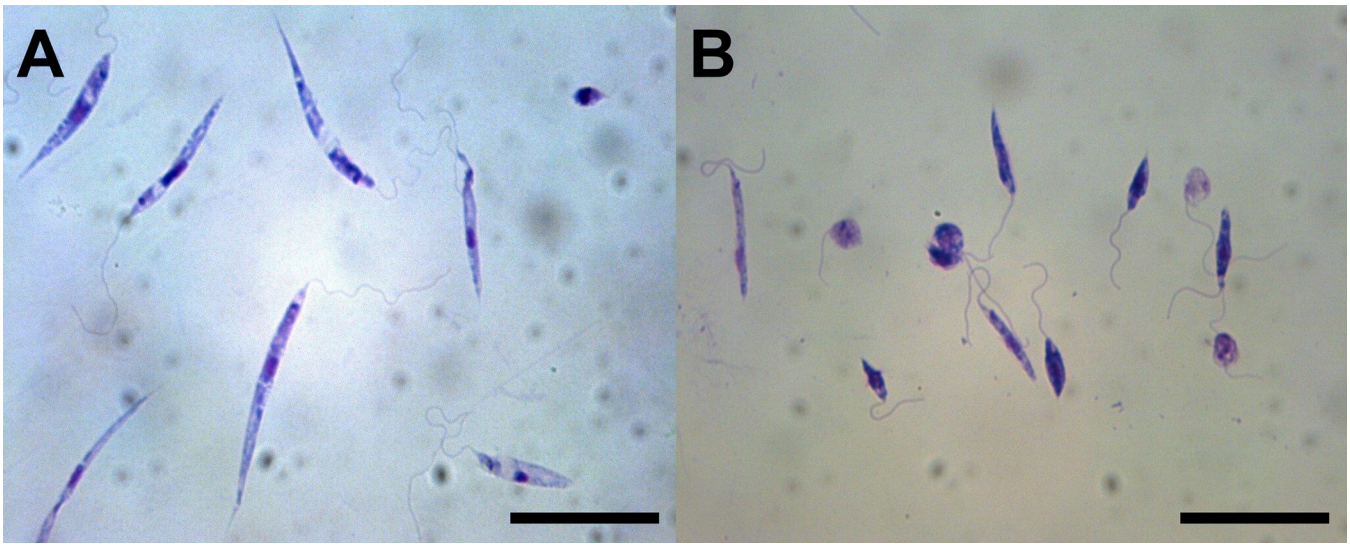

Fig 5. Cultured promastigotes of isolated strains. A) the strain RTAR/IT/21/RI325 isolated from a gecko *Tarentola mauritanica*, with longer body and flagella. B) the strain ISER/IT/21/SF178 isolated from a sand fly female *Sergentomyia minuta*. Scale bars A,B) 30 μm.

https://doi.org/10.1371/journal.pntd.0010650.g005

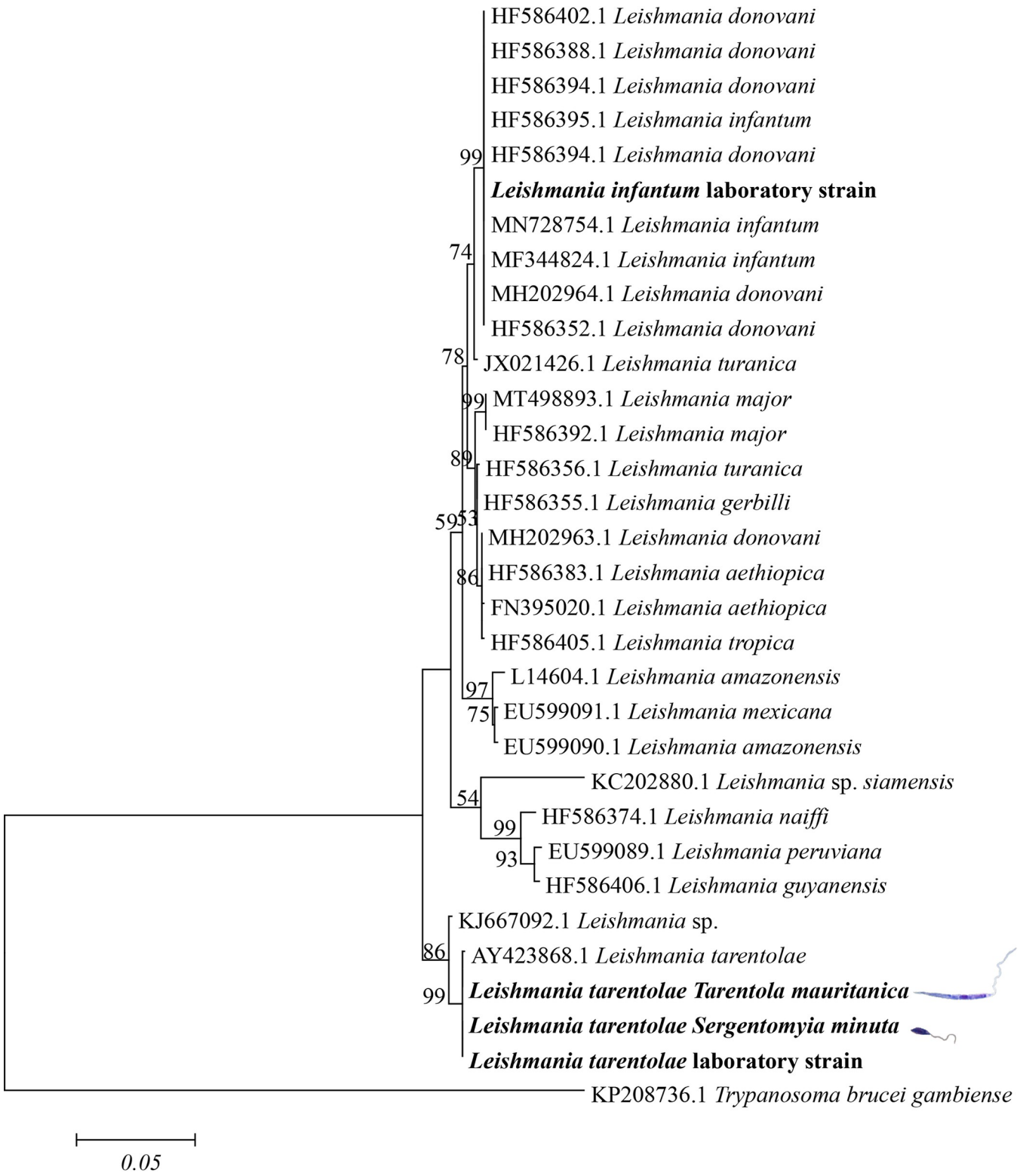

Fig 6. Phylogenetic tree based on *Leishmania hsp*70 sequences inferred using the maximum likelihood method based on the Kimura 3-parameter model. *Trypanosoma brucei gambiense* was used as an outgroup. Scale bar indicates nucleotide substitution per site. The sequences of *Leishmania* spp. obtained in this study are in bold.

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described (prevalence of 27.5%) co-infecting with *L. tarentolae*, in *T. mauritanica* geckoes captured in the surroundings of the Monopoli municipality [31], a city located in the same region (i.e., Apulia) of the present study sites, merely 40 km away. This trypanosomatid has posed an unexpected hindrance for the morphological study of *Leishmania* spp. in Mediterranean reptiles, being once synonymized with *L. tarentolae* as their developing forms in culture can be confused (i.e., trypomastigotes may develop into promastigotes after some passages; [22]). Although *L. infantum* has already been molecularly detected in reptiles [13], this is the first time that amastigote-like forms of this mammalian-associated *Leishmania* species have been observed in bone marrow aspirate by cytology and further confirmed molecularly. Therefore, these data suggest that reptiles may be infected with *L. infantum*, further supporting previous studies in which *Leishmania* spp. pathogenic to mammals were molecularly detected in reptiles [44–46]. Accordingly, efforts should be made to isolate *L. infantum* from reptiles to confirm their role as reservoirs of this medically and veterinary important protist.

The detected species composition and abundance of sand flies are in agreement with previous studies in endemic CanL areas [13,14,16,18], where *S. minuta* was the most abundant species, followed by *Ph. perniciosus*, the natural vector of *L. infantum*. The isolation of *Leishmania* parasites from sand flies confirmed the vector competence of *S. minuta* for *L. tarentolae* and represents a new wild type strain for this species [11,31]. Importantly, molecular positivity of *S. minuta* for *L. infantum* [13,18,19] and positivity for dog DNA in blood meal [16] may imply a putative role as a vector for this species. However, experimental studies are needed to verify the vector capacity of *S. minuta* for *L. infantum*.

The serological results in the studied canine population were consistent with the expected epidemiological scenario, also determined by the molecular detection of only *L. infantum* in dog blood. Moreover, *L. infantum* positive blood samples corresponded to animals that had antibody titers higher than 1:640, with lower Ct values in qPCR, which agrees with the higher sensitivity of minicircle qPCR [39]. Indeed, the usefulness of blood qPCR to detect *Leishmania* is correlated with antibody titration [47], and as high positive predictive value based on clinical evaluation [48]. Nonetheless, given the limited group of dogs selected, discarding a co-infection or a previous exposure to *L. tarentolae* is difficult. However, this study warrants the high possibility of cross-reactivity seen in most cases of sympatric occurrence of trypanosomatids using IFAT methods [49,50]. Thus, to distinguish the infecting species of *Leishmania*, specific confirmatory serological tests should be developed.

This study also represents the most recent isolation of *L. tarentolae* strains from both reptiles and sand flies, thus not suffering from genetic drift due to the long-term cultivation. Although the ITS1 and *hsp70* sequences were highly similar to those of the reference strains, such new strains may represent transiently infectious parasites that should be further molecularly characterized and subjected to phagocytosis and *in vitro* infectivity analyses [27,29]. Indeed, further attempts should be made to isolate *L. tarentolae* from other hosts (i.e., dogs and humans) to fully unravel the infection in mammalian hosts.

The epidemiological scenarios of canine leishmaniasis in the Mediterranean basin can change depending on multiple factors, such as geographic area, sand fly species, and/or canine populations. The scenario observed in the periurban areas of southern Italy adds to this factor the presence of often ignored sand fly, reptile, and *Leishmania* species, namely *S. minuta*, *T. mauritanica*, and *L. tarentolae*. Synanthropic omnipresent geckoes are exposed not only to their specific *Leishmania* species, but also to *L. infantum*, which can develop into amastigotes in the bone marrow. The studied sand fly species display different levels of anthropophilic feeding behavior. The isolation of new *L. tarentolae* strains may provide new information on transient infection, similar to the LEM125 strains. Confirming the isolation of *L. infantum*

from reptiles and *L. tarentolae* from mammals is needed to unravel the epidemiological context given by the sympatric occurrence of both *Leishmania* species.

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Experimental infections of sand flies and geckos with *Leishmania* (*Sauroleishmania*) *adleri* and *Leishmania* (*S.*) *hoogstraali*.

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Parasites & Vectors

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Experimental infections of sand flies and geckos with *Leishmania* (*Sauroleishmania*) *adleri* and *Leishmania* (*S.*) *hoogstraali*

Lucie Ticha[1*], Jovana Sadlova[1], Paul Bates[2] and Petr Volf[1]

Abstract

Background: Species belonging to the subgenus *Sauroleishmania* are parasites of reptiles, and traditionally considered to be non-pathogenic to mammals. Knowledge of the development of these parasites in sand flies and their mechanism of transmission is currently lacking. The main aim of this study was to test the susceptibility of various sand fly species to infection by two *Sauroleishmania* species, focusing on the localization of parasites in the sand fly intestinal tract.

Methods: The development of *Leishmania* (*Sauroleishmania* [*S.*]) *adleri* and *Leishmania* (*S.*) *hoogstraali* was studied in six sand fly species (*Phlebotomus orientalis*, *P. argentipes*, *P. sergenti*, *P. papatasi*, *P. duboscqi*, *Sergentomyia schwetzi*). Sand flies were fed through a chick-skin membrane on blood containing *Sauroleishmania* promastigotes, and they were dissected at various time intervals post blood meal (PBM). Guts were examined microscopically for the presence of parasites, and the intensity and localizations of infections were recorded. Morphological forms of both *Sauroleishmania* species developing in *P. orientalis* were analyzed. Experimental infections of geckos using sand fly-derived promastigotes were also performed, and the reptiles were repeatedly examined for *Sauroleishmania* infection by xenodiagnosis and PCR analysis.

Results: High infection rates for both *Sauroleishmania* species were observed in *P. orientalis* and *P. argentipes*, with the parasites migrating anteriorly and undergoing a peripylarian type of development, including colonization of the stomodeal valve. Conversely, the development of *L.* (*S.*) *adleri* in *P. sergenti*, *P. papatasi* and *Se. schwetzi* was restricted to the sand fly hindgut (hypopylarian type of development). Five morphological forms were distinguished for both *Sauroleishmania* species developing in *P. orientalis*. All experimentally infected geckos scored negative for *Sauroleishmania* based on xenodiagnosis and molecular analysis.

Conclusions: The results showed that *Sauroleishmania* promastigotes can undergo either a peripylarian or hypopylarian type of development in the sand fly intestinal tract, depending on the sand fly species infected. We demonstrated that *P. argentipes* and *P. orientalis*, two sand fly species known as permissive vectors for mammalian parasites of subgenus *Leishmania*, are also highly susceptible to *Sauroleishmania* as the parasites developed mature late-stage infections, including colonization of the sand fly stomodeal valve. Thus, the role of *Phlebotomus* sand flies in transmission of *Sauroleishmania* should be reconsidered and further investigated.

Keywords: *Sauroleishmania*, Sand flies, *Phlebotomus*, *Sergentomyia*, Geckos, Leishmaniasis

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Background

Protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are causative agents of leishmaniases. They are transmitted to vertebrate hosts by phlebotomine sand flies (Diptera: Psychodidae), with one exception, namely members of the subgenus *Mundinia*, for which biting midges (Diptera: Ceratopogonidae) are the main vectors [1]. The genus *Leishmania* is currently divided into four subgenera, of which three: *Leishmania*, *Viannia* and *Mundinia* include species infecting mammals, while the subgenus *Sauroleishmania* comprises of reptilian parasites [2, 3].

Various *Sauroleishmania* species have been found in reptiles of five families (Agamidae, Gekkonidae, Lacertidae, Scincidae and Varanidae) [4, 5], where they occur in two different forms: as free-living promastigotes and/or intracellular amastigotes [6]. Amastigote forms have been observed in different blood cells, mainly in monocytes or macrophages, but also in thrombocytes and erythrocytes [7–10].

The mechanism of *Sauroleishmania* transmission from sand flies to reptilian hosts is still being debated, as it has never been demonstrated under laboratory conditions [11]. Two possible modes of transmission are considered, either via sand fly bites and/or by ingestion of infected sand flies [6]. Sand flies of the genus *Sergentomyia*, which feed preferentially on cold-blooded animals, are generally accepted as the vectors of *Sauroleishmania* [12]. However, it has been reported that some *Sauroleishmania* species can cause late-stage infections in *Phlebotomus* sand flies; consequently, their possible involvement in *Sauroleishmania* transmission should also be considered [13, 14].

In the sand fly intestinal tract, the parasites initially multiply as promastigotes within the blood meal surrounded by a peritrophic matrix. Once the peritrophic matrix is broken, promastigotes migrate into various parts of the sand fly gut [14, 15] and, according to the description of Lainson and Shaw [16], undergo either hypopylarian, peripylarian or suprapylarian types of development. Hypopylarian development is confined to the hindgut (pylorus and rectum) and is considered typical of *Leishmania* (*Sauroleishmania* [*S.*]) species; peripylarian development includes a phase of development in the pylorus region of the hindgut followed by midgut and foregut colonization and is typical of *Leishmania* (*Viannia [V.]*) species, such as *L. braziliensis*; suprapylarian development occurs in the midgut and foregut only and is typical of *Leishmania* (*Leishmania* [*L.*]) species, such as *L. donovani*.

Leishmania (*S.*) *adleri* and *Leishmania* (*S.*) *hoogstraali* are two *Sauroleishmania* species distributed in sub-Saharan Africa. They were first isolated from the lacertid lizard *Latastia longicaudata* [17] and from the gecko *Hemidactylus turcicus* [18], respectively, but their vectors are unknown. The main aim of our study was to test the susceptibility of various sand fly species to *Sauroleishmania* infections. We investigated the development of *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* in six sand fly species differing in susceptibility to *Leishmania*, with the focus on localization of parasites in the sand fly intestinal tract. We also performed experimental infections of geckos using sand fly-derived parasites.

Methods

Parasites, sand flies and geckos

Two *Sauroleishmania* species, *Leishmania* (*S.*) *adleri* (RLAT/KE/LV30) isolated from a lizard (*Latastia* sp.) in Kenya and *Leishmania* (*S.*) *hoogstraali* (RHEM/SD/LV31) isolated from a gecko (*Hemidactylus* sp.) in Sudan were used in this study. Cryopreserved parasites were shipped from Lancaster to Prague, and low-passage parasites (< 5) were used for the experimental infections of sand flies. Promastigotes were cultivated at 23 °C in Medium 199 (Sigma-Aldrich, Prague, Czech Republic) supplemented with 20% heat-inactivated fetal calf serum (Gibco, Prague, Czech Republic), 1% Basal Medium Eagle vitamins (Sigma-Aldrich, Prague, Czech Republic), 2% sterile human urine and 250 µl amikacin (Medopharm, Pozorice, Czech Republic).

In the first series of experiments, three sand fly species, each with a different susceptibility to various *Leishmania* species, were selected: *Sergentomyia schwetzi* (refractory to all *Leishmania* species tested so far [19]; colony originating from Ethiopia), and two *Phlebotomus* species: *Phlebotomus papatasi* (natural vector of *Leishmania major* [20], colony originating from Turkey) and *Phlebotomus argentipes* (natural vector of *Leishmania donovani* [21], colony originating from India). In the second series of experiments, we tested the susceptibility of three *Phlebotomus* species sharing an overlapping geographical distribution with the parasites used in the study: *Phlebotomus duboscqi* (colony originating from Senegal), *Phlebotomus sergenti* (colony originating from Turkey) and *Phlebotomus orientalis* (colony originating from Ethiopia). All sand fly colonies were maintained under standard conditions (26 °C, 14/10-h light/dark photoperiod, 50% sucrose), as described previously [22].

Twelve specimens of the gecko *Hemidactylus turcicus* were used for experimental infections with *Sauroleishmania* parasites. They were maintained individually in plastic boxes (32.5 × 22 × 21 cm) equipped with sand substrate, shelters and water dish, under a 12/12-h light/dark photoperiod and constant temperature maintained by heating pads and cables. Geckos were provided with water ad libitum, and twice a week they were fed with

crickets (*Gryllus assimilis*) or mealworms (*Tenebrio molitor*) dusted in vitamins (Roboran, Univit, Czech Republic) to satiety.

Experimental infections of sand flies

Female sand flies (5–9 days old) were experimentally infected by feeding through a chick-skin membrane on heat-inactivated blood containing 5×10^6 promastigotes per milliliter. Engorged sand flies were then separated out, kept at 26 °C under standard conditions [22] and dissected at different time intervals post blood meal (PBM). The intensity of infections and localizations of parasites in the sand fly gut were examined under a light microscope. The intensity of infections was categorized according to Myskova et al. [23] as light/weak (< 100 parasites/gut), moderate (100–1000 parasites/gut) or heavy (> 1000 parasites per gut). All experiments were repeated at least twice for each *Sauroleishmania*-sand fly combination. Differences in infection rates were evaluated statistically by the Chi-square (χ^2) tests using the SPSS version 27 statistical software package (SPSS IBM Corp., Armonk, NY, USA).

Morphometry of parasites from gut smears

Sauroleishmania morphological forms were studied in *Phlebotomus orientalis* as this sand fly species was shown to be susceptible to both *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali*. Sand fly females were dissected on days 5, 7 and 9 PBM and their guts were used for analysis of morphological forms. Smears of sand fly guts positive for *Sauroleishmania* were fixed with methanol, stained with Giemsa staining solution and observed under a light microscope using an oil-immersion objective; promastigotes were photographed with an Olympus D70 camera (Olympus Corp., Tokyo, Japan). Body length, body width and flagella length of at least 140 randomly selected promastigotes from a minimum of three female sand flies were measured using ImageJ software and evaluated.

Morphological stages of the parasites were determined as described previously for members of the subgenus *Sauroleishmania* [14]: (i) long nectomonad promastigotes (body length ≥ 14 µm); (ii) short nectomonad promastigotes (body length < 14 µm and flagella length < 2-fold the body length); (iii) metacyclic-like promastigotes (body length < 14 µm and flagella length ≥ 2-fold body length); (iv) amastigote-like forms; and (v) haptomonad promastigotes. Differences in number of metacyclic-like stages were tested by the Chi-square (χ^2) tests using SPSS software version 27 (SPSS IBM Corp.).

Experimental infections of geckos

Geckos were infected with sand fly-derived parasites according to the methodology described for mammal-infecting *Leishmania* species [24] with a single modification: as the localization of metacyclic and reptile-infecting stages of *Sauroleishmania* have not been described yet, whole dissected sand fly guts (not only thoracic midguts) were used for the experimental infections. Briefly, two parasite-vector combinations displaying the highest infection rates and intensities of infections were chosen: *P. orientalis* for *L.* (*S.*) *adleri* and *P. argentipes* for *L.* (*S.*) *hoogstraali*. Sand fly females were infected by feeding through a chick-skin membrane with 10^7 promastigotes per milliliter, as described in preceding text, and maintained under standard conditions until day 7 PBM. Engorged sand flies were then dissected and their guts examined for the presence of parasites under a light microscope. Sand fly guts with high parasite loads were pooled and homogenized in sterile saline solution. Each gecko was infected with 10 µl of homogenate, which corresponds to 10 sand fly guts.

Twelve geckos were separated into two groups of six specimens each for experiments with *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali*, respectively. Three geckos from each group were infected intraperitoneally by insulin syringe and the remaining three in each group were infected via the oral route using pipette tips. For each gecko infected via the oral route, 90 µl of saline solution was added to the sand fly gut homogenate. The infection doses (calculated using a Bürker chamber) were determined as 3.39×10^5 for *L.* (*S.*) *adleri* and 7.5×10^4 for *L.* (*S.*) *hoogstraali*.

Infected geckos were monitored weekly for the external signs of the infection, and they were examined for the presence of parasites at different time intervals post-infection (p.i.) using xenodiagnosis. At the end of the experiments (21 weeks p.i.) they were sacrificed and dissected. Samples from the liver, skin, tail, feet and blood were stored at − 20 °C for subsequent molecular analysis. Other parts of these tissues were cultivated on SNB-9 blood agar [25] with M199 medium as an overlay supplemented with 20% heat-inactivated fetal calf serum (Gibco), 1% Basal Medium Eagle vitamins (Sigma-Aldrich), 2% sterile human urine, 250 µl amikacin (Medopharm) and 1.5 µg/ml of fluorocytosin (Sigma-Aldrich). Cultures were checked microscopically for the presence of parasites once a week for a total of 5 weeks.

Xenodiagnosis of geckos

Xenodiagnostic experiments were performed on weeks 3, 7, 12 and 18 p.i. using a laboratory-reared colony of *Se. schwetzi.* This was the only sand fly species which regularly fed on geckos in our laboratory. However, it does not support late-stage development of *Sauroleishmania* and

parasites could be found in its midgut only before defecation. Each gecko was placed in separated net containing 30–50 sand fly females (5–7 days old) that were allowed to feed on the gecko for a maximum of 2 h. Engorged sand flies were separated out, maintained in the nets for 2 days and then (before defecation) placed individually into microcentrifuge tubes with Tissue Lysis Buffer (Roche, Prague, Czech Republic) and stored at − 20 °C for subsequent DNA extraction and analysis by PCR.

PCR assay

Extraction of total DNA from sand flies and tissues of the geckos was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA was used as a template for PCR amplification targeting a region of the ribosomal internal transcribed spacer 1 (ITS1; approx. 300 bp) using the forward primer LITSR (5′-CTGGATCATTTTCCGATG-3′) and reverse primer L5.8S (5′-TGATACCACTTATCGCAC TT-3′) as described previously [26]. Reactions were performed with EmeraldAmp® GT PCR Master Mix at the following cycling conditions: denaturation at 95 °C for 3 min; 35 amplification cycles of 95 °C for 20 s, 53 °C for 30 s, 72 °C for 40 s; and a final cycle at 72 °C for 6 min. The PCR products were analyzed using a SYBR Green fluorescent probe on 1% agarose gels. DNA extracted from the cultures of *Sauroleishmania* spp. and *Leishmania major* were used as positive controls (in the preliminary experiment, we tested these primers with various *Leishmania* species: *Leishmania* (*L.*) *major*, *Leishmania* (*L.*) *amazonensis* and *Leishmania* (*S.*) spp.; all of them gave positive results and, therefore, for the main experiment we chose only *L. major*). Additionally, serial dilutions were performed to confirm the detection of a minimum of 10^2 parasites per sample.

Results

Experimental infections of sand flies I.

Development of *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* was studied in *Se. schwetzi*, *P. papatasi* and *P. argentipes* at two time points: day 1 PBM (before defecation) and day 7 PBM (late-stage infection). Altogether, 506 female sand flies were dissected and examined for the presence of parasites. Statistically significant differences in infection rates were observed between the two *Sauroleishmania* species (Table 1).

On day 1 PBM, in all three sand fly species tested, variable but relatively high infection rates (57–100%) were observed for *L.* (*S.*) *adleri* and very high infection rates (79–96%) for *L.* (*S.*) *hoogstraali* (Fig. 1). All parasites were present in the ingested blood meal within the peritrophic matrix. Statistically significant differences in infection rates were observed on day 1 PBM among sand fly species tested for *L.* (*S.*) *adleri* ($\chi^2 = 10.084$, $df = 2$, $P = 0.006$), but not significant among sand fly species tested for *L.* (*S.*) *hoogstraali* ($\chi^2 = 4.350$, $df = 2$, $P = 0.114$).

On day 7 PBM, after the defecation of blood meal remains, infection rates of both *Sauroleishmania* species were significantly reduced in *Se. schwetzi* and *P. papatasi*. *Leishmania* (*S.*) *adleri* was found in 20% of *Se. schwetzi* and 23% of *P. papatasi* females in which

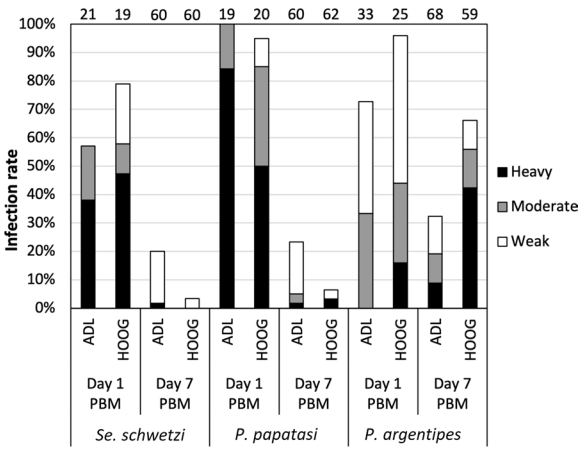

Fig. 1 Development of *Leishmania* (*Sauroleishmania* [*S.*]) *adleri* and *Leishmania* (*S.*) *hoogstraali* in three sand fly species: *Sergentomyia schwetzi*, *Phlebotomus papatasi* and *Phlebotomus argentipes*. Infection rates were examined on days 1 and 7 post blood meal. The intensities of infections were categorized as heavy (> 1000 parasites/gut), moderate (100–1000 parasites/gut) or weak/light (< 100 parasites/gut). Number of dissected sand flies is indicated above the bars. Abbreviations: ADL *L.* (*S.*) *adleri*; HOOG, *L.* (*S.*) *hoogstraali*; PBM, post blood meal

Table 1 Comparison of infection rates of *Leishmania* (*Sauroleishmania* [*S.*]) *adleri* and *Leishmania* (*S.*) *hoogstraali* in three sand fly species

Sand fly species	Day 1 PBM	Day 7 PBM
Sergentomyia schwetzi	$\chi^2 = 2.162$, $df = 1$, $P = 0.129$	$\chi^2 = 8.086$, $df = 1$, $P = 0.004$
Phlebotomus papatasi	$\chi^2 = 0.975$, $df = 1$, $P = 0.513$	$\chi^2 = 6.909$, $df = 1$, $P = 0.008$
Phlebotomus argentipes	$\chi^2 = 5.399$, $df = 1$, $P = 0.020$	$\chi^2 = 14.415$, $df = 1$, $P = < 0.001$

Statistical analysis was performed using the Chi-square (χ^2) test

PBM Post blood meal

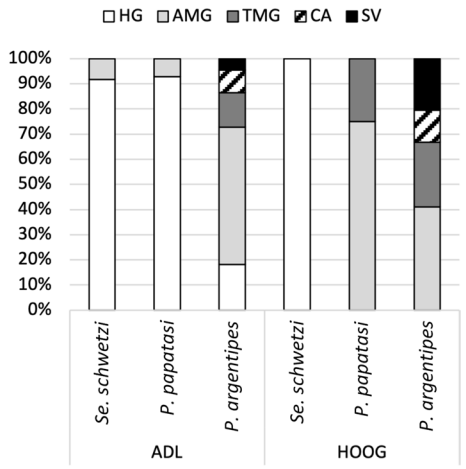

Fig. 2 Simplified localization of *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* in three sand fly species: *Se. schwetzi*, *P. papatasi* and *P. argentipes*. Localization of infections was evaluated microscopically on day 7 PBM. HG, hindgut; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. For more details, see Additional file 1: Table S1, Table S2

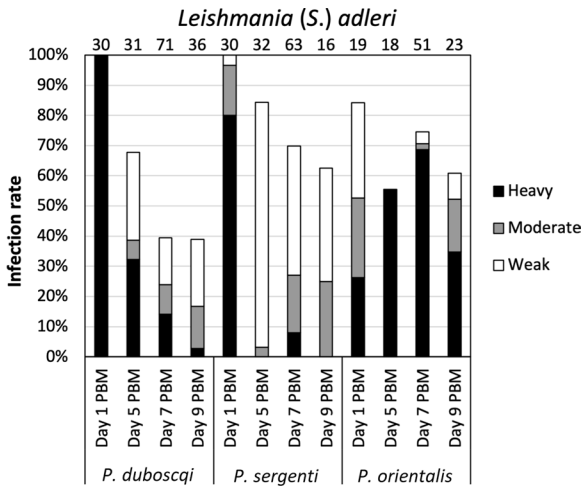

Fig. 3 Development of *L.* (*S.*) *adleri* in three sand fly species: *Phlebotomus duboscqi*, *Phlebotomus sergenti* and *Phlebotomus orientalis*. Infection rates were examined on days 1, 5, 7 and 9 PBM. The intensities of infections were categorized as heavy (> 1000 parasites/gut), moderate (100–1000 parasites/gut) or weak/light (< 100 parasites/gut). The number of dissected sand flies is indicated above the bars

infections of weak intensity prevailed. Parasites occupied the hindgut (mainly pylorus and ileum) (Fig. 2), where attached haptomonad promastigotes were the prevailing forms, but in a few females, long free-swimming flagellates were also present. Infection rates of *L.* (*S.*) *hoogstraali* in *Se. schwetzi* and *P. papatasi* were negligible, reaching 4% and 7%, respectively.

In contrast, higher infection rates were observed in *P. argentipes* on day 7 PBM when 32% of dissected sand flies were positive for *L.* (*S.*) *adleri* and 66% for *L.* (*S.*) *hoogstraali*. *Leishmania* (*S.*) *adleri* developed in the hindgut, but also migrated anteriorly into the *P. argentipes* midgut (82% of infected sand flies). In two sand fly females, promastigotes reached the cardia (i.e. part of the midgut immediately behind the stomodeal valve), and in a single female the stomodeal valve was successfully colonized.

Infections of *L.* (*S.*) *hoogstraali* in *P. argentipes* were the most successful, with the presence of promastigotes detected in 66% of dissected sand flies on day 7 PBM. In most cases, parasites developed heavy-intensity infections and underwent the peripylarian type of development. In addition to the hindgut, promastigotes were observed in the abdominal and thoracic midgut (41% and 26%, respectively), reaching the cardia and colonizing the stomodeal valve in 13% and 21% of infected females, respectively.

Infection rates between sand fly species on day 7 PBM were not significantly different for *L.* (*S.*) *adleri* ($\chi^2 = 2.782$, $df = 2$, $P = 0.249$), but they were significantly different for *L.* (*S.*) *hoogstraali* ($\chi^2 = 79.850$, $df = 2$, $P \leq 0.001$).

Experimental infections of sand flies II.

Development of *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* was studied in *P. duboscqi*, *P. sergenti* and *P. orientalis* at various time intervals, namely on days 1, 5, 7 and 9 PBM. In total, 783 sand flies were examined for the presence of parasites.

Development of L. (S.) adleri

Promastigotes of *L.* (*S.*) *adleri* multiplied abundantly in the ingested blood meal on day 1 PBM, and infection rates reached 84–100% in all three sand fly species tested, with statistically significant differences among the three sand fly species ($\chi^2 = 9.848$, $df = 2$, $P = 0.007$; Fig. 3). Infections of heavy intensity prevailed in *P. duboscqi* and *P. sergenti*, while the intensity of infection in *P. orientalis* was slightly lower. In all tested sand flies, *L.* (*S.*) *adleri* successfully survived defecation and developed late-stage infections. Significant differences were found in infection rates among sand fly species on day 7 PBM ($\chi^2 = 19.418$, $df = 2$, $P \leq 0.001$), while the differences were not significant on day 5 PBM ($\chi^2 = 5.074$, $df = 2$, $P = 0.079$) and day 9 PBM ($\chi^2 = 3.852$, $df = 2$, $P = 0.146$).

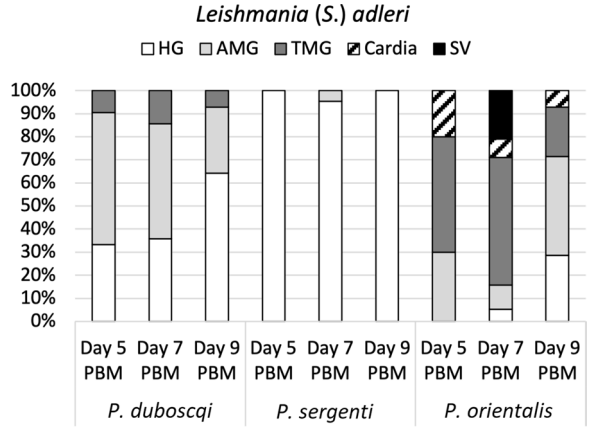

Fig. 4 Simplified localization of *L.* (*S.*) *adleri* in three sand fly species: *P. duboscqi, P. sergenti* and *P. orientalis*. Localization of infections was evaluated microscopically on days 5, 7 and 9 PBM. For abbreviations, see Fig. 2. For more details, see Additional file 2: Table S3

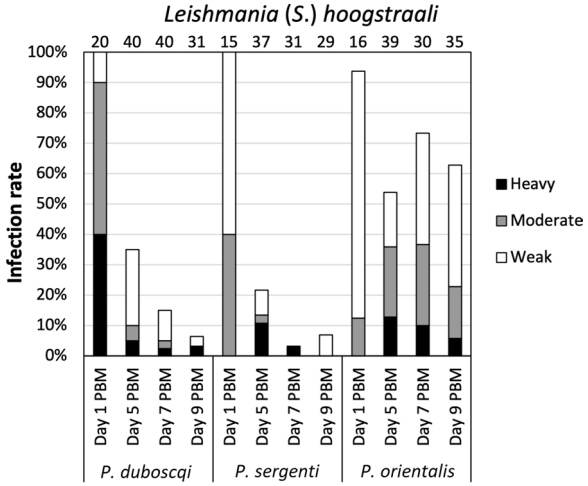

Fig. 5 Development of *L.* (*S.*) *hoogstraali* in three sand fly species: *P. duboscqi*, *P. sergenti* and *P. orientalis*. Infection rates were examined on days 1, 5, 7 and 9 PBM. The intensities of infections were categorized as heavy (> 1000 parasites/gut), moderate (100–1000 parasites/gut) or weak/light (< 100 parasites/gut). Number of dissected sand flies is indicated above the bars

In *P. duboscqi* the infection rate was almost 70% on day 5 PBM, then dropped to < 40% on days 7 and 9 PBM, with the majority of infections being of moderate and weak intensity. Promastigotes were localized in the hindgut and migrated to abdominal and thoracic midgut (peripylarian type of development) (Fig. 4). In the hindgut, haptomonad promastigotes were the most abundant forms, but free flagellates were also observed to a lesser extent.

Conversely, the hypopylarian type of development prevailed in *P. sergenti*. Relatively high infection rates were recorded at all designated time intervals (> 60%), and the intensity of most infections was moderate and weak/light. Parasites mainly occupied the hindgut (pylorus and ileum), with haptomonad promastigotes as the prevailing forms, while the presence of flagellates in the abdominal midgut was detected in only two *P. sergenti* females (5%).

In *P. orientalis,* heavy late-stage infections were observed on days 5 to 9 PBM, with > 50% positive sand flies, in which the peripylarian type of development prevailed. Promastigotes multiplied and migrated rapidly as they were present in thoracic midgut (50%) and cardia (20%) on day 5 PBM, and colonization of stomodeal valve had occurred in 21% of infected sand flies on day 7 PBM. Similar dynamics of the infections then persisted until day 9 PBM.

Development of L. (S.) hoogstraali

On day 1 PBM, high infection rates (94–100%) were reported in all three sand fly species, with no significant differences ($\chi^2 = 2.231$, $df = 2$, $P = 0.328$; Fig. 5). The intensities of infections were mostly weak/light or moderate, and parasites were present in the blood meal enclosed by peritrophic matrix (endoperitrophic space). After defecation, however, significant differences in infection rates were observed between sand fly species at all designated time intervals: day 5 PBM ($\chi^2 = 8.564$, $df = 2$, $P = 0.014$), day 7 PBM ($\chi^2 = 46.269$, $df = 2$, $P \leq 0.001$) and day 9 PBM ($\chi^2 = 35.113$, $df = 2$, $P \leq 0.001$).

In *P. duboscqi* and *P. sergenti* females, *L.* (*S.*) *hoogstraali* was not able to survive defecation. The number of positive sand fly females decreased over time, and only

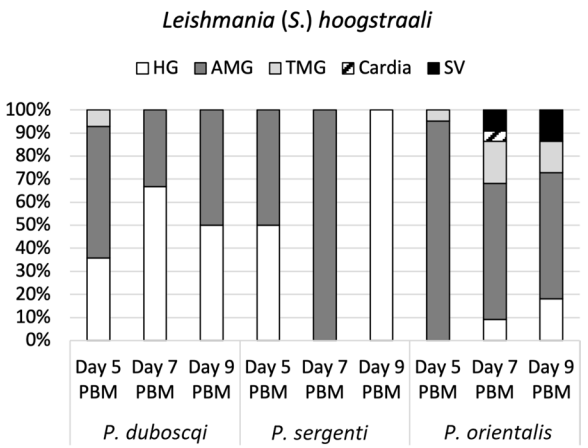

Fig. 6 Simplified localization of *L.* (*S.*) *hoogstraali* in three sand fly species: *P. duboscqi, P. sergenti* and *P. orientalis*. Localization of infections was evaluated microscopically on days 5, 7 and 9 PBM. For abbreviations, see Fig. 2. For more details, see Additional file 2: Table S4

Table 2 Differences in *L.* (*S.*) *adleri* and *L.* (*S*) *hoogstraali* development in various sand fly species

Leishmania species[a]	Sand fly species[b]					
	SCHW	PAP	ARG	DUB	SER	ORI
ADL	Hypopylarian	Hypopylarian	Peripylarian	Peripylarian	Hypopylarian	Peripylarian
HOOG	Hypopylarian	Peripylarian	Peripylarian	Peripylarian	Hypopylarian	Peripylarian

[a] *ADL Leishmania* (*S.*) *adleri*, *HOOG Leishmania* (*S.*) *hoogstraali*

[b] *SCHW Sergentomyia schwetzi*, *PAP Phlebotomus papatasi*, *ARG Phlebotomus argentipes*, *DUB Phlebotomus duboscqi*, *SER Phlebotomus sergenti*, *ORI Phlebotomus orientalis*

infections of weak intensity were observed. *Leishmania* (*S.*) *hoogstraali* migrated anteriorly in *P. duboscqi*: parasites colonized mainly the hindgut but were also present in the abdominal (57%) and thoracic (7%) midgut on day 5 PBM (Fig. 6). In contrast, *L.* (*S.*) *hoogstraali* development in *P. sergenti* was restricted to the hindgut (Table 2), and promastigotes were observed in the abdominal midgut only when the remnants of ingested blood were still present.

Leishmania (*S.*) *hoogstraali* successfully survived defecation and developed late-stage infections in *P. orientalis*, with infection rates reaching > than 50% at all designated time intervals. Both attached haptomonad promastigotes and free-swimming flagellates were observed in the hindgut, but *L.* (*S.*) *hoogstraali* more tended to acquire an anterior position in this sand fly species: promastigotes reached the cardia (5%) and colonized the stomodeal valve (10%) on day 7 PBM. A similar tendency was observed on day 9 PBM, when colonization of the stomodeal valve had occurred in 14% of dissected females.

Morphological transformations

Five morphological forms were observed in both *Sauroleishmania* species tested (Fig. 7). Long nectomonad promastigotes and short nectomonad promastigotes were the most abundant forms, while haptomonad promastigotes, metacyclic-like promastigotes and amastigote-like forms were presented to a lesser extent (Additional file 3: Table S5; Additional file 3: Table S7). Long nectomonad promastigotes prevailed in *L.* (*S.*) *adleri* (75%), whereas short nectomonad promastigotes were more frequent in *L.* (*S.*) *hoogstraali* (62%) (Fig. 8). Both long and short nectomonad promastigotes were present also in a variation with significantly shortened flagella (approx. 4 µm).

Metacyclic-like promastigotes were recorded at all designated time intervals, and these stages were

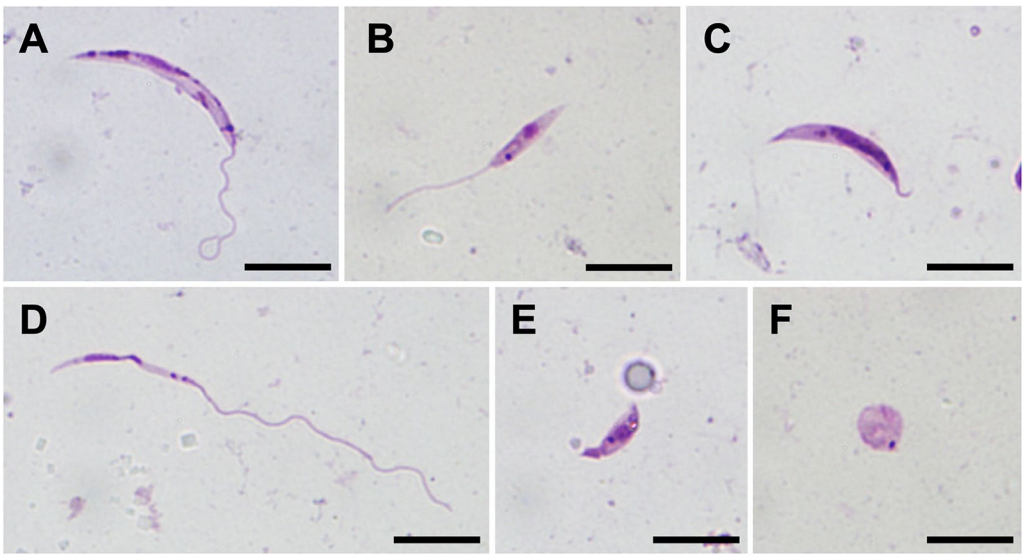

Fig. 7 *Sauroleishmania* morphological forms in sand flies. Morphological analysis was performed on *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* developing in *P. orientalis* on days 5, 7 and 9 PBM. **a** Long nectomonad promastigote, **b** short nectomonad promastigote, **c** long nectomonad promastigote with shortened flagella, **d** long slender metacyclic-like promastigote, **e** haptomonad promastigote, **f** amastigote-like form (stained by Giemsa, 1000× magnification, scale bars: 10 µm)

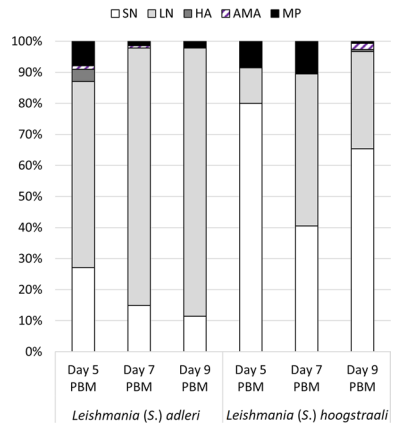

Fig. 8 Representation of *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali* morphological forms in *P. orientalis* on days 5, 7 and 9 PBM. Abbreviations: SN, Short nectomonad promastigotes; LN, long nectomonad promastigotes; HA, haptomonad promastigotes; AMA, amastigote-like forms; MP, metacyclic-like promastigotes

Fig. 9 *Sergentomyia* females feeding on gecko during xenodiagnosis

morphologically highly variable in terms of body length and width. We distinguished three cell types: short rounded, short slender and long slender metacyclic promastigotes. Moreover, some of these forms had remarkably elongated flagella (up to 50 µm). Statistical analysis showed that the number of metacyclic-like promastigotes was significantly different on day 7 PBM ($\chi^2 = 10.381$, $df = 1$, $P \leq 0.001$), but not on days 5 PBM ($\chi^2 = 0.045$, $df = 1$, $P = 0.494$) and 9 PBM ($\chi^2 = 1.204$, $df = 1$, $P = 0.279$).

Haptomonad promastigotes with typically leaf-shaped flagella were harder to detect as they are strongly attached to the cuticular lining of the sand fly gut and, therefore, their number is significantly underestimated. Rounded (amastigote-like) forms with very short or no flagella were also reported. Detailed measurements of individual morphological forms are summarized in Additional file 3: Table S6, Table S8.

Experimental infections of geckos and xenodiagnoses

No external signs of the infections were observed in any geckos. Xenodiagnostic experiments (Fig. 9) were performed on weeks 3, 7, 12 and 18 p.i., and among the 604 *Se. schwetzi* females tested, none were found to be positive (for more details see Additional file 4: Table S9). The experiment was terminated 21 weeks p.i. when geckos were sacrificed and dissected for tissue sampling. Nevertheless, *Sauroleishmania* DNA was not detected in any of the samples tested (i.e. liver, skin, tail, feet, and blood) and no parasites were observed in tissue cultures.

Discussion

In the present study we demonstrated that the ability to undergo different types of development in sand flies is typical for parasites of the subgenus *Sauroleishmania* and that this variability is influenced by sand fly vectors. It is interesting to note that none of the parasite-vector combinations tested showed suprapylarian development and that there was always hindgut involvement to varying degrees, indicating this may be a fundamental property of *L.* (*Sauroleishmania*) species.

Although it is generally accepted that *Sauroleishmania* parasites are transmitted by reptile-biting sand flies of the genus *Sergentomyia*, the role of other sand flies in *Sauroleishmania* transmission should be reconsidered. The susceptibility of *Phlebotomus* species to *Sauroleishmania* infections has been experimentally demonstrated by several authors [13, 14] and now confirmed in the present study. Some *Phlebotomus* species were reported to feed on reptiles [4, 13, 27], and recent molecular detection of *Leishmania* (*Sauroleishmania*) *tarentolae* in *Phlebotomus* spp. [28–30] further supports the hypothesis that these sand flies are alternative vectors of *Sauroleishmania* [14].

It has been assumed that *Sauroleishmania* development in sand flies is restricted to the hindgut and described as hypopylarian [16]. Therefore, infection per the oral route was considered as one of the possible modes of *Sauroleishmania* transmission to reptiles [6]. Conversely, some older studies reported *Sauroleishmania* promastigotes in the anterior midgut [11, 13, 31]. The tendency to obtain an anterior position in the sand fly gut suggests that members of this group may be transmitted via sand fly bites, in a manner similar to mammal-infecting *Leishmania* species [15]. Nonetheless, a recent study showed that *L.* (*S.*) *tarentolae* underwent both hypopylarian or peripylarian type of development depending on the sand fly species infected [14]; consequently, the mechanism of *Sauroleishmania* transmission from sand flies to reptilian hosts remains unclear.

Despite the proven role of members of the genus *Sergentomyia* as vectors of *Sauroleishmania*, the involvement of *Se. schwetzi* in the transmission of *L.* (*S.*) *adleri* is

unlikely as only 20% of females displayed the presence of parasites on day 7 PBM, with majority of infections being of weak/light intensity. It was also shown that *Se. schwetzi* is refractory to mammalian *Leishmania* spp. due to its delayed degradation of peritrophic matrix until the time of defecation, which does not provide sufficient time for promastigotes to escape the endoperitrophic space and attach to the midgut epithelium [19, 32].

Attachment of promastigotes to the sand fly gut is a key part of the *Leishmania* life-cycle as it prevents the expulsion of parasites during defecation [33]. The successful development of *L.* (*S.*) *adleri* in the hindgut of *P. papatasi* and *P. sergenti* may be due to the parasite's ability to attach to the cuticular lining of the hindgut but its inability to bind to the sand fly midgut. *Phlebotomus sergenti* is known to be a specific vector of *Leishmania tropica* [34], while *P. papatasi* is specific for *L. major* [20] and *Leishmania turanica* [35]. In specific vectors, the attachment of promastigotes to the midgut epithelium is mediated by species-specific surface lipophosphoglycan (LPG) [36]. Nevertheless, the role of LPG in the *Sauroleishmania* life-cycle is understudied and it has been reported that some *Sauroleishmania* spp. appear to lack LPG or certain enzymes involved in LPG modification [37, 38].

Conversely, *P. argentipes* and *P. orientalis* are known to be permissive vectors susceptible to multiple *Leishmania* spp. under laboratory conditions [33] in which promastigotes attach via a different, glycan-mediated, mechanism [39]. In both of these sand fly species, the highest infection rates and highest intensities of infections were recorded for *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali,* suggesting that some species of *Sauroleishmania* may non-specifically attach to the midgut of permissive vectors in a manner similar to mammalian *Leishmania*.

As *Sauroleishmania* transmission from sand flies to reptilian hosts has never been demonstrated under laboratory conditions, stages infectious for the reptiles are not known [15]. Only a few studies have described *Sauroleishmania* morphological forms produced in vectors [13, 14], assuming they do not differ from those described for mammalian *Leishmania*. In this study, we demonstrated the presence of stages morphologically identical to metacyclic promastigotes. Nevertheless, the metacyclogenesis of *Sauroleishmania* has not been studied and thus the potential infectiousness of these forms is unclear.

Although sand fly-derived parasites were used for the experimental infections of geckos, *Sauroleishmania* infection was not detected in any of the *H. turcicus* tested. Selection of the wrong host species is unlikely, as *L.* (*S.*) *hoogstraali* was primarily isolated from *H. turcicus* geckos and this species has also been shown to be susceptible to *L.* (*S.*) *adleri* [18]. Therefore, we assumed that one of the possible explanations of unsuccessful transmission may be the loss of infectivity of both *Sauroleishmania* strains. Most *Sauroleishmania* isolates were obtained decades ago and have since been passaged for long periods in media without the opportunity to undergo a complete life-cycle. It has been shown that prolonged cultivation results in genetic drift and noticeable changes in the mitochondrial genome [40] and, therefore, we consider it necessary to acquire new isolates for future research work.

Conclusions

This study provides experimental evidence that *Sauroleishmania* development in vectors is variable and significantly affected by sand fly species. Some *Phlebotomus* species, particularly *P. orientalis* and *P. argentipes*, are highly susceptible to *Sauroleishmania* infections and, therefore, the role of these sand flies in *Sauroleishmania* circulation should be reconsidered and further investigated. We also demonstrated the anterior migration of *Sauroleishmania* in their intestinal tract and confirmed the peripylarian type of development reported by several old studies.

Abbreviations

ITS1: Internal transcribed spacer 1; LPG: Lipophosphoglycan; PBM: Post-blood meal; p.i.: Post-infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05417-1.

Additional file 1: Table S1. Localization of *Leishmania* (*Sauroleishmania*) *adleri* promastigotes in three sand fly species differing in vector competence to *Leishmania*. **Table S2**. Localization of *Leishmania* (*Sauroleishmania*) *hoogstraali* promastigotes in three sand fly species differing in vector competence to *Leishmania*

Additional file 2: Table S3. Development of *Leishmania* (*Sauroleishmania*) *adleri* in three sand fly species sharing an overlapping geographical distribution. **Table S4**. Development of *Leishmania* (*Sauroleishmania*) *hoogstraali* in three sand fly species sharing an overlapping geographical distribution

Additional file 3: Table S5. Representation of individual morphological forms of *Leishmania* (*Sauroleishmania*) *adleri* developing in *Phlebotomus orientalis* on days 5 to 9 post blood meal. **Table S6**. Detailed measurements of individual forms of *Leishmania* (*Sauroleishmania*) *adleri* developing in *Phlebotomus orientalis* on days 5, 7 and 9 post blood meal. **Table S7**. Representation of individual morphological forms of *Leishmania* (*Sauroleishmania*) *hoogstraali* developing in *Phlebotomus orientalis* on days 5 to 9 post blood meal. **Table S8**. Detailed measurements of individual forms of *Leishmania* (*Sauroleishmania*) *hoogstraali* developing in *Phlebotomus orientalis* on days 5, 7 and 9 post blood meal

Additional file 4: Table S9. Xenodiagnoses of *Hemidactylus turcicus* geckos experimentally infected with *Leishmania* (*Sauroleishmania*) *adleri* and *Leishmania* (*Sauroleishmania*) *hoogstraali*

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Author contributions
LT carried out the experimental infections of sand flies and geckos, dissections of sand flies, morphometry of parasites, xenodiagnoses, and molecular analysis. JS contributed to dissections of sand flies, experimental infections of geckos and carried out the statistical analysis. Parasites were provided by PB. PV and JS participated in the design on the study and supervision. Article was drafted by LT and PV. JS and PB contributed with the revision of the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials
All the data are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on the Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All experiments were approved by the Committee on the Ethics of Laboratory Experiments of Charles University in Prague and were performed under permission of no. MSMT-11459/2019–4 of the Czech Ministry of Education of the Czech Republic. Investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

Consent for publication
Not applicable.

Competing interests
Authors declare that there are no competing interests.

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Publisher's Note

Experimental feeding of *Sergentomyia minuta* on reptiles and mammals: comparison with *Phlebotomus papatasi*.

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Parasites & Vectors

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Experimental feeding of *Sergentomyia minuta* on reptiles and mammals: comparison with *Phlebotomus papatasi*

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Abstract

Background *Sergentomyia minuta* (Diptera: Phlebotominae) is an abundant sand fly species in the Mediterranean basin and a proven vector of reptile parasite *Leishmania* (*Sauroleishmania*) *tarentolae*. Although it feeds preferentially on reptiles, blood meal analyses and detection of *Leishmania* (*Leishmania*) *infantum* DNA in wild-caught *S. minuta* suggest that occasional feeding may occur on mammals, including humans. Therefore, it is currently suspected as a potential vector of human pathogens.

Methods A recently established *S. minuta* colony was allowed to feed on three reptile species (i.e. lizard *Podarcis siculus* and geckos *Tarentola mauritanica* and *Hemidactylus turcicus*) and three mammal species (i.e. mouse, rabbit and human). Sand fly mortality and fecundity were studied in blood-fed females, and the results were compared with *Phlebotomus papatasi*, vector of *Leishmania* (*L.*) *major*. Blood meal volumes were measured by haemoglobinometry.

Results *Sergentomyia minuta* fed readily on three reptile species tested, neglected the mouse and the rabbit but took a blood meal on human. However, the percentage of females engorged on human volunteer was low in cage (3%) and feeding on human blood resulted in extended defecation times, higher post-feeding mortality and lower fecundity. The average volumes of blood ingested by females fed on human and gecko were 0.97 µl and 1.02 µl, respectively. *Phlebotomus papatasi* females readily fed on mouse, rabbit and human volunteer; a lower percentage of females (23%) took blood meal on the *T. mauritanica* gecko; reptilian blood increased mortality post-feeding but did not affect *P. papatasi* fecundity.

Conclusions Anthropophilic behaviour of *S. minuta* was experimentally demonstrated; although sand fly females prefer reptiles as hosts, they were attracted to the human volunteer and took a relatively high volume of blood. Their feeding times were longer than in sand fly species regularly feeding on mammals and their physiological parameters suggest that *S. minuta* is not adapted well for digestion of mammalian blood. Nevertheless, the ability to bite humans highlights the necessity of further studies on *S. minuta* vector competence to elucidate its potential role in circulation of *Leishmania* and phleboviruses pathogenic to humans.

Keywords Sand flies, *Sergentomyia*, *Phlebotomus*, Feeding preferences, *Leishmania*

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Background

Phlebotomine sand flies (Diptera: Psychodidae) are hematophagous insects of major medical and veterinary importance. Among more than 900 described sand fly species, approximately 100 are proven or suspected vectors of *Leishmania* protozoa, bacteria (*Bartonella* spp.) and sand fly-borne viruses [1]. In the Old World, *Phlebotomus* species act as major vectors of human diseases, while *Sergentomyia* species are mainly herpetophilic and have long been associated only with the reptilian parasites of subgenus *Sauroleishmania* [2]. Although some *Sergentomyia* species are currently suspected vectors of *Leishmania* pathogenic to humans [3], their capability to transmit human pathogens is yet to be revealed, as solid field and laboratory evidence is still lacking.

Sergentomyia minuta is one of the most abundant sand fly species in Mediterranean basin and the main vector of *Leishmania* (*Sauroleishmania*) *tarentolae*, a non-pathogenic parasite of geckos [4, 5]. Although *S. minuta* feeds preferentially on reptiles, blood meal analyses indicate it may occasionally feed on mammals, including humans [6–11]. Moreover, DNA of two mammalian parasites, *Leishmania* (*Leishmania*) *major* and *L.* (*L.*) *infantum* has been detected in *S. minuta* repeatedly [9, 12–17]. RNA of *Toscana phlebovirus* (TOSV), a causative agent of sporadic outbreaks of acute human encephalitis and meningoencephalitis in Mediterranean countries, was detected in wild-caught females of *S. minuta* in France [18]. Although molecular detection alone is not sufficient evidence to incriminate a sand fly as vector, all these findings raised questions about the spectrum of *S. minuta* hosts and its role in the transmission cycle of phleboviruses and *Leishmania* infecting humans and other mammals.

The aim of this study was to investigate the feeding behaviour of *S. minuta* in different reptilian and mammalian hosts. The effect of various blood sources on *S. minuta* mortality and fecundity was also studied. The results were compared with *Phlebotomus papatasi*, a species widespread in Europe, Africa and Asia and well known as human-biting pest [19].

Methods

Sand flies

The colonies of *Sergentomyia minuta* (originating from Portugal) and *Phlebotomus papatasi* (originating from Turkey) were established at the Department of Parasitology, Charles University, in 2019 and 2005, respectively. *Sergentomyia minuta* colony was maintained feeding on leopard geckos (*Eublepharis macularius*), while *P. papatasi* was routinely maintained on BALB/c mice. During the experiments, sand flies were kept in the insectaries of the Department of Parasitology, Charles University, and the Department of Veterinary Medicine, University of Bari. Colonies were maintained at 24–26 °C, 55–70% humidity, with 14 h light/10 h dark photoperiod, and offered 50% sucrose ad libitum, as described previously [20].

Mammals and reptiles

Three species of mammals were tested, including a human volunteer (co-author Volfova), mice and rabbits. BALB/c mice originating from AnLab s.r.o. (Harlan Laboratories, USA) were maintained in T3 breeding containers (Velaz) equipped with bedding (German Horse Span, Pferde a.s.) and breeding material (Woodwool) and provided with a standard feed mixture (ST-1, Velaz) and water ad libitum, with a 12 h light/12 h dark photoperiod, at 22–25 °C and 40–60% humidity. NZW rabbits (originating from AnLab s.r.o.) were kept in breeding boxes (Velaz) equipped according to guidelines and legislation, provided with a standard feeding mixture for rabbits (Biopharm), hay (Krmne smesi Kvidera) and water ad libitum.

Three reptile species were offered as hosts to test sand fly feeding, with two species of geckos (Moorish gecko *Tarentola mauritanica*; Mediterranean house gecko *Hemidactylus turcicus*) and a lacertid lizard (Italian wall lizard *Podarcis siculus*) being compared. Animals were captured and maintained at the Department of Veterinary Medicine, University of Bari, as part of a study on *Leishmania* spp. in Mediterranean reptiles [21].

Sergentomyia minuta feeding on a gecko and a human: assessment of blood meal volumes and sand fly fecundity

During the establishment of the *S. minuta* colony, several potential hosts for its blood-feeding were tested. Preliminary experiments revealed that, in addition to the geckos, *S. minuta* females also feed on human; therefore, its anthropophilic behaviour was further investigated. Routine maintenance of the *S. minuta* colony was done on leopard gecko (*E. macularius*), which is used in our faculty as a laboratory animal. However, its natural area of distribution differs from that of *S. minuta*; therefore, in feeding preferences experiments, we replaced it with three Mediterranean reptiles.

To compare blood meal volumes and fecundity on reptile versus mammalian blood, *S. minuta* females (5 days old) were fed either on a male leopard gecko (*E. macularius*) or on a forearm of the human volunteer. In human, a feeding chamber was used to increase numbers of *S. minuta* fully fed by human blood. The type of feeding chamber was described and depicted previously [22]. Briefly, 20 females were transferred into a plastic tube (diameter 3 cm) covered with fine gauze and placed onto an elbow area of a human arm for 2 h. The same relatively long exposure time (2 h) was used in gecko to allow the

females to feed to repletion. Two independent trials were performed.

Haemoglobinometry was used to measure the blood meal volumes taken by individual *S. minuta* females. During blood-feeding, sand flies concentrate ingested proteins because of prediuresis; thus, gravimetry might lead to underestimated results. Haemoglobinometry is independent of diuresis and provides more precise estimation of the ingested blood meal volume [23, 24]. For haemoglobin assay, individual guts without Malpighian tubules were dissected 1 h post-blood meal (PBM) and transferred into microtubes with 500 µl of distilled water in batches of ten guts per sample. The samples were stored at − 70 °C until use. After thawing the samples were thoroughly homogenized and then analysed using Haemoglobin Assay Kit (MAK115, Sigma-Aldrich) following the manufacturer's instruction. Afterwards, 50 µl of homogenate was loaded per well in triplications. The resulting haemoglobin content per gut was compared to the haemoglobin concentration measured in the host blood (same gecko and human individuals as used for experimental feeding).

The second group of females, fully fed on either gecko or human, was maintained in cages under standard conditions until defecation and dissected in buffered saline, and mature oocytes were counted under a Leica M205 FA stereomicroscope. The experiment was repeated twice.

Sergentomyia minuta and *Phlebotomus papatasi* feeding on reptiles and mammals: comparison of mortality and fecundity

In experiments with reptiles, sand fly females (i.e. $n = 50$, 5–7 days old) were separated into nylon cloth cages and left there for acclimatisation for 20 min. A small number of sand fly males (< 10) was used in each group. Reptiles were placed individually into cages, and sand flies were allowed to feed in darkness, at 23–26 °C, for 2 h. In mammalian experiments the methodology was modified in the following way: BALB/c mouse anaesthetized with ketamine/xylazine (62.5 mg/kg and 25 mg/kg, respectively), mechanically immobilized NZW rabbit and forearm of the human volunteer were positioned in the cages, and sand flies were allowed to feed on the hosts for 1 h (because of the use of anaesthesia in mice).

Approximately 2 h after the hosts were removed from the cages, the blood-fed females were separated into new cages, kept under standard conditions as described above, and their post-blood meal mortality was monitored until defecation of blood meal remains (day 4 PBM in *P. papatasi* and day 6 PBM in *S. minuta*). The surviving sand fly females were then anaesthetized on ice and dissected in saline solution. The number of mature oocytes from 10 sand fly females per group was counted under the stereomicroscope, and the experiment was repeated twice.

Statistical analysis

Differences in fecundity (oocytes numbers) of sand flies engorged on different hosts were tested by one-way ANOVA and multiple comparison of means using LSD post hoc test. Mortality and feeding were compared using Fisher's exact or Pearson's Chi-square test. All the statistical analyses were performed using SPSS software version 23.

Results

Life cycle parameters of *Sergentomyia minuta* colony

The whole development cycle of *S. minuta* in colony maintained on leopard geckos (*E. macularius*) at 26 °C was relatively fast; females laid first eggs 4–8 days post-blood meal (PBM) and first-instar larvae hatched 10–14 days PBM (Table 1). Development of four larval instars took about 2 weeks; first pupae were observed on days 23–28 PBM. Pupal period lasts for about 6 days and first adults emerged 4–5 weeks PBM. These life cycle parameters did not change during the maintenance of the colony, as they were almost the same in generations 1–4 and 23–25 (Table 1).

Table 1 Life cycle parameters of *Sergentomyia minuta* colony

		Days post-blood meal Mean (min/max)					
Year	Generation	Egg	1st instar larva	2nd instar larva	4th instar larva	Pupa	Adult
2019	1–4	6.75 (4–8)	11.50 (10–14)	16.10 (16–19)	22.00 (20–26)	26.20 (24–28)	32.40 (27–35)
2020	6–8	6.45 (4–7)	10.65 (9–12)	15.25 (13–19)	22.40 (21–26)	26.15 (23–30)	32.50 (30–37)
2022	23–25	6.30 (4–9)	10.65 (9–14)	16.30 (12–20)	22.05 (17–28)	27.60 (23–33)	33.30 (29–38)

Average intervals are given for three generations (with the range of average intervals for each generation)

The blood meal volumes of *Sergentomyia minuta* feeding on a gecko and a human

The prolonged time of exposure (2 h), together with the application of the feeding chamber, resulted in a relatively high feeding rate (60%) on human (24 out of 40 females), which allowed the study of blood meal volumes and fecundity of females. The feeding rate on leopard gecko in nylon cloth cage was > 70%, similar to the routine feeding on this gecko species during regular colony maintenance (Volfova, personal communication).

No visible skin reaction was observed in geckos, even after repeated exposure to *S. minuta* bites. The blood meal volumes were measured in two samples of 10 fully engorged *S. minuta* females fed on gecko and two samples of 10 fully engorged females fed on human. Volumes ingested by *S. minuta* fed on human arm and gecko were similar and ranged around 1 µl (0.97 ± 0.03 µl/female and 1.02 ± 0.05 µl/female, respectively). As the result of such relatively big blood meals, the fully fed females were scarcely able to fly and usually only crawled out of the host.

Considerable differences were, however, found in the feeding process; on the gecko, females started to feed within 5 min and the mean feeding time to repletion was 45 min. On the other hand, feeding on human was delayed; *S. minuta* females started to feed in an interval from 5 to > 60 min after beginning of exposure. Unlike feeding on the gecko, the females fed on the human often interrupted feeding and needed several attempts to full engorgement.

All females fed on the gecko defecated by day 4 PBM. In contrast, defecation of females fed on the human was delayed for 2 days (i.e. by day 6 PBM). Post-feeding mortality was also higher in the females fed on the human host than in those fed on the reptile (25% versus 15%).

Following defecation, ovaria were dissected, and mature oocytes were counted. All examined females (fed on either the reptile or the human host) developed mature oocytes (Fig. 1). Nevertheless, the oocyte numbers differed substantially between the experimental groups; females fed on reptile developed significantly higher numbers (average 73, range 45–108, median 76) than those fed on human (average 26, range 17–40, median 22). Interestingly, ascogregarine infection contaminating the colony was found markedly elevated in the sand fly females fed on human blood (Fig. 1B).

Mortality and fecundity of *Sergentomyia minuta* and *Phlebotomus papatasi* feeding on mammals

Sergentomyia minuta females were attracted to the hand and forearm of the human volunteer placed in a nylon cloth cage several minutes after exposure. Although numerous sand fly attempts of bite were recorded (Fig. 2A), only a negligible number of females (3%) engorged on the human volunteer under these conditions (Table 2, Fig. 3). Females were feeding mostly on parts with softer skin, typically on the back of the hand, on the elbow or between the fingers. Contrarily, no *S. minuta* females fed on mouse or rabbit, and these hosts were completely ignored by this sand fly species (Table 2, Fig. 3). *Sergentomyia minuta* bites did not cause any visible effects on naive individual (human); however, repeated exposure resulted in pronounced skin hypersensitivity with maximum reaction 24–72 h post-feeding (Fig. 2A).

The experiment confirmed that human blood had a negative effect on the digestion, mortality and fecundity of *S. minuta*. Females displayed a prolonged defecation period (i.e. 5–6 days post-blood feeding), their mortality post-feeding was increased to 30%, and number of

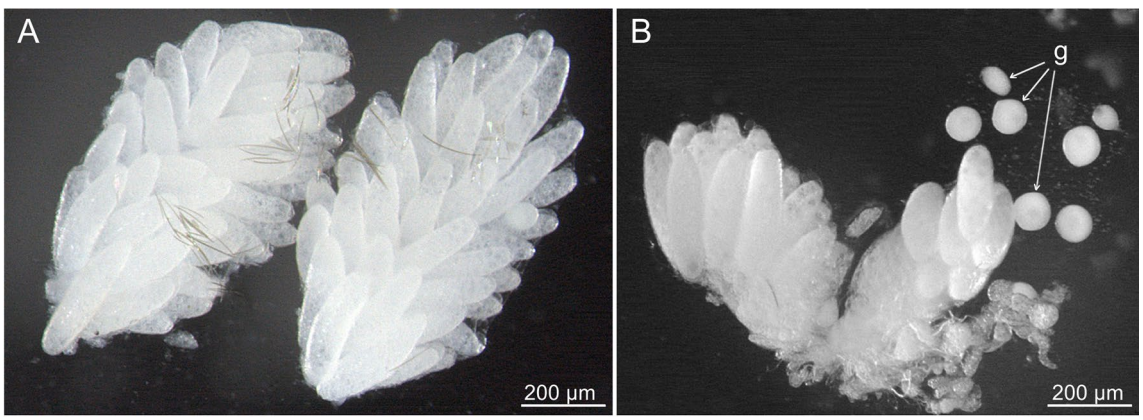

Fig. 1 Dissected ovaria of *Sergentomyia minuta* females fed on gecko (**A**) and human (**B**). The number of developing oocytes was high in females fed on reptile; gregarine gamonts (g) were frequently found in the females fed on human

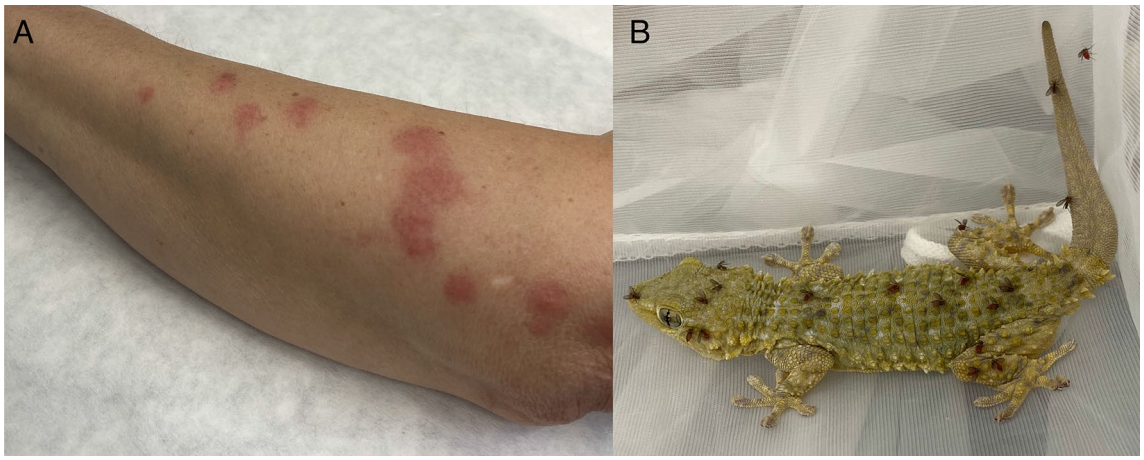

Fig. 2 Skin hypersensitivity reaction to *Sergentomyia minuta* bites in human volunteer repeatedly exposed to this sand fly species (reaction 24 h post-exposure) (**A**) *S. minuta* females feeding on *Tarentola mauritanica* gecko (**B**)

Table 2 Feeding of *Sergentomyia minuta* and *Phlebotomus papatasi* on mammals and reptiles: feeding rate and the effect on sand fly mortality and fecundity

Host species	Sand fly species	Sand fly feeding: *N* engorged/*N* (%)	Mortality post-feeding: *N* dying/*N* (%)	Number of oocytes: average (range; median)
Mouse	*S. minuta*	0/100 (0%)		
	P. papatasi	68/100 (68%)	2/68 (2.9%)	59.7 (26–96; 60)
Rabbit	*S. minuta*	0/100 (0%)		
	P. papatasi	72/100 (72%)	4/72 (5.5%)	57.8 (22–101; 53)
Human	*S. minuta*	3/100 (3%)	1/3 (30%)	15.5 (3–28; 15.5)
	P. papatasi	89/100 (89%)	4/89 (4.5%)	50.4 (27–85; 48)
T. mauritanica	*S. minuta*	62/100 (62%)	4/62 (6.4%)	64.3 (12–92; 67.5)
	P. papatasi	23/100 (23%)	3/23 (13%)	55.4 (11–83; 57)
H. turcicus	*S. minuta*	50/100 (50%)	3/50 (6%)	52.1 (22–73; 50.5)
	P. papatasi	1/100 (1%)	1/1 (100%)	
P. siculus	*S. minuta*	35/100 (35%)	5/35 (14.2%)	42.4 (27–66; 40)
	P. papatasi	0/100 (0%)		

developed oocytes (15,5 in average) was significantly lower compared to blood-feeding on reptilian hosts ($P < 0.001$, $F = 11.309$; Table 2; Additional file 1).

Phlebotomus papatasi females readily fed on mouse, rabbit and human volunteer with relatively high feeding rates (68, 72 and 89%, respectively; Fig. 3). The feeding process was considerably faster compared to *S. minuta* females. The blood meal source did not affect the mean number of *P. papatasi* mature oocytes ($P = 0.914$, $F = 0.012$); 56 and 55 oocytes were produced on average after feeding on mammals (mean of all three species tested) and *T. mauritanica* gecko, respectively (Table 2).

Mortality and fecundity of *Sergentomyia minuta* and *Phlebotomus papatasi* feeding on reptiles

Sergentomyia minuta readily fed on all three reptile species tested, with a slight preference of geckos over the lizard (Table 2, Fig. 2B; $P < 0.001$, Chi-square = 14.646, $df = 2$). This may be due to differences in host activity: both gecko species were relatively calm throughout the experiment, whereas the lizard was more active, and thus sand flies were more disturbed while feeding. Usually, reptiles did not show any defensive behaviour and only sporadic scratching was observed when many sand fly females were feeding at the same time. Sand fly females were attracted to reptilian hosts almost immediately

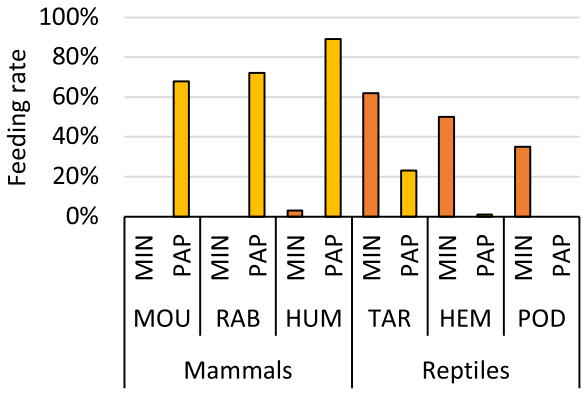

Fig. 3 Feeding rate of *Sergentomyia minuta* and *Phlebotomus papatasi* on different mammals and reptiles. MIN *Sergentomyia minuta*, PAP *Phlebotomus papatasi*, MOU BALB/c mouse, RAB NZW rabbit, HUM human volunteer, TAR *Tarentola mauritanica*, HEM *Hemidactylus turcicus*, POD *Podarcis siculus*

after the exposure, but their feeding behaviour varied: some started to feed within a few minutes while the others after dozens of minutes or even more than 1 h. Post-blood meal mortality of *S. minuta* was low (6–14%) and fecundity was relatively high (42–64 oocytes in average) (Table 2).

Phlebotomus papatasi females were regularly attracted to all reptilian hosts but only a small number initiated blood-feeding, as they were more easily distracted by host activity compared to *S. minuta* females. Of three reptile species tested, *P. papatasi* females took blood only on *T. mauritanica* gecko (feeding rate 23%), which was the calmest reptile tested.

Although reptilian blood increased *P. papatasi* mortality ($P=0.033$), it did not have a significant effect on sand fly fecundity ($P=0.914$, $F=0.012$); females fed on *T. mauritanica* gecko were able to develop oocytes in relatively high numbers (average 55.4). These numbers are fully comparable to *S. minuta* oocyte numbers after feeding on the same host (average 64.3) or to *P. papatasi* oocyte numbers after feeding on mammalian hosts (average 50.4–59.7) (Table 2).

Discussion

In this study, we demonstrated experimentally that *S. minuta* had an anthropophilic behaviour, being attracted to the human volunteer. Indeed, it is generally accepted that sand flies of the genus *Phlebotomus* are mostly mammalophilic and transmit *Leishmania* pathogenic to humans, while species of the genus *Sergentomyia* are referred as herpetophilic, being proven vectors of reptilian leishmaniasis [2]. However, some members of both genera have a broader host spectrum and thus availability of the hosts is an important factor to consider. For example, an extensive study in Paloich Area in the Sudan demonstrated that several *Sergentomyia* and *Phlebotomus* sand flies feed on both mammals and/or reptiles [25]. From laboratory experiments it is known that *Sergentomyia schwetzi* readily feeds on geckos but can feed and thrive also on mammals for many generations [26, 27]. Such an opportunistic behaviour may have important consequences, potentially opening new epidemiological scenarios for the transmission of vectored pathogens.

Feeding behaviour of *S. minuta* differed from that of *P. papatasi* and all other sand fly species tested so far. On both types of hosts (leopard gecko and human) the relatively small-sized *S. minuta* females were able to ingest the biggest volume of blood meal compared to other sand flies studied to date [24, 28]. In addition, the feeding was markedly prolonged when observed in other sand fly species, including *S. schwetzi* [23, 29, 30], which readily feeds on various mammals [26, 27]. This behaviour may reflect the adaptation of *S. minuta* to feeding on reptiles. In mosquitoes, similar long feeding time up to 40 min has been observed in *Culex territans* mosquitoes, which also primarily feed on cold-blooded vertebrates [31]. Due to the prolonged feeding time, *S. minuta* females might regulate and concentrate the imbibed large volume of a blood meal in a gut via prediuresis and thus supposedly compensate significantly lower haemoglobin content in reptilian erythrocytes.

Phlebotomus papatasi is well known for its aggressive behaviour in biting humans [19] and is a proven vector of *L.* (*L.*) *major* and viruses pathogenic to humans [1]. It is considered an opportunistic species, feeding on a variety of hosts, including mammals, birds and reptiles [25, 32–34]. Recently, *P. papatasi* was shown to be susceptible to *L.* (*S.*) *tarentolae* under laboratory conditions [35] and the demonstration of its ability to feed on *T. mauritanica* geckos, further supports the hypothesis of its involvement in *Sauroleishmania* transmission as a secondary vector [35, 36].

The colony of *S. minuta* thrives on leopard geckos (*E. macularius*); at standard temperature 26 °C the development of all life cycle stages was relatively fast. Comparison to other colonies maintained at the Department of Parasitology, Charles University, showed that *S. minuta* has the shortest generation time (i.e. 7–8 weeks). Accordingly, the larval period took approximately 2 weeks, which is about 1 week shorter than in other sand flies maintained in the same conditions [20]. In contrast to humans, no skin reactions were observed in geckos after repeated *S. minuta* bites.

Herpetophillic behaviour of *S. minuta* demonstrated in the experiments overlaps results of previous reports

from the field [10, 11, 17]. Among three reptile species tested, *S. minuta* readily fed on geckos but was also able to feed on *P. siculus* lizards, from which *L.* (*S.*) *tarentolae* DNA was recently isolated [16]. This *Sauroleishmania* species has so far been described in three species of geckos, namely *Tarentola mauritanica*, *T. annularis* and *Mediodactylus kotschyi* [5]. The ability of *S. minuta* to feed on *P. siculus* highlights the possibility that this common lizard is involved in circulation of *L.* (*S.*) *tarentolae* in Italy.

Even more interesting is, however, the experimental confirmation that *S. minuta* occasionally bites mammals, particularly humans. Although *S. minuta* prefers reptiles as a blood meal source, females were attracted to the forearm of the human volunteer placed in the nylon cloth cage and took a blood meal. These findings correspond to the results of field surveys showing this sand fly species occasionally feeds on human blood [6–11]. In *S. minuta*, humans were the most frequently detected hosts apart from reptiles in different catching sites [6, 8–11]. However, it was also reported that this species feeds to a lesser extent on a relatively wide range of mammalian hosts, including large ungulates, dogs and rabbits [9]. Our experiments showed that *S. minuta* took a blood meal on human volunteer but completely refused to feed on a rabbit or a mouse.

If allowed to feed ad libitum, *S. minuta* females were able to acquire almost the same volume of blood meal on the human as on the reptile host (approximately 1 µl). Nevertheless, the digestion of human blood was prolonged, post-feeding mortality was increased, while fecundity was decreased. Similar changes of physiological parameters were observed during an unsuccessful attempt to keep a *S. minuta* colony by feeding on humans: females had high mortality, low fecundity and the colony died out after two generations (Volfova and Volf, unpublished). All these results suggest that S. *minuta* is not adapted to feeding on mammals and cannot digest human blood properly. Consequently, feeding on humans is more likely an opportunistic behaviour of this sand fly species, which is in striking contrast to *S. schwetzi* where a lineage feeding exclusively on mice was successfully established [27], and females readily feed on humans (Volfova and Volf, unpublished).

Potential involvement of *Sergentomyia* as vectors of human pathogenic *Leishmania* spp. was mentioned repeatedly [3] but reliable evidence is still lacking and all *Leishmania* parasites isolated from *S. minuta* so far were typed as *L.* (*S.*) *tarentolae* [5]. Interestingly, this reptilian parasite was recently also detected in humans [11, 37] and dogs [16], and thus its pathogenic potential for mammals is currently unclear [5]. Demonstration of *S. minuta* feeding on humans may therefore explain how *L.* (*S.*) *tarentolae* was transmitted from geckos to humans and dogs.

Laboratory experiments are crucial for vector identification. Even though promastigotes were found and *L.* (*L.*) *infantum* DNA was detected in *S. schwetzi* [38], it was proved experimentally that this sand fly is refractory to mammal-infecting *Leishmania* spp. [39]. Early phase of *Leishmania* infection in sand flies is a non-specific process accompanied by rapid multiplication of promastigotes in the ingested blood meal; then, defecation of blood meal remnants represents the crucial barrier in unnatural parasite-vector pairs [40]. *Leishmania* (*L.*) *infantum* promastigotes were able to develop early-stage infections even in biting midges *Culicoides nubeculosus*, but they were, similarly to *S. schwetzi*, lost during defecation, although *Leishmania* DNA was detectable up to 7 days post-infection [41].

Unfortunately, the experiments with *S. minuta* are limited by the fact that females refused to feed through membranes. All attempts to perform experimental infections with this species failed, although various feeding conditions were tested repeatedly; these include the use of different blood sources (i.e. sheep, rabbit and chicken blood), membranes (i.e. chick skin, gecko skin, a membrane from pig intestine) and changes of temperature and humidity in the experimental box. Therefore, the field work accompanied by direct observation on natural promastigote infection (together with parasite isolation and its typing) remains the best way to prove the involvement of *S. minuta* in circulation of *L.* (*L.*) *infantum* and other species pathogenic to mammals.

Conclusions

Experimental data on the feeding behaviour of *S. minuta* were herein assessed for the first time. We demonstrated that *S. minuta* females readily took a blood meal on geckos and lizards and that the feeding times of *S. minuta* were significantly longer than those typical for sand fly species regularly feeding on mammals. Interestingly, despite the relatively small size of this sand fly species, the volume of ingested blood was higher than in other sand fly species tested so far. *Sergentomyia minuta* females refused to feed on mice and rabbits but were able to bite a human volunteer, causing pronounced skin hypersensitivity reaction in the volunteer repeatedly exposed. Digestion of human blood was prolonged, post-feeding mortality was high, and fecundity was reduced. All these findings suggest that *S. minuta* is not well adapted to feeding on humans and digesting human blood. However, the ability of *S. minuta* to bite humans raises questions about its potential role in circulation of various *Leishmania* parasites and phleboviruses.

Abbreviations

PBM	Post-blood meal
TOSV	*Toscana phlebovirus*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05758-5.

Additional file 1: Table S1. Number of developed oocytes of *Sergentomyia minuta* females feeding on different hosts. TAR, *Tarentola mauritanica*; HEM, *Hemidactylus turcicus*; POD, *Podarcis siculus*; HUM, human volunteer. **Table S2**. Comparison of *Sergentomyia minuta* fecundity after feeding on different hosts. TAR, *Tarentola mauritanica*; HEM, *Hemidactylus turcicus*; POD, *Podarcis siculus*; HUM, human volunteer.

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Author contributions

LT and VV carried out the experimental part of the project. PV, VV and CM helped with the establishment of *Sergentomyia minuta* colony. Reptiles were provided by JAMR, MABS and DO. PV designed and supervised the study. Article was drafted by LT, VV and PV. JS performed the statistical analysis. JAMR, MABS, CM, and DO contributed to the revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the data are included within the article and its additional files.

Declarations

Ethics approval and consent to participate

Mice, rabbits and leopard geckos were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University, Prague, and were performed under permissions of no. MSMT-8604/2019-6 and MSMT-11459/2019-4 of the Czech Ministry of Education of the Czech Republic. Investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic. Protocols for reptile collection were authorized by the Ministry for Environment, Land and Sea Protection of Italy (approval number 0073267/2019), the ***Societas Herpetologica Italica*** and the ***Istituto Superiore per la Protezione e la Ricerca Ambientale*** (approval number 71216). Reptile handling and maintenance were authorized by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 14/2022).

Consent for publication

Not applicable.

Competing interests

Authors declare that there are no competing interests.

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Publisher's Note

SUMMARY AND CONCLUSIONS

This Ph.D. thesis summarize results of the research project on reptile parasites of the subgenus *Sauroleishmania*, which was financially supported mainly by Grant Agency of the Charles University and resulted in five publications in peer-reviewed journals. The main objective of this study was to elucidate some missing aspects of *Sauroleishmania* life cycle. A major part of this project aimed to test the susceptibility of various sand fly species to different *Sauroleishmania* isolates and describe their development in the sand fly intestinal tract. Another part of the thesis was also devoted to the study of infection in reptiles.

Firstly, we focused on the development of *L.* (*S.*) *tarentolae*, which is one of the most studied *Sauroleishmania* species, but its life cycle in sand flies has not yet been properly documented (reviewed by Klatt et al., 2019). For our experiments, we selected three sand fly species of the genus *Phlebotomus* that differ in their specificity or permissiveness to mammalian *Leishmania* species (Volf and Myskova, 2007). Laboratory-bred sand flies were experimentally infected through a membrane on blood with a suspension of promastigotes, dissected at various time intervals post infection and the morphological forms of *Sauroleishmania* were distinguished.

Our results showed that *L.* (*S.*) *tarentolae* development differed between three *Phlebotomus* species tested. The highest infection rates and heavy parasite loads were present in *Phlebotomus pernicious*, sand fly permissive for various mammal-infecting *Leishmania* spp. (Maroli et al., 1994; Volf and Myskova, 2007). In this sand fly species, promastigotes developed in the hindgut, but also migrated anteriorly in the gut, often reached cardia and colonized the stomodeal valve. Similar findings were observed in *Phlebotomus papatasi*, which is a specific vector of *Leishmania* (*L.*) *major* (Pimenta et al., 1994) and *Leishmania* (*L.*) *turanica* (Chajbullinova et al., 2012). In contrast, infection rates were significantly lower in *P. sergenti*, a specific vector of *Leishmania* (*L.*) *tropica* (Kamhawi et al., 2000). Most infections were of light or moderate intensity, and although anterior migration of promastigotes was also observed, promastigote development was more restricted to the sand fly hindgut.

It is generally accepted that species of the subgenus *Sauroleishmania* undergo hypopylarian type of development in sand flies and their localization is restricted to the sand fly hindgut (Lainson and Shaw, 1987). Nevertheless, peripylarian type of the development was clearly demonstrated in all three sand fly species tested in our study. Promastigotes occupied both posterior and anterior parts of the gut and often colonized the stomodeal valve. Colonization of the stomodeal valve is one of the prerequisites for the successful transmission of mammalian leishmania to the vertebrate host (reviewed by Dostalova and Volf, 2012). These findings may therefore indicate that species of the subgenus *Sauroleishmania* could be transmitted similarly. Interestingly, in all *Sauroleishmania*-sand fly combinations, we observed colonization of Malpighian tubules (MTs). Promastigotes were often present in MTs in high numbers, and although this localization is rather unique among parasites of the genus *Leishmania*, it may be an essential part of *L.* (*S.*) *tarentolae* life cycle in sand flies (Ticha et al., 2021).

For better understanding of *Sauroleishmania* life cycle, it would be also important to describe the development of *L.* (*S.*) *tarentolae* in its natural vector, *Sergentomyia minuta* (Maroli et al., 1988; Bongiorno et al., 2021). Although colony of *Se. minuta* has been already established in our laboratory in Prague, females refused to feed through any type of the membrane (both chicken and lizard skins were tested). This behaviour of *Se. minuta* has been previously described by some authors (Wallbanks, 1982) and thus we assume that feeding on

infected geckos might be the only possibility how to study *L*. (*S*.) *tarentolae* development in this sand fly species. Nevertheless, our results from experiments with *Phlebotomus* spp. are in accordance with earlier findings of unspecified promastigote infection of *Sauroleishmania* in MTs of *Se. minuta* females collected in France (Killick-Kendrick, 1979), which suggests that colonization of Malpighian tubules is a regular part of *Sauroleishmania* life cycle in sand flies.

In the same study by Ticha et al. (2021), describing the development of *L*. (*S*.) *tarentolae* in *Phlebotomus* spp., five morphological stages of *Sauroleishmania* were distinguished for the first time. In addition, stages that were morphologically determined as metacyclic promastigotes have been recorded. However, the metacyclogenesis of *Sauroleishmania* has never been described and it is not clear if these forms are infectious to reptiles. Further laboratory experiments will be required to clarify whether these stages play the same role in the transmission as in *Leishmania* species infecting mammals (Ticha et al., 2021).

The successful development of *L*. (*S*.) *tarentolae* in *P. papatasi* was of particular interest as this sand fly species was previously considered as potential vector of some *Sauroleishmania* spp. (Adler and Theodor, 1929; Telford, 2009). Although *P. papatasi* feeds primarily on mammals, several studies have reported its opportunistic behaviour (Svobodova et al., 2003; Palit et al., 2005) and the ability of this species to feed on cold-blooded animals has been repeatedly demonstrated (Adler and Theodor, 1929; McMillan, 1965; Belova, 1971). Based on these findings and results of our study, we propose that some members of the genus *Phlebotomus* may be partially involved in the transmission of *Sauroleishmania* spp. as their alternative vectors. This hypothesis is further supported by several studies that have detected the presence of *Sauroleishmania* DNA in some species of the genus *Phlebotomus*. For example, DNA of *L*. (*S*.) *tarentolae* has been reported in wild-caught females of *Phlebotomus perfiliewi* (Pombi et al., 2020) and *P. perniciosus* (Mendoza-Roldan et al., 2021; Latrofa et al., 2021) in Italy.

Next, we investigated the development of two selected *Sauroleishmania* species, *Leishmania* (*S*.) *adleri* and *L*. (*S*.) *hoogstraali*, in various sand flies of the genera *Sergentomyia* and *Phlebotomus* (Ticha et al., 2022). In the first series of experiments, three sand fly species differing in susceptibility to mammalian *Leishmania* were tested: (1) *Sergentomyia schwetzi* as a potential natural vector of *Sauroleishmania*, but species refractory to all *Leishmania* spp. tested so far (Sadlova et al., 2013), (2) *P. papatasi* as a specific vector of *L*. (*L*.) *major* and *L*. (*L*.) *turanica* (Pimenta et al., 1994; Chajbullinova et al., 2012), and (3) *P. argentipes* as a vector permissive for several *Leishmania* spp. (Volf and Myskova, 2007; reviewed by Maroli et al., 2013). In the second series of experiments, the susceptibility of three sand fly species sharing an overlapping geographical distribution with *L*. (*S*.) *adleri* and *L*. (*S*.) *hoogstraali* were used: *P. duboscqi*, *P. sergenti* and *P. orientalis*. Sand fly females were infected through a chick-skin membrane on blood containing a promastigote suspension and dissected at various time points post blood meal. We again focused on the localization of parasites in the sand fly gut and their morphological forms.

Although sand flies of the genus *Sergentomyia* are considered as natural vectors of *Sauroleishmania*, the role of *Se. schwetzi* in the transmission of both tested *Sauroleishmania* species is unlikely. Infection rates of *L*. (*S*.) *hoogstraali* in *Se. schwetzi* were negligible and only 20 % of infected sand flies were positive for *L*. (*S*.) *adleri* on day 7 post blood meal with majority of light intensity infections. Development of promastigotes was limited to the sand fly hindgut, where they attached to its cuticular lining. Previously, it has been shown that *Se. schwetzi* has a delayed degradation of the peritrophic matrix associated with the time of defecation that does not provide sufficient time for the promastigotes to escape into the ectoperitrophic space and attach to the sand fly midgut. This resulted in the refractoriness of *Se.*

schwetzi to several mammalian *Leishmania* spp. (Sadlova et al., 2013; Sadlova et al., 2018) and may also explain the unsuccessful development of both *Sauroleishmania* spp. in this sand fly.

Leishmania (*S.*) *hoogstraali* infections in *P. papatasi* and *P. sergenti* were mostly lost after the defecation of blood meal remnants, whereas infections of *L.* (*S.*) *adleri* persisted until late stages. In these sand flies, *L.* (*S.*) *adleri* underwent a hypopylarian type of development and promastigotes were found exclusively in the sand fly hindgut.

Both *P. papatasi* and *P. sergenti* are known as specific vectors enabling the development of one or a few *Leishmania* species that specifically attach to their midgut epithelium (Volf and Myskova, 2007). Attachment of promastigotes is an essential part of the intravectorial development as it prevents the expulsion of parasites during defecation (reviewed by Dostalova and Volf, 2012). In specific vectors, the attachment is mediated by lipophosphoglycan (LPG), a species-specific surface molecule of parasites (Kamhawi, 2004). Some studies have reported that *Sauroleishmania* spp. appear to lack LPG, or the enzymes involved in its modification (Previato et al., 1997; Raymond et al., 2012), but it should be noted that LPG in *Sauroleishmania* is generally understudied. The successful development of *L.* (*S.*) *adleri* in *P. papatasi* and *P. sergenti* may be therefore due to its ability to non-specifically attach to the cuticular lining of the hindgut. In addition, these results showing susceptibility of *P. papatasi* to another *Sauroleishmania* species further support its role as an alternative vector of *Sauroleishmania* (Ticha et al., 2021).

Conversely, *P. argentipes* and *P. orientalis* are considered as permissive sand flies susceptible to various *Leishmania* spp. (reviewed by Dostalova and Volf, 2012). In these sand fly species, highest infection rates and heavy-intensity infections were observed for *L.* (*S.*) *adleri* and *L.* (*S.*) *hoogstraali*. Promastigotes were present in the hindgut, but also migrated anteriorly in the sand fly intestinal tract and underwent peripylarian development. In mammalian *Leishmania* spp., the attachment of promastigotes to the midgut of permissive vectors is LPG-independent and determined *via* glycan-glycan interactions (Volf and Myskova, 2007; reviewed by Dostalova and Volf, 2012; Myskova et al., 2016; Hall et al., 2020). Based on our results, we suppose that some *Sauroleishmania* spp. may also non-specifically attach to the midgut of permissive sand fly species and thus persist into the late-stage infections.

More importantly, we demonstrated that the same *Sauroleishmania* species can undergo both hypopylarian and peripylarian type of development, depending on sand fly species. None of the *Sauroleishmania*-sand fly combinations showed suprapylarian development and there was always hindgut involvement to varying degrees. Thus, colonization of the sand fly hindgut might be an essential part of the *Sauroleishmania* life cycle in sand flies.

This variability in intravectorial development is influenced by sand fly species, and the ability of *Sauroleishmania* to undergo different types of development may explain the contradictory data observed by some authors in older studies (Killick-Kendrick, 1979). For better understanding of *Sauroleishmania* life cycle it would be also important to study their development in sand flies of the genus *Sergentomyia* in more detail. However, studies on natural infections of *Sauroleishmania* spp. in wild-caught sand fly females are few. In terms of experimental infections, most of them were performed with sand flies of the genus *Phlebotomus*, as laboratory colonies of *Sergentomyia* were almost lacking and/or colonized sand flies refused to feed under laboratory conditions (Killick-Kendrick et al., 1979; Wallbanks, 1982).

Due to the hypopylarian development of *Sauroleishmania* it was believed that ingestion of infected sand fly or contaminative way of transmission are probable (Killick-Kendrick et al., 1986). The tendency of several *Sauroleishmania* spp. to obtain an anterior position in the sand fly gut contrarily suggest that transmission may occur *via* sand fly bite, in a manner similar to mammalian *Leishmania* species (reviewed by Bates, 2007). Even though experimental

infections of reptiles with cultures of *Sauroleishmania* promastigotes have been rarely reported (McMillan, 1965; Dollahon and Janovy, 1974), the methods used in these studies were very far from the natural mode of transmission. The successful transmission from sand flies to reptile hosts has never been demonstrated under laboratory conditions and is yet to be revealed.

Therefore, experimental infections of the Mediterranean house geckos (*Hemidactylus turcicus*) were performed in our study (Ticha et al., 2022) to investigate the development of *Sauroleishmania* in reptiles. Geckos were experimentally infected with *L*. (*S*.) *adleri* or *L*. (*S*.) *hoogstraali*, either *via* oral route or injected intraperitoneally, and monitored by xenodiagnoses for several months. Although sand fly-derived parasites were used, none of the geckos became positive for *Sauroleishmania*. All tested sand fly females from xenodiagnoses were negative for the presence of *Sauroleishmania* promastigotes, and similar results were obtained by cultivation of blood and internal organs of geckos.

To the best of our knowledge, this was the first attempt to infect reptiles with promastigotes derived directly from sand flies, and despite the negative results, we found it important to include these data in this publication. We assume that selection of the wrong host species is unlikely, as *L*. (*S*.) *hoogstraali* was isolated from *H. turcicus* geckos, which were also shown to be susceptible to *L*. (*S*.) *adleri* (McMillan, 1965). A possible explanation may be improperly chosen route of infection or loss of infectivity of the *Sauroleishmania* isolates used. We suppose that the latter is more likely. The vast majority of *Sauroleishmania* isolates were obtained decades ago and since then have often been maintained in cultures for long periods of time without the opportunity to undergo their whole life cycle under its selective pressure. It has been reported that the prolonged cultivation may result in noticeable changes in the mitochondrial genome (reviewed by Klatt et al., 2019) and acquiring new *Sauroleishmania* isolates is therefore currently one of the biggest challenges in this research area.

For many years, parasites of subgenus *Sauroleishmania* were considered as non-pathogenic to humans and some of them have been often used in biotechnology due to their easy handling (reviewed by Klatt et al., 2019). However, the close relationship between reptilian and mammalian species results in the ability of some *Sauroleishmania* spp. to infect mammals, at least transiently. Some strains of *L.* (*S*.) *tarentolae* have been shown to invade mammalian cells and differentiate into amastigote-like stages (Breton et al., 2005; Taylor et al., 2010). DNA of the same *Sauroleishmania* species was also unexpectedly detected in a human mummy (Novo et al., 2015). Similarly, *L.* (*S*.) *adleri* was capable to develop transient skin reactions in human volunteer inoculated with promastigote culture (Manson-Bahr and Heisch, 1961). Transient infections of the same *Sauroleishmania* sp. were also observed in hamsters and mice (Adler, 1963), and its DNA was isolated from asymptomatic rodent in Ethiopia (Coughlan et al., 2017).

Among 21 described *Sauroleishmania* species, *Leishmania* (*S*.) *tarentolae* is the best-studied and commonly used as a model organism, which made a significant contribution to the study of kinetoplast DNA (kDNA) or RNA editing (Blum et al., 1990; Aphasishev et al., 2003; reviewed by Aphasishev and Aphasizheva, 2014). It has also been considered as promising expression system for human recombinant proteins and has potential applications in the immunotherapy of mammalian leishmaniasis (Breton et al., 2005; Taylor et al., 2010; Abdossamadi et al., 2017; Montakhab-Yeganeh et al., 2017; reviewed by Klatt et al., 2019). The commercially available isolates of *L*. (*S*.) *tarentolae* are frequently used in laboratories and the above-mentioned findings should be thus of particular concern as they raise questions about the potential infectivity of these strains and the current biosafety level (reviewed by Klatt et al., 2019).

Although *L*. (*S*.) *tarentolae* was isolated from geckos over a century ago, some basics aspects of its life cycle are still unknown. Recently, DNA of this species has been found in the canine and human blood in Italy (Pombi et al., 2020; Mendoza-Roldan et al., 2021), and both dogs and humans have also been serologically tested positive (Iatta et al., 2021). At the same time, *Leishmania* (*S*.) *infantum* DNA was detected in *Se. minuta* in Italy in areas where *L*. (*S*.) *infantum* and *L*. (*S*.) *tarentolae* occur in sympatry (Mendoza-Roldan et al., 2021; Mendoza-Roldan et al., 2022c), which supports the previously reported mammalophilic behaviour of this species and possible mammalian exposure to *L*. (*S*.) *tarentolae*. All these findings have once again drawn attention to the research of reptilian leishmania.

In the study of Mendoza-Roldan et al. (2022c), we investigated the occurrence of *L*. (*S*.) *tarentolae* and *L*. (*S*.) *infantum* in geckos, dogs and sand flies in Italy. First part of this project aimed to screen naturally infected reptiles. Altogether, 37 specimens of three reptile species were tested: two *Hierophis carbonarius* snakes, seven *Podarcis siculus* lizards and 28 *Tarentola mauritanica* geckos. Small amount of blood was obtained from each specimen for detection of parasites on smears, by culture and by PCR. Axenic culture of *L*. (*S*.) *tarentolae* was obtained from *T. mauritanica* gecko. Examination of blood smears did not reveal the presence of any *Leishmania* spp., but reptile trypanosomes were detected in three (8 %) *T. mauritanica* geckos.

Detection of another reptilian trypanosomatid was not surprising, as *T. mauritanica* geckos are known to carry both *Sauroleishmania* and *Trypanosoma* spp., often also in co-infections (Adler and Theodor, 1935; Gramiccia et al., 1989). Although *Trypanosoma* sp. found in *T. mauritanica* gekos has not been further identified, it can be assumed that these were trypomastigotes of *Trypanosoma platydactyli*. This species is widely distributed in the Mediterranean basin, sharing the same host and vector with *L*. (*S*.) *tarentolae* (Gramiccia et al., 1989).

Out of 37 reptile blood samples examined by real-time PCR (qPCR) and duplex real-time PCR (dqPCR), two (5 %) *T. mauritanica* scored positive for *L*. (*S*.) *tarentolae* and another two (5 %) for *L*. (*L*.) *infantum*. Subsequently, bone marrow biopsy of the femur was performed in geckos positive for mammalian *Leishmania* sp., and amastigote forms were found in bone marrow films. The identification of *L*. (*L*.) *infantum* amastigotes in bone marrow of geckos was further confirmed molecularly. Although DNA of *L*. (*L*.) *infantum* has previously been detected in geckos in Italy (Mendoza-Roldan et al., 2022a), the presence of its amastigote forms in bow marrow was here reported for the first time. Similar findings were observed by researchers in China, who found DNA of several *Leishmania* (*L*.) spp., in local reptiles (Chen et al., 2019; Zhang et al., 2019). Nevertheless, it should be noted that further studies are needed to investigate whether reptiles can indeed be infected and may possibly serve as regular hosts for mammal-infecting *Leishmania* species. We propose that mainly the isolation of vivid parasites from reptiles would be of particular importance for clarifying the hypothesis.

In the second part of the project, sheltered dogs from the same area were serologically examined. A complete physical examination of dogs was conducted and whole blood was sampled. Dog sera were tested by the immunofluorescence antibody test (IFAT) and out of 19 dogs, 12 (63 %) were positive for both *L*. (*L*.) *infantum* and *L*. (*S*.) *tarentolae* in May and 11 (58 %) in November (as one of the dogs died). These data indicate that dogs are exposed to both species of *Leishmania*, further supporting the results of the previous study in southern Italy (Mendoza-Roldan et al., 2021). However, serological cross-reaction has often been observed between various trypanosomatids when using IFAT methods (Badaró et al., 1983; Paz et al., 2018). Thus, the serological cross-reactivity between *Leishmania* and *Sauroleishmania* cannot be excluded and more specific methods should be used to distinguish the infecting species.

Entomological survey has also been included in this study. Sand flies were collected using sticky traps and CDC light traps, stored in 70% ethanol and morphologically determined. Alive sand fly females were dissected, examined under a microscope and positive sand fly guts were cultivated in a modified Tobie-Evans medium. Four *Sergentomyia minuta* displayed the presence of promastigotes, and axenic culture of *L.* (*S.*) *tarentolae* have been established from single *Se. minuta* female.

From a total of 716 sand flies, 294 females of three species were collected: 231 *Se. minuta* (79 %), 52 *Phlebotomus perniciosus* (18 %) and 11 *Phlebotomus neglectus* (3 %). Sand fly females were tested by dqPCR and 24 specimens (8 %) were positive. *Leishmania* (*S.*) *tarentolae* infection was found in 21 specimens of *Se. minuta* (88 %) and two of *P. perniciosus* (8 %), while *L.* (*L.*) *infantum* was detected in one *P. perniciosus* female (4 %).

Detection of *L.* (*S.*) *tarentolae* in *P. perniciosus* has been previously reported (Mendoza-Roldan et al., 2021; Latrofa et al., 2021), and the same sand fly species has been shown highly susceptible to *L.* (*S.*) *tarentolae* under laboratory conditions (Ticha et al., 2021). *Phlebotomus perniciosus* is an opportunistic feeder (Bongiorno et al., 2003; Rossi et al., 2008; Remadi et al., 2020), but the willingness of this species to feed on cold-blooded animals has not been studied yet. Although *P. perniciosus* is widely distributed in areas where *Se. minuta*, *L.* (*S.*) *tarentolae* and geckos occur in sympatry, vivid *Sauroleishmania* isolates have never been obtained from this sand fly species. All promastigote cultures isolated from *P. perniciosus* were repeatedly typed as *L.* (*L.*) *infantum* (Bettini et al., 1986; Maroli et al., 1988; Maroli et al., 1994; Rossi et al., 2008; Bongiorno et al., 2014). Vectorial role of *P. perniciosus* in the transmission of *Sauroleishmania* is still unclear but proves that more attention should be paid to *Phlebotomus* spp. as potential alternative vectors of *Sauroleishmania*.

As a part of entomological survey, engorged sand fly females were tested for the host species determination and blood meal was successfully analysed in five out of ten sand flies (50 %). Reptile and canine blood were detected in *Se. minuta*, two females of *P. perniciosus* were positive for human blood and one *P. neglectus* fed on the dog. These data are consistent with earlier findings, both *Phlebotomus* species feed preferentially on mammals, while *Se. minuta* is known for its herpetophilic and occasionally mammalophilic behaviour.

To summarize the results of this study, (1) two new isolates of *L.* (*S.*) *tarentolae* from reptile and sand fly have been isolated, which formed the basis for further experiments, particularly those on possible infectivity to mammals; the isolation of *L.* (*S.*) *tarentolae* from *Se. minuta* further confirm its vector competence for this *Sauroleishmania* species; (2) Mammalophilic behaviour of *Se. minuta* has been repeatedly recorded, but its putative role as a vector of mammalian *Leishmania* spp. is still unclear; (3) amastigote forms of *L.* (*L.*) *infantum* were found in bone marrow of geckos suggesting that reptiles may also be infected by mammalian *Leishmania* spp.; (4) dogs are exposed to *L.* (*S.*) *tarentolae* but the potential infectivity of this reptilian parasite to mammals has not yet been revealed.

Finally, all these recent findings have also led us to summarize in a review Mendoza-Roldan et al. (2022b) the current knowledge on the history, life cycle and potential applications of *L.* (*S.*) *tarentolae*.

In the study of Ticha et al. (2023), we investigated the host feeding preferences of *Sergentomyia minuta* and compared it with *Phlebotomus papatasi*. Relatively recently, the colony of *Se. minuta* has been established in our laboratory in Prague. This sand fly species has been reported as natural vector of *L.* (*S.*) *tarentolae* (reviewed by Klatt et al., 2019) but the possible involvement of this sand fly in the transmission of human pathogenic *Leishmania* spp. has also been discussed (reviewed by Maia and Depaquit, 2016). *Sergentomyia minuta* feeds

readily on reptiles, but blood meal analyses indicate it occasionally bites mammals, including humans (Maia et al., 2015; Bravo-Barriga et al., 2016; Bennai et al., 2018; Abbate et al., 2020; González et al., 2020; Pombi et al., 2020). Detection of *Leishmania* (*L*.) *major* and *Leishmania* (*L*.) *infantum* DNA in wild-caught *Se. minuta* females potentially implicating this species in the transmission of human pathogens (Campino et al., 2013; Jaouadi et al., 2015; Pereira et al., 2017; Latrofa et al., 2018; Abbate et al., 2020; Mendoza-Roldan et al., 2021).

The aim of this study was to examine the willingness of *Se. minuta* to feed on various hosts, both reptiles and mammals. In addition to being a potential vector of human leishmaniases, it is also a suspected vector of *Toscana phlebovirus* (TOSV), a causative agent of acute human encephalitis and meningoencephalitis in the Mediterranean basin. The viral RNA has been detected in wild-caught females of *Se. minuta* in France (Charrel et al., 2006) and investigating the feeding behaviour of this sand fly is therefore of particular interest.

Three reptile species were chosen as potential hosts of *Sauroleishmania* (i.e., *Tarentola mauritanica*, *Hemidactylus turcicus*, *Podarcis siculus*) and three species of mammals were tested (i.e., mouse, rabbit, human). Sand fly mortality and fecundity were studied in engorged females and the results were compared with *Phlebotomus papatasi*, a vector of *L*. (*L*.) *major* and *L*. (*L*.) *turanica* (Pimenta et al., 1994; Chajbullinova et al., 2012), and potential alternative vector of *Sauroleishmania* spp. (Ticha et al., 2021).

Some studies have previously described occasional herpetophilic behaviour of *P. papatasi* (Adler and Theodor, 1929; Quate, 1964). It has been reported that this sand fly species feeds on a wide range of the hosts, including mammals, birds and reptiles (Adler and Theodor, 1929; Quate, 1964; Svobodova et al., 2003; Palit et al., 2005). *Phlebotomus papatasi* has also been shown susceptible to some *Sauroleishmania* spp. under laboratory conditions (Adler and Theodor, 1929; Ticha et al., 2021). Among three reptile species tested, *P. papatasi* fed relatively readily on *T. mauritanica* geckos, from which *L*. (*S*.) *tarentolae* has been isolated (reviewed by Klatt et al., 2019). The ability of *P. papatasi* to feed on these geckos thus further supports the hypothesis of its role as alternative vector of *Sauroleishmania* (Ticha et al., 2021).

As expected, *Sergentomyia minuta* fed readily on all reptiles tested, including *Po. siculus* lizards. Although so far *L*. (*S*.) *tarentolae* has only been isolated from geckos, DNA of this species has recently been detected in *Po. siculus* lizards in Italy (Mendoza-Roldan et al., 2021). Molecular detection together with the willingness of *Se. minuta* to feed on lizards may indicate that these common lizards may also serve as hosts of *L*. (*S*.) *tarentolae* in Italy. To confirm this, it will be also necessary to isolate live parasites from these lizards.

The experimental part with *Se. minuta* and mammalian hosts yielded more interesting results. This sand fly species completely refused to feed on mice and rabbits, but *Se*. *minuta* females were attracted to and fed on the forearm of human volunteer. Blood meal surveys carried out at various catching sites showed that, apart from reptile blood, humans were the most frequently detected hosts (Maia et al., 2015; Bennai et al., 2018; Abbate et al., 2020; González et al., 2020; Pombi et al., 2020). Although *Se. minuta* prefers reptiles as a blood source, anthropophilic behaviour of this species was experimentally demonstrated in this study and these data are consistent with the results of field surveys.

In addition to host preferences, feeding behaviour of *Se. minuta* was observed and blood meal volumes were measured by haemoglobinometry. Two host species, a gecko (*Eublepharis macularius*) and a human volunteer, were compared. Feeding behaviour of *Se. minuta* differs from that of *P. papatasi* and other tested sand flies in our laboratory so far. Females took large amounts of blood in proportion to their relatively small-sized body, and the feeding period was significantly prolonged compared to other sand fly species (Sadlova et al., 1998; Sant'anna et al., 2010; Roby et al., 2015). A relatively long feeding time was previously recorded in *Culex territans* mosquitoes, which also feed on cold-blooded animals (Reinhold et al., 2021). This

behaviour of *Se. minuta* may thus result from an adaptation to feed on cold-blooded animals. Due to the prolonged feeding, sand fly females might regulate the large volume of a blood and compensate the lower haemoglobin levels that are typical for reptile blood.

Sergentomyia minuta was able to intake similar volumes of gecko and human blood (0.97 ± 0.03 µl/female and 1.02 ± 0.05 µl/female, respectively). Nevertheless, the digestion of human blood was problematic for this sand fly species. Digestion of blood meal was delayed and was also accompanied by higher mortality of females. Moreover, human blood had negative effect on female fecundity, which was significantly lower compared to reptile blood. These data correspond to the unsuccessful attempts to establish a *Se. minuta* colony feeding on mammals in our laboratory in Prague. The colony of *Se. minuta* is normally maintained on leopard geckos but was unable to survive into the next generation when feeding on mammalian hosts (Volfova and Volf, personal communication). Although *Sergentomyia schwetzi* successfully thrives on mammalian blood (Polanska et al., 2020), our data indicates that *Se. minuta* is not adapted well to digest human blood and its anthropophilic behaviour is likely opportunistic. Feeding of *Se. minuta* females also caused pronounced skin hypersensitivity reaction in a human volunteer after repeated exposure but no skin reaction was observed in geckos.

Although our study provides interesting results, the potential role of *Se. minuta* as a vector of mammalian leishmaniases has yet to be revealed. Reliable evidence is still lacking as molecular detection alone is not sufficient to incriminate a sand fly species as vector (reviewed by Maia and Depaquit, 2016). Human pathogenic *Leishmania* spp. have been molecularly detected in wild-caught females, but all promastigotes isolated from these sand flies so far have been always typed as *L.* (*L.*) *tarentolae* (reviewed by Mendoza-Roldan et al., 2022b). Laboratory experiments may be therefore crucial for investigating the vectorial competence of this sand fly species.

In *Sergentomyia schwetzi*, promastigote infection and DNA of *L.* (*L.*) *infantum* has been found (Senghor et al., 2016) but laboratory experiments demonstrated that this species is refractory to mammalian *Leishmania* spp. (Sadlova et al., 2013). It has been shown that *Leishmania* promastigotes can proliferate abundantly in the early phases of infection, even in unsuitable vector species. Although the living parasites were defecated, their DNA was detectable up to 7 days post-infection (Seblova et al., 2012). In unnatural *Leishmania*-vector combinations the barrier is formed by inability of promastigotes to attach in the sand fly gut; this attachment is a crucial part of *Leishmania* life cycle as it prevents the expulsion of parasites with blood meal remnants and allows their survival into the late phases of infection (reviewed by Dostalova and Volf, 2012).

Despite considerable effort, some of our studies on *Sauroleishmania* have not yet been finished and published. We would like to summarize them below to point the direction of future research and to inspire those who might be interested to continue our experimental studies.

Our colony of *Se. minuta* continuously refuses to feed experimentally through a membrane. Various feeding conditions have been repeatedly tested, i.e. different types of blood and membranes together with changes in temperature and humidity, but all attempts failed. Such behaviour seems to be characteristic for this sand fly species, as it was previously reported by some other authors (Adler and Theodor, 1929; Wallbanks, 1982). We are concerned that only direct observation on natural promastigote infection may elucidate the potential vectorial role of *Se. minuta* in the transmission of human pathogenic *Leishmania* species.

Attempts to experimentally infect *Se. minuta* females under laboratory conditions failed and therefore we used naturally infected *T. mauritanica* geckos to study the development of *Sauroleishmania* spp. in reptiles. The captured geckos were first examined by PCR for the presence of infection. Specimens molecularly positive for *L.* (*S.*) *tarentolae* were then used for

the xenodiagnoses. Sand fly females were fed on geckos and dissected at various time point post blood meal. Out of four geckos used in xenodiagnoses, three displayed the presence of parasites.

Parasites occupied the whole sand fly gut, i.e. hindgut, midgut but also migrated to the thoracic parts where often colonized the stomodeal valve. Moreover, heavy parasites loads were present in Malpighian tubules. This finding confirms the results of our previous study where *L.* (*S.*) *tarentolae* promastigotes developed in Malpighian tubules of *Phlebotomus* spp. sand flies (Ticha et al., 2021). The colonization of MTs is thus fundamental property of this *Sauroleishmania* species in both *Phlebotomus* and *Sergentomyia* vectors.

The geckos were examined during autumn (October-December 2021) and although they were kept under constant conditions, the infection gradually disappeared. We assumed that there was some sort of dormant phase of infection over the winter and therefore we decided to continue monitoring the geckos through the whole sand fly season. Geckos were xenodiagnosed in February, April and then in September 2022. Unfortunately, the infection did not re-appear in any of the gecko tested.

Another complication in these experiments was the frequent presence of co-infections with *Leishmania* (*S.*) *tarentolae* and *Trypanosoma platydactyli*. In addition to promastigotes, other stages of trypanosomes have been found in sand flies, most probably epimastigotes and trypomastigotes. Moreover, these forms were often present in anterior parts of the gut colonizing the stomodeal valve, while *Sauroleishmania* promastigotes more tended to posterior parts of the gut, mainly Malpighian tubules. It should be noted that distinguishing the two parasites under the microscope was extremely complicated. Therefore, we decided to use molecular methods to analyse co-infections. We have tried different PCR methods, several combinations of primers but all attempts were unsuccessful. This hindrance possibly stemmed from a non-specific binding of primers utilized, which appeared to interact with elements present in the sand flies' genetic material and therefore we have not yet been able to distinguish the infections.

The fact that *L.* (*S.*) *tarentolae* and *Tr. platydactyli* sharing the same vectors and hosts has been previously reported (Gramiccia et al., 1989) and it is interesting how these two parasites have learned to coexist. Since colonization of Malpighian tubules has not been described in other *Sauroleishmania* spp., this unique localization may be a consequence of the coexistence of these two species and interspecific competition for the *niche* in sand fly digestive tract. Experiments with fluorescent labelled parasites would certainly provide answers to this hypothesis.

The data from this unpublished study also provided other important information. We tried different diagnostic methods to detect the presence of parasites in geckos and xenodiagnosis using *Se. minuta* proved to be the most reliable. In addition, *P. papatasi* has also been used in xenodiagnostic experiments and it has been shown that this sand fly species can also harbour infection of *L.* (*S.*) *tarentolae* and *Tr. platydactyli*. Trypomastigotes were observed colonizing the stomodeal valve of *P. papatasi* on day 7 post blood meal and *Tr. platydactyli* development in this sand fly species is worth of further research.

We assume that our research project has substantially advanced the knowledge of the parasites of the subgenus *Sauroleishmania*. We have elucidated several aspects of the life cycle of these parasites, but many questions have also arisen, and further experimental work is necessary to clarify them.

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APPENDIX

Curriculum Vitae - Lucie Tichá

EDUCATION

2019-present	Ph.D. study Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic Thesis: *Sauroleishmania*-sand fly interactions Supervisor: prof. RNDr. Petr Volf, CSc.
2017-2019	Master's degree in Parasitology Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic Thesis: Development of *Sauroleishmania* in sand flies and geckos Supervisor: prof. RNDr. Petr Volf, CSc.
2014-2017	Bachelor's degree in Biology Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic Thesis: Attachment of trypanosomatids in the insect vector Supervisor: RNDr. Jitka Myskova, Ph.D.

CERTIFICATES

2020	Certificate of professional competence to design experiments and experimental projects under Section 15d (3) of Act No. 246/1992 Coll., on the protection of animals against cruelty, no. CZ03756 (Ministry of Agriculture, Czech Republic)

INTERNSHIPS

2021	University of Bari Aldo Moro, Valenzano, Italy Department of Veterinary Medicine Laboratory of prof. Domenico Otranto Duration: 14 weeks Topics: study of naturally infected reptiles, xenodiagnostic experiments, development of parasites in sand flies, host preferences of sand flies

INTERNATIONAL CONFERENCES

1st ALIVE, Animal Leishmaniosis International Veterinary Event (31st March-2nd April 2022; Málaga, Spain);

Oral communication

Ticha L., Mendoza-Roldan J. A., Bezerra-Santos M. A., Maia C., Volfova V., Otranto D., Volf P. Experimental infection of *Sergentomyia minuta* with *Leishmania* (*Sauroleishmania*) *tarentolae* using xenodiagnosis of naturally infected geckos.

Leishmaniasis 2022, 3rd International Caparica Congress on Leishmaniasis (24th-26th October, 2022; Caparica, Portugal);

Poster and shotgun presentation

Ticha L., Mendoza-Roldan J. A., Vomackova Kykalova B., Bezerra-Santos M. A., Sadlova J., Gramiccia M., Gradoni L., Otranto D., Volf P. The role of *Sergentomyia minuta* and *Phlebotomus papatasi* as vectors of *Leishmania* (*Sauroleishmania*) *tarentolae*

SCIENTIFIC PROJECTS

2022-2023	Investigator in the project **HORIZON Europe** 101057690 „CLIMOS – Climate Monitoring and Decision Support Framework for Sand Fly-borne Diseases Detection and Mitigation with Cost-benefit and Climate-policy Measures“.
2021-2023	Investigator in the project **GAČR 21-15700S** „*Leishmania*-sand fly interaction: new approaches to answer old questions“
2021-2023	Investigator in the project **START/SCI/083** „Development of *Mundinia* in vectors and hosts: comparison with other *Leishmania* subgenera“.
2021-2022	Investigator in the project **OPVVV,** Czech Ministry of Education, ERD funds CZ.02.1. 01/0.0/0.0/16_019/0000759 „Centrum výzkumu patogenity a virulence parazitů“,
2020-2022	**Principal investigator** in the project **GAUK 180220** „Development of *Sauroleishmania* in sand flies and geckos“.
2020-2022	Investigator in the project **GAUK 1380120** „Investigation of sand fly midgut immune response to *Leishmania“.*
2019	Investigator in the project **GAUK 288217** „Comparison of different rodent species as hosts of *Leishmania“.*

PUBLICATIONS

Kykalová, B., **Ticha, L.**, Volf, P., & Loza Telleria, E. (2021). *Phlebotomus papatasi* antimicrobial peptides in larvae and females and a gut-specific defensin upregulated by *Leishmania major* infection. *Microorganisms*, *9*(11), 2307.

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Mendoza-Roldan, J. A., Votypka, J., Bandi, C., Epis, S., Modry, D., **Ticha, L.**, Volf, P. & Otranto, D. (2022). *Leishmania tarentolae*: A new frontier in the epidemiology and control of the leishmaniases. *Transboundary and Emerging Diseases*, *69*(5), e1326-e1337.

Mendoza-Roldan, J. A., Zatelli, A., Latrofa, M. S., Iatta, R., Bezerra-Santos, M. A., Annoscia, G., Gernone, F., Votypka, J., Modry, D., **Ticha, L.**, Volf, P. & Otranto, D. (2022). *Leishmania* (*Sauroleishmania*) *tarentolae* isolation and sympatric occurrence with *Leishmania* (*Leishmania*) *infantum* in geckoes, dogs and sand flies. *PLoS Neglected Tropical Diseases*, *16*(8), e0010650.

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Klocek, D., Grybchuk, D., **Ticha, L.,** Votypka, J., Volf, P., Kostygov, A. Y., & Yurchenko, V. (2023). Evolution of RNA viruses in trypanosomatids: new insights from the analysis of *Sauroleishmania*. *Parasitology Research*, *122*(10), 2279-2286.

Becvar, T., Vojtkova, B., Pacakova, L., Vomackova Kykalova, B., **Ticha, L.,** Volf, P., & Sadlova, J. (2024). Steppe lemmings and Chinese hamsters as new potential animal models for the study of the leishmania subgenus *Mundinia* (Kinetoplastida: Trypanosomatidae). *bioRxiv preprint*, 2024-01.