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Comparison of different rodent species as hosts of human pathogenic *Leishmania*

Srovnání různých druhů hlodavců jako hostitelů leishmanií patogenních pro člověka

Ph.D. Thesis / Disertační práce

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

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Prague, 28. 2. 2022

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I declare that Barbora Vojtková substantially contributed to the experimental work in the four projects presented in her thesis and had a principal role in writing two of the four publications presented.

Prohlašuji, že se Barbora Vojtková významně podílela na experimentální práci na čtyřech projektech shrnutých v této dizertační práci a je hlavní autorkou textu dvou publikací.

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ABSTRACT

Leishmania parasites (Kinetoplastida: Trypanosomatidae) are causative agents of leishmaniases, a group of vector-borne diseases with various manifestations and complex epidemiology. Principal vectors are sand flies (Diptera: Psychodidae) and among mammalian hosts, rodents are predominantly represented. Rodents are also the most common laboratory models for the research of leishmaniases. However, reservoirs of most *Leishmania* species are still poorly recognized and the scope of standard model hosts is still insufficient to mimic well the wide spectrum of leishmaniases. This PhD thesis summarizes three different topics, all dealing with experimental infections of rodents with important human parasites – *Leishmania major* and *L. donovani*. The first topic was focused on the effect of sand fly saliva on the development of cutaneous leishmaniasis. BALB/c mice infected intradermally with *L. major* were repeatedly bitten by *P. duboscqi* females every two weeks. The multiple and repeated sand fly bites significantly enhanced the development of cutaneous lesions and increased parasite load. The second topic was aimed at enrichment of the spectrum of laboratory model animals for *Leishmania* research. Three Asian rodent species (*Cricetulus griseus*, *Lagurus lagurus* and *Phodopus sungorus*) were infected with *L. major* and *L. donovani* and the course of the disease was compared with a commonly used laboratory model animals (BALB/c mice and *Mesocricetus auratus*). All three Asian rodent species were highly susceptible to *L. major* offering the model of both the healing (*C. griseus* and *L. lagurus*) and the non-healing (*P. sungorus*) phenotype. Both *C. griseus* and *L. lagurus* were also highly susceptible to *L. donovani* parasites with higher parasite load and wider parasite distribution and infectiousness than standard model animals. The third topic was focused on the evaluation of the host competence of wild Sub-Saharan rodents. *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis* were experimentally infected with *L. major*, *L. donovani* and *L. (Mundinia) sp.* from Ghana. *Leishmania major* persisted in all 3 tested wild rodent species for 20–25 weeks and hosts were infectious to the natural vector *P. duboscqi*. Inoculated animals showed no skin lesions but only temporary swellings or changes of pigmentation at the site of inoculation. The results support the involvement of *Mastomys* and *Arvicanthis* in the life cycle of *L. major* in Sub-Saharan Africa. On the other hand, none of all animals infected with *L. donovani* was infectious to *P. orientalis* females although, in some animals, parasite DNA was detected even 30 weeks p. i. These results suggest that wild *Arvicanthis* and *Mastomys* found infected with *L. donovani*, most probably represent incidental hosts without an important role in parasite transmission. Similarly, poor survival of *Leishmania* parasites in the rodent´s bodies and negative xenodiagnoses do not support the involvement of *Arvicanthis* and *Mastomys* in the transmission cycle of *L. sp.* from Ghana.

ABSTRACT IN CZECH

Leishmanie (Kinetoplastida: Trypanosomatida) jsou parazitičtí prvoci vyvolávající různé formy onemocnění, souhrnně nazývaných leishmaniózy. Parazité kolují mezi dvoukřídlým krevsajícím hmyzem a savčími hostiteli. Hlavními přenašeči jsou flebotomové (Diptera: Psychodidae), zatímco spektrum savčích rezervoárových hostitelů je velmi široké. Nejvíce zastoupenými druhy RH jsou hlodavci, právě oni jsou i nejčastěji používanými modelovými zvířaty pro výzkum leishmnióz v laboratořích. Rezervoároví hostitelé mnoha druhů leishmanií ale nejsou stále známi a také běžně používané druhy laboratorních zvířat nepokrývají dostatečně široké spektrum klinických příznaků leishmanióz. Tato disertační práce shrnuje tři rozdílná témata zabývající se experimentálními infekcemi různých druhů hlodavců leishmaniemi patogenními pro člověka, druhy *Leishmania major* a *L. donovani*. První téma bylo zaměřené na vliv sání flebotomů na rozvoj kožní leishmaniózy. Myši kmene BALB/c byly infikovány intradermální cestou *L. major* a poté každé dva týdny vystaveny sání flebotomů druhu *P. duboscqi*. Opakované sání flebotomů významně podpořilo dynamiku růstu kožních lézí a zvýšilo počty parazitů v hostitelské tkáni. Cílem druhého tématu bylo obohatit druhové spektrum laboratorních modelových zvířat pro výzkum leishmanióz. Jedinci tří asijských druhů hlodavců (*Cricetulus griseus*, *Lagurus lagurus* and *Phodopus sungorus*) byli nakaženi kožní *L. major* a viscerální *L. donovani* a projevy onemocnění byly srovnávány s běžně používanými laboratorními zvířaty (BALB/c myši a *Mesocricetus auratus*). Všechny tři asijské druhy byly vysoce vnímavé k infekci *L. major*, přičemž vykazovaly buď hojící se (*C. griseus* a *L. lagurus*), nebo nehojící se fenotyp (*P. sungorus*). Dva druhy (*C. griseus* and *L. lagurus)* projevily vysokou vnímavost také k vicserální *L. donovani*. Ve srovnání se standardně používanými modely byl u obou druhů pozorován vyšší počet parazitů a jejich rozsáhlejší diseminace do různých tkání, což bylo spojené i s vyšší infektivitou pro flebotomy. V rámci třetího tématu jsme hodnotili hostitelskou kompetenci hlodavců žijících v subsaharské Africe. *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis* byli experimentálně nakaženi *L. major, L. donovani* a *L. (Viannia) sp.* z Ghany. Druh *L. major* byl detekováni ve tkáních hlodavců i 20–25 týdnů p. i. a hostitelé byli infekční pro přirozeného přenašeče *P. duboscqi*. Nakažená zvířata vykazovala pouze mírné projevy onemocnění, byly pozorovány jen drobné otoky nebo změny pigmentace. Tyto výsledky podporují zapojení těchto druhů hlodavců do životního cyklu *L. major*. Na druhou stranu, zvířata nakažená *L. donovani* nebyla infekční pro flebotomy druhu *P. orientalis*, i když parazit byl přítomný ve tkáních některých hostitelů dokonce i 30. týden p. i.. Občas popisované nákazy Arvicanthis a *Mastomys* druhem *L. donovani* jsou tedy spíše jen případy náhodných hostitelů, kteří se neúčastní dalšího přenosu parazita. Hlodavci rodů *Arvicanthis* a *Mastomys* zřejmě nefigurují ani v životním cyklu *L. sp*. z Ghany, protože parazit přežíval jen v malén podílu zvířat a tato zvířata nebyla infekční pro flebotomy.

INTRODUCTION

1. *Leishmania* parasites

Leishmania spp. are parasitic protozoa, responsible for a group of human diseases collectively known as leishmaniases. One billion people are at risk of infection across 98 countries worldwide, with over 1.5 million new cases and around 40 thousand deaths reported each year (Alvar et al. 2012, WHO 2022). They belong to the family Trypanosomatidae within the order Kinetoplastida (Akhoundi et al. 2016). Together with *Trypanosoma spp.*, they comprise the medically most relevant dixenous parasites that switch between blood-feeding arthropods and vertebrate hosts. In the new taxonomy, *Leishmania* species are divided into four subgenera: *Leishmania, Viannia, Sauroleishmania* and the most recently established subgenus *Mundinia*. About 50 species are recognized and more than 20 *Leishmania* species are pathogenic to humans (Espinosa et al. 2018, Akhoundi et al. 2016). The leishmaniases are vector-borne diseases with complex epidemiology, each parasite species possessing different mammalian hosts and being transmitted with a different spectrum of insect vectors. Principal vectors are sand flies (Diptera: Psychodidae) of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Among over 800 sand fly species, 98 are proven or suspected vectors (Maroli et al. 2013). Except for sand flies, also biting midges (Diptera: Ceratopogonidae) have been recently confirmed as vectors of *Leishmania* parasites (Dougall 2011, Becvar et al. 2021).

1.1 Life cycle

The *Leishmania* life cycle (Figure 1) includes morphologically and physiologically distinct forms, flagellated extracellular promastigotes in the insect vector and non-flagellated intracellular amastigotes in the mammalian host. Intracellular amastigotes are ingested with blood meal by a sand fly female. Within the peritrophic matrix produced by the epithelial midgut cells and surrounding the blood meal, amastigotes transform into motile procyclic promastigotes with the short external flagellum and rapidly divide by binary fission. After several days, promastigotes transform to elongated nectomonads that escape through the broken peritrophic matrix. The next part of the life cycle depends on the species of parasites. Species with suprapylarian development (classified in the subgenus *Leishmania*) develop in the abdominal midgut where they attach to the epithelium to prevent expulsion during defecation. Species with peripylarian development (classified in the subgenus *Viannia)* move to the hindgut where they transform into round flagellated promastigotes and attach to the wall by hemidesmosomes (reviewed in Bates, 2007; Ramalho-Ortigao & Saraiva,

2010; Dostálová & Volf, 2012). After defecation, actively multiplying leptomonads migrate to the thoracic midgut and produce the promastigote secretory gel (PSG), which plugs the anterior part of the sand fly gut (Rogers et al. 2002 and Kamhawi 2006). In the late- stage, non-motile haptomonads attach to the chitinous lining of the stomodeal valve (SV) and highly motile metacyclic forms adapted for successful transmission to the mammalian host are produced. The damage of the SV by leishmania chitinase and blockage of the cardia region by the plug, formed by parasites and PSG, alters the sand fly feeding behaviour forcing the vector multiple feeding attempts, thereby regurgitating parasites into the biting site (Rogers et al. 2002, Sack et al. 2008). Complete *Leishmania* development in the vector takes 6 – 9 days, depending on the species (Kamhawi 2006). Sand flies, like the most other hematophagous arthropods, take multiple blood meal during their lifetime. Serafim et al. (2018) showed that subsequent blood meal significantly amplifies *Leishmania* parasites in the sand fly gut and enhance their infectiousness. Reverse metacyclogenesis occurs after every subsequent blood meal, where metacyclic promastigotes dedifferentiate to retroleptomonads. Retroleptomonads rapidly multiply and dedifferentiate back to metacyclic promastigotes (Serafim et al 2021). However, it is not clear how these retroleptomonads differ from leptomonads, authors did not provide any morphologic or molecular evidence for this. After injection of parasites into the skin, an acute inflammatory response develops, neutrophils, monocytes and macrophages migrate into the feeding site and take up the metacyclic promastigotes that transform into amastigotes. Together with *Leishmania*, also *Leishmania*-derived exosomes and PSG and sand fly saliva as well as gut bacteria are introduced to the host skin and modulate its immune response (Atayde et al., 2015; Gomes and Oliveira, 2012; Rogers, 2012). Amastigotes multiple inside the phagolysosome of the host cells, then rupture the cell and infect next macrophages, which also marks the starting point for an onset of the disease (Handman and Bullen 2002).

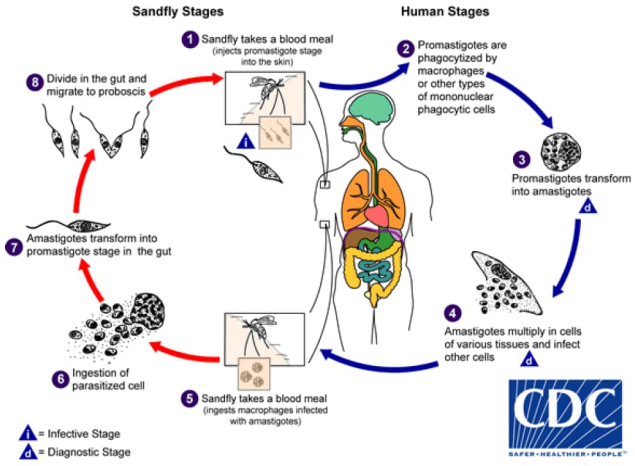


Figure 1: Life cycle of *Leishmania* parasites

Taken from http://www.cdc.gov/parasites/leishmaniasis

1.2 Effect of sand fly saliva on the development of *Leishmania* infection in vertebrate hosts

The hematophagous arthropods secrete the saliva into the wound to facilitate bloodmeal intake. Their saliva contains a wide array of anti-haemostatic, anti-inflammatory and immunomodulatory peptides and proteins that help to create an optimum environment during feeding. In addition, saliva is also involved in the transmission and establishment of pathogens in the host (reviewed by Fontaine et al. 2011). The same is true for sand fly saliva and *Leishmania* parasites. Sand fly feeding provides a suitable environment for the survival and multiplication of parasites. Sand fly saliva is chemotactic for immune cells (mainly for neutrophils and macrophages) and allows parasites to enter into these cells faster. This chemotactic effect is important especially at the early phase of infection when salivas are co-inoculated together with *Leishmania* parasites (Anjili et al. 1995, Teixeria et al. 2005, Peters et al. 2008, de Moura et al. 2010, Prates et al. 2012). Promastigotes must invade macrophages as quickly as possible. Otherwise, they are rapidly degraded by the cytotoxic activity of natural killer cells, neutrophils, and eosinophils in the vertebrate host. The study of Zandbergen et al. (2004) suggests that infected apoptotic neutrophils can serve as "Trojan horses", because they can transfer parasites silently to macrophages inside them. Later studies propose a little different scenario (hypothesis of "Trojan rabbits"), where amastigotes escape from dying neutrophils just before ingestion by macrophages (Peters et al. 2008, Ritter et al. 2009).

Besides the chemotactic effect, sand fly saliva has various ways how to modulate the immune response of the mammalian host. These functions were examined mainly for *P. papatasi* (the Old World species) and *Lu. longipalpis* (the New World species). Saliva components of *P. papatasi* (5´AMP and adenosine) inhibit the ability of IFN-γ to activate macrophages to the production of NO (Hall et al. 1995). The ability of both tested species to decrease the secretion of pro-inflammatory cytokines (IL-4, IL-8) and to enhance the production of anti-inflammatory cytokines (IL-12, IL-10), was described (Belkaid et al. 1998). They also induce apoptosis of neutrophils (Prates et al. 2011) and inhibit the ability of dendritic cells to present antigens (Carregaro et al. 2008). All these effects can either enhance or aggravate the development or course of *Leishmania* infection, depending on the history of exposure to the vectors. In hosts without pre-exposure history, sand fly saliva co-inoculated together with infectious inoculum exacerbates the infection. This effect was confirmed on the CL laboratory model *P. papatasi – L. major* – BALB/c mice, where addition of the salivary gland lysate resulted in more severe disease symptoms (Theodos et al. 1991) and has been proven on various *Leishmania* – sand fly combinations, for example *Lu*. *longipalpis* - *L*. *braziliensis, Lu*. *Longipalpis* - L. *mexicana (Samuelson et al. 1991)*, and *P*. *duboscqi* - *L*. *major* (Rohousova et al. 2011).

On the other hand, laboratory animals repeatedly exposed to the salivary glands lysate or to bites of uninfected sand fly females were protected against severe leishmaniasis (review by Lestinova et al. 2017). The protective immune response lies on the activation of host immune cells by salivary-related antigens, and it can be efficient also against *Leishmania* parasites. It is a delayed-type hypersensitivity (DTH) specific to sand fly saliva that is characterized by the influx of leucocytes and activated macrophages into the bite site and by increased production of pro-inflammatory cytokines IFN-γ and IL-12. This DTH culminates 48 hours after the contact with sand fly saliva and at this time, parasites are prone to faster degradation in the host´s skin (Titus and Ribeiro 1988, Theodos et al. 1991, Kamhawi et al. 2000a, Gomez and Oliveira 2012, Carregaro et al. 2015, Leštinová et al. 2017). The composition of sand fly saliva varies among species, which leads to high specific immune response to the particular sand fly species (Thiakaki et al. 2005, Leštinová et al. 2017).

While the above-described mechanisms have been are properly studied on several models, so far, only the study of Valverde et al. (2017) focused on the effect of sand fly saliva in hosts previously infected with *Leishmania*. These authors described that the multiple exposure of infected hamsters to *Lu. Longipalpis* increased the transmissibility of *L. donovani* parasites.

2. Manifestation of leishmaniases

Manifestation of leishmaniases varies in severity and is intimately linked to the parasite species as well as the genetic background and immune response of the vertebrate host (Burza et al. 2018, Mann et al. 2021). The main three phenotypic categories of human leishmaniases are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and the most serious visceral leishmaniasis (VL). On the other hand, many vertebrate hosts do not show any symptoms of the disease, even though they maintain parasitic infectious agents in their bodies. We call them asymptomatic hosts. Asymptomatic individuals with persistent *Leishmania* parasites in the blood or skin can also be a significant source of infection for vectors (Quinnell and Courtenay, 2009, Stauch et al. 2011, Singh et al. 2014).

2.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is the most frequent and the least severe form of the disease affecting mammalian hosts worldwide, characterized by a skin lesion at or near the site of insect bite. CL is principally caused by *L. major, L. tropica* and *L. aethiopica* in the Old World and *L. mexicana, L. braziliensis, L. amazonensis, L. venezuelensis, L. guyanensis* and *L. peruviana* in the New World (Akilov

et al. 2007, McGwire and Satoskar 2014). This form of the disease is further divided into several sub-forms (Figure 2), namely localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL), disseminated leishmaniasis (DL) and post Kala-azar dermal leishmaniasis (PKDL) (Akilov et al. 2007, Mann et al. 2021). LCL usually manifests as small papulae that progressively enlarge to lesions. Lesions are either wet and ulcerative (*L. major* and the New World species) or remain dry (*L. tropica*, *L. aethiopica, L. donovani* complex); both types can spontaneously heal over months to years. DCL, local dissemination of acute CL associated mainly with parasites of the *Leishmania* subgenus, is usually caused by the spread of amastigotes or their antigens from primary lesions to other body parts, where they form satellite non-ulcerative nodular lesions of varying severity (Bailey and Lockwood 2007). DL is a type of non-contiguous pleomorphic lesions often accompanied by mucosal involvement in immunocompetent hosts with a tendency toward chronicity and relapse as well as resistance to standard treatment, caused mainly by parasites of the subgenus *Viannia* (Hashiguchi et al 2016). PKDL is a chronic skin rash occurring after an episode of VL (mainly caused by *L. donovani,* less often by *L. infantum*), commonly found on the face and potentially spreading to other body parts. Contrary to visceral leishmaniasis, PKDL is not fatal, and patients do not show signs of disease, but they can still play a role in the transmission of the parasite (WHO 2012, Mukhopadhyay et al. 2014). Despite is CL the least severe form of the disease, patients often suffer from stigmatization and social exclusion, women usually being much more affected than men (Erber 2017).



Figure 2: Subforms of cutaneous leishmaniasis

A - Localized CL caused by *L. major;* B, C - Diffuse CL caused by *L. (Viannia) guyanensis/panamensis;* D, E - Disseminated CL case caused by *L. (Viannia) guyanensis/panamensis*; F –A papular rash (PKDL) caused by *L. donovani*, G - A macular rash (PKDL) caused by *L. donovani* (modified from Hashiguchi et al. 2016, Haouas et al. 2017, Zijlstra et al. 2019)

2.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (ML) is a more severe form of the disease, which can occur months to years after the healing of primary lesions. Parasites escape the host´s immune response in the skin, disseminate throughout the body by the lymphatic or haematogenous system and produce metastasis in the mucous tissue (Reithinger et al. 2007). ML often displays disfiguring infections of the nose and upper respiratory tract (Figure 3). In the case of "Espundia" (severe ML), amastigotes also attack ears, mouth, oro- and nasopharynx as well as eyelids and can progress to affect respiratory function and ingestion. This clinical form is difficult to treat and there is a high risk of secondary bacterial infections. Pathogenesis of ML is poorly understood and most likely associated with a complex interplay between parasite and host factors (Marsteller et al. 2011, Brodskyn and de Oliveira 2012). Mucocutaneous leishmaniasis, confined to the New World, is caused by *L. (Viannia) brazilliensis* or, less frequently, *L. (V.) guyanensis* and *L. (V.) panamensis*.

2.3 Visceral leishmaniasis

The major causative agents of visceral leishmaniasis are *L. donovani* occurring in the Old World and *L. infantum* occurring in both the Old World and the New World, both species belonging to the *L. donovani* species complex (Figure 3). VL caused by *L. donovani* in the Indian peninsula and East Africa is the most lethal and it is also known as kala-azar ("Kala" is a Hindi word for black, "Azar" a Persian word for disease). The incubation period typically ranges from a few weeks to 18 months, but VL symptoms can take years to appear. VL is a systemic infection affecting mainly the liver, spleen haematogenous and lymphatic system as well as bone marrow. The first symptoms are fever, nausea, diarrhoea, weight loss. Around 10 % of cases are clinically manifested and more than 75 % of untreated VL cases end in death (John and Petri 2006, Barrett and Croft 2012, McGwire and Satoskar 2014, Mann et al.2021).



Figure 3: Mucocutaneous leishmaniasis and visceral leishmaniasis

A - mucosal leishmaniasis caused by *L braziliensis*, B – massive hepatosplenomegaly (VL) caused by *L. donovani* (taken from and modified Reithinger et al. 2007, Sangenis et al. 2014)

3. Model animal species in leishmaniases research

As reviewed in the previous chapter, human leishmaniases have a wide array of clinical manifestations ranging from asymptomatic infections to mild cases of skin ulcers to severe visceral forms. Not surprisingly, highly variable are also courses of experimental infections of model animals and the way in which the disease manifests depend largely on the species and virulence of the *Leishmania* parasite, but the important role also plays the host genetic background and immune response (Loría-Cervera et al. 2014). Therefore, careful consideration is required when choosing the model animal species for leishmaniasis research.

The laboratory mouse (*Mus musculus domesticus*) has become the most prevalent biological model in leishmaniasis research (as well as in the field of biomedicine at large) thanks to its easy maintenance and the availability of many inbred strains. Mouse models allowed researchers to discover the roles played by the various types of immune cells, cytokines, signalling cascades, and effector mechanisms in the immune response to leishmania infections. Experimental infections of mice belonging to susceptible strains (BALB/c) and resistant strains (C57BL/6) with *L. major* helped to reveal the mechanism of resistance to the disease. Whereas the Th2-type response is manifested in the susceptible mice, the healing process in resistant mice is correlated with the Th1-type immune response (macrophage activation and toxic NO production) (Sacks et al. 2002). Mouse model research also helped explain the role of sand fly saliva in the proliferation of infection. Co-inoculation of naïve hosts with saliva results in an enhancing effect leading to increased spread of lesions as well as an increase in leishmania load, whereas exposing animals to uninfected sand fly females or vaccinating the animals with saliva/salivary proteins before inoculation with leishmania provides protection to the animals (reviewed by Lestinova et al. 2017). The research on the mouse model also revealed that the immunity response in the same mouse strain varies across *Leishmania* species, e.g., C57BL/6 mice resistant to *L. major* develop chronic lesions when infected with *L. amazonensis* without any Th2-type response and BALB/c mice susceptible to *L. major* are capable of developing an effective Th1-type immune response to *L. donovani* (Loría-Cervera and Andrade-Narváez, 2014).

The Syrian hamster (*Mesocricetus auratus*) is highly susceptible to the visceral leishmaniasis caused by *L. donovani* and *L. infantum*. This host is frequently used as a model animal for immune response research related to these two parasite species because the infection progress has similarly pathological characteristics as progressive visceral leishmaniasis in humans (Wilson et al. 2005). The cytokines produced in hamsters infected with *L. donovani* correspond to the Th1-type immune response, although there is no NO synthase activation within macrophages and, therefore, the main effector mechanism inhibiting the infection in mice is not present (Melby et al. 2001, Perez et al. 2006).

Also, the protective effect of sand fly saliva to the fatal course of the infection caused by *L. infantum* was demonstrated on this model (Gomes et al. 2008).

The Guinea pig (*Cavia porcellus*) is used as a biological model in the study of cutaneous leishmaniasis caused by *L.* (*Mundinia*) *enriettii*. The skin lesions heal over time similarly to CL in humans (El-On et al. 1986). On the other hand, the guinea pig appears to be resistant to another *Mundinia* species as well as to *L. braziliensis (*Paula et al. 2005, Bečvář et al 2020*)*.

Dogs (*Canis lupus f. familiaris*), together with wild canines, are the main reservoir hosts of *L. infantum* and are therefore used as animal models for visceral leishmaniasis research. Canine VL's clinical symptoms are highly variable – the infection can be asymptomatic, mild (oligosymptomatic), or fatal (Maia et al. 2010). Understanding the immune response mechanisms against VL was significantly enhanced by looking into the roles of the various immune cells and their products in asymptomatic and symptomatic dogs (Pinelli et al. 1994, Menezes-Souza et al. 2011).

Non-human primates have been used as well, primarily for their similarities to humans in terms of anatomy, physiology, and immunity. Macaques (rhesus macaque, *Macaca mulatta*), for example, are susceptible to leishmania, and lesions develop in individuals infected with *L. amazonensis*, *L. braziliensis*, and *L. major* in a very similar way to such lesions in humans (Amaral et al. 2000 and 2001, Probst et al. 2001). Of New World monkeys, the three-striped night monkey (*Aotus trivirgatus*) has been found to be susceptible to VL caused by *L. donovani* while the common squirrel monkey (*Saimiri sciureus*) is resistant (Broderson et al. 1986, Dennis et al. 1986). Experiments on primates are rare due to ethical as well as financial concerns, which is why the immune responses of simians and prosimians to leishmania infections are not as well documented as those of the model animals described above. Despite this, non-human primates remain a frequent final biological model for the testing of various types of leishmaniasis vaccines (Loria-Cervera et al. 2014).

The trend in recent years is to use wild rodents, especially those species that play the role of leishmania reservoir hosts in endemic areas. These rodents have a polymorphous genetic background - just as humans have - and, therefore, can be used to study the natural infection dynamism as well as the relationship between the host and the pathogen, i.e., the natural balance between the immune response of the host and the reproduction and successful survival of the *Leishmania* parasites (Loria-Cervera et al. 2014). As these animals are not commercially available and their breeding is not as easy compared to those of the animals mentioned above, few species have been studied for this purpose.

The hispid cotton rat (*Sigmodon hispidus*) from Central America, Mexico, and the Southeastern parts of the USA has been relatively often used for studies of various infectious pathogens, from viruses to helminths. Based on this line of research, many specific and commercially available reagents (cell lines and monoclonal antibodies specific for their cytokines and chemokines) have been developed,

and the gene sequences of several related genes have been published (Niewiesk and Prince 2002). These rodents have been found highly susceptible to both the CL-causing *L. mexicana* and the VL-causing *L. donovani.* Infections develop progressively but, unlike in hamsters, are not fatal to them (Laison and Strangways-Dixon 1964, Fulton et al. 1950).

The Brazilian rodents *Thrichomys laurentius* have been experimentally infected with *L. infantum* and *L. braziliensis*. It is of note that both these species developed in *T. laurentius* similar infection patterns as in human patients: The amastigotes persisted in the skin and viscera for several months without causing any lesions or other pathological changes to the animals (Roque et al. 2010).

A new model Yucatan deer mouse (*Peromyscus* yucatanicus) has been established for purpose of research of CL caused by *L. mexicana*. The clinical and histopathological characteristics of the disease are similar to CL in humans. In contrast to the laboratory mouse model, the NO synthase activity within the macrophages of this deer mouse does not lead to the healing phenotype (Roque et al. 2010).

Experimental infections of Chinese hamsters (*Cricetulus griseus*) had begun as early as in the 1930s (Hindale and Patton 1926, Hindale and Tomson 1928, Adler and Theodor 1931). These hamsters were previously described as highly susceptible to *Leishmania* infections, with their histopathological symptoms being similar to humans. Later, they fell out of use as model animals for leishmaniasis research, despite the fact that they are both commercially available and easy to keep. In recent years though, they saw resurgence in popularity as model animals for VL research in China (Li-ren et al. 1995).

Using new animal models for *Leishmania* research has demonstrated that immune responses to any *Leishmania* species vary wildly across host organisms and that the mechanisms that work in the seemingly standard model (inbred laboratory mouse) are far from universally applicable.

Animals suitable as biological models should display such pathological characteristics and the immune response to the disease that would ideally closely match those in humans or reservoir hosts. Therefore, one or two models cannot be sufficient for the study of a wide array of leishmaniases forms. This finding is further supported by information from vaccine testing. One of the main issues raised by "The Working Group on Research Priorities for Development of Leishmaniasis Vaccines" is the study of natural hosts and models as working on these models may help to answer important questions like What is the significance of subclinical infections? What kind of immune response is protective? Are antibody responses part of a protective response? What is the role of co-infections and nutritional status in the immune responses to *Leishmania* and to vaccines? And many others.. (Costa et al. 2011), Therefore, it is necessary to look for, create, and test new animal models that would most closely match the pre-set criteria.

4. *Leishmania* reservoir hosts

It is a well-documented fact that *Leishmania* spp. are parasites with a dixenous cycle alternating between blood-feeding arthropods and vertebrate hosts including human (Figure 4). The development of the parasite in vectors takes as little as between a week and a month, whereas in the so-called reservoir hosts (RH), the leishmania can persist for much longer - months to years. Most leishmaniases are categorised under zoonotic diseases and humans become infected randomly. The only anthroponotic cycles represent infections caused by *L. donovani* and *L. tropica*, and even then, the anthroponotic transmission is geographically restricted. The largest representation of RH can be found among rodents and canines, although the disease is far from unknown to other mammalian orders either (reviewed by Ashford 1996 and 2000).



Figure 4: Anthroponosis versus zoonosis

VL caused by *L. donovani* is shown on the left, where human is the main host (anthroponosis). CL caused by *L. aethiopica* is on the right, infections alternate between hyraxes and vectors and humans become infected accidentally (zoonosis)

Photo © Jovana Sádlová, picture was taken in Leishmaniasis Clinical Research Centre in Arba Minch, Ethiopia

The risk of infection depends heavily on the biology and epidemiology of RHs and vectors; hence it is important to identify not only vector organisms but reservoir hosts as well. Definition of RH has changed over time. According to Ashford (1996), an RH is an "ecological system in which the infectious agent survives indefinitely". Ashford proposed several guidelines to identify RH: RH must be abundant in the endemic area and form a large proportion of the mammalian biomass. RH must be sufficiently "long-lived" to allow the parasite population to survive any non-transmission season. RH should remain infected for a long time without acute disease and parasites should be present in the skin or

blood to be accessible to the vectors. Ashford (1996) also classified hosts as primary reservoirs (ensuring long-term persistence of the parasite), secondary reservoirs (the liaison between primary reservoirs and incidental hosts) and incidental hosts (irrelevant for long-term persistence of leishmaniasis but able to occasionally transmit Leishmania). However, Haydon et al (2002) and Chaves et al. (2007) have introduced a new concept that discriminates only so-called "source hosts", which can transmit the infection to other hosts, and "sink hosts", which do not play a part in further transmission of the disease. Chaves et al (2007) argued also against the additional criteria raised by Silva et al (2005) for identification of RH: (i) overlap between the geographical and temporal distribution of vector and hosts, (ii) presence of the same parasite in 'reservoirs' and humans and (iii) infection prevalence higher than 20%. The first two criteria do not distinguish RH and incidental hosts, the third – the value of *Leishmania* prevalence - vary seasonally and is affected by measurement errors. Summa summarum, RH is, according to the recent definition, a host with dynamic feedback in the transmission of a pathogen, as it can transmit the pathogen to new hosts (Chaves et al. 2007). For a mammalian population to be considered an RH, it is necessary to confirm that the parasite population is dependent on the host species. Such a confirmation requires thorough field and laboratory research. When testing host populations, it is important to realize that the synanthropic and the sylvatic cycles may co-occur in a particular region, which means that there could coexist more than one RH population in one place (Quinnell and Courtney 2009).

In laboratory conditions, the "reservoir potential" of animal species is tested using experimental infections and xenodiagnosis. Xenodiagnosis is a method employing a naïve vector fed on infected hosts to the detection of the presence of the infectious agent. In the past, xenodiagnosis was the main method used to confirm the presence of parasites in the blood of patients with Chagas disease, because the rate of detection of *T. cruzi* promastigote forms under the light microscope is very low (Bronfen et al., 1984; Dias, 1940; Perlowagora-Szumlewicz and Muller, 1982; Schenone, 1999). Over time, the method has been replaced by modern alternatives (PCR, *etc*.) (Mullis 1990). Today, the method appears to be useful in testing the infectiousness of a host to a vector, which is why it is frequently used in studying leishmaniasis in domestic and wild animals that are suspected of transmitting various leishmaniases in endemic areas (Quinnell and Courtney 2000).

OBJECTIVES

Among all mammalian groups, rodents possess the largest populations of reservoir hosts and, simultaneously, rodents are the most common laboratory models for the research of leishmaniases. However, reservoirs of most *Leishmania* species are still poorly known and the scope of standard model hosts is still insufficient to mimic well the wide spectrum of leishmaniases. My PhD project targeted both establishments of new animal models and the incrimination of rodent reservoir hosts using experimental infections of various rodent species with *L. major* and *L. donovani* and detailed between-species comparisons and analyses of infection patterns. Also, the effect of repeated sand fly biting on the progress of cutaneous leishmaniasis was examined.

The study had three major aims:

1. **Evaluation of the effect of repeated sand fly biting on the manifestation of cutaneous leishmaniasis.** The first aim was performed on the commonly used laboratory model. BALB/c mice infected intradermally with sand fly derived *Leishmania* were repeatedly bitten by *Phlebotomus duboscqi* females every two weeks. The lesion development was recorded weekly for ten weeks post-infection and parasite load and distribution in various organs were tested *post mortem* using qPCR.

2. **Introduction of new rodent models for leishmaniasis research.** The previous experience with the standard model was used to evaluate new potential animal models. Three Asian rodent species *Cricetulus griseus, Lagurus lagurus* and *Phodopus sungorus* were infected with *L. donovani* and *L. major* and the external manifestation of the disease, parasite load and distribution in the host body, as well as infectiousness to natural sand fly vectors, were compared with the standard models. Also, effects of the infectious dose and the type of inoculum (sand fly - vs. culture-derived promastigotes) were evaluated and three different methods for detecting parasites in the host´s body were used.

3. **Evaluation of host competence of African rodents *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis* for human pathogenic *Leishmania***. Experimental infections were initiated either with sand fly-derived or with culture-derived *Leishmania* promastigotes. Inoculated rodents were monitored for several months and tested by xenodiagnoses for their infectiousness to natural vectors of *L. major* and *L. donovani* in Sub-Saharan Africa. The distribution and load of parasites were determined post mortem using qPCR from the blood, skin and viscera samples. The attractiveness of *Arvicanthis* and *Mastomys* to natural vectors was tested by pair-wise comparisons.

PUBLICATIONS

1. **Vojtkova, B.,** Frynta, D., Spitzova, T., Lestinova, T., Votypka, J., Volf, P., & Sadlova, J. (2021). Repeated sand fly bites of infected BALB/c mice enhance the development of *Leishmania* lesions. *Frontiers in Tropical Diseases. 2:* 745104

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4. Sadlova, J., **Vojtkova, B.,** Becvar, T., Lestinova, T., Spitzova, T., Bates, P., & Volf, P. (2020). Host competence of the African rodents *Arvicanthis neumanni*, *A. niloticus* and *Mastomys natalensis* for *Leishmania donovani* from Ethiopia and *L*. (*Mundinia*) sp. from Ghana. *International Journal for Parasitology: Parasites and Wildlife*, *11*, 40-45.

Repeated Sand Fly Bites of Infected BALB/c Mice Enhance the Development of *Leishmania* Lesions

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Sand fly saliva has considerable immunomodulatory effects on *Leishmania* infections in mammalian hosts. Studies on several *Leishmania* – sand fly - host combinations have demonstrated that co-inoculation with *Leishmania* parasites enhances pathogenicity, while pre-exposure of hosts to sand fly bites provides significant protection against infection. However, the third scenario, the effect of sand fly saliva on parasite development in hosts infected before exposure to sand flies, remains an understudied aspect of *Leishmania*–host–vector interaction. Here we studied the effect of exposure of *L. major*-infected BALB/c mice to repeated sand fly bites. Mice infected intradermally with sand fly-derived *Leishmania* were repeatedly bitten by *Phlebotomus duboscqi* females every two weeks. The lesion development was recorded weekly for ten weeks post-infection and parasite load and distribution in various organs were tested *post mortem* using qPCR. Repeated sand fly bites significantly enhanced the development of cutaneous lesions; they developed faster and reached larger size than in unexposed mice. Multiple sand fly bites also increased parasites load in inoculated ears. On the other hand, the distribution of parasites in mice body and their infectiousness to vectors did not differ significantly between groups. Our study provides the first evidence that multiple and repeated exposures of infected BALB/c mice to sand fly bites significantly enhance the progress of local skin infection caused by *Leishmania major* and increase tissue parasite load, but do not affect the visceralization of parasites. This finding appeals to adequate protection of infected humans from sand fly bites, not only to prevent transmission but also to prevent enlarged lesions.

Keywords: *Leishmania major*, *Phlebotomus*, sand flies, vector saliva, cutaneous lesions, BALB/c mice

INTRODUCTION

Leishmaniases are diseases caused by protozoan *Leishmania* parasites (Kinetoplastida: Trypanosomatidae) transmitted by insect vectors, phlebotomine sand flies (Diptera: Psychodidae). Leishmaniases are endemic in 98 countries with 350 million people worldwide living at risk and 20 – 40 000 dying annually (1). More than 20 *Leishmania* species are pathogenic to

humans, and each *Leishmania* species is transmitted with a different spectrum of vector species (2). Among over 800 sand fly species, 98 are proven or suspected vectors (3). The species-specific complex interactions between the host, parasite and sand fly result in the broad spectrum of clinical manifestations typical for human leishmaniases. Cutaneous leishmaniasis is the most prevalent clinical form characterized by localized, diffuse, or disseminated skin lesions, mucocutaneous leishmaniasis with progressive destruction of oronasopharyngeal mucosa and cartilaginous structures is disfiguring and potentially life treating and visceral leishmaniasis is the most severe form, often fatal if left untreated, characterized by fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy, and anaemia (4, 5).

Sand fly saliva contains a mixture of at least 20 diverse proteins with antihemostatic, anti-inflammatory, and immunomodulatory properties. This cocktail is obligatorily inoculated into the host skin during blood feeding of sand fly female and can help spread *Leishmania* parasites in naive, previously non-bitten, hosts. This phenomenon is called enhancing effect and can be caused also with some individual salivary proteins or even salivary peptides produced in recombinant form. On the other hand, preimmunization of mice with salivary glands lysates or their pre-exposure to uninfected sand fly bites provided significant protection against infection (reviewed by 6). However, only a single study has been concerned on the effect of saliva in hosts previously infected by *Leishmania:* on the hamster model, increased transmissibility of *L. donovani* to vectors after multiple exposures of the host to sand fly bites was reported (7).

In this study, we exposed BALB/c mice infected with sand fly-derived *L. major* to repeated bites of *Phlebotomus duboscqi* and provide evidence that vector saliva influences substantially the outcome of *L. major* infection.

MATERIALS AND METHODS

Sand flies, Parasites, and Mice

Phlebotomus duboscqi colony (originating from Senegal) was maintained under standard conditions (26°C on 50% sucrose and 12 h light/12 h dark photoperiod) as described previously (8).

Leishmania major (MHOM/IL/81/Friedlin/VI; FVI) was cultured in M199 medium (Sigma) containing 10% heat-inactivated foetal calf serum (Gibson) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma), 2% sterile urine and 250 μg mL−1 amikacin (Amikin, Bristol-Myers Squibb).

BALB/c mice originated from AnLab. s.r.o. (Harlan Laboratories, USA, Nederland). Animals were maintained in T3 breeding containers (Velaz) equipped with bedding German Horse Span (Pferde) and breeding material (Woodwool), provided with a standard feed mixture ST-1 (Velaz) and water ad libitum, with a 12 h light/12 h dark photoperiod, temperature 22–25°C and humidity 40–60%.

Infection of BALB/c Mice With Sand Fly-Derived *Leishmania*

Rodents were infected with sand fly-derived *Leishmania* according to Sadlova et al. (9). *Phlebotomus duboscqi* females were infected with *L. major* promastigotes (10^6 parasites/ml) by feeding through chick-skin membranes and engorged females were maintained under standard conditions till day 8 post blood meal (PBM). Their midguts were checked microscopically for the presence of promastigotes and thoracic midguts with good density of parasites were pooled in sterile saline: 80 infected thoracic midguts were homogenized into 40 μl of sterile saline. Dissected salivary glands (SG) of naïve *P. duboscqi* were pooled in sterile saline (10 glands per 10 μl of saline) and stored at −20° C. Before mice inoculation, SG was disintegrated by 3 successive immersions into liquid nitrogen and 4 μl added to homogenized guts. Immediately, BALB/c mice anaesthetized with ketamine/xylazine (62.5 mg and 25 mg/kg, respectively) were injected with 5.5 μl of the suspension intradermally into the inner side of the ear pinnae using a syringe. The number of parasites was calculated using the Bürker apparatus and the proportions of metacyclic forms were identified on Giemsa stained smears based on morphological criteria described previously (10). In two repeats of experiments, *L. major* numbers in the inoculums were 8.5 x 10^4 per mouse, metacyclics comprised 72% of all forms. Mice were checked weekly for external signs of the diseases till week 10 post-infection (p.i.). At the end of the experiment (10 weeks p.i.), the blood and tissue samples were collected for qPCR analysis. In each of the two independent experiments, 12 mice were infected and 4 mice were used as non-infected controls (NONI group).

Mice Exposure to *P. duboscqi* Bites

Half of the mice in each experiment were exposed to sand flies on weeks 2, 4, 6, 8 and 10 p.i. (EXPOSED group) while remaining mice were exposed only at the end of experiments on week 10 p. i. (UNEXPOSED group). Anaesthetized mice were inserted into the cotton bag, inoculated ears were removed from the bag and 5 to 7-day-old *P. duboscqi* females were allowed to feed on both sides of ear pinnae. Engorged sand fly females were separated and maintained under standard conditions. Eight days PBM, females were dissected, their guts were examined under the light microscope and intensity and localization of infections were evaluated as described previously (10, 11).

Mice Sampling and Quantitative PCR

Mice were euthanized by cervical dislocation under anaesthesia 10 weeks p. i. Both ears (inoculated and contralateral), both ear-draining lymph nodes, all four paws, tail, spleen, liver and blood were stored in - 20°C. Extraction of total DNA from mice tissues was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer´s instructions. Quantitative (q) PCR for detection and quantification of *Leishmania* DNA was performed in Bio-Rad iCycler&iQ Real-Time PCR Systems using the SYBR Green detection method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical analyses of weight gains, lesion development, infectiousness and percentage of infected organs were carried out using the R software (http://cran.r-project.org). The continuous response variable (weight, lesion size) and their relationship with categorical (EXPOSED, UNEXPOSED or NONI group) and continuous (week p.i.) explanatory variables were examined fitting multilevel linear regression models (package "nlme"), taking into account the correlation between repeated measures of the same animal over time. The model used included the interaction term between categorical and continuous explanatory variable. The infectiousness was analysed by proportional test. Percentage of infected organs and parasite load were analysed with GLM with a binomial distribution and negative binomial distribution, respectively. Differences in lesion size in respective weeks were tested by one-tailed T tests. The between-group differences in lesion size were tested with nonparametric Independent Samples Median Test (medians) and Independent Samples Mann-Whitney U Test (distributions) using SPSS software version 23. A P-value of < 0.05 was considered to indicate statistical significance.

Ethical Consideration

Animals were maintained and handled in the animal facility of Charles University in Prague following 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 in Prague and were performed under permit no. MSMT-10270/2015-5 of the Ministry of the Education, Youth and Sports of the Czech Republic. Investigators were certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

RESULTS

Infection Course and Cutaneous Lesions Development

Mice were infected with intradermal inoculation of 8.5×10^4 sand fly–derived parasites, with a prevalence (72%) of metacyclic forms. The course of infections was observed in 24 mice in two independent experiments. In each experiment, infected mice were divided into the EXPOSED group exposed in 2-week intervals to *P. duboscqi* bites and the UNEXPOSED group, left without exposure. In the first experiment, one mouse in the UNEXPOSED group died before the end of the experiment. In addition, 8 mice (4 in each experiment) were used as the non-infected control (NONI group).

Infected mice did not show significant weight loss, there were no statistical differences in weight gains between uninfected control mice (NONI group) and both infected groups (**Supplementary Table S1**). In all groups in both experiments, the bodyweight increased significantly with the age of mice ($P < 0.0001$).

Lesions developed in all infected mice with exception of 2 animals in the UNEXPOSED group in the first experiment. They appeared on week 5 p.i. in the first experiment and on week 2 p.i. in the second experiment. Importantly, lesions appeared one week earlier and they developed to a large size faster in the EXPOSED group compare to the UNEXPOSED group in both experiments ($P = 0.048$, **Figures 1A, B** and **Supplementary Table S2**).

At the end of the experiments on week 10 p.i., lesions reached a significantly larger size in the EXPOSED group compared to the UNEXPOSED group ($P = 0.012$, **Figure 2A**). The mean values were 8.3 mm for the EXPOSED group and 4.3 mm for the UNEXPOSED group in the first experiment and 8.1 mm for the EXPOSED group and 5.8 mm for the UNEXPOSED group in the second experiment (**Figures 3C, D**).

Infectiousness to *Phlebotomus duboscqi*

Phlebotomus duboscqi is a natural vector of *L. major* (3) and the colonized flies have been shown to support the development and transmission of the parasite (12). In mice exposed to *P. duboscqi* females in two-week intervals (EXPOSED group), the relative proportion of mice infectious to sand flies as well as the percentage of infected vectors increased with time till week 6 p.i. in the first experiment and week 4 p.i, in the second experiment. From these time points, all tested mice were infectious, including those two mice without lesions in the UNEXPOSED group (**Table 1**). On week 10 p.i., the proportion of infected sand flies did not differ significantly between the EXPOSED group and the unexposed UNEXPOSED group, $P = 0.259$.

Parasite Load and Anatomical Distribution in BALB/c Mice

Parasites were distributed in the same spectrum of tissues in both groups: inoculated ears were positive in all animals including two mice without apparent lesions, parasites were often found also in contralateral ears and draining lymph nodes of inoculated ears. In some animals of both groups, also paws and tail were infected while blood, draining lymph nodes of contralateral ears, liver and spleen were negative (**Table 2**). The proportion of infected organs did not differ significantly between the EXPOSED and the UNEXPOSED group ($P = 0.130$).

Parasite load detected *post mortem* by qPCR is also summarized in **Table 2**. The numbers of *Leishmania* were higher in mice exposed to sand flies according to the higher representation of heavy and moderate parasite load (red and orange coloured) and lower representation of weak infections (green coloured) in respective tissues. The numbers of *Leishmania* in inoculated ears in the EXPOSED group (mean value 1.3x10*6, median 7.5x10*5) were about twice higher than numbers in the UNEXPOSED group (mean value 5.3x10*5, median 4.0x10*5), and the difference is on the border of statistical significance ($P = 0.066$, **Figure 2B**). The two experiments differed in this respect (**Figures 3A, B**): the EXPOSED group showed significantly higher

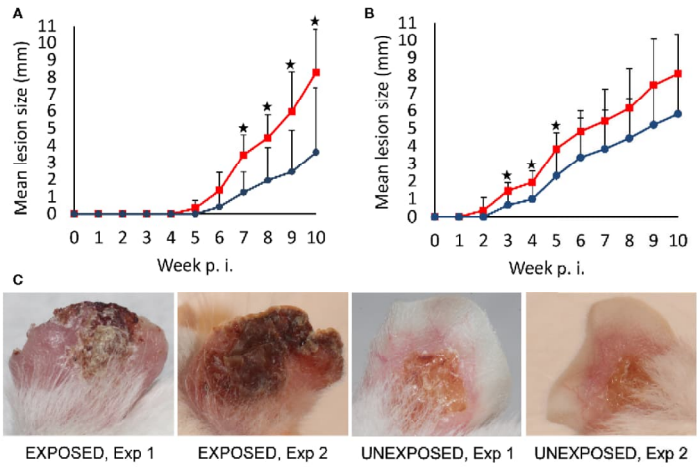


FIGURE 1 | Lesion development in BALB/c mice infected with *L. major*. Mice infected with intradermal inoculation of sand fly–derived parasites were divided into the EXPOSED group (exposed in 2-week intervals to *P. duboscqi* bites) and the UNEXPOSED group (left without exposure). The course of infections was observed for 10 weeks in 24 mice in two independent experiments. Between-group differences were tested with multilevel linear regression model on entire data set (P = 0.048). Significant differences between experimental groups in respective weeks, revealed by T tests (one-tailed), are marked by asterisks **(A)** experiment 1; **(B)** experiment 2. Red squares, EXPOSED group, blue circles, UNEXPOSED group. **(C)** typical lesion appearance in BALB/c mice on week 10 p.i., Exp 1, Experiment 1; Exp 2, Experiment 2.

parasite load than the UNEXPOSED group in the first experiment (P = 0.019) while the differences were not significant in the second experiment (P = 0.195). These results are not surprising considering earlier lesions development in the second experiment: the ear pinnae were more necrotic and a smaller part of the ears remained available for the parasite growth on week 10 p.i. (**Figure 1C**).

DISCUSSION

This study addresses the substantial question if repeated sand fly bites influence the clinical manifestation of cutaneous leishmaniasis caused by *L. major*. We used the model of BALB/c mice to compare the disease progress in mice exposed to *P. duboscqi* females in 2 weeks intervals (EXPOSED group) with mice left without sand fly bites (UNEXPOSED group) and provide the first evidence that repeated sand fly bites significantly enhance the progress of cutaneous lesions and increase tissue parasite load.

The development of *Leishmania* infection in the host body is a complex process depending on many parasites- and host-associated factors. Different *Leishmania* species cause different disease forms in the same model animal (reviewed by 13) and even various strains of *Leishmania major* differ in virulence for the host, as has been demonstrated also for *L. major* (14, 15). Simultaneously, the genetic background and the immune response of the mammalian host play important role in disease progress (16). BALB/c mouse is the model animal highly susceptible to *L. major* infection, with the immunity polarized to strong Th2, driven largely by IL-4, that is responsible for suppressing Th1-cell development and inhibiting the secretion of IFN-γ that is required to activate infected macrophages for parasite killing (17–19).

The clinical manifestation of the disease in experimental infections is substantially influenced by methodological parameters like parasite dose and type (procyclic/metacyclic promastigotes or amastigotes) and inoculation route and site (reviewed by 20). In this study, we used the intradermal route of inoculation, which is closest to the natural mode of transmission since parasites are exposed to the localized immune responses in the skin (21). Parasites used for inoculation were predominantly metacyclic infective forms pooled from thoracic midguts of laboratory infected *P. duboscqi*. The parasite load (8.5×10^4 parasites/mouse) did not exceed the maximal number of *L. major* parasites transmitted by *P. duboscqi* females (22). The knowledge and control of parasite load is a big advantage of the used method compare to natural infections by sand fly bite - in the second case, the numbers of transmitted parasites differ in several orders between individual vector females (22–24). To mimic the natural transmission, the homogenate of salivary glands of *P. duboscqi* females were added to the inoculum. This standardized procedure resulted in the symptomatic course of the disease in BALB/c mice.

It should be noted that in this study, infected mice were exposed to sand flies in two-week intervals while in the real

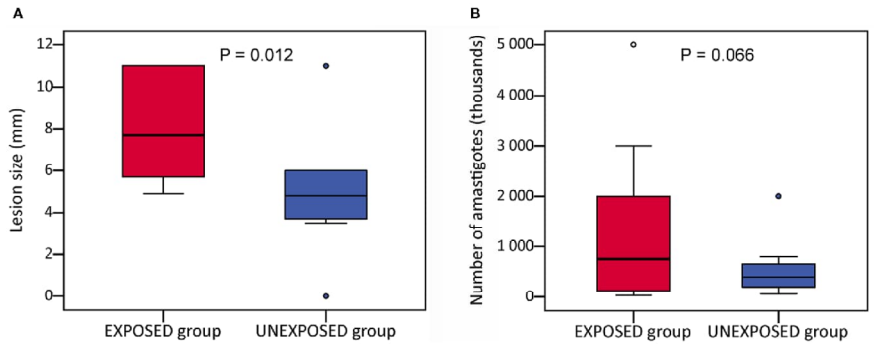


FIGURE 2 | Infection parameters in BALB/c mice on week 10 p.i. Mice infected with intradermal inoculation of sand fly–derived parasites were divided into the EXPOSED group (exposed in 2-week intervals to *P. duboscqi* bites) and the UNEXPOSED group (left without exposure). The course of infections was observed for 10 weeks in 24 mice in two independent experiments. Parasite load was evaluated *post mortem* by qPCR. The data presented here is sum from both experiments. **(A)** the final lesion size; **(B)** parasite load in infected ears. In the boxplots, the box is bordered by upper and lower quartile (IQR, interquartile range), the horizontal line denotes the median value, whiskers denote 1.5 times IQR and circles denote outliers. Significance of between-group differences in lesion size were tested with nonparametric Independent Samples Median Test, parasite loads were analysed with GLM with a negative binomial distribution.

world, sand fly biting is continuous. However, mimicking a real scenario in the laboratory is not possible as the animals cannot be subjected to anaesthesia so often. It is also quite difficult to assess the exact sand fly biting rate on rodent reservoirs of *L. major* in nature. Turner and Hoogstraal (25) reported the numbers corresponding to 51, 45, 41 and 89 fed *Phlebotomus* females per two weeks on *Tatera*, *Acomys*, *Mastomys* and *Arvicanthis* in Sudan. While experimental exposure of rodents used as bait in

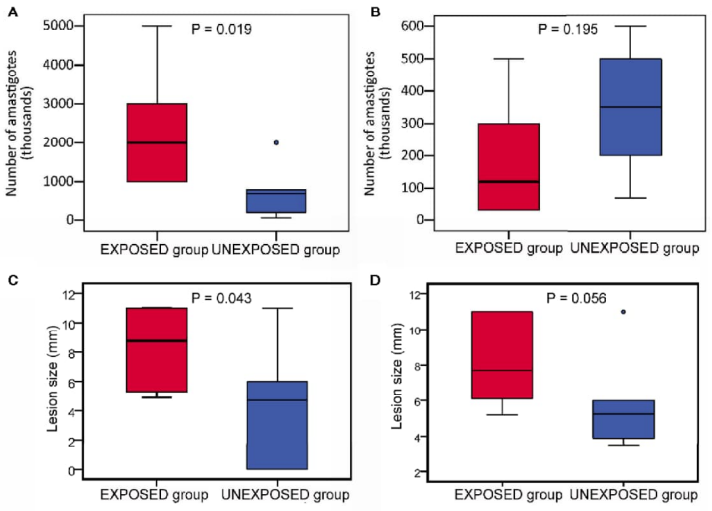


FIGURE 3 | Infection parameters in BALB/c mice on week 10 p.i. in two independent experiments. The parasite load **(A, B)** and final lesion size **(C, D)** in infected ears. **(A, C)** experiment 1; **(B, D)**, experiment 2. In the boxplots, the box is bordered by upper and lower quartile (IQR, interquartile range), the horizontal line denotes the median value, whiskers denote 1.5 times IQR and circles denote outliers. Significance of differences betweed mice exposed to sand flies (EXPOSED group) and control mice (UNEXPOSED group) were analysed with GLM with a negative binomial distribution and one tailed T test.

TABLE 1 | Xenodiagnosis of *L. major* in BALB/c mice: feeding of *P. duboscqi* on inoculated ears.

Week p.i.	Group	Mouse	No. of fed females	No of dissected females	No and (%) of positive sand flies	Mouse	No. of fed females	No of dissected females	No and (%) of positive sand flies
			Experiment 1					**Experiment 2**	
2	XENO	1	77	45	0	1	48	27	2 (7.4)
		2	55	16	0	2	45	24	6 (25.0)
		3	78	21	1 (4.7)	3	40	28	1 (3.6)
		4	67	23	0	4	44	30	0
		5	54	19	0	5	37	28	5 (17.9)
		6	52	20	0	6	42	26	0
		Σ	**383**	**144**	**1 (0.7)**	**Σ**	**256**	**84**	**5 (5.95)**
4	XENO	1	71	31	0	1	45	30	1 (3.3)
		2	69	37	3 (8,1)	2	48	31	3 (9.7)
		3	42	4	0	3	49	32	1 (3.1)
		4	92	40	2 (5.0)	4	44	34	4 (11.8)
		5	78	36	4 (11.1)	5	42	29	7 (24.1)
		6	105	57	2 (3.5)	6	46	38	3 (7.9)
		Σ	**457**	**205**	**11 (5.4)**	**Σ**	**274**	**101**	**14 (14.6)**
6	XENO	1	97	59	2 (3.4)	1	50	40	14 (35.0)
		2	82	53	13 (24.5)	2	50	40	10 (25.0)
		3	78	46	4 (8.7)	3	52	40	5 (12.5)
		4	56	21	4 (19.0)	4	50	37	2 (5.4)
		5	59	18	5 (27.8)	5	48	40	7 (17.5)
		6	71	18	1 (5.6)	6	47	41	3 (7.3)
		Σ	**443**	**215**	**29 (13.5)**	**Σ**	**297**	**118**	**12 (10.1)**
8	XENO	1	77	47	3 (6.4)	1	37	34	10 (29.4)
		2	95	61	7 (11.5)	2	40	25	7 (28.0)
		3	73	42	15 (35.7)	3	42	38	10 (26.3)
		4	48	20	3 (15.0)	4	39	28	4 (14.3)
		5	79	56	4 (7.1)	5	30	22	3 (13,6)
		6	105	89	4 (4.5)	6	31	25	2 (8.0)
		Σ	**477**	**315**	**36 (11.4)**	**Σ**	**219**	**75**	**9 (12.0)**
10	XENO	1	105	77	16 (20.8)	1	28	19	3 (15.8)
		2	102	68	6 (8.8)	2	27	19	1 (5.3)
		3	108	70	12 (17.1)	3	28	18	3 (16.7)
		4	67	41	3 (7.3)	4	31	21	6 (28.6)
		5	65	31	6 (19.4)	5	35	21	4 (19.0)
		6	66	38	3 (7.9)	6	30	25	5 (20.0)
		Σ	**513**	**325**	**46 (14.2)**	**Σ**	**179**	**67**	**15 (22.5)**
	non-XENO	1	83	46	8 (17.4)	1	30	21	7 (33.3)
		2	119	85	16 (18.8)	2	30	23	7 (30.4)
		3	121	86	16 (18.6)	3	25	16	3 (18.7)
		4	55	32	3 (9.4)	4	28	23	3 (13.0)
		5	58	46	3 (6.5)	5	23	20	5 (25.0)
		6	–	–	–	6	30	19	5 (26.3)
		Σ	**476**	**307**	**49 (17.8)**	**Σ**	**166**	**62**	**13 (21.4)**

In bold: No. of fed females: Number of P. duboscqi females fed on anaesthetized mice. No of dissected females: Number of engorged females that survived till dissection and microscopy. No of positive sand flies: Number of females with Leishmania infection determined by microscopy.

traps may not exactly reflect the natural situation, influencing their movement, temporal activity pattern, burrowing, aggregation, etc., the natural biting rate may be accurately measured on humans. According to the study on the biting behaviour of *P. argentipes* in Sri Lanka, the biting rate on humans was 8.4 (range 2-25 bites) per night (26) which would represent 117 (17-210) in two weeks. Although these numbers vary with sand fly species, host, locality, season and many other factors, it is at least a rough estimate of the natural biting rate and in our experiments, bite numbers lied within this range.

It is a well-known phenomenon that salivary molecules are an obligatory part of feeding sand flies on mammalian hosts and they have an immunomodulatory effect on *Leishmania* infection (27). Like other blood-feeding arthropods, sand flies need to secrete saliva into the wound to facilitate blood-feeding. Sand fly saliva is composed of multiple proteins and peptides with diverse functions that evolved mainly to counteract the host's hemostatic system. However, salivary proteins are also capable of inducing a strong anti-inflammatory immune response in naïve hosts, which is beneficial for *Leishmania* parasites (reviewed by 6, 28). Therefore, it is not surprising, that co-injection of *Leishmania* parasites with salivary gland lysates (SGL) or the recombinant salivary peptides has been shown to enhance *Leishmania* pathogenicity for mice on many combinations of *Leishmania* and sand fly species (reviewed by 6). However, to our knowledge, only a single study has been concerned on the additional effect of

TABLE 2 | Presence and amount of *L. major* DNA in BALB/c mice acquired by qPCR.

Group	Exp.	Mouse	B	IE	CE	DN-IE	DN-CE	FP	HP	T	S	L
EXPOSED	1	1	0	2x10^6	7x10^3	4x10^3	0	5x10^4	0	0	0	0
		2	0	10^6	0	7x10^3	0	3x10^3	4x10^2	0	0	0
		3	0	10^6	0	2x10^3	0	0	0	0	0	0
		4	0	2x10^6	10^4	6x10^4	0	0	0	6x10^4	0	0
		5	0	3x10^6	10^5	7x10^5	0	10^4	0	2x10^4	0	0
		6	0	5x10^6	2x10^5	5x10^4	0	5x10^4	10^4	10^4	0	0
	2	1	0	5x10^5	10^3	0	0	2x10^3	0	0	0	0
		2	0	4x10^4	0	0	0	10	0	0	0	0
		3	0	3x10^5	2x10^4	4x10^2	0	6x10^2	0	0	0	0
		4	0	3x10^4	2x10^4	0	0	10	0	0	0	0
		5	0	3x10^4	10^3	0	0	2x10^3	10^2	2x10^3	0	0
		6	0	2x10^5	5x10	0	0	0	0	0	0	0
UN-EXPOSED	1	1	0	6x10^4	0	10^3	0	0	0	0	0	0
		2	0	2x10^5	0	3x10^3	0	0	0	10^3	0	0
		3	0	7x10^5	0	7x10^2	0	0	0	0	0	0
		4	0	8x10^5	0	5x10^3	0	0	0	0	0	0
		5	0	2x10^6	0	8x10^3	0	0	0	0	0	0
	2	1	0	3x10^5	4x10^2	0	0	3x10^2	0	0	0	0
		2	0	5x10^5	6x10^4	0	0	2x10^3	0	0	0	0
		3	0	6x10^5	9x10^3	0	0	2x10^2	4x10^2	10^2	0	0
		4	0	7x10^4	10^3	0	0	0	0	0	0	0
		5	0	4x10^5	7x10	0	0	10	0	10	0	0
		6	0	2x10^5	0	0	0	10	0	0	0	0

IE, inoculated ear; CE, contralateral ear; DN-IE, draining lymph nodes of the inoculated ear; DN-CE, draining lymph nodes of the contralateral ear; FP, forepaws; HP, hindpaws; T, tail; L, liver; S, spleen; B, blood; green colour, ≤ 10^2 parasites; yellow colour, 10^2–10^4 parasites; orange colour, > 10^4 parasites, red colour, > 10^6 parasites.

saliva in hosts previously infected by *Leishmania*; Valverde et al. (7) described increased transmissibility of *L. donovani* to vector after multiple exposures of hamsters to sand fly bites.

The current study demonstrated that repeated exposure of the infected host to sand fly bites has an onward "enhancing effect" on the lesion growth to the co-injection of *Leishmania* with salivary gland lysates. Lesions developed faster and reached a higher size in mice exposed to repeated sand fly bites. Apart from the enhanced lesion development, also tissues parasite load was higher in mice exposed to repeated bites. This differs from the effect of sand fly exposure on *L. donovani* load in the hamster model; here, repeated sand fly bites enhanced significantly parasite numbers in the peripheral blood but not in the skin (7). This difference may reflect the species-specific organ tropism of different *Leishmania* - the visceral species *L. donovani* circulates in the blood, while cutaneous *L. major* remains localized predominantly in the skin and local lymph draining nodes (29).

In our experiments, the anatomical distribution of parasites was not affected by repeated bites of uninfected sand flies. In both experimental groups, parasites migrated from the site of inoculation (left ear) to its draining lymph nodes and skin on other body parts (contralateral ears, paws and tail) but were never detected in blood or viscera. This corresponds to the previously published pattern of development of *L. major* in BALB/c mice (30) while visceralisation occurs in BALB/c mice in case of unnatural intravenous or subcutaneous initiation of *L. major* infection or overdose of the intradermal infection (reviewed by 31). For unknown reasons, migration to ear-draining lymph nodes was reduced in the second experiment in both experimental groups.

Similarly, infectiousness to sand flies did not differ significantly between exposed and unexposed mice in this study. On week 10 p.i., all 24 BALB/c mice infected with *L. major* were infectious to *P. duboscqi* females and the mean percentage of infected sand flies varied from 14.2 to 22.5% in respective groups. Similar infectiousness was described for symptomatic dogs infected with *L. infantum* exposed to *Lu. longipalpis* (13 - 28%) (32–34), for BALB/c mice infected with *L. donovani* exposed to *P. orientalis* (19%) (9) or hamsters infected with *L. donovani* exposed to *L. longipalpis* (24%) (7). In the latter study, the infectiousness of hamsters increased significantly post exposures to sand flies while this effect was not observed in our experiments. Here, the differences in infectiousness between exposed and unexposed mice were evaluated at the end of experiments, two weeks post previous exposure of the EXPOSED group while Valverde et al. (7) tested the infectiousness of hamsters just 24h post previous exposure. Therefore, we cannot exclude that if there was a similar short-term effect of saline on infectiousness in order of hours or days, it was not detected due to the experimental design. Similarly, it was not possible to directly compare infectiousness in early weeks p.i. in this study.

Sand fly feeding provides a suitable environment for the survival and multiplication of *L. major* parasites. Sand fly saliva has chemotactic activities for both neutrophils and macrophages which accelerate the entry of parasites into these cells in the early days (35–39). Besides this effect, sand fly saliva modulates the host immune system in many ways, advantageous for the parasite: inhibits macrophage activation to the production of NO (40), modulates the activity of T cells and macrophages by decreasing the secretion of pro-inflammatory

cytokines and enhancing the production of anti-inflammatory cytokines (41), induces apoptosis of neutrophils (42) and inhibits the ability of dendritic cells to present antigens (43). All these effects favor the establishment of *Leishmania* infection and enhance parasite survival in naive, previously non-bitten hosts. Nevertheless, here we showed a type of onward "enhancing effect" when the disease outcome was significantly exacerbated after further bites of uninfected sand fly females. In our opinion, this effect could be caused by enzyme hyaluronidase present in *P. duboscqi* saliva (44). Hyaluronidase cleaves hyaluronic acid, a major component of vertebrate extracellular matrix, facilitating the spread of other pharmacologically active compounds of saliva. Hyaluronidase activity was shown to exacerbate skin lesions caused by *L. major* in concentration-dependent manner but did not affect visceralization (45) and therefore we think that repeated exposure to uninfected bites multiplies the effect of hyaluronidase activity. In addition, several other vector –derived components like proteophosphoglycans, exosomes or sand fly microbiota, co-inoculated with saliva and *Leishmania* into the host skin, have been shown to modulate host immune response, promote infection and enhance disease pathology (46–48). Presumably, these components inoculated by uninfected sand flies into the skin with *Leishmania* infection may also contribute significantly to the "enhancing effect".

In conclusion: our study provides first evidence that sand fly saliva inoculated repeatedly to infected hosts substantially influences the outcome of *L. major* infection. This finding appeals to adequate protection of infected humans from sand fly bites not only to prevent transmission but also to prevent enlarged lesions

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Ethics of Laboratory Experiments of the Charles University in Prague.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology and supervision: JS. Investigation: BV, TL, and JV. Statistical analysis: DF and TS. Writing-original draft preparation: BV and JS. Writing-review and editing: PV. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2021.745104/full#supplementary-material

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Central Asian Rodents as Model Animals for *Leishmania major* and *Leishmania donovani* Research

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Abstract: The clinical manifestation of leishmaniases depends on parasite species, host genetic background, and immune response. Manifestations of human leishmaniases are highly variable, ranging from self-healing skin lesions to fatal visceral disease. The scope of standard model hosts is insufficient to mimic well the wide disease spectrum, which compels the introduction of new model animals for leishmaniasis research. In this article, we study the susceptibility of three Asian rodent species (*Cricetulus griseus*, *Lagurus lagurus*, and *Phodopus sungorus*) to *Leishmania major* and *L. donovani*. The external manifestation of the disease, distribution, as well as load of parasites and infectiousness to natural sand fly vectors, were compared with standard models, BALB/c mice and *Mesocricetus auratus*. No significant differences were found in disease outcomes in animals inoculated with sand fly- or culture-derived parasites. All Asian rodent species were highly susceptible to *L. major*. *Phodopus sungorus* showed the non-healing phenotype with the progressive growth of ulcerative lesions and massive parasite loads. *Lagurus lagurus* and *C. griseus* represented the healing phenotype, the latter with high infectiousness to vectors, mimicking best the character of natural reservoir hosts. Both, *L. lagurus* and *C. griseus* were also highly susceptible to *L. donovani*, having wider parasite distribution and higher parasite loads and infectiousness than standard model animals.

Keywords: *Phodopus sungorus*; *Cricetulus griseus*; *Lagurus lagurus*; model animals; cutaneous leishmaniasis; visceral leishmaniasis; infectiousness; xenodiagnosis; *Phlebotomus*; sand fly

1. Introduction

Leishmania parasites (Kinetoplastida: Trypanosomatidae) are causative agents of leishmaniases, a group of diseases prevalent worldwide in 98 countries with more than 350 million people considered at risk, more than 1 million new cases occurring every year, and more than 50 thousand deaths annually, due to the visceral form [1,2]. The epidemiology and ecology of leishmaniases are exceptionally complex—at least 20 *Leishmania* species are pathogenic to humans, each of them possessing different mammalian reservoir hosts, as well as insect vectors [3].

This species diversity is reflected in the broad spectrum of clinical manifestations of human leishmaniases, which results from interactions between the parasite species and the host immune

responses. Cutaneous leishmaniasis (CL), transmitted by diverse sand fly vector species, is caused principally by *L. major*, *L. tropica*, and *L. aethiopica* in the Old World and *L. mexicana*, *L. venezuelensis*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, and *L. peruviana* in the New World. Infection with CL may be characterized by localized, diffuse, or disseminated skin lesions [4]. Metastatic mucocutaneous leishmaniasis (MCL), confined to the New World, is due to *Leishmania (Viannia) braziliensis* or, less frequently, *L. (V.) panamensis* and *L. (V.) guyanensis*. Visceral leishmaniasis (VL), caused by *L. donovani* and *L. infantum*, is the most severe form, often fatal if left untreated, characterized by fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy, and anemia [5].

Experimental animal models are expected to mimic the specific features of the variety of human leishmaniases. Many immunological aspects of the disease have been studied using standard laboratory models, such as mice, hamsters, domestic dogs, and non-human primates. However, none of them accurately reproduces the outcome of human *Leishmania* infection [6]. The major advantage of inbred mouse models is their controlled genetic background and well-defined immune response. On the other hand, replication and spread of the pathogen in mice are far from the natural pattern. The relative lack of genetic polymorphism in laboratory mice has been specifically overcome by using stocks derived from recently trapped wild progenitors belonging to different taxa of the genus *Mus* [7]. An alternative approach is the use of genetically polymorphic wild rodents as experimental animal models for host-parasite relationships studies. These models allow a better understanding of the dynamics and range of infection, including mechanisms of parasite amplification, their availability for transmission, and the natural regulation of the immune response [6].

More than 20 rodent species have been used for experimental infections with *Leishmania* parasites. The extensive research in this field was mainly done during the first half of the last century. However, many of the tested species are protected or difficult to breed in the laboratory, and many of them have been shown to be resistant to infection (reviewed in [8]). More recently, two New World wild rodent species *Thrichomys laurentius* and *Peromyscus yucatanicus* have been used for the study of *L. infantum*, *L. braziliensis*, and *L. mexicana* infections, respectively [9,10].

Our study was aimed at evaluation of susceptibility of three Central Asian rodent species, *Cricetulus griseus*, *Phodopus sungorus*, and *Lagurus lagurus* to two human pathogenic *Leishmania* species. The three rodent species were chosen as they are commercially available and relatively easy to breed. Moreover, several publications from the beginning of the last century showed the susceptibility of *C. griseus* to *Leishmania donovani* [11–14], while *L. lagurus* was used for testing of VL in China (reviewed in [15]). Here, rodents were infected with *L. major* and *L. donovani*, using different inoculum types (sand fly vs. culture-derived parasites). Fluorescence detection and quantitative PCR were applied to evaluate the load of parasites and their anatomical distribution in the rodent.

2. Materials and Methods

2.1. Sand Flies, Parasites, and Rodents

The sand fly colonies of *Phlebotomus duboscqi* (originating from Senegal) and *Phlebotomus orientalis* (originating from Ethiopia) were maintained in the insectary of the Department of Parasitology, Charles University in Prague, under standardized conditions (26 °C, fed on 50% sucrose, and with 14 h light/10 h dark photoperiod) as previously described [16].

Two *Leishmania* strains were used for infection of sand flies and rodents: *L. major* (MHOM/IR/-/173) and *L. donovani* (MHOM/ET/2009/AM459) with DsRed and RFP red fluorescent markers, respectively, prepared according to the methodology described by Sadlova et al. [17]. Promastigotes were cultured in M199 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FBS Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2% sterile human urine, supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma-Aldrich, St. Louis, MO, USA) and 250 µL/mL amikacin (Medopharm, Prague, Czech Republic).

Five rodent species were used: *Cricetulus griseus* (Chinese hamsters), *Lagurus lagurus* (Steppe lemmings), *Phodopus sungorus* (Djungarian hamsters), *Mesocricetus auratus* (Golden hamsters), and *Mus musculus* (BALB/c mice), all originating from commercial sources. BALB/c mice and *M. auratus* originated from AnLab s.r.o. (Prague, Czech Republic), *C. griseus*, *Phodopus sungorus*, and *L. lagurus* from Karel Kapral s.r.o. (Prague, Czech Republic). Breeding containers T2 and T3 (Velaz s.r.o., Prague, Czech Republic) were used for maintaining BALB/c mice; the remaining species were maintained in breeding containers 3/H3 and T4 (Velaz s.r.o., Prague, Czech Republic). Containers were equipped with German Horse Span bedding (Pferde a.s., Prague, Czech Republic), breeding material (Woodwool, Miloslav Vlk s.r.o., Ratibor, Czech Republic), hay (Krmne smesi Kvidera s.r.o, Nezvestice, Czech Republic), and the standard feed mixture ST-1 (Velaz s.r.o., Prague, Czech Republic). The feed mixture MOK (Biopharm s.r.o., Kraluv Dvur, Czech Republic) without animal protein content was used for *L. lagurus*. Food and water were provided *ad libitum*, and the animals were maintained in standard conditions (12 h dark/12 h light photoperiod, at a temperature of 22–25 °C and humidity of 40–60%).

2.2. Experimental Infections of Sand Flies

Promastigotes from log-phase cultures were resuspended in heat-inactivated defibrinated rabbit blood at a concentration of 1×10^6 promastigotes/mL. Sand fly females were infected by feeding through a chick-skin membrane, and engorged specimens were maintained in the same conditions as the colony.

2.3. Infections of Rodents

Two methods for infection of rodents were used: Infections initiated with sand fly-derived promastigotes (SDP) according to Sadlova et al. [18] and infection initiated with stationary phase culture-derived promastigotes (CDP), to include infective metacyclic forms. For the first method, sand fly females experimentally infected with *Leishmania* parasites were dissected on day 8 post bloodmeal, when mature infections and accumulation of metacyclic forms had developed in the thoracic midguts (TMG). Pools of 80 freshly dissected TMG with a good density of parasites were homogenized in 40 µL of sterile saline. For the inoculation of rodents with CDP, stationary-phase culture was washed twice in saline. For the selection of *L. major* metacyclic forms, 50 µL/mL of peanut agglutinin lectin (PNA) was added, which agglutinated the procyclic forms with galactosyl side-chains of LPG. The non-agglutinated metacyclic forms were then separated using centrifugation (200 G/5 min, 4 °C) [19]. Parasites were counted using a Bürker chamber, and pools of 10^6 or 10^8 promastigotes (*L. donovani*), or 10^6 metacyclic forms (*L. major*) were resuspended in 50 µL of saline. Dissected salivary glands of *P. duboscqi* and *P. orientalis* females (SG) were pooled (10 glands per 10 µL of saline) and stored at −20 °C. Prior to the inoculation of mice, SG were frozen in liquid nitrogen and thawed three times, and the resulting lysate was added to the inoculum.

Rodents were anesthetized with a mixture of 66 mg/kg ketamine and 26 mg/kg xylazine. Then, 5.5 µL of inoculum (mixed parasites with SG suspension) were injected intradermally into the ear pinnae. Therefore, the inoculum dose per animal with CDP comprised of 10^5 or 10^7 promastigotes (*L. donovani*) or 10^5 selected metacyclic forms (*L. major*). Numbers of SDP were calculated using a Bürker chamber, and the proportion of metacyclic forms was identified on Giemsa stained smears based on morphological criteria described previously [20]. *Leishmania major* numbers ranged from 5.6×10^4 to 7.6×10^4 per animal with metacyclics comprising 69–73% of all forms, *L. donovani* numbers ranged from 5.0×10^4 to 6.39×10^5 per animal with 29–46% of metacyclic forms. Post inoculation (p.i.), rodents were checked weekly for external signs of the disease and killed at week 9–15 p.i. or 30 p.i. for groups infected with *L. major* and *L. donovani*, respectively. The experiments were performed in two runs in all rodent species.

2.4. Xenodiagnosis

Five to seven-day-old sand fly females were used for xenodiagnosis experiments. *Phlebotomus duboscqi* females (natural vectors of *L. major* [21]) were allowed to feed on the site of inoculation of *L. major* (ear pinnae) of anesthetized rodents; the rodent bodies were covered with a cotton bag, so that only the left ear pinnae were accessible to sand flies. *Phlebotomus orientalis* females (natural vectors of *L. donovani* [22]) were allowed to feed on the whole body of the anesthetized rodents infected with *L. donovani (*the smaller ear pinnae of *L. lagurus* did not produce sufficient access to initiate the feeding behavior of *P. orientalis* females). The rodents were placed into a small cage (20 × 20 × 20 cm) and 30–40 sand fly females were allowed to feed for one hour. The fed sand fly females were separated and maintained at 26 °C on 50% sucrose. On day 7–10 PBM, the females were dissected, and their guts were examined under a light microscope. The intensities and locations of infections were evaluated, as described previously [19].

2.5. Tissue Sampling and Fluorescence Detection of Leishmania Post-Mortem

Rodents were killed by cervical dislocation under anaesthesia. Both ears (inoculated and contralateral), both ear-draining lymph nodes, spleen, liver, paws, and tail, were removed and placed on Petri dishes. Fluorescence detection of *Leishmania* was done immediately post killing using the In Vivo Xtreme optical display (Bruker Biospin, Ettlingen, Germany) at the Center of Advanced Imaging Methods at the First Faculty of Medicine, Charles University. Excitation and emission wavelengths were 570 nm and 700 nm. *Leishmania* positivity was evaluated as the color difference against the negative controls, i.e., organs and tissues of uninfected animals. After that, half of all the left and right ears were used for parasite detection by flow cytometry. The second half of each ear and other organs were stored at −20 °C for quantitative PCR (q PCR).

2.6. Flow Cytometry and qPCR

The preparation procedure was identical for all rodent groups. Half of the ear was homogenized into small pieces in the 1 mL well on the plate. Then 500 µL saline, 42 µL liberase (Sigma-Aldrich, St. Louis, MO, USA), and 2 µL DNAse (Top-bio s.r.o., Vestec, Czech Republic) were added. The plate with the samples was incubated for 50 min (37 °C, 5% CO_2). After stopping tissue digestion with 10% heat-inactivated fetal calf serum (FBS Gibco, Thermo Fisher Scientific, Waltham, MA, USA), the samples were filtered through fine gauze into a cold tube and centrifuged (8 min/175 G, 8 °C). The pellet was resuspended in 800 µL saline. 200 µL of the suspension from each sample was used for parasite detection by flow cytometry using the cytometer LRS II (BD Bioscience, Franklin Lakes, NJ, USA) and the software Flowlogic 7.3. Before measurement, Hoechst dye 33258 (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample to identify dead cells. Before tissue analysis, the sensitivity of flow cytometry was tested and scaled with culture-derived *Leishmania* samples (with a range of 10^1–10^8 cells). The final results are expressed per the whole volume of the suspension and corresponding to half of the ear pinna.

For q PCR, extraction of total DNA from rodent tissues and sand flies was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Q PCR for detection and quantification of *Leishmania* parasites was performed in a Bio-Rad iCycler and iQ Real-Time PCR Systems using the SYBR Green detection method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA, USA) as described [20].

2.7. Statistical Analysis

Statistical analyses were carried out using the R software (http://cran.r-project.org/), and the results were graphically visualized using the "ggplot2" package in the same software. A p-value of < 0.05 was considered to indicate statistical significance. The relationship between response variables (weight, lesion size, number of parasites, percentage of infected organs, and infectivity

to sand flies) and explanatory variable (infection with CDP or SDP) were tested for each rodent species separately. In the case of nonsignificant differences between the infection methods, the data were summarized for the subsequent analysis. The between-species differences were tested with the BALB/c set as the reference level for the variable rodent species. The continuous response variable (weight, lesion size) and their relationship with categorical (infection method, rodent species) and continuous (week p.i.) explanatory variables were examined fitting multilevel linear regression models (package "nlme"), taking into account the correlation between repeated measures of the same animal over time. The model used included the interaction term between categorical and continuous explanatory variables. The association between numbers of parasites in inoculated ear and infection method and between numbers of parasites in inoculated ear and rodent species (between-species differences) were analyzed with GLM with a negative binomial distribution. Wilcoxon signed-rank test was used to compare the numbers of parasites in inoculated ear measured by two methods, qPCR and flow cytometry. The test was run for each rodent species separately. The influence of the infection method (SDP, CDP- infection dose 10^5 and 10^7) on the percentage of *L. donovani* infected organs in rodent bodies was measured by 2- and 3-sample test for equality of proportions. The relationship between the percentage of sand flies infected by feeding on rodent host (infectiousness) and between percentages of sand flies infected by feeding on rodent host and rodent species (between-species differences) were tested with GLM with a quasi-binomial distribution.

2.8. Animal Experimentation Guidelines

Animals were maintained and handled in the animal facility of Charles University in Prague following institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under permission no. MSMT-38031/2016-2 of the Ministry of Education, Youth and Sports. Investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

3. Results

3.1. Development of L. major in Four Rodent Species

In total, 12 BALB/c mice, 12 *P. sungorus*, 16 *C. griseus*, and 16 *L. lagurus* were infected; half of them with 1×10^5 culture-derived parasites (CDP) selected with PNA for high representation of metacyclic forms and the second half with $6–7 \times 10^4$ sand fly-derived parasites (SDP), where metacyclics comprised 69–73% of all morphological forms. The numbers of SDP were derived from 10 dissected sand fly females, without any adjustments, to keep the character of the inoculum as natural as possible.

All four rodent species showed stable growth of the body weight during the experiments, and no significant differences in weight gains were found between the groups infected with CDP and SDP (Table S1). In addition, the lesion growth was very similar for the two inoculum types in BALB/c mice, *P. sungorus*, and *L. lagurus*. In *C. griseus*, lesions in the SDP inoculated group developed more slowly, but the difference against the CDP infected group did not reach the statistical significance (Figure 1 and Table S2).

Lesions appeared very early in BALB/c mice (already on week 2 p.i.), one week later in *P. sungorus* and *L. lagurus* (in week 3 p.i.), and even later in *C. griseus* (week 4 p.i.). Compared to susceptible BALB/c mice, the growth of lesions was very similar in *P. sungorus* ($p = 0.08$); the lesions have an ulcerative character in both species (Figure 2a,b), and their size increased progressively until the end of the experiment. In some *P. sungorus*, the skin surrounding ears was also affected by redness and exuviation (Figure 2c). Experiments with BALB/c mice and *P. sungorus* were, therefore, finished on week 9–11 p.i. to avoid excessive distress to the animals. In *L. lagurus*, the lesion growth was significantly slower

than in BALB/c mice ($p < 0.0001$), and although the character of lesions was initially also ulcerative (Figure 2d); their size increased only to week 7 p.i. and then began to reduce. These animals were able to resolve lesions by necrosis of affected parts, resulting in a reduction of the size of ear pinnae (Figure 2e). In *C. griseus*, lesion development was also significantly slower than in BALB/c mice ($p = 0.0001$); lesions were not ulcerative and were fully healed in some (3 out of 13) animals (Figure 2f, Table S2).

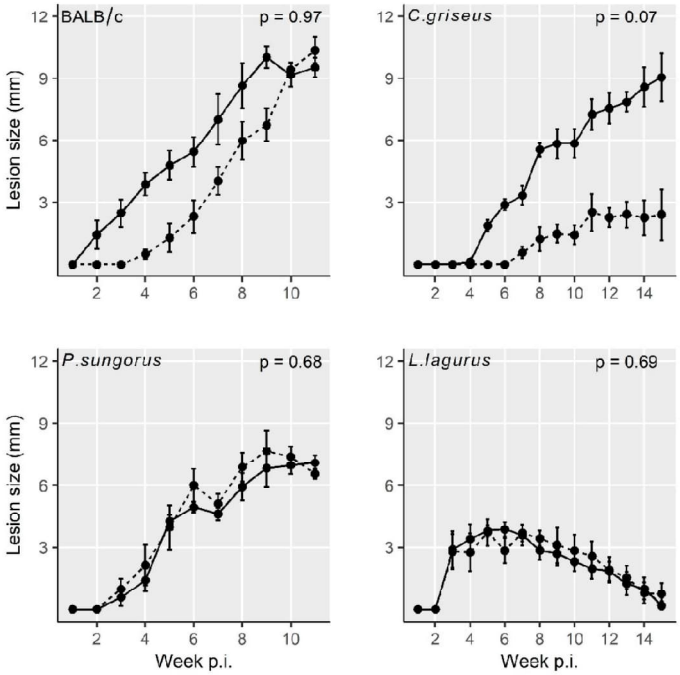


Figure 1. Lesion growth in animals inoculated with *L. major* using the two infection modes. Solid line = infection with culture-derived promastigotes (CDP); dashed line = infection with sand fly-derived promastigotes (SDP). *p* values indicate the statistical difference between the two infection types. Data are presented as the means ± standard errors of the means; 15% of the variance was explained by individual variability.

Numbers of parasites in inoculated ears were evaluated from the samples taken post-mortem using two different methods—qPCR and flow cytometry. Similar to the previous analyses, qPCR did not reveal significant differences between animals inoculated with CDP and SDP; therefore, results were summarized for both infection modes. Parasite loads in inoculated ears generally ranged between 10^2 and 10^7 parasites with a median of 10^3–10^4 in *L. lagurus* and 10^5–10^6 in the remaining three species. Both quantification methods showed similar numbers of parasites in inoculated ears of BALB/c mice and *P. sungorus* (Table 1 and Figure S1). Based on the data from qPCR, significantly lower parasite loads in comparison with numbers in BALB/c mice were detected in *C. griseus* ($p = 0.03$), and the parasite load was lowest in *L. lagurus* ($p = 0.0003$). Based on data from flow cytometry, the difference between the four species was insignificant in this respect. The direct comparison of the two quantification methods in individual species revealed no significant differences in BALB/c mice ($p = 0.6221$), *P. sungorus* ($p = 0.4357$), and *C. griseus* ($p = 0.08$), while in *L. lagurus* qPCR gave significantly lower numbers than flow cytometry ($p = 0.011$).

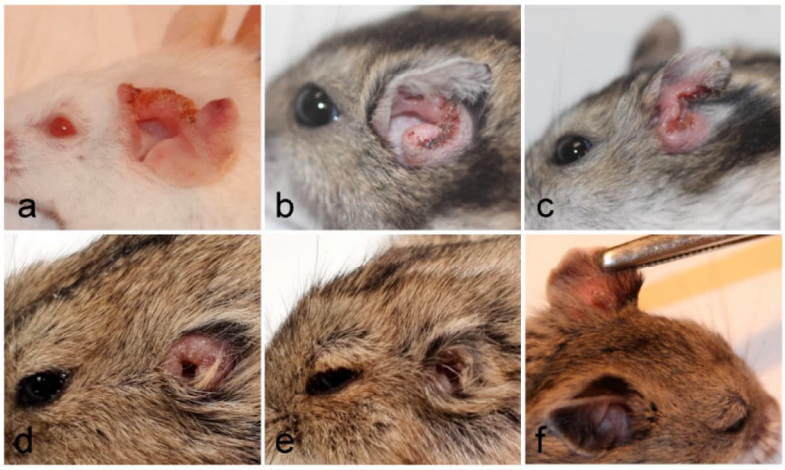


Figure 2. Inoculated ears of BALB/c mice (**a**), *P. sungorus* (**b**,**c**), *L. lagurus* (**d**,**e**), and *C. griseus* (**f**) showing external manifestation of *L. major* infections at the end of experiments.

Table 1. Comparison of *L. major* numbers detected in inoculated ears by flow cytometry (FC) and qPCR (PCR). The numbers represent parasite loads in half of the ear pinna.

Rodent Species	No. of Samples		Median (in Thousands)		Minimum (in Thousands)		Maximum (in Thousands)		*p* Values [1]	
	FC	PCR	FC	PCR	FC	PCR	FC	PCR	FC	PCR
BALB/c mice	12	12	430	438	2	0.3	2756	14,270	-	-
P. sungorus	5	11	732	293	28	46	820	2240	0.93	0.19
C. griseus	13	16	744	152	6	0	1947	2240	0.89	0.03
L. lagurus	16	16	7	3	0.04	0.1	6982	974	0.09	0.0003

[1] significance of the differences against the values in BALB/c mice.

The distribution of parasites in rodent bodies was evaluated using the PCR and the fluorescence detection with In Vivo Xtreme (Table S3, Figure 3a, and Appendix A). In BALB/c mice, parasites remained restricted to both ears (inoculated and contralateral) and their draining lymph nodes. Only in one animal, the liver was also infected. In all remaining species, *Leishmania* parasites were detected in all the tested organs and tissues. In *P. sungorus*, the infection rates in all these tissues were the highest, and these hamsters were also the sole species where blood was also infected. Generally, the PCR was more sensitive than fluorescence detection. For example, numbers of parasites detected in contralateral ears by qPCR reached up to 86 thousand, 56 thousand, 31 thousand, and 15 thousand in BALB/c mice, *P. sungorus*, *C. griseus*, and *L. lagurus*, respectively, but the ears did not produce higher fluorescence signal than negative controls. In addition, the fluorescence detection was not applicable to densely haired paws of *P. sungorus* and *L. lagurus*, where even the control animals produced too strong a fluorescence signal (Appendix A). On the other hand, this method gave a good spatial picture of the parasite distribution. In ear pinnae, the fluorescence signal mostly corresponded to areas affected by skin lesions (Figure 3b). In the liver, the signal came either from the single site (typically in BALB/c mice or *L. lagurus*), or there were several smaller fluorescence centers dispersed over the whole organ (apparent in *P. sungorus* infected with CDP, Appendix A).

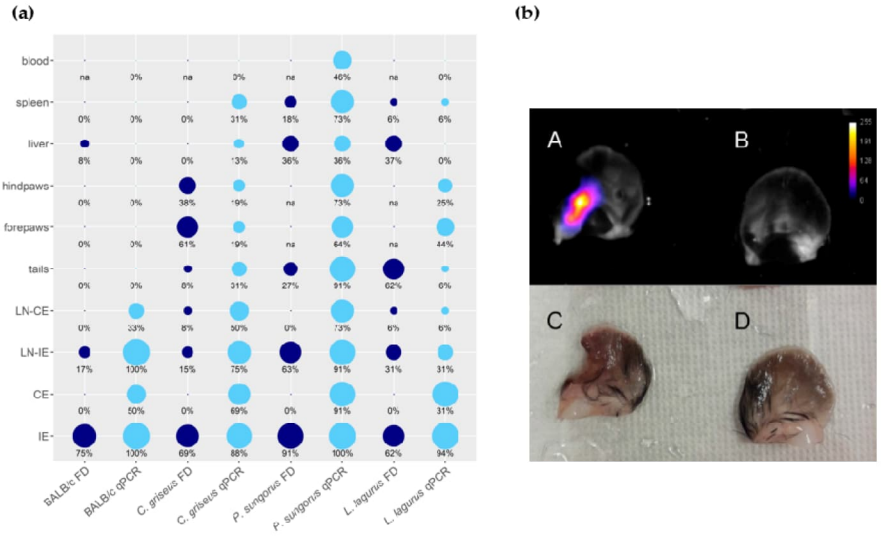


Figure 3. Anatomical distribution of *L. major* in four rodent species. (**a**) Results of fluorescence detection (FD, dark blue) and qPCR (light blue) presented in the balloon graph where the size of the balloon corresponds to the infection rate, i.e., the percentage of *L. major*-infected organs from the total sum of tested organs of the same type. IE = inoculated ears; CE = contralateral ears; LN-IE = draining lymph nodes of the inoculated ears; LN-CE = draining lymph nodes of the contralateral ears; na = not analyzed. (b) Ears of the *C. griseus* wetted with saline, photographed by the end of the experiment at week 15 p.i. A, B, images from In Vivo Xtreme optical display; C, D, images taken by Canon EOS 60D camera with Canon MP-E 65 mm f/2,8 1–5× Macro objective. A, C—inoculated left ear with ulcerative lesion and 2240 thousand parasites detected by q PCR. B, D—contralateral right ear with no external manifestation of the disease and 16.8 thousand parasites according to qPCR.

Infectiousness of animals, tested by xenodiagnosis experiments with *P. duboscqi* females, was, again, very similar in the groups infected with CDP and SDP (Table 2). Infectiousness corresponded very well to parasite loads in inoculated ears: The values in *P. sungorus* and *C. griseus* (29% and 11% of sand flies infected, respectively) did not differ significantly from infectiousness of BALB/c mice (14% of sand flies infected, $p = 0.19$ and $p = 0.54$, respectively), while *L. lagurus* infected significantly fewer sand fly females than BALB/c mice (8% of sand flies infected, $p = 0.006$).

3.2. Development of *L. donovani* in Four Rodent Species

In total, 14 *C. griseus*, 12 BALB/c mice, 12 *L. lagurus*, and 12 *M. auratus* were infected with *L. donovani*; 3 *L. lagurus* and 3 *M. auratus* with 10^5 CDP, three individuals of each species with 10^7 CDP, and 32 animals with 5–63.9 × 10^4 SDP, where metacyclics comprised 29–46% of all morphological forms.

All the rodent species showed stable weight gain during the experiment, and the weight did not differ between the CDP and SDP groups (Table S4). None of the inoculated animals developed lesions or other external signs of the disease throughout the entire experiment. Nevertheless, qPCR performed at the end of the experiment (on week 30 p.i.) revealed the presence of *L. donovani* DNA in various tissues and organs of infected rodents (Table S5). The size of the inoculum (10^5 vs. 10^7 parasites) considerably influenced the outcome of the infection. In both species inoculated with 10^5 CDP (*M. auratus* and *L. lagurus*), only one of 3 individuals became infected, dissemination through the body was limited, and only low parasite loads (<10^3) were found in tissues of these animals (Figure 4a). Higher infection rates and parasite loads were detected in animals infected with 10^7 CDP and SDP, and no significant

differences were found between these two groups (Table S5). Therefore, the data from 10^7 CDP and SDP inoculated animals were combined for further analyses.

Table 2. Results of xenodiagnosis experiments performed with *P. duboscqi* and the four rodent species infected with *L. major*.

Rodent Species	Rodent Numbe [1]	No. of Sand Fly Females	No. and (%) of Positive Females	Rodent Species	Rodent Number	No. of Sand Fly Females	No. and (%) of Positive Females
BALB/c mice $p = 0.58$	C1	30	0	*P. sungorus* $p = 0.80$	C1	33	5 (15)
	C2	32	7 (21)		C2	25	5 (20)
	C3	24	3 (12)		C3	22	6 (27)
	C4	30	4 (13)		C4	24	11 (46)
	C5	29	8 (27)		C5	22	8 (36)
	C6	30	5 (17)		**Σ**	**126**	**35 (28)**
	Σ	**175**	**27 (15)**		S1	32	6 (19)
	S1	32	6 (18)		S2	25	9 (36)
	S2	30	5 (17)		S3	33	9 (27)
	S3	31	8 (26)		S4	22	10 (45)
	S4	34	0		S5	23	6 (26)
	S5	39	5 (13)		**Σ**	135	40 (30)
	S6	33	0		Total	**261**	**75 (29)**
	Σ	**199**	**24 (12)**	*L. lagurus* $p = 0.36$	C1	4	0
	Total	**374**	**51 (14)**		C2	2	0
C. griseus $p = 0.84$	C1	23	6 (26)		C3	2	0
	C2	24	3 (13)		C4	20	2 (10)
	C3	27	2 (7)		C5	25	0
	C4	23	1 (4)		C6	23	1 (4)
	C5	23	0		C7	24	2 (8)
	C6	24	5 (21)		**Σ**	100	**5 (5)**
	C7	20	0		S1	3	0
	Σ	164	17 (10)		S2	2	2 (100)
	S1	28	0		S3	2	0
	S2	15	5 (33)		S4	22	2 (9)
	S3	26	0		S5	18	1 (6)
	S4	23	8 (35)		S6	13	3 (23)
	S5	15	2 (13)		S7	26	3 (12)
	S6	19	0		S8	22	0
	Σ	**126**	**15 (12)**		**Σ**	**108**	**11 (10)**
	Total	290	32 (11)		Total	208	16 (8)

[1] CX, rodents inoculated with CDP, SX, rodents inoculated with SDP. *p*, the significance of the difference between CDP and SDP groups.

In *M. auratus*, 7/9 animals infected with 10^7 CDP and SDP maintained the parasite till the end of the experiment. However, *Leishmania* parasites were present in low numbers (<10^3 per sample) and remained restricted to inoculated ears or, in one specimen, draining lymph nodes of the inoculated ear (Figure 4a). In BALB/c mice, 8/12 individuals showed infection and the parasite visceralized into various tissues and organs except for the tail and blood, but again, the parasite numbers did not exceed 10^3. In *C. griseus*, *Leishmania* were detected in 12/14 animals and spread to all the tested tissues and organs except for the tail and blood. Contrary to *M. auratus* and BALB/c mice, parasite loads per sample were often higher than 10^3 or 10^4. All *L. lagurus* inoculated with 10^7 CDP and SDP were *Leishmania*-positive, parasites were detected in all the tested tissues except blood, and high parasite loads (>10^4) prevailed. Infected spleens showed distinct enlargement (Figure 4a,b).

Infectiousness to sand flies was tested from weeks 15 to 30 p.i. *Phlebotomus orientalis* females were allowed to feed on the whole body of anesthetized animals (Table 3). All the 514 and 207 *P. orientalis* females fed on BALB/c mice and *M. auratus*, respectively, were negative. On the contrary, for *C. griseus*, sand fly females became infected at week 25, and the infectiousness persisted until the end of the experiment in both 10^7 CDP and SDP groups. In total, 144 *P. orientalis* females were used for xenodiagnosis with *L. lagurus*. In this case, considerable differences appeared between the three groups. Specimens inoculated with 10^5 CDP were not infectious to the vector during the whole experiment, while in both remaining groups, sand flies became infected 25 weeks p.i. (25% of sand flies infected) and the infectiousness was still high (18.5%) or even increased (57.1%) 30 weeks p.i. in animals inoculated with SDP and 10^7 CDP, respectively.

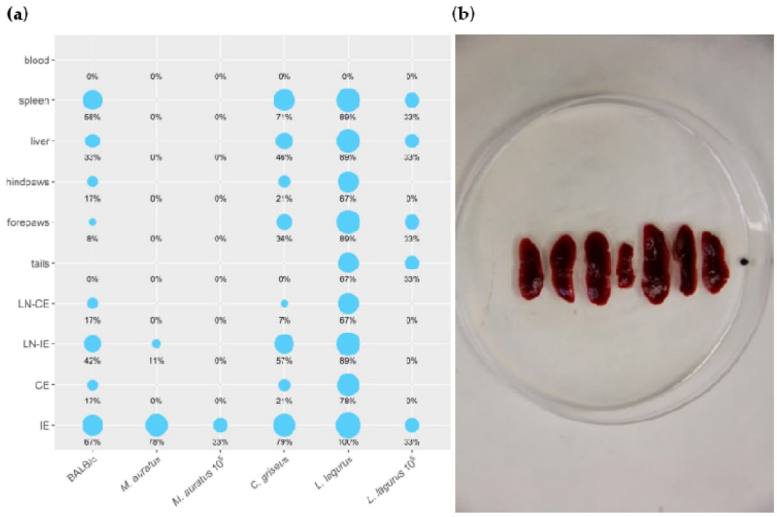


Figure 4. (**a**) Anatomical distribution of *L. donovani* in rodent species determined by qPCR. Results are presented by the balloon graph where the size of the balloon corresponds to the percentage of *L. donovani* infected organs from the total sum of tested organs of the same type. IE = inoculated ears; CE = contralateral ears; LN-IE = draining lymph nodes of the inoculated ears; LN-CE = draining lymph nodes of the contralateral ears. 10^5: Animals inoculated with 10^5 CDP. Animals that are not 10^5–labeled were inoculated with 10^7 CDP or SDP (**b**) Splenomegaly in *L. lagurus* infected with *L. donovani* in comparison with uninfected control animal (in the centre).

Table 3. Results of xenodiagnosis experiments performed with *P. orientalis* in the four rodent species infected with *L. donovani*.

Rodent Species	Week p.i.	No. of Animals Exposed			No. of Sand Fly Females			No. and % of Positive Females		
		10^5 CDP	10^7 CDP	SDP	10^5 CDP	10^7 CDP	SDP	10^5 CDP	10^7 CDP	SDP
BALB/c mice	10	-	4	4	-	21	32	-	0	0
	15	-	7	7	-	73	79	-	0	0
	20	-	4	4	-	55	76	-	0	0
	25	-	3	4	-	45	49	-	0	0
	30	-	2	4	-	25	59	-	0	0
	Σ					219	295		0	0
C. griseus	10	-	4	4	-	11	7	-	0	0
	15	-	7	7	-	22	33	-	0	0
	20	-	4	4	-	11	28	-	0	0
	25	-	4	4	-	24	23	-	1 (4.2	1 (4.4)
	30	-	4	4	-	25	22	-	2 (8.0)	1 (4.6)
	Σ					93	113		**3 (3.2)**	**2 (1.8)**
M. auratus	15	3	3	3	3	7	25	0	0	0
	20	3	3	3	26	20	NA [1]	0	0	NA
	25	3	3	3	6	4	29	0	0	0
	30	3	3	6	13	14	60	0	0	0
	Σ				48	45	114	0	0	0
L. lagurus	15	3	3	3	9	17	4	0	0	0
	20	3	3	3	14	3	NA	0	0	NA
	25	3	3	3	14	12	16	0	3 (25.0)	4 (25.0)
	30	3	3	6	14	14	27	0	8 (57.1)	5 (18.5)
	Σ				51	46	47	0	**11 (23.9)**	**9 (19.1)**

[1] not analyzed.

4. Discussion

Experimental infections of *C. griseus*, *L. lagurus*, and *P. sungorus* were performed to establish new laboratory models for studying leishmaniasis. Three Asian rodent species were compared here with the most widely used laboratory animals in leishmaniasis research—BALB/c mice and golden hamsters. All the tested Asian rodent species were highly susceptible to both *Leishmania* species, but showed different disease phenotypes. All Asian species showed high susceptibility to *L. major*, surpassing BALB/c mice with the wider anatomical distribution of the parasites and offering the model of both the healing phenotype (*L. lagurus* and *C. griseus*) and the non-healing phenotype (*P. sungorus*). *Lagurus lagurus* also exhibited excellent susceptibility to *L. donovani*, presenting giant parasite loads in all the tested tissues and organs.

Chinese hamsters, *C. griseus*, were already introduced to the study of leishmaniasis in 1924 by Smyly and Young [11]. All ten animals in their experiments were successfully infected with intraperitoneal inoculation of large amounts of liver and splenic emulsion from kala-azar patients. This study was continued, and in 1926, the authors described a high rate of *C. griseus* infections (129 from 182 animals), and although hepatosplenomegaly was developed, the animals remained in good condition up to a year [12]. Hindle and Patton showed, in the same year, that *C. griseus* were also susceptible to intraperitoneal inoculation of promastigote forms, while intradermal and subcutaneous inoculation of amastigotes or promastigotes were not successful [13]. In our experiments, initiated with intradermal inoculation of promastigotes, *C. griseus* was confirmed as highly susceptibility to *L. donovani*—86% of animals were successfully infected. Although no external signs of the disease were apparent, parasites disseminated to almost all tested organs and tissues with the highest presence in inoculated ears (79% of animals) and spleen (71% of animals). Infectiousness to sand flies was confirmed from week 25 p.i. In addition, *C. griseus* were also susceptible to *L. major*. The parasites persisted in inoculated ears in 88% of animals, disseminated to all tested skin samples (contralateral ears, paws, tail), and to a lesser extent, also reached visceral organs (13% and 31% of liver and spleen samples, respectively). The ear skin lesions were not ulcerative, well-tolerated, or even healed, and animals were highly infectious to sand flies, of the tested species most accurately mimicking the features of the natural host of *L. major*. In natural reservoirs, visceralisation of *L. major* was observed repeatedly—bone marrow, lymph node and spleen of *Psammomys obesus* and spleen and liver of *C. gundi* caught in Tunisia were identified as positive by ITS1-PCR [23,24] and spleen and liver of gerbils and *Rattus rattus* were shown to be PCR- positive in southern Iran [25,26].

Similar to *C. griseus*, *L. lagurus* also tended to heal skin lesions caused by *L. major*. Parasites were detected in 94% of inoculated ears and 44% of forepaws and were also present in other skin samples (contralateral ears, hindpaws, tail), but only exceptionally visceralized. Concerning VL, these rodents have been used as animal models in few studies performed in the 1990s in China (reviewed in [15]). Our results proved higher susceptibility of *L. lagurus* to *L. donovani* compared to both golden hamsters and BALB/c mice. High loads of parasites were detected in both external and visceral tissues and organs, and the infectiousness to sand flies was the highest among the tested species. Except for splenomegaly, the animals did not show signs of suffering, which favors *L. lagurus* as the optimal model for progressive disseminated visceral leishmaniasis.

Finally, *P. sungorus* showed exceptional susceptibility to *L. major*. The growth of skin lesions was progressive, similar to BALB/c mice and the parasite numbers in inoculated ears were also comparably high. Parasites disseminated to all tested tissues and organs, including blood and viscera (71% of spleen samples infected), and of all the tested species, the highest infection rates, and infectiousness to sand flies were observed. Therefore, *P. sungorus* may serve as a genetically polymorphic animal model for testing drug resistance or vaccine efficacy, an alternative to inbred lines of susceptible mice.

The three Asian rodent species share distribution with human pathogenic *L. donovani*, *L. infantum*, and *L. major*, but reports of their involvement in the life cycle of the parasites are lacking. To our best knowledge, the only indirect indication is that the related species *Cricetulus migratorius* was reported to be infected with *L. donovani* species complex in Iran [27,28]. In nature, the three rodent species included

in this study prefer arid habitats like steppes, steppe forests, and semi-deserts. *Lagurus lagurus* are widely distributed from Ukraine to north-west China, *C. griseus* range from Kazakhstan and west Siberia to Mongolia, north-east China, and the Korean peninsula, and *P. sungorus* are restricted to Kazakhstan and south of west and central Siberia [29]. In theory, all species fulfill the criteria postulated by Ashford (1996) [30] for the reservoir host—in their habitats, they are abundant, forming a large proportion of the mammalian biomass during population outbreaks, and are sufficiently long-lived to allow *Leishmania* surviving through the non-transmission season. As shown in this laboratory study, their response to infection also meets Ashford's criteria—a large proportion of individuals are infected; they remain infected for a long time without the acute disease, and the parasites are in the skin accessible to feeding sand fly vectors.

The development of *Leishmania* infection in the host body is a complex process that depends on many factors, the most important being the *Leishmania* species and virulence of the parasite strain on one side and secondly on the genetic background and the immune response of the mammalian host. In addition, the clinical manifestation of the disease is influenced substantially by the experimental design, especially the infection dose, type of inoculum (procyclic/metacyclic promastigotes or amastigotes), and inoculation route [8,31–33]. In the mouse model, it has been shown that the delivery of the parasites by sand flies makes a substantial contribution to *Leishmania* infection in comparison with syringe inoculation [34]. The significant influence of the inoculation route on disease outcome was also observed in the hamster model: Disease progressions were slower, when *L. braziliensis*, *L. mexicana*, *L. donovani* or *L. infantum* infections were initiated by a sand fly bite or intradermal inoculation. Infections initiated with intracardiac or subcutaneous inoculation showed unnatural rapid visceralisation without the formation of skin lesions [33,35,36].

Initiation of infection with sand fly bite comprises several factors exacerbating the disease progression, specifically, sand fly saliva components (reviewed in [37]), parasite-derived exosomes [38], and promastigote secretory gel [34]. However, using this natural infection route, the inoculum dose cannot be controlled—the numbers of parasites transmitted by sand fly bite differ in several orders of magnitude between individual females [39–41]. Therefore, we used natural parasite forms derived from thoracic midguts of experimentally infected sand flies with late-stage infection (SDP), which were homogenized, counted, and inoculated by syringe. The inoculum size of SDP ($5–8 \times 10^4$) in *L. major* did not exceed the upper numbers delivered to host tissues by *P. duboscqi* bite (1×10^5) [39], and the percentage of metacyclic forms (69–73%) corresponded to the average percentage of metacyclic forms in thoracic midguts of *P. duboscqi* [42]. In *L. donovani*, the infectious dose of SDP was slightly higher (5×10^4–6.4×10^5), but in this case, a lower percentage of metacyclic forms was present in the inoculum (29–46%). The disease progression in animals inoculated with SDP was compared with animals infected with promastigotes from stationary-phase cultures (CDP). Homogenates of sand fly salivary glands were co-inoculated with both inoculum types to enhance the development of infection [43], and the intradermal route of infection was used to ensure parasite exposure to the local immune response in the skin [44]. No significant differences in lesion growth, infectiousness to sand flies, and parasite loads were found between animals infected with SDP or CDP in any rodent, and *Leishmania* species. These results suggest that culture-derived parasites with sufficient representation of metacyclic forms co-inoculated with sand fly saliva intradermally can adequately simulate the natural features of the disease.

On the other hand, the progression of *L. donovani* infections in *M. auratus* and *L. lagurus* was significantly affected by the infectious dose—lower infection rates, dissemination, and parasite loads were detected in rodents infected with 10^5 *L. donovani* CDP compared to animals infected with 10^7 *L. donovani* CDP and SDP. This is in accordance with previous studies with various *Leishmania* species using the mouse model [34,39,45,46].

Distributions and numbers of parasites in rodent bodies were evaluated from samples taken post-mortem using three different methods—quantitative PCR, flow cytometry, and the fluorescence detection with In Vivo Extreme. For this purpose, *L. major* and *L. donovani* parasites were marked

with DsRed and RFP, respectively. Polymerase chain reaction (PCR) is being increasingly used for leishmaniasis diagnosis, characterization, and identification of *Leishmania* species, due to the high sensitivity for the detection and identification of *Leishmania* species (reviewed in [47]). Quantitative PCR is a commonly used method for determining the quantity of *Leishmania* DNA in various tissues of laboratory animals experimentally infected with *Leishmania* parasites, asymptomatic humans from endemic areas, and also for testing putative reservoir hosts [18,48–50]. Flow cytometry-based on the use of fluorescent probes is a relatively quick and cheap method that has been introduced to detect various protozoan parasites in the host cells [51–54]. When combined with the vital DNA Hoechst dye [55], this method is effective for visualizing both the living and dead populations of *Leishmania* parasites in the sample. In our study, both quantification methods were very specific and showed similar numbers of parasites in all rodent species, with the exception of *L. lagurus*. However, in this species, the preparation of samples for quantitative analyses was more difficult because of the much-reduced size of ear pinnae at the end of the experiment, which might influence the analyses.

In addition to PCR, the parasite distributions in rodent bodies were visualized by fluorescence detection with In Vivo Xtreme. In vivo imaging is a novel non-invasive procedure, which can be used for analyzing and monitoring small animal models of leishmaniasis [56,57]. If parasites were engineered to express luciferase, their numbers and distribution in individual animals could be imaged multiple times during longitudinal studies [56]. In this study, *Leishmania* parasites were marked with the red fluorescence protein suitable for flow cytometry, but the fluorescence signal with In Vivo Xtreme was not sensitive enough to detect parasites without killing of animals. Therefore, the analysis was performed post-mortem. While qPCR revealed parasite loads in infected organs, the fluorescence method provided valuable information about the *Leishmania* spatial distribution within these organs. Surprisingly, between-species differences were observed in this respect. In the liver, fluorescence signal came from the single spot in BALB/c mice and *L. lagurus*, while there were several smaller amastigote centers dispersed over the whole organs in *P. sungorus*. However, lower parasite loads were not detectable, and this method was not applicable to densely haired paws of *P. sungorus* and *L. lagurus*, due to high autofluorescence of the background.

Laboratory-bred golden hamsters are highly susceptible to visceral leishmaniasis [33,58]. They often exhibit severe clinical signs and symptoms during visceral infection (hypergammaglobulinemia, hepatosplenomegaly, anemia, cachexia) that is initiated by the intracardiac or the intraperitoneal route (reviewed in [59]). In this study, *L. donovani* infections were initiated intradermally, and parasites remained restricted to inoculated ears, in low loads. Mild disease outcome and a delay in disease progression in hamsters after intradermal inoculation of both *L. donovani* and *L. infantum* has been repeatedly described [60,61]. In addition, it has been demonstrated that the immune response to sand fly salivary protein protects hamsters against the fatal outcome of VL [62]. This study showed that, contrary to golden hamsters, the *L. lagurus* model enables the study of disseminated VL initiated via a natural intradermal route. Such a model is, therefore, closer to the active human disease and should be favored for drug and vaccine testing.

Inbred BALB/c mice are highly susceptible to *L. major* showing a non-healing phenotype associated with polarization to a Th2 response, which is not effective against intracellular parasites (reviewed in [63]). On the other hand, BALB/c mice are able to control *L. donovani* and *L. braziliensis* infection [64–66]. In this study, the tissue specificity was different for the two parasite species in BALB/c mice. *Leishmania major* amastigotes remained localized in inoculated ears and draining lymph nodes, without visceralisation. Mice infected with *L. donovani* showed spleen and liver infections in 58% and 33% of mice by week 30 p.i., respectively. This corresponds to general observation of murine visceral leishmaniasis with both *L. donovani* and *L. infantum*—the liver serves as the site for initial parasite expansion with fast parasite resolution, while in the spleen, visceralising *Leishmania* can persist for the life of the animal (reviewed in [31,59,67]). Although inbred mouse models have been invaluable for research on the principles of the mammalian immune response, wild rodents provide a more natural model of parasite-host interactions as, like humans, they are genetically polymorphic [6]. A larger

spectrum of model species, covering the whole picture of human leishmaniasis, is also a prerequisite to facilitate vaccine development [68]. In this respect, *L. lagurus* and *C. griseus* may be used in research on the healing CL phenotype, while *P. sungorus* provides the non-healing CL phenotype. All three species showed higher dissemination of *L. major* in the tissues and organs than BALB/c mice.

This study provides experimental infections of three Asian rodent species to enrich the animal model spectrum available to both CL and VL research. *Cricetulus griseus, P. sungorus*, and *L. lagurus*, although all are susceptible to both *L. major* and *L. donovani*, differ in the infection outcomes, which enable their application to various research objectives. Further studies are necessary to better understand the immunological mechanisms involved in the response of these rodents to *Leishmania*.

5. Conclusions

1. Infections initiated with intradermal inoculation of 10^5 culture-derived metacyclic *L. major* or with 10^7 culture-derived non-selected *L. donovani*, co-inoculated with 0.5 sand fly SG, were fully comparable in all disease manifestations to infections initiated with sand fly-derived parasites (10 sand fly thoracic midguts and 0.5 sand fly SG per animal).

2. *Lagurus lagurus* represented the healing phenotype with *L. major* infections. In addition, this rodent species showed higher susceptibility to *L. donovani* than golden hamsters and BALB/c mice—parasites were present in high loads in all the tested tissues and organs and the infectiousness to sand flies was the highest among tested species; therefore, these animals may best model disseminated visceral leishmaniasis.

3. *Cricetulus griseus* tolerated well or even healed *L. major* lesions, while parasites remained widely distributed through the body, and animals were highly infectious to sand flies, mimicking best the features of the natural host of the parasite. These rodents were also highly susceptible to *L. donovani*, similarly to *L. lagurus*.

4. *Phodopus sungorus* showed high susceptibility to *L. major* with the non-healing phenotype manifested in the progressive growth of ulcerative lesions and massive parasite loads in inoculated ears. Dissemination of parasites through the body and infectiousness to sand flies was even higher than in immunodeficient BALB/c mice.

5. Both qPCR and flow cytometry are highly sensitive methods for the evaluation of parasite loads in host tissues. Fluorescence detection using In Vivo Xtreme, although less sensitive and not suitable for accurate quantification of parasites, gives valuable spatial data for parasite distribution.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/9/1440/s1, Figure S1: Numbers of *L. major* parasites in inoculated ears evaluated by qPCR (A) and flow cytometry (B), Table S1: Weight gains in four rodent species inoculated with *L. major*, Table S2: Lesions diameters (mm) in animals inoculated with *L. major*, Table S3: Parasite loads and distribution determined by qPCR in animals inoculated with *L. major*, Table S4: Weight gains in four rodent species inoculated with *L. donovani*, Table S5: Parasite loads and distribution determined by qPCR in animals inoculated with *L. donovani*.

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

Appendix A

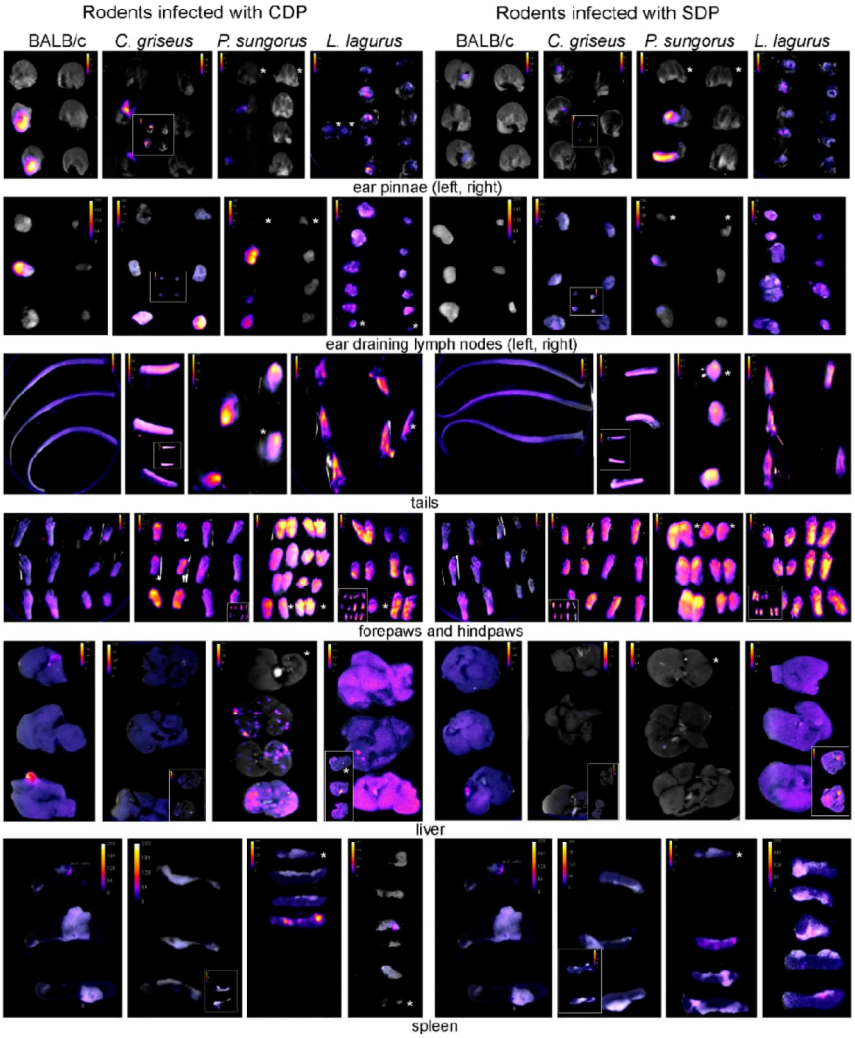


Figure A1. Tissues of infected rodents photographed with In Vivo Xtreme optical display post-mortem during the first run of the experiments.

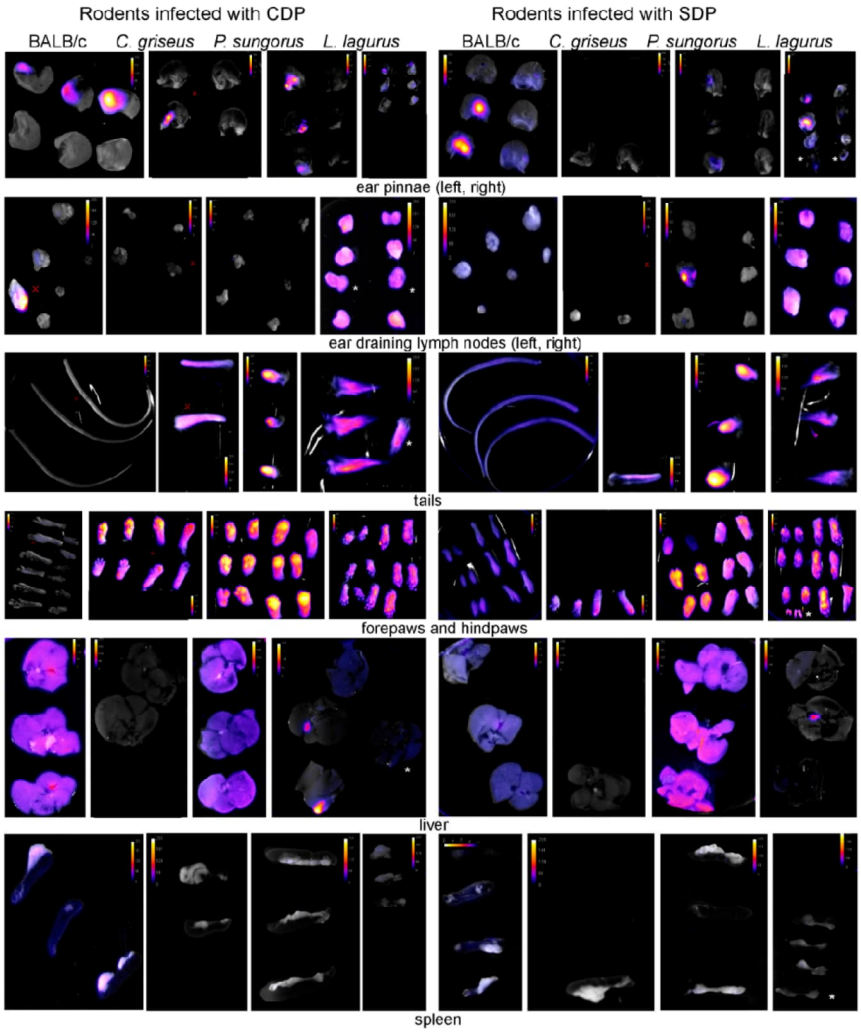


Figure A2. Tissues of infected rodents photographed with In Vivo Xtreme optical display post-mortem during the second run of the experiments. Asterisks indicate control animals.

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Host competence of African rodents *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis* for *Leishmania major*

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ABSTRACT

Cutaneous leishmaniasis caused by *Leishmania major* is a typical zoonosis circulating in rodents. In Sub-Saharan Africa the reservoirs remain to be identified, although *L. major* has been detected in several rodent species including members of the genera *Arvicanthis* and *Mastomys*. However, differentiation of true reservoir hosts from incidental hosts requires in-depth studies both in the field and in the laboratory, with the best method for testing the infectiousness of hosts to biting vectors being xenodiagnosis.

Here we studied experimental infections of three *L. major* strains in *Arvicanthis neumanni*, *A. niloticus* and *Mastomys natalensis;* the infections were initiated either with sand fly-derived or with culture-derived *Leishmania* promastigotes. Inoculated rodents were monitored for several months and tested by xenodiagnoses for their infectiousness to *Phlebotomus duboscqi*, the natural vector of *L. major* in Sub-Saharan Africa. The distribution and load of parasites were determined *post mortem* using qPCR from the blood, skin and viscera samples. The attractiveness of *Arvicanthis* and *Mastomys* to *P. duboscqi* was tested by pair-wise comparisons.

Three *L. major* strains used significantly differed in infectivity: the Middle Eastern strain infected a low proportion of rodents, while two Sub-Saharan isolates (LV109, LV110) infected a high percentage of animals and LV110 also produced higher parasite loads in all host species. All three rodent species maintained parasites of the LV109 strain for 20–25 weeks and were able to infect *P. duboscqi* without apparent health complications: infected animals showed only temporary swellings or changes of pigmentation at the site of inoculation. However, the higher infection rates, more generalized distribution of parasites and longer infectiousness period to sand flies in *M. natalensis* suggest that this species plays the more important reservoir role in the life cycle of *L. major* in Sub-Saharan Africa. *Arvicanthis* species may serve as potential reservoirs in seasons/periods of low abundance of *Mastomys*.

1. Introduction

Leishmania (Kinetoplastida: Trypanosomatidae) are parasites with a digenetic life cycle, alternating between blood feeding insects - sand flies (Diptera: Psychodidae) and mammalian hosts including humans. *Leishmania major* is a causative agent of human cutaneous leishmaniasis (CL) affecting thousands of people in the Old World. It is transmitted by sand flies of the genus *Phlebotomus*. Proven vectors are *P. papatasi,* a species with wide distribution from North Africa and Southern Europe to India, and *P. duboscqi*, a species occurring in a wide belt through Sub-Saharan Africa ranging from Senegal and Mauritania in the west to Ethiopia and Kenya in the east (Maroli et al., 2013).

CL caused by *L. major* is a typical zoonosis maintained in reservoir rodent hosts. Humans are infected incidentally; lesions appear at the site of insect bite and heal without treatment after about three months. The short duration of the disease precludes survival of the parasite in humans through any non-transmission season (Ashford, 2000). Proven reservoir hosts are the Fat Sand-Rat *Psammomys obesus* and gerbils of the genus *Meriones* in North Africa and the Middle East, and the Great Gerbil *Rhombomys opimus* in Central Asia. On the other hand, reservoir rodent species in Sub-Saharan Africa remain to be confirmed. *Leishmania major* has been isolated from several rodent species in this region; most isolates have been made from Grass Rats *Arvicanthis* spp. and Multimammate Mice *Mastomys* spp. which live in the immediate vicinity of humans, and are the most dominant rodents in many Sub-Saharan endemic localities of CL (reviewed by Ashford, 1996; Ashford,

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2000; Desjeux, 1996). *Arvicanthis* and *Mastomys* belong to the same large subfamily Murinae, but are separated into different tribes – Arvicanthini and Praomyini, respectively (Lecompte et al., 2008). The origin of both tribes was estimated to be about 10.2 Mya. Recently, the genus *Arvicanthis* was reported to include seven species and the genus *Mastomys* eight species (Granjon and Ducroz, 2013; Leirs, 2013).

Identification of reservoir hosts is essential for the control of zoonoses. However, it requires longitudinal in-depth studies both in the field and in the laboratory. True reservoir hosts must satisfy many parameters - the most important being longevity sufficient to sustain parasitemias during the non-transmission season, high population density of the host, and the location of the parasite within the host suitable for transmission by pool-feeding sand flies. In addition, the infection is likely to be sufficiently benign (or too infrequent) to exert a regulatory effect on host populations (Ashford, 1997, 2000). Finding PCR positive animals does not necessarily mean they serve as parasite reservoirs for biting sand flies (Silva et al., 2005). Indeed, such animals may simply serve as parasite sinks, i.e. animals upon which infected sand flies feed but do not contribute to vector infection and transmission to the next host (Chaves et al., 2007). The best method for testing the infectiousness of hosts to biting vectors is by xenodiagnosis, i.e., feeding of laboratory reared insects on the infected host with subsequent examination of the insects for presence of parasites.

The main aim of this laboratory study was to contribute to analysis of the host competence of the African rodents *Arvicanthis neumanni* (Neumann's Grass Rat), *A. niloticus* (Nile Grass Rat) and *Mastomys natalensis* (Natal Multimammate Mouse) for *L. major*. *Arvicanthis neumanni* is the smallest *Arvicanthis* species, ranging from Ethiopia to Kenya; *A. niloticus* is widespread from the Nile Delta to Kenya and West Africa and *Mastomys natalensis* widely distributed in almost all Sub-Saharan Africa throughout many biotic zones (Granjon and Ducroz, 2013; Leirs, 2013). Their response to the infection and ability to present the parasites to feeding sand flies were tested using experimental infections and xenodiagnoses. Feeding rates of *P. duboscqi* on these rodents were tested by host-choice experiments.

2. Material and methods

2.1. Sand flies, parasites and rodents

The colony of *P. duboscqi* originating in Senegal was maintained in the insectary of the Department of Parasitology, Charles University in Prague, under standard conditions (26 °C on 50% sucrose, humidity in the insectary 60–70% and 14 h light/10 h dark photoperiod) as described previously (Volf and Volfova, 2011).

Three *L. major* strains were used: MHOM/IL/81/Friedlin, a human isolate from Israel, and two strains isolated in Senegal by Ranque - MARV/SN/XX/RV24; LV109 and MHOM/SN/XX/BO-DK; LV110. The identity of the Senegalese strains was confirmed by sequencing of the RPL23a intergenic sequence (Dougall et al., 2011). Promastigotes were cultured in M199 medium (Sigma) containing 10% heat-inactivated fetal bovine calf serum (FBS, Gibco) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma), 2% sterile human urine and 250 μg/ml amikacin (Amikin, Bristol-Myers Squibb).

Breeding colonies of *A. neumanni* and *A. niloticus* (originating from Prague Zoo and Pilsen Zoo, respectively) and *M. natalensis* (originating from a commercial source, Karel Kapral s.r.o.) were established in the animal facility of the Department of Parasitology. BALB/c mice originated from AnLab s.r.o. Animals were maintained in T IV breeding containers (Velaz) equipped with bedding German Horse Span (Pferde), breeding material (Woodwool) and hay (Krmne smesi Kvidera), provided with a standard feed mixture ST-1 (Velaz) and water ad libitum, with a 12 h light/12 h dark photoperiod, temperature 22–25 °C and humidity 40–60%.

2.2. Experimental infection of sand flies

Promastigotes from log-phase cultures (day 3–4 in culture) were washed twice in saline and resuspended in heat inactivated rabbit blood (LabMediaServis) at a concentration of 5×10^6 promastigotes/ml. Sand fly females (5–9 days old) were infected by feeding through a chick-skin membrane (BIOPHARM) on the promastigote-containing suspension. Engorged sand flies were maintained under the same conditions as the colony.

2.3. Infections of rodents

Two methods of rodent infections were used – infections initiated with sand fly-derived *Leishmania* according to Sadlova et al. (2015) and infections initiated with culture-derived promastigotes. For the first method, *P. duboscqi* females experimentally infected with *L. major* (for details see above) were dissected on day 10 or 12 post bloodmeal (PBM); their midguts were checked microscopically for the presence of promastigotes, and thoracic midguts (the site of accumulation of metacyclic forms) with a good density of parasites were pooled in sterile saline. Pools of 100 freshly dissected thoracic midguts were homogenized in 50 μl of saline.

For inoculation of rodents with culture-derived promastigotes, stationary-phase promastigotes (day 7 post inoculation) were washed twice in saline and counted using a Burker apparatus. Pools of 10^8 promastigotes were resuspended in 50 μl of saline.

Dissected salivary glands of *P. duboscqi* females (SG) were pooled in sterile saline (10 glands per 5 μl of saline) and stored at −20 °C. Prior to mice inoculation, SG were disintegrated by 3 successive immersions into liquid nitrogen and added to both types (sand fly - and culture-derived) of promastigote suspensions.

Rodents anaesthetized with ketamin/xylazine (33 mg and 13 mg/kg in *A. neumanni*, 62 mg and 7 mg/kg in *A. niloticus*, 50 mg and 20 mg/kg in *M. natalensis*, 62 mg and 25 mg/kg in mice, respectively) were injected with 5.5 μl of the mixed parasite and SG suspension intradermally into the ear pinnae. Therefore, the inoculum of culture-derived promastigotes comprised 10^7 parasites. Numbers of sand fly - derived parasites stages were calculated using a Burker apparatus, and the proportions of metacyclic forms were identified on Giemsa stained smears based on morphological criteria described previously (Sadlova et al., 2010). The inoculum of sand fly-derived parasites was 3.6×10^4 with LV 110 strain (35% metacyclic forms) and ranged between 3.5×10^4–7×10^4 parasites/rodent with FVI strain (23–69% metacyclic forms) and 4.1×10^4–$5.4 \times 10^4$with LV109 strain (43–68% metacyclic forms). Animals were checked weekly for external signs of the disease until week 20–35 post infection (p.i.) when they were sacrificed.

2.4. Xenodiagnosis

Five to seven-day-old *P. duboscqi* females were allowed to feed on the site of inoculation of *L. major* (ear pinnae) of anaesthetized rodents between weeks 2 and 25 p.i. Smaller size rodents *M. natalensis* and *A. neumanni* were covered with the cotton bag, so that only the left ear pinnae were accessible to sand flies, placed into a small cage (20 × 20 × 20 cm) and 40–70 sand fly females were allowed to feed for one hour. In the larger sized *A. niloticus*, the xenodiagnoses were made using small plastic tubes with 30 sand fly females covered with fine mesh. The tubes were held on the ear of the anaesthetized animal for one hour (Fig. 1A). Fed sand fly females were separated and maintained at 26 °C on 50% sucrose. On day 7–10 PBM, females were dissected and their guts examined under the light microscope. Intensities and locations of infections were evaluated as described previously (Sadlova et al., 2010).

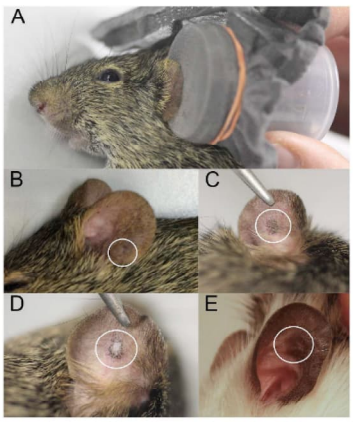


Fig. 1. Xenodiagnosis and external manifestation of *L. major* in rodents. Direct xenodiagnosis with *P. duboscqi* in plastic tubes covered with fine mesh held on the ear of the anaesthetized *A. niloticus* (A) and external manifestation of *L. major* LV109 in ear pinnae (site of inoculation) of *A. neumanni* by week 10 p.i., (B); *A. niloticus* by week 30 p.i. (C, D) and *M. natalensis* by week 19 p.i. (E).

2.5. Tissue sampling and quantitative PCR

Rodents were sacrificed at different weeks p.i by cervical dislocation under anesthesia. Both ears (inoculated and contralateral), both ear-draining lymph nodes, spleen, liver, paws and tail were stored at −20 °C for qPCR. Extraction of total DNA from rodent tissues (on equal weight samples) and sand flies was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Quantitative PCR (Q-PCR) for detection and quantification of *Leishmania* parasites was performed in a Bio-Rad iCycler & iQ Real-Time PCR Systems using the SYBR Green detection method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA) as described (Sadlova et al., 2010). Infectious load was scored using a scoring table considering the number of parasites detected: < 100 was evaluated as low dose infection; 100–1000 as medium dose infection; > 1000 as high dose infection.

2.6. Host choice experiments and assessment of mortality and fecundity of sand flies fed on different hosts

Pair-wise comparisons between two types of hosts were performed using a row of three connected small cages (20 × 20 cm). *P. duboscqi* females (200 specimens) were placed into the central cage and left for habituation for 20 min. Anaesthetized animals were placed in each of the lateral cages and partitions with the central cage were opened. After one hour, the cages were separated and closed, host animals removed and the numbers of blood-fed sand flies in each host cage were counted. *Arvicanthis neumanni* and *M. natalensis* are species of comparable size (60–80 g) and therefore one animal each was placed in cages. For comparison between mice and *Arvicanthis* or *Mastomys*, two mice were used against one *Arvicanthis* or *Mastomys* to counterbalance biomass differences between these host types. Each pair of hosts was tested four times, with the hosts alternated between lateral cages in each repeat. Experiments were conducted in darkness at 24–26 °C.

Fed females were maintained under the same conditions as the colony and their mortality was recorded for 4 days post-feeding. Then, females were introduced individually into small glass vials equipped with wet filter papers, closed with fine gauze and allowed to oviposit (Killick-Kendrick and Killick-Kendrick, 1991). Small pieces of cotton wool soaked in sugar solution (50% sucrose) were placed on the mesh and changed every second day. All vials were placed into a single plastic box with its base filled with the wet filter paper to ensure a uniform microclimate. The humidity was checked and numbers of laid eggs were recorded daily.

2.7. Statistical analysis

Statistical analyses were carried out using R software (http://cran.r-project.org/). The differences in feeding preferences, mortality and fecundity *of P. duboscqi* females fed on different host species were analyzed by Chi-square test. The differences in numbers of eggs laid by *P. duboscqi* females fed on different hosts were tested by nonparametric Mann Whitney *U* test.

2.8. Animal experimentation guidelines

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under permit no. MSMT-10270/2015–5 of the Ministry of the Environment of the Czech Republic. Investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

3. Results

3.1. Experimental infections and xenodiagnosis with A. neumanni

In total, 33 females of *A. neumanni* were infected by three different *L. major* strains, most of them (30) using sand fly-derived *Leishmania*. The strain Friedlin originating from the Middle East showed only very weak infectivity for *A. neumanni* (Table 1). None of 12 female *A. neumanni* inoculated with sand fly-derived *Leishmania* developed lesions. Q-PCR revealed presence of *Leishmania* in 1 specimen only, with parasites localized in the inoculated ear pinnae in very low numbers (less than 100). All 532 *P. duboscqi* females exposed to biting sand flies at different times p.i. for xenodiagnoses, were negative (Table 2).

The Sub-Saharan strain LV110 originating from Senegal infected all six female *A. neumanni* inoculated with sand fly-derived *Leishmania* (Table 1), but animals did not show any external signs of the disease throughout the entire experiment. Q-PCR revealed the presence of parasites in left ear pinnae (site of inoculation) in all the six animals, however, the numbers of parasites were very low and all 442 females *P. duboscqi* used for xenodiagnoses were negative (Table 2).

The second Sub-Saharan strain LV109 originating from Senegal was inoculated into 15 *A. neumanni* (Table 1); 12 with sand fly-derived *Leishmania* (experimental groups A and B) and 3 with culture-derived promastigotes (experimental group C). Wet skin lesions did not develop, but hyper-pigmentations of left ear pinnae (sites of inoculation) were observed in 3 animals, two from group A and one from group C (Fig. 1B). PCR showed presence of parasites in 7 from 15 animals. They were localized mostly in the left ear (site of inoculation) and once in the blood. Interestingly, the numbers of detected *Leishmania* were higher (hundreds to thousands) in 3 animals, two of which also showed hyperpigmentation of the ear. All three animals with hyperpigmentation were infective to sand flies, two by week 5 and the third by week 10 p.i. In total, 0.4% of 748 *P. duboscqi* females tested were positive (Table 2).

3.2. Experimental infections and xenodiagnosis with A. niloticus

Twelve *A. niloticus* of both sexes were inoculated with the strain LV109 originating from Senegal. Six *A. niloticus* (3 males and 3 females)

Table 1
Presence and amount of *L. major* DNA in *A. neumanni*, *A. niloticus* and *M. natalensis* and their infectiousness to *P. duboscqi*. Group A, rodent infections initiated with sand fly-derived *Leishmania* and animals exposed to sand fly bites; Group B, rodent infections initiated with sand fly-derived *Leishmania* and animals not exposed to sand flies; Group C, rodent infections initiated with culture-derived promastigotes and animals exposed to sand flies. IE, inoculated ear; CE, contralateral ear; DN-IE, draining lymph nodes of the inoculated ear; DN-CE, draining lymph nodes of the contralateral ear; FP, forepaws; HP, hindpaws; T, tail; L, liver; S, spleen; B, blood; *, < 100 parasites; **, 100–1000 parasites; ***, > 1000 parasites; H-Pi, hyper-pigmentation. A1-A6 and C1-C5 - individual marks of animals referring to Tables 3 and 4.

Rodent species	*L. major* strain	Experimental group	Week p.i.	No of animals tested	No of PCR positive animals (%)	Location (No) of parasites determined by qPCR in individual animals	External signs of the disease (on the inoculated ear)	No of animals infective for sand flies
A. neumanni	Friedlin	Group A	20	6	1	IE*	No	0
		Group B	10	2	0	–	No	not tested
			15	2	0	–	No	not tested
			20	2	0	–	No	not tested
		Total		12	1 (8.3%)			0
	LV110	Group A	5	1	1	IE*	No	0
			10	2	2	IE*	No	0
						IE*	No	0
			15	1	1	IE*	No	0
			20	2	2	IE*	No	0
						IE*	No	0
		Total		6	6 (100%)			0
	LV109	Group A	20	6	4	IE*	No	0
						IE**	H-Pi	1
						IE**	H-Pi	1
						IE**	No	0
		Group B	10	2	0	–	No	not tested
			15	2	1	IE*	No	not tested
			20	2	1	B*	No	not tested
		Group C	15	3	1	IE*	H-Pi	1
		Total		15	7 (47%)			3 (33%)[a]
A. niloticus	LV109	Group A	25	6	1	A5:FP*	H-Pi	1
		Group C	12	1	1	C1: IE***, CE**, HP*	H-Pi	not tested
			25	4	2	C2: IE**	H-Pi	1
						C4: CE**, T*, HP*	H-Pi	0
		Total		11	4 (37%)			2 (20%)[b]
M. natalensis	Friedlin	Group A	35	6	3	IE*	No	0
						CE* and L*	No	0
						FP*	No	0
		Group B	10	2	0	–	No	not tested
			15	2	2	IE*	No	not tested
						IE*, CE*	No	not tested
			20	2	1	IE*, CE*	No	not tested
			35	1	0	–	No	not tested
		Total		13	6 (46%)			0
	LV109	Group A	20	5	5	A1: IE**	Swelling, H-Pi	1
						A2: IE**, DN-CE**, HP***	Swelling	0
						A3: IE*, S**	Swelling, H-Pi	0
						A5: IE**, FP***,HP****,T***	Swelling, H-Pi	0
						A4: IE***, DN-IE*	Swelling	0
		Group C	15	3	3	C1: IE**, T***	Swelling	0
						C4: IE**	Swelling	0
						C5: IE*	Swelling	0
			25	2	2	C2: IE***, FP***, HP**	Swelling	1
						C3: IE**	Swelling, H-Pi	0
		Total		10	10 (100%)			2 (20%)

[a] 9 tested animals.
[b] 10 tested animals.

were infected with sand fly-derived *Leishmania* (experimental group A) and the same numbers of animals were infected with culture-derived promastigotes (experimental group C), but one animal from group C died early during the experiment and thus was not evaluated. In both groups, the first external signs of the disease appeared on inoculated ear pinnae on week 6 p.i. The affected area was characterized by mild flaking of the skin and hyper-pigmentation (Fig. 1C). The pigmentation was lost in the centre while the borders remained hyper-pigmented in some of the animals (Fig. 1D, Table 3). These dry lesions increased to 3–4 mm by weeks 12–14 p.i; then, in 3 animals the lesion size remained

Table 2
Direct xenodiagnosis of *L. major* in *A. neumanni*, *A. niloticus* and *M. natalensis*: feeding of *P. duboscqi* on inoculated ears.

Rodent species	*L. major* strain	Experimental group	Week p.i.	No of animals exposed	No of dissected sand flies	No and (%) of positive sand flies
A. neumanni	Friedlin	Group A	2	6	124	0
			5	6	179	0
			10	6	95	0
			15	5	54	0
			20	5	80	0
			Total		532	0
	LV110	Group A	5	6	143	0
			10	5	177	0
			15	3	105	0
			20	2	17	0
			Total		442	0
	LV109	Group A	5	6	85	1 (1,2)
			10	6	287	1 (0,3)
			15	5	78	0
			20	5	148	0
		Group C	5	3	98	1 (1,0)
			15	3	52	0
			Total		748	3 (0,4)
A. niloticus	LV109	Group A	5	2	30	3 (10.0)
			10	2	33	2 (6.1)
			15	2	63	0
			20	2	31	0
			25	6	108	0
		Group C	5	3	49	2 (4.1)
			10	2	18	1 (5.6)
			15	3	66	0
			20	2	31	0
			25	4	47	0
		Total			476	5 (1.1)
M. natalensis	Friedlin	Group A	2	6	126	0
			5	6	130	0
			10	6	166	0
			15	6	150	0
			20	6	66	0
		Total			638	0
	LV109	Group A	15	5	145	1 (0.7)
			25	4	61	2 (3.3)
		Group C	15	5	136	0
			25	2	24	1 (4.1)
		Total			366	4 (1.1)

Group A, rodent infections initiated with sand fly-derived *Leishmania*; Group C, rodent infections initiated with culture-derived promastigotes.

constant until the end of the experiment by week 25 p.i., while in the others, lesions decreased or completely disappeared (Table 3).

PCR confirmed the presence of *Leishmania* in 4 of 11 animals, with localization in ears, forepaws, hindpaws and tail (Table 1). The numbers of detected parasites were higher (hundreds to thousands) in the animal killed on week 12 p.i., while no parasites or only low numbers (around one hundred) were present in organs dissected on week 25 p.i. (at the end of the experiment). This fact corresponds with results of xenodiagnoses: like in *A. neumanni*, the period of infectiousness of *A. niloticus* to *P. duboscqi* was restricted to weeks 5 and 10 p.i. (4.1% and 10.0% of sand fly females became infected, respectively) while no females developed *Leishmania* infection in feeding experiments on weeks 15–25 p.i. (Table 2).

3.3. Experimental infections and xenodiagnosis with M. natalensis

In total, 23 *M. natalensis* were inoculated with two *L. major* strains. Thirteen *M. natalensis* were all inoculated with sand fly–derived promastigotes of the Israeli strain Friedlin. Q-PCR revealed presence of *L. major* in 46% of the animals (Table 1). However, none of the 13 *M. natalensis* tested developed lesions or other external signs of the disease. *Leishmania* were localized mostly in the inoculated ear pinnae (4 animals), less often in the contralateral ear pinnae (3 animals) and exceptionally also in a forepaw (1 animal) and liver (1 animal). However, since parasites were present in very low numbers (less than 100) animals were not infectious to feeding sand flies (Tables 1 and 2).

Ten *M. natalensis* were experimentally infected with the LV109 strain (Table 1), 5 with sand fly-derived *Leishmania* (experimental group A) and 5 with culture-derived promastigotes (experimental group C). Skin swellings developed at the site of inoculation (left ear pinnae) in animals of both experimental groups approximately 10 weeks p. i. (Table 4, Fig. 1E). Prior to the swelling the affected site usually reddened, which was observed more often in specimens of the group C. The size of the swelling increased gradually to 6–8 mm, then decreased and

Table 3
Time-course of the external manifestation of *L. major* LV109 in ear pinnae (site of inoculation) of *A. niloticus*. Animals C1-C5 were infected with culture-derived promastigotes (Group C), animals A1-A5 were infected with sand fly-derived *Leishmania* (Group A). Black colour – hyper-pigmentation, grey colour – depigmentation in the centre surrounded with hyper-pigmented borders. The numbers are the length of the affected area in mm. *, animal died by week 10 p.i.

Animals	Weeks post infection											
	2	4	6	8	10	12	14	16	18	20	22	24
C1*			2	3	4	X	X	X	X	X	X	X
C2					1	2.5	3.5	3.5	4	4	4	4
C3					1	2.5	3.5	3.5	4	4	4	4
C4			1	1	1	1	3.5	3.5	4	4	4	3
C5				1	1	2.5	3.5	4	4	4	4	3
A1					1	2	2	2.5	3	3	3	3
A2			1	1	2	4	4	3	3	3	3	3
A3				1	1	4	4	2	1	1	1	1
A4					3	3	2	2	2	2	1	1
A5			1	1	2.5	2.5	3	1.5	1.5	1.5	1	1
A6			1	2	2.5	3	3	1.5	1			

finally disappeared. Hyper-pigmentation often accompanied healing of the swellings (Table 4) and it mostly persisted until the end of the experiments.

Parasites were detected by Q-PCR in all tested animals and they disseminated to draining lymph nodes, forepaws, hindpaws and tail in several animals and also to the spleen in one specimen (Table 1). Infectiousness to sand flies was tested at weeks 15 and 25 p.i.: 0.7% of females from group A became infected after feeding on week 15 p.i., while 3.3% and 4.1% of females from the group A and C, respectively, were infected feeding on week 25 p.i. (Table 2).

3.4. Host choice experiments with P. duboscqi

Two potential host species were offered to *P. duboscqi* females in each pair-wise comparison. Preliminary experiments showed that *P. duboscqi* did not distinguish between males and females of *A. neumanni* and both species of the genus *Arvicanthis* (smaller *A. neumanni* and bigger *A. niloticus*). Then, different host genera (represented by *A. neumanni*, *M. natalensis* and BALB/c mice) were compared: each host combination was tested twice with hosts alternating between lateral cages. Sand fly females showed a high feeding rate on all tested rodents: 40.5–80.5% of females took bloodmeals during experiments (Table 5). The only significant preference was observed when *Arvicanthis* was compared with BALB/c mice – sand flies preferred *Arvicanthis* more than BALB/c mice. On the other hand, no difference was observed between *Mastomys* and *Arvicanthis* or *Mastomys* and BALB/c mice.

Engorged females that took blood meals on different hosts were further followed for comparison of mortality and fecundity. Mortality was assessed until day 4 post bloodmeal and ranged between 5% and 27%, but was not significantly influenced by host types (Table 5). Four days PBM, females were allowed to oviposit in small glass vials where they were kept individually. Blood source did not influence significantly either the fecundity of fed *P. duboscqi* females (Table 5) or the numbers of eggs laid by individual females (Table 6).

4. Discussion

The present study is, to our knowledge, the first one assessing the importance of Sub-Saharan rodents as hosts of *L. major* based on experimental infections of animals and testing of their infectiousness to

Table 4
Time-course of the external manifestation of *L. major* LV109 in ear pinnae (site of inoculation) of *M. natalensis*. Animals C1-C5 were infected with culture-derived promastigotes (Group C), animals A1-A5 were infected with sand fly-derived *Leishmania* (Group A). Light grey colour – red macula, dark grey colour – swelling, black colour – hyper-pigmentation of the site where swelling had healed. The numbers are the length of the swelling area in mm. *, animals killed by week 15 p.i.

Animals	Weeks post infection																	
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
C1*				1	1	1	1	1	5.8	X	X	X	X	X	X	X	X	X
C2			1	1.7	1.7	4	4.9	5.3	6.8	7	7	7	7	5	5	5	5	1
C3				1	1	1	1	1	2.8	3.4	5.2	5.4	5.6	2				
C4*				1	2.9	3	4.7	5	5	X	X	X	X	X	X	X	X	X
C5*				1	1	2	3.3	3.8	4	X	X	X	X	X	X	X	X	X
A1				2	2	2	3	4.6	5.2	6	6	6	6	3.7				
A2				1	1	1	2	2	4	5.2	5.2	5.2	5.2	5.2	5.2	5.2	3	3
A3					3			3	4.2	5.9	5.9	5.9						
A4				1	3	3.5	4.7	6.3	6.3	6.3	6.8	7	8	5.2	2.4	1		
A5		1	1	1	1	1	1	5	6.3	7	6.5	5.8	5	1				

Table 5
Feeding preferences, mortality and fecundity of *P. duboscqi* females fed on different host species. The between-species differences were tested by the Chi-squared test.

Host combination	Host	N (%) of fed sand flies	Significance of between-species differences	Mortality post feeding: N dying/N (%)	Significance of between-species differences	Fecundity N lying eggs/N (%)	Significance of between-species differences
Arvicanthis vs. BALB/c mouse	*Arvicanthis*	161 (80.5%)	$\chi2 = 17.015$, $P < 0.0001$	12/161 (7.4%)	$\chi2 = 0.118$, $P = 0.472$	26/76 (34.2%)	$\chi2 = 0.119$, $P = 0.432$
	BALB/c mouse	95 (47.5%)		6/95 (6.3%)		24/76 (31.6%)	
Arvicanthis vs. *Mastomys*	*Arvicanthis*	94 (47.0%)	$\chi2 = 0.129$, $P = 0.719$	25/94 (26.6%)	$\chi2 = 0.007$, $P = 0.534$	20/28 (71.4%)	$\chi2 = 0.012$, $P = 0.582$
	Mastomys	81 (40.5%)		22/81 (27.2%)		14/20 (70.0%)	
Mastomys vs. BALB/c mouse	*Mastomys*	134 (67.0%)	$\chi2 = 0.055$, $P = 0.808$	6/100 (6.0%)	$\chi2 = 0.787$, $P = 0.132$	18/20 (90.0%)	$\chi2 = 0.784$, $P = 0.661$
	BALB/c mouse	135 (67.5%)		10/200 (5.0%)		16/20 (80.0%)	

Table 6
Numbers of eggs laid by *P. duboscqi* females fed on different hosts. The differences were tested by the nonparametric Mann Whitney *U* test.

Host combination	Host	Number of eggs		Significance of between-species differences in distribution and means
		N	Median (Min, Max)	
Arvicanthis vs. BALB/c mouse	*Arvicanthis*	26	21 (2, 75)	$P = 0.426$, $P = 0.777$
	BALB/c mouse	24	13 (1, 54)	
Arvicanthis vs. *Mastomys*	*Arvicanthis*	20	45 (15, 75)	$P = 0.290$, $P = 0.727$
	Mastomys	14	40 (3, 70)	
Mastomys vs. BALB/c mouse	*Mastomys*	33	20 (4, 81)	$P = 0.379$, $P = 0.190$
	BALB/c mouse	13	31 (5, 72)	

sand flies.

Rodents of the genera *Arvicanthis* and *Mastomys* have been frequently found infected with *Leishmania major*: infections of *A. niloticus* have been reported from the NW and SW of Ethiopia, from Kenya, Senegal and Sudan, infections of *M. natalensis* from Kenya and *M. erythroleucus* from Senegal (reviewed by Desjeux, 1996). The fact that only *A. niloticus* (and no other species of the genus *Arvicanthis*) have been mentioned could be explained by the poorly understood taxonomy of the genus. Only recently have investigations using cytogenetic and molecular data revealed the presence of at least three sibling species in western and central Africa where the single species *A. niloticus* was previously reported (Granjon and Ducroz, 2013). In Ethiopia, which is situated in the centre of the range of *A. niloticus* (Dobigny et al., 2013), even four species of the genus are now recognized, including *A. niloticus* and *A. neumanni* (Granjon and Ducroz, 2013).

Frequent field findings of *L. major* in *Arvicanthis* and *Mastomys* have been reported, and the eco-etiological and physiological characteristics of these rodents match the requirements essential for reservoirs: they live in colonies with high population numbers in the vicinity of humans in endemic localities, and they have sufficient longevity. These characteristics encouraged us to perform laboratory experiments which can help to confirm or exclude their reservoir role. The results revealed the importance of the *L. major* strain used for the experiments. Substantial differences were observed in the infectivity of *L. major* strains isolated from the Middle East and Sub-Saharan Africa. The Sub-Saharan strain LV109 persisted in all three tested rodent species for several months and, importantly, the parasites were infective to *P. duboscqi* females. On the other hand, the Middle Eastern strain FV1 produced only poor infections in *A. neumanni* and *M. natalensis*, parasites were present in low numbers and the animals were not infectious to sand flies. These differences correspond with results of the study of Elfari et al. (2005) testing cross-infectivity of three *L. major* strains differing in geographical origin in three rodent species – *Psammomys obesus*, *Rhombomys opimus* and *Meriones libycus*. No infections were detected in *R. opimus* when infected with the African or Middle Eastern strains and no signs of disease were seen in any *P. obesus* infected with a Central Asian strain (Elfari et al., 2005).

Important methodological points influencing results of experimental infections are the size and nature of the inocula and the infection route (reviewed by Loría-Cervera and Andrade-Narváez, 2014). It has been shown repeatedly that the number of parasites transmitted by sand flies to the host is highly variable but it does not exceeded 10^5 parasites inoculated per bite (Kimblin et al., 2008; Maia et al., 2011; Secundino et al., 2012). Here we used an intradermal route of inoculation which is close to the natural mode of transmission, since parasites are exposed to the localized immune responses in the skin (Belkaid et al., 1998, 2002). Infections were initiated with either $3–7 \times 10^4$ of sand fly-derived parasites or with 10^7 of parasites derived from stationary-phase promastigote cultures. The former inocula comprised mainly metacyclic stages present in thoracic regions of sand fly midguts during the late stage infections. Rodent infections initiated in our study by a typical dose of sand fly derived *Leishmania*, showed the same outcome as those initiated with an unnaturally large inoculum of cultured parasites. Dissemination of parasites in the host's body as well as infectiousness to sand flies were very similar with both types of infection.

Infection rates, the percentage of sand flies that became infected while biting on experimental animals, ranged between 0 and 1.2% in *A. neumanni*, 0–10% in *A. niloticus* and 0–4.1% in *M. natalensis*. Similarly low infection rates were detected previously: 0–7% in *P. sergenti* feeding on rats (*Rattus rattus*) experimentally infected with *L. tropica* (Svobodova et al., 2013), 0–5% in *Lu. youngi* feeding on *Proechimys semispinosus* experimentally infected with *L. panamensis* (Travi et al., 2002) or 0–11% in *P. perniciosus* feeding on hares (*Lepus granatensis*) naturally infected with *L. infantum* (Molina et al., 2012). Higher infection rates have been reported more rarely, for example 19% of *P. orientalis* feeding on BALB/c mice experimentally infected with *L. donovani* (Sadlova et al., 2015) or up to 27–28% of *L. longipalpis* feeding on symptomatic dogs infected with *L. infantum* in Brazil (Michalsky et al., 2007; Courtenay et al., 2002).

External clinical manifestations of *L. major* observed in ears of infected rodents in this laboratory study (changes in pigmentation in *Arvicanthis* and swellings, redness and hyper-pigmentation in *Mastomys*) appeared 6 and 10 weeks post infection, respectively. They generally resembled natural manifestation of *L. major* infections in *Psammomys obesus* and *Meriones shawi* described from Sidi Bouzid in Tunisia: hyperpigmentation, depilation, inflammation and edema of the ears were found frequently in both these North African reservoir hosts (Ghawar et al., 2011). Changes in pigmentation and swellings were often accompanied by the presence of high numbers of parasites in our experiments. This is important as only animals with high numbers of parasites at the site where sand flies fed infected the vector. It was also pointed out by Courtenay et al. (2017) that among dogs infected with *L. infantum*, only some were "super-spreaders", while others contributed little to transmission (15%–44% of dogs were responsible for > 80% of all sand fly infections). Based on the model proposed by Miller et al. (2014) only 3.2% of the asymptomatic people infected with *L. donovani* in Ethiopia were responsible for of 53–79% of infections in the sand fly population.

One of the important prerequisites of the involvement of any rodent species in the life-cycle of *Leishmania* parasites is its attractiveness to

sand flies. It is also known from laboratory colonies that some sand fly species are opportunistic and readily feed on mice, while the others, like species in the subgenera *Larroussius* and *Adlerius*, prefer hamsters or rabbits (Volf and Volfova, 2011). Since the blood of vertebrate species varies in several properties influencing its nutritive value (Harrington et al., 2001), host choice affects the fitness of fed females as was repeatedly demonstrated in mosquitoes (Lyimo and Ferguson, 2009). In the neotropical sand fly *Lutzomyia longipalpis* significant differences in the numbers of eggs laid among flies fed on various hosts were reported (Macedo - Silva et al., 2014), and in fleas significant differences in the energetic cost of blood digestion were found even at the level of two rodent species from the same family (Sarfati et al., 2005). On the other hand, studies on the Old World sand fly species *P. papatasi* and *P. halepensis* revealed no appreciable differences between the fecundity of females fed on human blood and different animal blood sources (Hare et al., 2001; Sadlova et al., 2003). In our experiments, *P. duboscqi* females manifested as opportunistic feeders, being ready to feed on all offered rodent species, although they preferred *Arvicanthis* over laboratory mice. Mortality and fecundity of *P. duboscqi* females were comparable post feeding on all rodents tested. This is in accordance with a study from Kenya where *P. duboscqi* also showed opportunistic behavior, being attracted to wild rats, chickens, mongooses, dogs and goats (Mutinga et al., 1986).

The definition of reservoir hosts in leishmaniasis has changed in recent years. Ashford (1996, 1997) originally distinguished primary reservoirs (species ensuring long-term persistence of the parasite) and secondary reservoir hosts (species acting as liaison between primary reservoirs and incidental hosts), but this division was assessed to be arbitrary by Chaves et al. (2007), as hosts may vary locally and seasonally with the dynamics of transmission. According to the widely accepted ecological concept of Pulliam (1988), populations generally exhibit source – sink dynamics, where sources sustain exponential growth and are characterized by emigration while sinks operating under worse conditions demonstrate positive immigration. Chaves et al. (2007) applied this concept to reservoirs of leishmaniasis and proposed to recognize reservoir host as "a host with dynamic feedback in the transmission of a pathogen, that is, it can transmit the pathogen to new hosts". Incidental hosts lack such a dynamic feedback and cannot transmit the pathogen to new hosts. In this light, our results suggest that both *Mastomys* and *Arvicanthis* have the potential to be effective reservoirs (sources of the parasite) as both are able to maintain parasites for several months and infect the vector without apparent health complications. However, the higher infection rates, more generalized distribution of parasites and longer period of infectiousness to sand flies in *M. natalensis*, suggest that this species plays the more important reservoir role in transmission dynamics of this parasite in Sub-Saharan Africa. *Arvicanthis* species may serve as potential reservoirs in seasons/periods of low abundance of *Mastomys*.

Both *Arvicanthis* and *Mastomys* are known to undergo enormous abundance fluctuations: they are able to breed very rapidly and their population numbers may become very large when environmental conditions are favorable but with deteriorating conditions the numbers decline very rapidly (Granjon and Ducroz, 2013; Leirs, 2013). In the same locality, the Paloich district in Sudan, numbers of *Arvicanthis* and *Mastomys* alternated in two consecutive years (Hoogstraal and Dietlein, 1964). Therefore, the scenario that these species maintain the parasite alternatively is highly likely: in localities/seasons with a low abundance of *Mastomys, Arvicanthis* could serve as source of the parasite and vice versa. A similar scenario, alteration of *L. major* between two host species *P. obesus* and *M. shawi*, was proposed in Central Tunisia (Ghawar et al., 2011). Involvement of another rodent species in maintenance of *L. major* in Sub-Saharan region is also not excluded - it was suggested in Kenya where *Tatera robusta* possessed higher infection rates of *L. major* than *A. niloticus* and *M. natalensis* (Githure et al., 1996). Moreover, a high prevalence of *L. major* in invasive *Rattus rattus* was recently described in the southern part of Senegal (Cassan et al., 2018).

In conclusion, the results of this laboratory study support the field findings and give further support to the involvement of *Arvicanthis* and *Mastomys* spp. in the life cycle of *L. major* in Sub-Saharan Africa. This information is essential for any proposed control efforts against the human infection. However, more studies concerning other rodent species are needed to reveal the whole complexity and diversity of the epidemiology of *L. major* in this region.

Declarations of interest

None.

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Host competence of African rodents *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis Leishmania donovani* from Ethiopia and *L. (Mundinia) sp.* from Ghana

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Host competence of the African rodents *Arvicanthis neumanni, A. niloticus* and *Mastomys natalensis* for *Leishmania donovani* from Ethiopia and *L.* (*Mundinia*) sp. from Ghana

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ABSTRACT

Visceral leishmaniasis caused by *Leishmania donovani* is regarded as mostly anthroponotic, but a role for animal reservoir hosts in transmission has been suggested in East Africa. Field studies in this region have shown the presence of this parasite in several mammalian species, including rodents of the genera *Arvicanthis* and *Mastomys*. Further, the natural reservoirs of *Leishmania* (*Mundinia*) sp. causing human cutaneous disease in Ghana, West Africa, are unknown. This study assessed the potential role of the Sub-Saharan rodents *Arvicanthis neumanni*, *A. niloticus* and *Mastomys natalensis* as hosts of *L. donovani* and *L. sp.* from Ghana, based on experimental infections of animals and xenodiagnoses. The distribution and load of parasites were determined *post mortem* using qPCR from the blood, skin and viscera samples. The attractiveness of *Arvicanthis* and *Mastomys* to *Phlebotomus orientalis* was tested by pair-wise comparisons. None of the animals inoculated with *L. donovani* were infectious to *P. orientalis* females, although, in some animals, parasites were detected by PCR even 30 weeks post infection. Skin infections were characterized by low numbers of parasites while high parasite burdens were present in spleen, liver and lymph nodes only. Therefore, wild *Arvicanthis* and *Mastomys* found infected with *L. donovani*, should be considered parasite sinks rather than parasite reservoirs. This is indirectly supported also by results of host choice experiments with *P. orientalis* in which females preferred humans over both *Arvicanthis* and *Mastomys*, and their feeding rates on rodents ranged from 1.4 to 5.8% only. Therefore, the involvement of these rodents in transmission of *L. donovani* by *P. orientalis* is very unlikely. Similarly, poor survival of *Leishmania* parasites in the studied rodents and negative results of xenodiagnostic experiments do not support the involvement of *Arvicanthis* and *Mastomys* spp. in the transmission cycle of *L. sp.* from Ghana.

1. Introduction

Leishmania (Kinetoplastida: Trypanosomatidae) are parasites alternating between blood feeding sand flies (Diptera: Psychodidae) and vertebrate hosts, including humans and various other mammals. Visceral leishmaniasis (VL) is a severe disease caused by parasites of the *L. donovani* complex; specifically by *L. donovani* in Asia and East Africa and by *L. infantum* in Asia, the Middle East, Europe and Latin America. VL caused by *L. infantum* is typically a zoonosis, where domestic dogs serve as principal reservoirs, although high prevalence of infection has been reported in some other mammalian species (reviewed by Quinnell and Courtenay, 2009). In contrast, VL caused by *L. donovani* is regarded as mostly anthroponotic; however, in East Africa a role for animal reservoir hosts has been suggested for many years and various findings suggest that reservoir animals may contribute to the transmission of *L. donovani* during initial outbreaks (reviewed by Ashford, 2000). In Sudan, high levels of seroprevalence and confirmed infections with *L. donovani* were found in dogs (Dereure et al., 2003), and serious risk of infection in some uninhabited areas indicates a strong probability that *L. donovani* can be zoonotic (Elnaiem et al., 1998). Indeed, previous field studies on wild mammals in East Africa have shown the presence of *L. donovani* in small carnivores (Elnaiem et al., 2001; Hoogstraal and Heyneman, 1969) and several rodent species: *Arvicanthis niloticus* (Hoogstraal and Heyneman, 1969; Elnaiem et al., 2001; El-Hassan et al., 1993; Chance et al., 1978), *Mastomys natalensis* (Elnaiem et al., 2001), *Acomys* sp. (Hoogstraal and Heyneman, 1969; Chance et al.,

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1978) and *Rattus rattus* (Hoogstraal and Heyneman, 1969; Chance et al., 1978). Recently, our team identified *L. donovani* in southern Ethiopia, using PCR and DNA sequencing of the ITS1 gene, in *Arvicanthis* sp., *Mastomys erythroleucus* and *Gerbilliscus nigricaudus* (Kassahun et al., 2015).

A *Leishmania* parasite causing human cutaneous disease in Ghana was identified as a member of the *L. enriettii* species complex in 2015 (Kwakye-Nuako et al., 2015). The species complex was classified as a new subgenus *Mundinia* one year later (Espinosa et al., 2016). The five known members of this subgenus (*L. enriettii*, *L. macropodum*, *L. martiniquensis*, *L. orientalis* and *L.* sp. originating from Ghana) are geographically widely dispersed and vary substantially in their potential to cause human disease. Although medically important, the biology of these *Leishmania* species is poorly understood and information on their natural reservoir hosts as well as vector species is scarce. Identification of reservoir hosts and vector species of Ghanaian *Leishmania* species is, therefore, a significant research challenge.

Dense populations of the rodents of the genera *Arvicanthis* and *Mastomys* live in close vicinity of humans in Sub-Saharan Africa. We recently studied the susceptibility of *Arvicanthis neumanni* (Neumann's Grass Rat), *A. niloticus* (Nile Grass Rat) and *Mastomys natalensis* (Natal Multimammate Mouse) to *Leishmania major* and demonstrated that the latter may serve as a reservoir host for this parasite (Sadlova et al., 2019). This study is a follow-up analysis of the host competence of these three rodent species for *L. donovani* and *L.* (*M.*) sp. from Ghana. *Arvicanthis niloticus* and *M. natalensis* are widespread in almost all Sub-Saharan Africa (Granjon and Ducroz, 2013; Leirs, 2013) while *Arvicanthis neumanni* with a range restricted to East Africa (absent in Ghana) was tested only for *L. donovani*. The response of these rodents to infection and their ability to infect sand flies were evaluated by experimental infections and xenodiagnosis. In addition, feeding rates of *P. orientalis* on these rodents were tested by host-choice experiments.

2. Materials and methods

2.1. Sand flies, parasites and rodents

The colonies of *P. orientalis* (originating from Ethiopia) and *P. duboscqi* (originating from Senegal) were maintained in the insectaries of the Department of Parasitology, Charles University in Prague, under standard conditions as described previously (Volf and Volfova, 2011). Two Sub-Saharan *Leishmania* strains were used: *L. donovani* strain MHOM/ET/2010/GR374 (a human isolate from Ethiopia) and *L.* (*M.*) sp. strain MHOM/GH/2012/GH5; LV757 (a human isolate from Ghana; Kwakye-Nuako et al., 2015). Promastigotes were cultured in M199 medium (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco) supplemented with 1% BME (Basal Medium Eagle, Sigma) vitamins, 2% sterile human urine and 250 μg/ml amikacin (Amikin, Bristol-Myers Squibb). To obtain higher densities of *L. donovani* metacyclic forms in the stationary-phase culture for rodent infections, a mixture of salts (sodium urate 10 mg/ml, uric acid 10 mg/ml and cysteic acid 50 mg/ml, all Sigma) was added to the medium according to Howard et al. (1987). Breeding colonies of *A. neumanni*, *A. niloticus* and *M. natalensis* were established and maintained at the animal facility of the Department of Parasitology as described previously (Sadlova et al., 2019).

2.2. Rodent infections and xenodiagnosis

Two methods were used for infecting rodents with *L. donovani* – infections initiated with sand fly-derived *Leishmania* according to Sadlova et al. (2015) and infections initiated with culture-derived promastigotes. For the first method, *P. orientalis* females, experimentally infected with *L. donovani* as described previously (Sadlova et al., 2017), were dissected on day 8 post bloodmeal (PBM), when mature infections with accumulation of metacyclic forms in the thoracic midguts (TMG) were observed. Pools of 100 freshly dissected TMG with a good density of parasites were homogenized in 50 μl of saline.

The natural vector of *L.* sp. from Ghana is not known; therefore, only culture-derived promastigotes were used for rodent infections with this *Leishmania* species. For this purpose, stationary-phase promastigotes (day 7 post inoculation) were washed twice in saline and counted using a Burker apparatus. Pools of 10^8 promastigotes were resuspended in 50 μl of saline.

Dissected salivary glands (SG) of *P. orientalis* (for *L. donovani* infections) and *P. duboscqi* females (for infections with *L.* sp. from Ghana) were pooled in sterile saline (10 glands per 10 μl of saline) and stored at − 20 °C. Prior to mice inoculation, SG were disintegrated by 3 successive immersions into liquid nitrogen and added to both types (sand fly - and culture-derived) of promastigote suspensions.

Rodents were infected as described previously (Sadlova et al., 2019). Briefly, 5.5 μl of the suspension of parasites and SG were injected intradermally into the ear pinnae. The inoculum of culture-derived promastigotes comprised 10^7 parasites with 73% of metacyclic forms in *L. donovani* and 33–42% of metacyclic forms in *L.* sp. from Ghana. The inoculum of sand fly-derived *L. donovani* was 3–6 × 10^4 with 65–90% of metacyclic forms; the proportions of metacyclic forms were identified on Giemsa stained smears based on morphological criteria described previously (Sadlova et al., 2010). Animals were checked weekly for external signs of the disease until week 20–30 post infection (p.i.) when they were sacrificed.

Five to seven-day-old *P. orientalis* females (natural vectors of *L. donovani*) were allowed to feed on the site of *L. donovani* inoculation (ear pinnae) of anaesthetized rodents at 5 weeks-intervals as described previously for *L. major* infections (Sadlova et al., 2019). Fed sand fly females were separated and maintained at 26 °C on 50% sucrose until day 10–12 PBM, when females were dissected and their guts examined under the light microscope for presence and quality of *Leishmania* infections. As natural vectors for xenodiagnoses of *L.* sp. from Ghana are not available, animals infected with this *Leishmania* species were exposed to the unnatural vector *P. duboscqi* which supports the infection only until defecation of blood remnants on day 4–5 PBM (JS and TB, unpublished data). Therefore, parasite presence was determined on day 3 PBM, allowing multiplication of parasites before loss of infections, using PCR in pools of 5 whole female bodies.

2.3. Tissue sampling and quantitative PCR

Rodents were euthanized by cervical dislocation under anesthesia. Samples from both ears (inoculated and contralateral), both ear-draining lymph nodes, spleen, liver, paws and tail were stored at − 20 °C for qPCR. Extraction of total DNA from rodent tissues (on equal weight samples) and sand flies was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Quantitative PCR (Q-PCR) for detection and quantification of *Leishmania* parasites was performed in a Bio-Rad iCycler & iQ Real-Time PCR Systems using the SYBR Green detection method (SsoAdvanced™ Universal SYBR®, Bio-Rad, Hercules, CA). Infectious loads in rodent tissues were scored using a scoring table considering the number of parasites detected: < 1000 was evaluated as low parasite loads; 1000–10000 as medium parasite loads; > 10000 as high parasite loads.

2.4. Host choice experiments and assessment of mortality and fecundity of sand flies fed on different hosts

Pair wise comparisons between two types of hosts were performed using the same equipment as described previously (Sadlova et al., 2019). Briefly, 200 *P. orientalis* females were placed into the central cage from a row of three connected small cages and left for habituation for 20 min. Then, anaesthetized animals were placed or the forearm of a human volunteer positioned in each of the lateral cages for 1 h, after which sand flies were collected. Hosts alternated between lateral cages in each repeat and each pair of hosts was tested four times. Experiments were conducted in darkness at 24–26 °C. Fed females were maintained under the same conditions as the colony and their mortality was recorded for 4 days post-feeding. Then, females were introduced individually into small glass vials lined with wet filter papers and covered with fine mesh. A small ball of cotton wool soaked with 50% sugar solution was placed on the mesh. The numbers of eggs oviposited were compared to determine the effect of the host blood source host on *P. orientalis* fecundity (Killick-Kendrick and Killick-Kendrick, 1991).

2.5. Statistical analysis

Statistical analyses were carried out using R software (http://cran.r-project.org/). The differences in feeding preferences, mortality and fecundity of *P. orientalis* females fed on different host species were analysed by the Chi-square test. The differences in numbers of eggs laid by females fed on different hosts were tested by the nonparametric Mann Whitney U test. The relationships between weight and groups (infected and non-infected animals) and time were tested by fitting multilevel linear regression models (package "nlme"), taking into account the correlation between repeated measures of the same animal over time. The model used included interaction term between groups (categorical variable) and time (continuous independent variable).

2.6. Animal experimentation guidelines

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which 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 in Prague and were performed under permit no. MSMT-10270/2015–5 of the Ministry of the Education, Youth and Sports of the Czech Republic. Investigators were certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

3. Results

3.1. Experimental infections with L. donovani

In total, 8 *M. natalensis*, 7 *A. neumanni* and 10 *A. niloticus* were infected by *L. donovani;* all *M. natalensis*, all *A. neumanni* and five *A. niloticus* with sand fly-derived *Leishmania*, while five *A. niloticus* were infected with culture-derived *Leishmania*. Two animals of each species served as controls inoculated with sterile saline.

None of the inoculated animals developed lesions or other external signs of the disease throughout the entire experiment. The weight of animals did not differ significantly between infected animals and uninfected controls (P = 0.126, P = 0.446 and P = 0.382 in *A. niloticus*, *A. neumanni* and *M. natalensis*, respectively) (S1 Table). All 100, 194 and 251 *P. orientalis* females used at different times p.i. for xeno diagnoses on *M. natalensis*, *A. neumanni* and *A. niloticus*, respectively, were *Leishmania*-negative.

Nevertheless, PCR performed at the end of the experiment, on week 30 p.i., revealed presence of *L. donovani* in different tissues and organs of infected rodents; the quantities of parasites were subsequently determined using qPCR. High parasite loads were found in the lymph node draining the inoculated ear of one *A. neumanni* and low parasite loads in forepaws and the inoculated ear of three *A. niloticus* (Table 1). The parasite loads in *M. natalensis* were the highest among tested rodent species. Half of the animals maintained *L. donovani* until week 30 p.i., with parasites localized mostly in liver, spleen and paws. An inoculated ear was positive in one specimen and draining lymph nodes in two animals. High parasite burdens were detected only in the spleen and liver. No parasites were detected in the right ear, tail or blood (Table 1).

3.2. Experimental infections with L. sp. from Ghana

Six *A. niloticus* (3 males and 3 females) and six female *M. natalensis* were inoculated with culture-derived promastigotes, but one *M. natalensis* died before the end of the experiment. Six animals of each species were used as controls inoculated with sterile saline. No external signs of infection were apparent on infected animals throughout the experiment. The weight of infected *A. niloticus* did not differ significantly from control animals (P = 0.98) but in *M. natalensis*, infected animals gained significantly less weight than controls (P = 0.01) (S2 Table).

Xenodiagnoses with *P. duboscqi* did not reveal *Leishmania* presence in sand flies tested by PCR – all 95 and 87 pools of sand flies used for xenodiagnoses on inoculated ears of *A. niloticus* and *M. natalensis,* respectively, on different week p.i., were negative. By the end of the experiment, on week 25 p.i., PCR failed to detect *Leishmania* DNA in any *A. niloticus*. In *M. natalensis*, the presence of *Leishmania* was confirmed in a single animal on week 20 p.i., with localization in the inoculated ear and its draining lymph nodes, the contralateral ear and forepaws. Based on qPCR, all these tissues possessed only low parasite loads.

3.3. Host choice experiments with P. orientalis

Two potential rodent hosts were offered to *P. orientalis* females in each pair-wise comparison, and each host combination was tested four times with hosts alternating between lateral cages. *Phlebotomus orientalis* females showed very low feeding rate on all tested rodents: 1.4–5.8% of females took bloodmeals during experiments (Table 2). No difference was observed in attractiveness of *A. niloticus* (the bigger species) and *A. neumanni* (the smaller species), or between *Mastomys* and *Arvicanthis* (represented by *A. neumanni)*. On the other hand, significant preferences were observed when human forearm was offered as an alternative to rodents: sand flies preferred human forearms to both *Arvicanthis* and *Mastomys* (Table 2). Engorged females that took blood meals on different hosts were further followed for comparison of mortality and fecundity. Mortality (until day 4 PBM) ranged between 15.2% and 54.5%, but was not significantly influenced by host types (Table 2). Four days PBM, females were allowed to oviposit in small glass vials where they were kept individually. Blood source did not influence significantly either the proportion of females laying eggs or the numbers of eggs laid by individual females. Median numbers of eggs per female were 36–53, depending on the experiment (Table 2).

4. Discussion

Zoonotic transmission of *L. donovani* has long been suggested in

Table 1
Presence, amount and location of *L. donovani* DNA determined by qPCR in various tissue of individual animals. IE, inoculated ear; CE, contralateral ear; DN-IE, draining lymph nodes of the inoculated ear; DN-CE, draining lymph nodes of the contralateral ear; FP, forepaws; HP, hindpaws; T, tail; L, liver; S, spleen; B, blood; -, negative results, +, < 1000 parasites; ++, 1000–10000 parasites; +++, > 10000 parasites.

Rodent species	Individual marks of animals	IE	CE	DN-IE	DN-CE	FP	HP	T	L	S	B
A. niloticus	A1	–	–	–	–	–	–	–	–	–	–
	A2	–	–	–	–	+	–	–	–	–	–
	A3	–	–	–	–	–	–	–	–	–	–
	A4	–	–	–	–	–	–	–	–	–	–
	A5	–	–	–	–	–	–	–	–	–	–
	A6	–	–	–	–	–	–	–	–	–	–
	A7	+	–	–	–	+	–	–	–	–	–
	A8	–	–	–	–	+	–	–	–	–	–
	A9	–	–	–	–	–	–	–	–	–	–
	A10	–	–	–	–	–	–	–	–	–	–
M. natalensis	M1	–	–	–	–	–	–	–	–	–	–
	M2	–	–	–	–	+	++	–	++	+++	–
	M3	–	–	++	–	+	+	–	++	+++	–
	M4	–	–	–	–	–	–	–	–	–	–
	M5	–	–	++	+	+	–	–	+++	+++	–
	M6	+	–	–	–	–	–	–	–	–	–
	M7	–	–	–	–	–	–	–	–	–	–
	M8	–	–	–	–	–	–	–	–	–	–

Sudan, based on repeated outbreaks of the disease in people who had visited uninhabited areas and findings of the parasite in wild animals. However, despite a considerable effort over many years no reservoir host has been proven (reviewed by Ashford, 2000; Ashford, 1996). Here we undertook testing of the reservoir role of rodents in the genera *Arvicanthis* and *Mastomys*, as *L. donovani* parasites were repeatedly found in these rodent species during several field studies performed in East Africa (El-Hassan et al., 1993; Elnaiem et al., 2001), since studies in the 1960s when the highest prevalence was found in *A. niloticus* in the southern part of Sudan (Hoogstraal and Heyneman, 1969). Recently, the presence of *L. donovani* was reported in *Arvicanthis* and *Mastomys* in south-western Ethiopia (Kassahun et al., 2015).

The results of our laboratory study are in accordance with the above cited field studies, since we confirmed that *L. donovani* parasites survive in some individuals of *Arvicanthis* and *Mastomys* for several months. Parasites were localized not only in the viscera, but in some animals also in external organs - ears and paws, therefore, theoretically available for transmission by biting sand flies. However, all xenodiagnoses performed on ears of animals were negative. This is in contrast with results of our previous study performed with the same Ethiopian *L. donovani* strain on BALB/c mice (Sadlova et al., 2015). Mice were infected using the same method – intradermal inoculation of sand fly derived parasites in the ear pinnae. Mice did not show any signs of disease in our previous study, but 19% of the *P. orientalis* females that fed at the site of inoculation, became infected (Sadlova et al., 2015).

Parasites of the *Mundinia* subgenus from Ghana still lack their scientific name and there is no current information on their natural reservoir hosts and vector species. Our experimental infections of *A. niloticus* and *M. natalensis*, rodents present in endemic localities, did not confirm their host competence for this parasite. *Leishmania* were mostly completely lost and survived only in a single *M. natalensis* till week 20 p.i. In this animal, *Leishmania* disseminated to different tissues (draining lymph nodes of the inoculated ear, the contralateral ear and forepaws), but were present only in low parasite loads.

Generally, a failure of infectivity to sand flies can be explained by absence or low numbers of parasites in peripheral blood or in the skin. In dogs infected with *L. infantum*, high parasite numbers in skin (and blood) have been shown to be the best markers of infectiousness to sand fly vectors (Courtenay et al., 2014; Borja et al., 2016). In our study, high parasite loads were present only in the spleen and liver of *M. natalensis* and lymph nodes of *A. neumanni* infected with *L. donovani,* these being visceral organs where the parasites were not accessible to sand fly borne transmission. Skin samples derived from whole ears and paws revealed 10–700 amastigotes, compared with 14–80 thousand present in lymph nodes and viscera. A similar phenomenon was observed in the study of host competence of *Mastomys* and *Arvicanthis* for *L. major* – the *L. major* strains which produced poor skin infections in animals were not infectious to sand flies (Sadlova et al., 2019).

The main vector of *L. donovani* in Sudan and Ethiopia is *Phlebotomus* (*Larroussius*) *orientalis* (Ashford, 2000). Host choice experiments reported here showed that this sand fly species was not strongly attracted to rodents. Females clearly preferred humans as a source of blood meals. Preference of *P. orientalis* for humans and large domestic animals was also observed during field studies with animal baited traps and blood meal analyses in Ethiopia (Gebresilassie et al., 2015a, 2015b; Yared et al., 2019). Similarly, the very low engorgement rates of *P. orientalis* on rodents in our experiments (1.4–5.8%) are in accordance with the results of the study of Gebresilassie et al. (2015b), where only 1.08% of females took blood meals on small wild animals compared to 30.53% feeding on larger domestic animals. The likelihood of *L. donovani* being maintained in rodents in areas where *P. orientalis* acts as the main vector is, therefore, very low.

Taken together, the results of this study suggest that rodents infected with *L. donovani* in East Africa most probably represent parasite sinks on which the infected sand fly occasionally feed but which do not contribute to vector infection and transmission to the next host (Chaves et al., 2007). True animal reservoirs of *L. donovani* in East Africa must be confirmed with further studies, and good candidates may be small carnivores like the Egyptian mongooses, 14% of which were found infected in Dinder National Park (Elnaiem et al., 2001), or Senegal genet (*Genetta G. senegalensis*), Sudanese serval (*Felis serval phillipsi*) or White-tailed mongooses (*Ichneumia albicauda*) reported to be infected with *L. donovani* in southern Sudan and Ethiopia, respectively (Hoogstraal and Heyneman, 1969; Kassahun et al., 2015). For the *Mundinia* parasites causing cutaneous leishmaniasis in Ghana we do not have any indication from the field for identification of the reservoir host; therefore, any such findings would be highly valuable to enable further experimental research.

Table 2
Feeding preferences, mortality and fecundity of *P. orientalis* females fed on different host species. The between-species differences were tested by the Chi-squared test. The differences in numbers of eggs laid were tested by the nonparametric Mann Whitney U test.

Host combination	Host	N (%) of fed sand flies	Significance of between-species differences	Mortality post feeding: N dying/N (%)	Significance of between-species differences	Fecundity N eggs-lying/N (%)	Significance of between-species differences	Number of eggs Median (Min, Max)	Significance of between-species differences
A. neumanni vs. *A. niloticus*	*A. neumanni*	22 (1.4%)	$\chi2 = 1.231$ P = 0.267	12/22 (54.5%)	$\chi2 = 0.746$ P = 0.483	10/10 (100%)	$\chi2 = 0.405$ P = 0.600	36 (5, 60)	P = 1.000
	A. niloticus	30 (2.0%)		15/30 (50.0%)		14/15 (93.3%)		38 (2, 70)	
A. neumanni vs. *M. natalensis*	*A. neumanni*	46 (2.3%)	$\chi2 = 0.095$ P = 0.758	7/46 (15.2%)	$\chi2 = 0.259$ P = 0.192	37/39 (94.9%)	$\chi2 = 0.115$ P = 0.115	49 (1, 67)	P = 0.966
	M. natalensis	49 (2.4%)		12/49 (24.5%)		31/37 (83.8%)		48 (1, 75)	
A. neumanni vs. man	*A. neumanni*	17 (3.4%)	$\chi2 = 14.0625$ P = 0.0002	7/17 (41.2%)	$\chi2 = 0.922$ P = 0.577	10/10 (100%)	$\chi2 = 0.376$ P = 0.527	50 (26, 64)	P = 0.552
	man	47 (9.4%)		20/47 (42.6%)		25/27 (92.3%)		53 (2, 78)	
M. natalensis vs. man	*M. natalensis*	29 (5.8)	$\chi2 = 5.582$ P = 0.0182	7/29 (24.1%)	$\chi2 = 0.989$ P = 0.597	19/22 (86.4%)	$\chi2 = 0.718$ P = 0.510	52 (15, 66)	P = 0.918
	man	50 (10.0)		12/50 (24.0%)		34/38 (89.5%)		51 (5, 76)	

In conclusion, the results of this laboratory study do not support the involvement of *Arvicanthis* and *Mastomys* spp. in the transmission cycle of *L. donovani* in East Africa nor *L. sp.* in Ghana. In contrast, these rodent species most probably do comprise important reservoir hosts of *L. major* in this region (Sadlova et al., 2019).

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.12.002.

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SUMMARY AND DISCUSSION

This dissertation thesis summarises the results of four projects involved in my project, supported by Grant Agency of the Charles University since 2016. I have performed experimental infections of several species of both laboratory and wild rodents from endemic areas with human pathogenic *Leishmania* to target three particular aims - to evaluate the effect of repeated sand fly biting on the progress of cutaneous leishmaniasis, to introduce new animal models for leishmaniasis research, and to test the reservoir potential of putative hosts of *L. major* and *L. donovani* in Sub-Saharan Africa.

All three studies are based on the proper execution of experimental infections as methodological parameters used are known to influence substantially the outcome of infections. The clinical manifestation of the disease is influenced by parasite dose and developmental form (procyclic/metacyclic promastigotes or amastigotes) as well as by the inoculation route and site (reviewed by Loria-Cervera and Andrade –Narveaz 2014). In our experiments, we used the intradermal route of inoculation, which is closest to the natural mode of transmission since parasites are exposed to the localized immune responses in the skin (Belkaid et al 1998, 2002). Parasites used for inoculation were predominantly sand fly-derived, pooled from thoracic midguts of laboratory infected natural vectors. This unique method, which mimics a natural route of infection, was established by Sadlova et al. (2015). In thoracic midguts of infected sand flies, infective metacyclic *Leishmania* forms prevail and, in addition, also other factors known to promote host infection like leishmania-derived proteophosphoglycans and exosomes or sand fly microbiota are present (Atayde et al 2016, Rogers 2012, Dey et al 2018). We have used natural parasite load (10^4 - 10^5parasites/rodent) which did not exceed the reported maximal number of parasites transmitted by natural vectors (Kimblin et al 2008, Maia et al 2011, Secundino et al 2012). The knowledge and control of the parasite load is a big advantage of the used method compared to natural infections by sand fly bite where the numbers of transmitted parasites differ in several orders between individual vector females (Kimblin et al 2008, Maia et al 2011, Secundino et al 2012). Also, the homogenate of salivary glands of vector females, influencing the immune response of the host, have been added to the inoculum.

Saliva components are an obligatory part of interactions between blood-feeding arthropods and vertebrate hosts, containing multiple proteins and peptides with diverse functions and targeting the host haemostatic and immune system. Sand fly saliva has multiple immunomodulatory effects on *Leishmania* infection in mammalian hosts (reviewed by Lestinova et al. 2017). The enhancing effect of sand fly saliva co-inoculated with parasites on disease progression is a well-known phenomenon (Kamhawi et al. 2000). Another well-reported phenomenon is the protection against infection in hosts preimmunized with salivary glands lysates or pre-exposed to uninfected sand fly bites. However, the

effect of sand fly bites in hosts previously infected by *Leishmania* is less understood – only a single study reported increased transmissibility of *L. donovani* to vectors after multiple exposures of the hamster's host to sand fly bites (Valverde et al 2017). Therefore, the aim of our first study was the evaluation of the effect of sand fly saliva on the manifestation of cutaneous leishmaniasis caused by *L. major*. For this purpose, we used the standard laboratory model of BALB/c mice. Infected mice were divided into 2 groups to compare the disease progress in mice exposed to *P. duboscqi* females every 2 weeks with mice left as unexposed control. Our results demonstrated that the repeated sand fly bites of the infected host have a similar "enhancing effect" on the lesion growth as the co-infection of *Leishmania* parasites with SGL. Cutaneous lesions formed at the injected site developed faster and reached a higher size in the group exposed to repeated sand fly bites. In our opinion, this effect could be caused by the enzyme hyaluronidase present in *P. duboscqi* saliva (Cerna et al. 2002). Hyaluronidase cleaves hyaluronic acid, a major component of vertebrate extracellular matrix, facilitating the spread of other pharmacologically active compounds of saliva and hyaluronidase activity was shown to exacerbate skin lesions caused by *L. major* in a concentration-dependent manner (Volfova et al. 2008). However, besides saliva, also other above-mentioned sand fly-derived components - proteophosphoglycans, exosomes and vector microbiota, inoculated together with saliva and parasites into the bite site, might more or less contribute to the observed effect.

In our study, repeated exposure to uninfected *P. duboscqi* females also increased tissue parasite load in BALB/c mice. In the hamster model infected with *L. donovani* (Valverde et al. 2017), repeated *Lu. longipalpis* bites enhanced significantly parasites numbers in the peripheral blood but not in the skin. This difference may reflect the species-specific organ tropism of visceral and cutaneous *Leishmania* (Zhang et al. 2003). On the other hand, mice´s infectiousness to vectors and distribution of parasites in the host body did not differ significantly between experimental groups in this study. At the end of the experiment, 10 weeks p.i., all 24 BALB/c mice infected with *L. major* were infectious to *P. duboscqi* females and the mean percentage of infected sand flies ranged between 14.2 and 22.5% in respective groups. Similar infectiousness has been reported for symptomatic dogs infected with *L. infantum* exposed to *Lu. longipalpis* (13 – 28 %, Travi et al. 2002, Michalsky et al. 2007), BALB/c mice infected with *L. donovani* exposed to *P. orientalis* (19 %, Sadlova et al. 2015) or hamsters infected with *L. donovani* exposed to *Lu. longipalpis* (24 %, Valverde et al. 2017). In the last study, the infectiousness of hamsters increased significantly post exposures to sand flies while this effect was not observed in our study. This difference could be due to the different methodology, the infectiousness of hamsters was tested 24 hours after the last exposure (Valverde et al. 2017) while in our experiments, we used the two weeks time intervals. Therefore, we can´t exclude that if there was a similar short-term effect of sand fly saliva on infectiousness, it was not detected due to the experimental design of the study.

The second study (Vojtkova et al 2020) was focused on establishing new laboratory models for studying leishmaniasis. Three Asian rodent species (*C. griseus*, *L. lagurus* and *P. sungorus*) infected with *L. major* or *L. donovani* were compared with commonly used laboratory animals in *Leishmania* research (BALB/c mice and *M. auratus*). Genetically polymorphic wild rodents had been used as experimental animal models for host-parasite relationships studies in the last decades. These models allow a better understanding of the infection dynamics and the natural regulation of the immune response (Loría-Cervera and Andrade-Narváez 2014). The Asian rodent species were chosen because they are commercially available and easy to breed and older observations (Hindle and Patton 126, Young et al. 1926) as well as more recent studies from China (Lun et al. 2015) suggested their high susceptibility to *Leishmania* parasites. Also, these rodent species share the geographical distribution with *L. major, L. donovani* and *L. infantum*, but reports of their involvement in the life cycle of the parasites are lacking. As far as we know, only the related species *Cricetulus migratorius* has been reported to be infected with *L. donovani* in Iran (Mohebali et al. 1995, Mohebali et al. 2018). All Asian species involved in the study showed high susceptibility to *L. major*, offering the model of both the healing phenotype (*L. lagurus* and *C. griseus*) and the non-healing phenotype (*P. sungorus*). Both, *L. lagurus* and *C. griseus* also exhibited excellent susceptibility to *L. donovani*, having higher parasite loads in most tested tissues and viscera than standard model animals.

Chinese hamsters (*C. griseus*) were confirmed as highly susceptible to *L. donovani*. Without external signs of disease, 86 % of individuals were successfully infected with the dissemination of amastigote to almost all tested organs and tissues. The highest presence of amastigotes was detected in inoculated ears (79 % of animals) and spleen (71 % of animals) and infectiousness to *P. orientalis* was confirmed from week 25 p. i. A similar susceptibility was described also for *L. major*. The parasites were detected in all tested skin samples (inoculated and contralateral ears, paws and tail) and to a lesser extent, also in visceral organs (mainly in the spleen). The cutaneous lesions formed on inoculated ears were not ulcerative, well-tolerated, or even healed and rodents were highly infectious to *P. duboscqi* females. Therefore, Chinese hamsters mimic best the features of the natural host of *L. major*. In reservoir hosts of *L. major*, visceralization was observed repeatedly, *Leishmania* DNA was determined by PCR in bone marrow, lymph nodes and spleen of *P. obesus*, liver and spleen of *C. gundi* and *R. rattus* caught in Tunisia and southern Iran, respectively (Jaouadi et al. 2011, Parhizkari et al. 2011, Davami et al. 2014, Ghawar et al. 2018).

Steppe lemmings (*L. lagurus*) also represented the healing phenotype with *L. major* infection. The amastigotes were mainly detected in inoculated ears (94 % of animals) and forepaws (44 % of animals) but rarely disseminated into the visceral organs. In addition, this rodent species showed the higher susceptibility to *L. donovani* than golden hamsters and BALB/c mice, high loads of parasites

were present in all the tested skin a viscera samples and infectiousness to *P. orientalis* was the highest (from 19.1 to 23.9 % infected sand flies) among tested rodent species. Except for splenomegaly, steppe lemmings did not show any pathological signs , which favour them as the optimal model for progressive VL.

Finally, Djungarian hamsters (*P. sungorus*) infected with *L. major* presented a non-healing phenotype with progressive ulcerative skin lesions. Parasite loads in inoculated ears were comparable to standard laboratory models, parasites were detected in all tested tissues and organs, including blood and viscera (71 % of hamster´s spleens infected). In addition, the highest infection rates and infectiousness from all tested species were observed. Therefore, Djungarian hamsters seem to be an optimal genetically polymorphic animal model for testing drug resistances or vaccine efficacy.

This study provided also a comparison of different types of inoculums and three different methods for detecting parasites in the host´s body. No significant differences were observed in parasite loads, disease progress and infectiousness between rodents infected with sand fly-derived parasites and culture-derived parasites in any *Leishmania* species. Therefore, also cultured parasites with sufficient representation of metacyclic forms intradermally inoculated with sand fly saliva can simulate the natural features of the disease. On the other hand, the infectious dose significantly affected *L. donovani* infections in *M. auratus* and *L. lagurus:* lower infection rates, dissemination, and parasite loads were detected in rodents infected with 10^5 compared to 10^7 *L. donovani*. This is in accordance with previous studies with various *Leishmania* species using the mouse model (Rogers et al 2004, Kimblin et al 2008, Ong et al 2020). Parasite loads and distribution in rodent bodies were evaluated from samples taken *post-mortem* using 3 different methods – qPCR, flow cytometry and fluorescent detection with In Vivo Extreme. For this purpose, *Leishmania* parasites were marked with red fluorescent markers. Quantitative PCR (qPCR) is a commonly used method for detecting of *Leishmania* DNA in various samples of laboratory animals infected with *Leishmania* parasites, testing putative reservoir hosts and vectors, and also asymptomatic humans from endemic areas (Fagundes et al. 2010, Sadlova et al. 2015, Conter at al. 2015). Flow cytometry (FC) based on the use of fluorescent probes is a relatively cheap and quick method able to detect various protozoan parasites in the host cells (Barbieri et al. 1993, Staalsoe et al. 1999, Neumann et al. 2000, Di Giorgio et al. 2000). *In vivo* imaging is a novel non-invasive method, which can be used for monitoring smaller animals with leishmaniasis (Melby et al. 2001, Thalhofer et al. 2017). Better results were obtained if parasites express luciferase than they were marked with fluorescent signal because of low sensitivity and high autofluorescence signal of hairy parts of animal body (Thalhofer et al. 2017). In our study, both qPCR and FC were highly sensitive methods for the evaluation of parasite loads in tissue samples. Fluorescence detection using In Vivo Extreme, although less sensitive and not suitable for accurate

quantification of *Leishmania*, gave valuable spatial data for parasite distribution. For example, fluorescence signals came from the single spot in the liver of *L. major* infected BALB/c mice and *L. lagurus*, while there were several smaller amastigote centres dispersed over the whole organs in *P. sungorus*.

The last two studies involved in this thesis (Sadlova et al 2019, 2020) evaluated the host competence of three African rodent species to *L. major, L. donovani* and *L. (Mundinia)* from Ghana. We tested the potential of *Arvicanthis niloticus*, *A. neumanni* and *Mastomys natalensis*, to maintain *Leishmania* parasites and their attractiveness and infectiousness to sand flies.

In Sub-Saharan Africa, reservoirs of *Leishmania* spp are unknown. CL caused by *L. major* is a typical zoonosis circulating between rodents and sand flies, in contrast to the mainly anthroponotic transmission of VL caused by *L. donovani* (WHO 2010). However, the role of small carnivores and several rodent species in the transmission of *L. donovani* has been suggested in East Africa (Chance et al. 1978, El-Hassan et al. 1993, Elnaiem et al. 2001). Candidate reservoirs among wild rodents are species of genera *Arvicanthis* and *Mastomys* because *L. major and L. donovani* parasites (respective parasite DNA) were repeatedly found in these rodent species during several field studies. The presence of *L. donovani* was detected by PCR in *A. niloticus* in Sudan (Hoogstraal and Heynemann 1969) and in *Arvicanthis* and *Mastomys* in Ethiopia (Kassahun et al. 2015). *Leishmania major* infection of *A. niloticus, M. natalensis* and *M. erythroleucus* was reported from Kenya, Senegal, and Ethiopia (reviewed by Desjeux 1996). The eco-etiological, epidemiological and physiological characteristics of these wild rodent species match the requirements essential for RH: *Leishmania* parasites were frequently found in their body, they live in colonies with high population numbers near human villages in endemic areas, and they are sufficiently long-lived. Our laboratory study was aimed to reveal if these rodent species are only incidental hosts or have the potential to serve as reservoirs of *Leishmania* parasites in Sub-Saharan Africa.

We confirmed that *L. donovani* amastigotes survived in some individuals of *Arvicanthis* and *Mastomys* for several months. *Leishmania* DNA was detected not only in visceral organs, but in a few animals also in the skin of ears and paws, therefore theoretically available to transmission to vectors. However, all xenodiagnoses using natural vector *P. orientalis* were negative. This contrasts with our previous work performed with the same Ethiopian *L. donovani* strain on BALB/c mice (Sadlova et al. 2015), where the infectiousness of host to *P. orientalis* females reached 19 %. The failure of infectivity to sand flies could be explained by the absence or very low numbers of parasites in the peripheral blood and/or in the skin. In dogs infected with *L. infantum*, numbers of amastigotes in skin and blood correlated with infectiousness to vectors (Courtenay et al. 2014, Borja et al. 2016). In our study, high parasite loads were present only in the liver and spleen of *M. natalensis* and draining lymph nodes of

A. neumanni, where the amastigotes were not accessible to vector-borne transmission. Therefore, *Arvicanthis* and *Mastomys* naturally infected with *L. donovani* in East Africa most probably represent incidental hosts. These hosts serve as parasite sinks - infected sand flies occasionally feed on them, but animals do not contribute to vector infection and transmission to the next host (Chaves et al. 2017). The true reservoir host of *L. donovani* in East Africa must be confirmed with further investigations. Besides rodents, small carnivores like Egyptian mongoose, White-tailed mongoose, Sudanese felis, or Senegal genet may be next candidates (Hoogstraal and Heynemann 1969, Elnaiem et al. 2001, Kassahun et al. 2015).

Results of *L. major* infection confirmed the importance of using a proper combination of *Leishmania* strain and the mammalian host. Significant differences were obtained in the infectivity of *L. major* strain from the Middle East and Sub-Saharan Africa. The Sub-Saharan strain LV109 persisted in all 3 tested wild rodent species for several months and hosts were infectious to vectors, while the Middle East strain FVI, highly infective for commonly used BALB/c mice, produced only poor infection with low parasite loads in African rodent species and they were not infectious to *P. duboscqi* females. Specificity of the host-parasite relationship reported for *L. major* also Elfari et al. (2005): infections failed in *Rhombomys opimus* (Central Asian reservoir) infected with African and the Middle East strains as well as in *Psammomys obesus* (North African reservoir) infected with Central Asian parasite.

In our experiments, *Arvicanthis* and *Mastomys* infected with the Sub-Saharan *L. major* showed only redness, temporary swelling or pigmentation changes in inoculated ears. These small cutaneous changes resembled natural manifestation of *L. major* infections (hyperpigmentation, depilation, inflammation and oedema of ears) in *M. shawi* and *P. obesus*, reservoir hosts of cutaneous leishmaniasis in Tunisia (Ghawar et al. 2011). Swelling and changes in pigmentation were often accompanied by the presence of high parasite loads in our study and only wild rodents with high numbers of parasites at the site exposed to sand flies were able to infect vectors. Similar findings have been shown in dogs infected with *L. infantum* and humans infected with *L. donovani* (Miller et al. 2014, Courtenay et al. 2017), only some individuals were “super – spreaders”, while others contributed little to transmission. Only around 15 % of dogs were responsible for more than 80 % of all sand fly infections and only 3.2 % of the asymptomatic people were responsible for 53 – 79 % of infections in the sand fly population.

Infectiousness of rodents to vectors was tested by xenodiagnoses using *P. duboscqi* females. Infection rates ranged between 0 – 1.2 % in *A. neumanni,* 0 – 10 % in *A. niloticus* and 0 – 4.1 % in *M. natalensis*. Low infection rates have been reported comparatively often in similar studies, e.g., 0.7 % *P. sergenti* on *Rattus rattus* experimentally infected with *L. tropica* (Svobodova et al., 2006), 0–5 % *Lu. youngi* feeding on *Proechimys semispinosus* experimentally infected with *L. panamensis* (Travi et al.,

2002) or 0–11% *P. perniciosus* feeding on *Lepus granatensis* naturally infected with *L. infantum* (Molina et al., 2012).

Attractiveness to vectors is an important prerequisite of the involvement of any vertebrate hosts in the life – cycle of *Leishmania* parasites. Host choice can also affect the nutritive value of blood meal, fecundity and fitness of fed females (Harringhton et al. 2001, Lyimo and Ferguson 2009, Macedo – Silva et al. 2014). Host choice experiments performed in this study showed that *P. orientalis*, the main vector of *L. donovani* in Sudan and Ethiopia, was not strongly attracted to rodents but preferred humans as a source of blood meals. Similar observation described field studies in Ethiopia (Gebresilassie et al., 2015a, 2015b; Yared et al., 2019), where *P. orientalis* females preferred humans and larger domestic animals. On the other hand, *P. duboscqi*, a proven vector of *L. major* in Sub-Saharan Africa, manifested opportunistic behavior, being ready to feed on all offered species, although preferred *Arvicanthis* over laboratory mice. Our laboratory results are similar to field observations of Mutinga et al. (1986) - in Kenya, *P. duboscqi* females were attracted to a wide spectrum of wild and domestic animals.

As described in more detail in the introduction, the definition of reservoir host (RH) in leishmaniasis has been revisited in recent years. The division of RH on primary, secondary and incidental hosts (Ashford 1996, 1997) was assessed to be arbitrary (Haydon et al 2007) and was replaced by concept of Chaves et al. (2007), recognizing reservoir host (RH) as "a host with dynamic feedback in the transmission of a pathogen, that is, it can transmit the pathogen to new hosts" and incidental hosts lacking such dynamic feedback. According to this division, our results suggested that both *Mastomys* and *Arvicanthis* have a reservoir potential for *L. major*, may be a source of the parasite. Higher infection rates, more generalized distribution of parasites and a longer period of infectiousness to vectors favour *Mastomys natalensis* to play the more important role in the transmission dynamic of *L. major* in Sub-Saharan Africa than *Arvicanthis*, but the latter may serve as potential RH in seasons of low abundance of *Mastomys*. In contrast, none of these rodents seems to be a reservoir of *L. donovani*.

Finally, we have used parasites of the *Mundinia* subgenus from Ghana which still lack their scientific name and cause human cutaneous disease in Ghana (Kwakye – Nuako et al. 2015). Although the tested rodent genera are present in Ghanaian endemic localities, infections of *M. natalensis* and *A. niloticus* did not confirm their host competence for this *Leishmania* species. Infections were mostly lost and parasites survived only in a single *M. natalensis* till week 20 p. i., but in very low parasites loads in ears, draining lymph node of inoculated ear and forepaws. Therefore, reservoir hosts for this unnamed species from Ghana remains unknown.

CONCLUSIONS

1) The study Vojtkova et al. (2021) provided the first evidence that **multiple and repeated sand fly bites significantly enhance the outcome of *L. major* infection**. This finding appeals to adequate protection of infected humans from sand fly bite not only to prevent transmission but also to prevent enlarged lesions.

2) The study Vojtkova et al (2020) **enriched the animal model spectrum available to both CL and VL research**. All three tested Asian rodent species (*L. lagurus*, *C. griseus* and *P. sungorus*) showed high susceptibility to *L. major*, surpassing the standard model BALB/c mice with the wider anatomical distribution of the parasites and offering the model of both the healing phenotype (*L. lagurus* and *C. griseus*) and the non-healing phenotype (*P. sungorus*). Both, *L. lagurus* and *C. griseus* were also highly susceptible to *L. donovani*, the first showing wider parasite distribution and higher parasite loads and infectiousness than standard model animals (golden hamsters).

3) The studies Sadlova et al. (2019) and Sadlova et al. (2020) **assessed the importance of wild Sub-Saharan rodent species as hosts of *L. major*, *L. donovani* and *L.* (*Mundinia*) from Ghana** based on experimental infections of animals and testing of their infectiousness to sand flies. The results support the involvement of *Mastomys* and *Arvicanthis* in the life cycle of *L. major* in Sub-Saharan Africa. In contrast, their involvement in the transmission cycle of *L. donovani* in East Africa and *L.* sp in Ghana is very unlikely.

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