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# Experimental animal models and vectors of *Leishmania* (*Mundinia*)

# Experimentální zvířecí modely a přenašeči leishmanií podrodu *Mundinia*

Ph.D. thesis/Dizertační 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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**Declaration of supervisor/ Prohlášení školitele**

I declare that Tomáš Bečvář substantially contributed to the experimental work in the projects presented in his thesis and had a principal role in writing in three of the five publications presented.

Prohlašuji, že se Tomáš Bečvář významně podílel na experimentální práci na projektech shrnutých v této dizertační práci a je hlavním autorem textu tří publikací ze pěti uvedených.

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# Abstract

*Leishmania* are vector-borne dixenous protozoan parasites of vertebrates causing diseases collectively called leishmaniases, which threaten more than 1 billion people mainly in tropical and subtropical regions. These parasites are divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania,* and *Mundinia*. The last named are geographically widely dispersed and their distribution covers all continents except Antarctica. Although their medical and veterinary importance is increasing, there is currently almost no information on natural reservoir hosts, vector species, and experimental research options due to the very limited range of model animals. This thesis summarizes our efforts to unravel possible vectors and laboratory models for this subgenus. In the first three studies, we focused on potential models; therefore, we experimentally infected guinea pigs (*Cavia porcellus*), BALB/c mice, Chinese hamsters (*Cricetulus griseus*), and steppe lemmings (*Lagurus lagurus*) with five species of *Mundinia* and we also tested reservoir potential of African grass rat (*Arvicanthis niloticus*) and Natal multimammate mouse (*Mastomys natalensis*) to *Leishmania chancei*. The second part focused on potential vectors of the parasites. We experimentally infected biting midges *Culicoides sonorensis* and sand flies sharing geographical distribution with respective *Mundinia* species to test their susceptibility and vector competence by transmission experiments. We proved that *C. porcellus* are a good model for *L. enriettii*, but their utilisation for other species is limited since only animals infected with *L. enriettii*, *L. orientalis* and *L. martiniquensis* developed temporary pathological changes on the ears. BALB/c mice and *A. niloticus* are not good models for *Mundinia* as they have been proven resistant to the infection. In contrast, *M. natalensis* cannot be excluded from possible involvement in circulation of *L. chancei* in nature because parasites sporadically disseminated to different tissues and survived up to 5 months, albeit in relatively small numbers. *Cricetulus griseus* and *L. lagurus* were proved susceptible to *Mundinia.* While *C. griseus* were asymptomatic but infectious to sand flies and DNA of parasites was detected in animals by the end of experiment 20 weeks post infection, *L. lagurus* exhibited signs of cutaneous and visceral leishmaniases and were also infectious to sand flies during the whole experiment. The second part of our study proved that *C. sonorensis* support the development of *Mundinia* and three human-infecting species, *L. martiniquensis*, *L. orientalis,* and *L. chancei,* can be transmitted to the naïve host by bites of these insects. On the other hand, sand flies sharing geographical distribution with tested *Mundinia* did not transmit parasites to mammals and only *L. martiniquensis* and *L. orientalis* developed late stage infections in *P. argentipes*. These data support the hypothesis of the involvement of biting midges in circulation of *Mundinia*. If these findings will be supported by field studies, it may ultimately lead to redefinition of the entire *Leishmania* genus, where sand flies are dogmatically considered the sole vectors or the removal of *Mundinia* to a separate genus.

# Abstrakt

Leishmanie jsou dvojhostitelští a hmyzem přenášení parazité obratlovců, kteří způsobují onemocnění označované jako leishmanióza, ohrožující více než miliardu lidí po celém světě. Tito parazité jsou aktuálně rozděleni do čtyř podrodů: *Leishmania*, *Viannia*, *Sauroleishmania* a *Mundinia*. Zástupci posledního jmenovaného podrodu se vyskytují na všech kontinentech kromě Antarktidy. Ačkoliv jejich medicínský i veterinární význam stále roste, o jejich rezervoárových hostitelích a přenašečích se ví jen velmi málo, stejně tak jako o možných modelových organismech využitelných pro jejich výzkum. Tato práce shrnuje výsledky naší snahy o rozšíření znalostí o tomto podrodu. V prvních třech studiích jsme se zaměřili na potenciální modelové hostitele a postupně jsme experimentálně infikovali morčata (*Cavia porcellus*), BALB/c myši, křečíky čínské (*Cricetulus griseus*) a pestrušky písečné (*Lagurus lagurus*) celkem 5 druhy leishmanií z podrodu *Mundinia*, a navíc jsme testovali rezervoárový potenciál afrických hlodavců myši nilské (*Arvicanthis niloticus*) a krysy mnohobradavkaté (*Mastomys natalensis*) pro *L. chancei*. Čtvrtá studie byla zaměřena na přenašeče. Provedli jsme experimentální infekce flebotomů, sdílejících rozšíření s danými druhy leishmanií, a tiplíků *Culicoides sonorensis*, abychom mohli sledovat vývoj leishmanií v tomto hmyzu. Navíc jsme u druhů podporujících vývoj leishmanií provedli přenosové experimenty, kdy byli hlodavci vystaveni sání nakažených přenašečů. Dokázali jsme, že ačkoliv jsou morčata dobrým modelem pro výzkum *L. enriettii*, jejich využití pro ostatní druhy není vhodné, neboť dočasné kožní příznaky se objevily pouze u zvířat nakažených *L. orientalis* a *L. martiniquensis*. BALB/c myši nejsou vhodným modelem pro výzkum podrodu *Mundinia*, neboť jsou, stejně jako *A. niloticus* rezistentní vůči infekci. Naopak *M. natalensis* nelze vyloučit z možného zapojení do koloběhu *L. chancei*, neboť parazité ojediněle diseminovali do různých tkání a přežívali i 5 měsíců, byť v relativně malých počtech. Křečíci čínští a pestrušky písečné se ukázaly být jednoznačně nejvhodnějšími modely. Křečíci mohou sloužit jako model pro asymptomatický průběh, protože ač nevykazovali během experimentu žádné příznaky onemocnění, byli infekční pro flebotomy a DNA leishmanií byla na konci pokusu detekována u většiny testovaných zvířat. Pestrušky písečné mohou sloužit jako model pro kutánní i viscerální formy onemocnění, navíc byly silně infekční pro flebotomy v průběhu celého pokusu a DNA leishmanií byla detekována u většiny zvířat v různých částech těla. Naše poslední studie prokázala, že pravděpodobnějším přenašečem *Mundinií* budou spíše tiplíci než flebotomové. Zatímco u testovaných kombinací flebotom x parazit došlo k vývoji pozdní fáze infekce pouze u *P. argentipes* infikovaných druhy *L. orientalis* a *L. martiniquensis*, u testovaných tiplíků *C. sonorensis* došlo k vývoji pozdních forem infekce u všech druhů leishmanií, z nichž 3 byly navíc přeneseny na myši. Tato data podporují hypotézu o zapojení tiplíků jako přenašečů leishmanií, a pokud budou podpořena dalším terénním výzkumem, mohou vést k redefinici rodu *Leishmania*, kde jsou flebotomové chápáni jako výhradní přenašeči nebo dokonce k zavedení samostatného rodu *Mundinia*.

# 1. Introduction

## 1.1 *Leishmania* parasites

*Leishmania* are dixenous protozoan parasites of vertebrates transmitted by sand flies (Diptera: Phlebotominae) or possibly biting midges (Diptera: Ceratopogonidae), which cause diseases collectively called leishmaniases [1]–[3]. These parasites cluster with trypanosomes, creating the family Trypanosomatidae, which belongs to the order Kinetoplastida, typical by the presence of a kinetoplast. This unique structure created from mitochondrial DNA is present in most kinetoplastids and is responsible for RNA editing, which is necessary for functional activation of mitochondria allowing the development of the extracellular and extramammalian part of the life cycle. Parasites which lost the kinetoplast e.g. *Trypanosoma evansi* or *T. equiperdum* also lost the ability to develop in the insect vector, thus are limited to transmission by contaminative way (sexually or by passive transfer in/on vector mouthparts) [4], [5].

*Leishmania* are now divided into four subgenera: *Leishmania*, *Viannia*, *Mundinia,* and *Sauroleishmania*. The first three named are parasites of mammals, but *Sauroleishmania* infect mainly reptiles. Approximately 50 species of leishmania are described, and more than 20 of these can infect humans. The majority of human cases are caused by four parasite species belonging to the subgenus *Leishmania*: *L. donovani*, *L. major, L. tropica* and *L. infantum*. Less frequent but also medically important are parasites of the subgenus *Viannia*, mainly *L. braziliensis* endemic in South America, and *L. martiniquensis* from the subgenus *Mundinia,* which causes disease of humans in South East Asia and disease of horses and cattle in the USA and central Europe [6], [7].

## 1.2 Life cycle

The life cycle of *Leishmania* can be divided into four main steps: a) development in the host, b) transmission from the host to the insect vector, c) development in the vector, and d) transmission from the vector to the vertebrate host.

In mammals, parasites live intracellularly within macrophages, monocytes, and neutrophiles in the form of amastigotes (short, round form without visible flagella). Amastigotes multiply inside the phagolysosome of the parasitised cell, causing its rupture which allows parasites to evade and infect other cells. Thanks to this life strategy, leishmania can spread within the host's body hidden from its immune system. During blood feeding, intracellular and extracellular amastigotes present in the tissues and blood are uptaken by the vector.

In the gut of vectors, females of sand flies or biting midges, the peritrophic matrix is formed within a few hours after blood feeding. Parasites escape from macrophages and undergo morphological changes, developing into procyclic promastigotes (prolonged form with visible short flagella).

Subsequent development depends on the parasite species. We distinguish three main groups by their development strategy: peripylarian, suprapylarian and hypopylarian. Peripylarian species, belonging to the subgenus *Viannia*, move to the hindgut and transform into round flagellated promastigotes that attach to the hindgut wall. Suprapylarian species (subgenera *Leishmania*, *Mundinia*) develop in the abdominal midgut attached to its epithelium, which prevents their expulsion during defecation, and hypopylarian species (subgenus *Sauroleishmania*) develop in hindgut [8]–[10]. Because this thesis focuses mainly on *Leishmania* and *Mundinia* subgenera, their suprapylarian development in vectors will be described in more details below.

After defecation of the vector and attachment to the abdominal midgut, parasites migrate anteriorly to the thoracic midgut and transform into short leptomonads, which multiply and subsequently give rise to two stages: attached haptomonads interacting with chitinase layer of the stomodeal valve and free swimming, non-multiplying metacyclic promastigotes infective to mammalian hosts [11]. During colonisation of the anterior parts of vector's gut (cardia and stomodeal valve), the parasites start to prepare for transmission by destroying the stomodeal valve [12] and by production of a promastigote secretory gel which blocks the gut and prevents the vector from continuous blood feeding [13]. For this reason, vectors are forced to feed repeatedly and regurgitate parasites (plus their exosomes and gut microbiota) into the host's skin. Co-inoculation of various components from the vector 's gut and saliva has also been shown to have an enhancing effect on parasite survival and propagation in the host [14]–[19]. Depending on the parasite/vector combination, development of late stage infections takes 5-8 days [20]. Serafim et al. [21] showed that after a second blood meal, a part of the metacyclic promastigotes transforms into a non-infectious stage called retroleptomonads, which can multiply again. This step should contribute to an increase in the number of parasites in the gut and lead to a higher number of metacyclic promastigotes present during the next blood feeding, but experiments conducted by Ashwin et al. do not support this hypothesis [22], thus further studies are necessary to confirm this phenomenon. After regurgitation of metacyclic promastigotes into the skin, parasites invade the host's immune cells and transform into amastigotes, and the whole cycle begins again.

## 1.3 Leishmaniases

Leishmaniases are present in nearly 100 countries around the world with 1 billion people living in endemic areas. Almost 1 million new cases are described annually, of which more than 30 000 are lethal. Furthermore, a significant part of cases is not recorded since leishmaniases occur dominantly in third world countries where the level of health care and infrastructure is low. Taken together, leishmaniases are the second most prevalent vector-borne disease in the world after malaria, causing tremendous economic and life losses [2].

## 1.4 Forms of the disease

Three basic forms of the disease are described: visceral, mucocutaneous, and cutaneous. The last one can be further divided into several subforms, see below. Progress and overall manifestation generally depend on endo- and exogenous factors such as parasite species, strain and virulence, the infectious dose, type of inoculum, the host immune response, and other biological and environmental factors [23]–[27].

### 1.4.1 Cutaneous form

Cutaneous leishmaniases (CL) are the most frequent main form characterised by development of localized skin nodules, ulcers or lesions close to the vector bite site, which can spontaneously heal, persist or progress to other forms in some cases [28]. Due to various pathological signs of cutaneous leishmaniases, we distinguish four main forms of CL: localised CL (LCL), diffuse CL (DCL), disseminated CL (DL), and post kala-azar dermal leishmaniasis (PKDL) [25]. The main causative agents of CL are *L. major*, *L. tropica*, *L. aethiopica* in the Old World and *L. mexicana* or *L. braziliensis* in the New World [29]. It usually causes only a little burden in the terms of pathogenicity to humans, but stigma and socioeconomic burden on affected people is much higher as lesions are usually present on body parts exposed to the vector bite (face, arms, legs) [30]. LCL is the most common and least severe form of leishmaniasis, manifesting in the early stages of infection as a single localised papule that may enlarge and develop into dry or wet lesion. So called “dry lesions” are usually caused by *L. tropica,* while wet ulcerative lesions are often caused by *L. major* or *L. mexicana*. Both can heal spontaneously within a few weeks or months from the onset of the first symptoms or various healing techniques (*in situ* drug application or cryotherapy) can be used. DCL can develop from LCL when parasites or their antigens spread through the body of the host and form non-ulcerative nodular metastatic lesions, which may appear even many months after healing of the primary lesion [31]. DL is usually caused by parasites of the subgenus *Viannia*. It manifests mainly as discontinuous pleomorphic lesions which are often associated with the development of mucosal symptoms, relapses and resistance to standard treatment even in immunocompetent hosts [32]. Visceral leishmaniasis, mainly caused by *L. donovani*, which will be described in details later, can be followed by the development of a cutaneous form called PKDL, which manifests as multiple nodules or papulae affecting parts or the entire host body. In contrast to VL, PKDL is not fatal and does not cause serious pathological signs, but the host can still serve as a source of infection for vectors, and the visual appearance of the disease leads to serious social exclusion and stigmatisation [30], [33].

### 1.4.2 Mucocutaneous form

Mucocutaneous leishmaniasis is caused mainly by New World species e. g. *L. braziliensis*, *L. panamensis,* and *L. amazonensis* or *L. macropodum* from the subgenus *Mundinia* and is characterized by invasion of soft tissues such as the nose, ears, eye lids, lips, or inner mouth parts and is often accompanied by secondary bacterial infections that enhance pathological effect of the disease. Espundia, as MCL is called in endemic localities, can occur even years after the primary lesion since parasites escape the host's immune system and covertly spread throughout the body via the lymphatic system or immune cells [34]. The immune background is poorly understood and consists of complex interplay between the parasite and the host [35].

### 1.4.3 Visceral form

The most severe form, called visceral leishmaniasis, is caused mainly by *L. donovani*, *L. infantum,* and *L. martiniquensis*. The majority of cases come from the Indian peninsula (India, Bangladesh) and Eastern Africa (Sudan) and the disease is called kala-azar ("Kala" – Hindi word for black; "Azar" – Persian word for fever). The first symptoms, which are high fever, poor appetite, weight loss, nausea, and diarrhoea, usually appear in a period of 2 weeks to 18 months post bite of infectious vector. Within the body of the host, parasites spread and proliferate in the mononuclear phagocyte system (macrophage, neutrophils) and cause hepatosplenomegaly, and infection of the bone marrow leading to pancytopenia. Only about 10 % of the infected individuals are symptomatic, but approximately 75-95% of these cases are lethal, if not treated. High-sensitivity diagnostic methods for parasite detection in asymptomatic patients are developed and used in the field to prevent spread of parasites, as asymptomatic carriers can also serve as a source of infection [36]–[38].

## 1.5 Vectors

Sand flies (Diptera: Phlebotominae) are considered the main vectors of leishmania. We distinguish more than 800 species belonging to this group and from these approximately 100 species can transmit parasites, while only 30 are responsible for spread of the human pathogens (for example *P. papatasi*, *P. argentipes*, *P. sergenti*, *P. pernicious,* and *Lu. longipalpis*) [39]. Sand flies are widespread in tropical, subtropical, and warm areas of temperate climate zones. Old-World species of genera *Phlebotomus* and *Sergentomyia* often inhabit arid and semi-arid ecosystems, while New World species (genus *Lutzomyia*) are mostly typical for forest and rainforest areas. Conversely to mosquitos, sand fly larvae do not develop in the water environment, but use moisturised soil with high amount of organic material to rear (especially rodent burrows, nests, termite mounds and manure) [40], [41]. Adults do not fly far from their hatcheries, so the presence of burrows and nests of potential

reservoir hosts near human dwellings and outside working places is crucial for successful transmission of parasites to human hosts from wild reservoirs [42], [43].

Due to the development of more sensitive methods of molecular biology, alternative vectors of *Leishmania* have been suggested in the last several years, because the parasite DNA has been detected in various blood-feeding arthropods (ticks, fleas, and biting midges). It is crucial to keep in mind that the sole presence of parasite DNA is not reliable proof of vector competence, since *Leishmania* survive non-specifically in blood remnants during the first days after feeding and although the infection may be lost with defecation of the arthropod, parasite DNA can be still detectable [44]–[46]. That is why several criteria were established to evaluate the vector potential of suspected arthropods by Killick-Kendrick [47] and later updated by Ready [48]:

- On more than one occasion, promastigotes are isolated and/or typed from several unambiguously identified wild female flies that did not contain recent blood meals (less than 36 h).
- Infective forms or 'luxuriant growth' of *Leishmania* are observed in the anterior midgut and in the stomodeal valve of naturally infected female flies or of colony flies after xenodiagnosis.
- A sand fly species is attracted to humans and any reservoir host and bites them.
- Strong ecological associations, including seasonality, are shown between the fly, humans, and any reservoir host.
- Experimental transmission is achieved after infection from a natural host species or an equivalent laboratory model.
- Using retrospective data, mathematical modelling demonstrates that the vector is essential for maintaining transmission with or without the involvement of other vectors.
- Mathematical modelling based on a planned control programme shows that disease incidence significantly decreases following a significant decrease in the biting density of the specific vector.

The last two presumed criteria are more or less hypothetical, and their practical usage is so far very limited.

Experiments with ticks and fleas did not so far prove their involvement in circulation of parasites, even though these arthropods can influx parasites during blood feeding [49]–[54]. On the contrary, biting midges have been shown to have a high potential to transmit *Leishmania*. Dougall et al. [3] described successful colonisation of guts and stomodeal valves of Australian biting midges (Forcipomyia: Lasiohelea) by *L. macropodum* responsible for kangaroo mucocutaneous leishmaniases [55]. These findings inspired other researchers to test the vector potential of biting

midges under laboratory conditions, and later Seblova et al. [44] showed that *L. enriettii* is capable of developing in *Cu. Sonorensis*, the North American biting midge.

## 1.6 Reservoir hosts

Infectious diseases of humans can be divided into two big groups according to the source of pathogen: zoonoses and anthroponoses. For leishmaniases, the zoonotic cycle is typical as parasites circulate between animal hosts (mainly rodents and canines) and vectors, while humans are only accidental hosts. However, the anthroponotic cycle is also present in some regions with a high prevalence, usually with *L. tropica* and *L. donovani* as causative agents [56].

A basic premise for identifying the reservoir host of pathogen is that it occurs in the endemic localities, supports long term parasite survival, and may serve as a source of infection to vectors. In most cases, true reservoirs do not show serious signs of infection or are asymptomatic thanks to the long-term evolutionary development of the parasite-host relationship, which also helps the parasite to survive for long periods of time in the host body, as a commensal rather than a parasite [57].

Other criteria must be carefully interpreted and are often modified to account for specific differences between endemic localities and causative species. The list of criteria is dynamic and varies regarding to authors opinions. For example, Ashford suggested that reservoirs should form a large mammalian biomass in region and lives long enough to surpass the “non-transmission season” [57]. Silva et al. claim that the prevalence must be above 20%, which is also highly questionable since these statistics can be biased by the sampling method or depending on the season of capture [58]. In recent years the distinction between so-called ‘source hosts’ and “sink hosts” has been widely accepted. The source host can serve as a source of infection to vectors that transmit pathogens to other individuals and sinks can be infected, but they do not spread the parasites [59]. The potential for “source or sink” can be tested in laboratory experiments, called xenodiagnoses, where experimentally infected or captured animals are exposed to naïve vectors to test whether parasites can be transmitted [27].

## 1.7 Animal model organisms

As already described, leishmaniases cannot be understood as a standard disease with well specified signs and course of infection; more precisely, it is a group of diseases caused by similar organisms. There are huge differences even between symptoms caused by the same parasite strain in various hosts. A good animal model should mimic the desired group of pathological features and immunological responses observed in humans when exposed to different parasite species/strains, therefore a wide range of model organisms should be used [60].

The laboratory mouse (*Mus musculus domesticus*) is the most exploited animal model in the biological and biochemical field. These mammals are easy and unexpensive to maintain and there is an immense variation of inbred strains with different, but well defined spectre of traits enabling huge scope of experiments [61]. Research in mice, for example, has enabled the role of various cytokines, immune cells, mechanisms of resistance or disease course to be discovered [62], but it has also played a major role on "the other side of the circle' by revealing the role of sand fly saliva before and after parasite inoculation [63], [64].

On the other hand, the greatest strength of inbred models is also their greatest weakness. In nature, parasites are exposed to huge host variability and inbred strains cannot cover the whole spectrum of signs, therefore polymorphic or especially wild rodents are valuable models that more precisely mimic the natural situation thanks to their genetic polymorphism [62].

Guinea pigs are also often used as a laboratory model for biological/biochemical research, but their use in field of leishmaniases is limited since they have so far found susceptible only to infection caused by *L. enriettii* that causes cutaneous lesions with healing phenotype [65]–[69].

In recent years, Chinese hamsters (*Cricetulus griseus*) are becoming popular model animals in the leishmaniases field. These rodents were first used in 1930's [70]–[72] and they were described as extraordinarily susceptible to infection with histopathological signs similar to those of humans. Afterwards, they have been neglected as model animals, although they are commercially available and easy to maintain. In recent years, they were used to study visceral leishmaniasis caused by *L. donovani* and cutaneous leishmaniasis caused by *L. major* [73]. Similarly to Chinese hamsters, steppe lemmings (*Lagurus lagurus*) have also been found extremely susceptible to visceral leishmaniasis caused by *L. donovani* and *L. major*, suggesting a huge potential for future studies [73].

As previously written, wild, genetically polymorphic animals are becoming more important in research due to their better imitation of the real situation. Ideally, animals that serve as reservoir hosts for leishmania are used as models. These organisms allow us to study the natural dynamics of infection and the parasite-host relationship. In reservoir hosts, parasites often cause very mild symptoms or the infection is asymptomatic, although parasites can be found in organs exposed to vector bites; this contrasts with classical models like BALB/c mice, where parasites usually cause serious pathological symptoms and multiply in tissues to unnatural densities. On the other hand, most of the wild rodents are not commercially available and their breeding is difficult, so only a limited number of species have been studied, such as *Sigmodon hispidus*, *Trichomys laurentius*, *Peromyscus yucatanicus* [62], *Arvicanthis niloticus* and *A. neumanni* [74] or *Meriones shawi* [75].

## 1.8 *Mundinia*

Review summarizing basic information about the subgenus *Mundinia* is one of publications included in these theses, nevertheless it is good to mention here some more details.

*Mundinia* is the most recently described *Leishmania* subgenus [6], currently consisting of six species. While *L. enriettii*, *L. macropodum,* and *L. procaviensis* are parasites of wild mammals [55], [66], [76], the other three species, *L. martiniquensis*, *L. orientalis,* and *L. chancei,* can also infect and cause disease in humans [77]–[79]. These parasites are found on all continents except Antarctica, which is hypothesised to be caused by their ancestral origin and spread after the breakup of Gondwana [80].

*Leishmania enriettii*, a parasite isolated only from domestic guinea pigs in Brazil, was discovered in 1948 [66]. The long-time gaps and geographical diversity between individual cases contributed to the fact that natural reservoirs and vectors are still unknown even 80 years after parasite discovery. These parasites cause cutaneous and visceral forms of leishmaniases in guinea pigs *Cavia porcellus* and golden hamsters *Mesocricetus auratus* [65], [67], [81]. The identity of vectors remains uncertain, but general knowledge about *Mundinia* and works published by Seblova [81] and Dougall [3] suggest that biting midges may play a major role in their transmission in nature, but the involvement of sand flies living in endemic localities like *Lu. monticola* [82] cannot be excluded. The same uncertainty exists in the case of natural reservoirs, since experimental infections of mice, dogs, rats, Rhesus macaque and wild guinea pigs *Cavia aperea* have failed [66].

*Leishmania macropodum* is the only known leishmania from Australia and, unlike other *Mundinia*, causes the mucocutaneous form of the disease with lesions spontaneously healing over time [55], [83]. The most probable vectors are biting midges of the genus *Forcipomyia* (*Lasiohelea*), since heavy mature infections were observed in a wild-caught defecated specimen [3]. These parasites were discovered in captive red kangaroos *Macropus rufus* and later isolated from other kangaroo species kept in captivity (*Macropus robustus woodwardi, Macropus bernardus,* and *Macropus agilis agilis)*, so the involvement of wild kangaroos in parasite circulation is very probable.

The last animal-infecting species is *L. procaviensis,* isolated from hyrax (*Procavia capensis*) in Namibia [76], [84].

Three other *Mundinia* species, *L. martiniquensis*, *L. orientalis,* and *L. chancei,* can infect humans. Symptoms range from a single spontaneously healing cutaneous lesion to a visceral form, depending on the species/strain of the parasite and endogenous host factors. *Leishmania martiniquensis* was first isolated from an HIV-positive human on the Martinique island [85], but most human cases were reported from Thailand [86]. The pathogenicity of this species is not limited to HIV-positive patients, since it has also been isolated from an HIV-negative patient and caused a single cutaneous lesion [87], and asymptomatic infections are assumed to be present in immunocompetent individuals [88]. However, the majority of the described symptomatic cases are

from the HIV-positive community [89]–[91] . The parasites have also been isolated from cutaneous lesions of horses in Florida, Germany, Switzerland, the Czech Republic, and cows from Switzerland [92]–[94]. Due to geographical and host differences, searching for reservoir hosts and vectors is difficult. Based on data acquired so far, we assume the involvement of domestic and wild cattle or rodents. In the case of vectors, it seems most likely that, as with other *Mundinia*, biting midges are the responsible agents, but we cannot rule out local sand fly species [95]–[98].

*Leishmania orientalis* has so far been isolated only from humans. Although there are not many described cases, the true prevalence is probably higher, since research in a HIV-positive community in Trang province (Thailand) revealed a 25% prevalence of leishmaniases caused mainly by *L. martiniquensis* and *L. orientalis*, although most infections are asymptomatic, even in HIV-positive patients. Natural reservoirs and vectors are unknown, but most probably, biting midges play the major role in the circulation of these parasites since *L. orientalis* DNA was detected in *Culicoides* species and development of *L. orientalis* was also observed in laboratory experiments [86], [99], [100].

The last currently described species is *Leishmania chancei,* causing self-healing cutaneous leishmaniasis in children, young people and newcomers in the Volta region of Ghana, where more than 8000 cases have been recorded. In the native language, the disease is called „agbamekamu", which can be translated as „a gift from somebody who has returned from a journey" because locals believe the disease was brought from neighbouring Togo with the new arrivals. With respect to the identity of the natural reservoirs and the vectors, the same uncertainty applies here as with other *Mundinia* species [79], [101].

# 2. Objectives

*Mundinia* is the most enigmatic of all the *Leishmania* subgenera. It's widespread distribution and large range of potential hosts and vectors bring many questions for researchers. The topic of this thesis is to illuminate some aspects of the biology of these parasites. We focused on establishment of mammalian laboratory models for the study of *Mundinia* and on laboratory testing of various vector species that can be maintained in the insectary of the Department of Parasitology.

1) **Establishment of laboratory models for *Mundinia* research.** A good model animal should mimic similar pathological signs and immune response to the parasite species present in humans. The immune response against the same parasite species varies in different organisms and the mechanisms described in the standard inbred mouse model are not universally applicable, so the only way to find a proper model organism is to study a wider range of potential models. We, therefore, decided to compare the development of *Mundinia* in four rodent species: the classic BALB/c mouse and guinea pigs (*Cavia porcellus*) models and

not commonly used Chinese hamsters (*Cricetulus griseus*) and steppe lemmings (*Lagurus lagurus*). We also tested rodent species *Mastomys natalensis* and *Arvicanthis niloticus,* abundant in endemic sites, for their reservoir potential for *L. chancei.*

2) **Comparison of *Mundinia* in various insect species.** While *Leishmania* and *Viannia* are certainly transmitted by phlebotomine sand flies (Diptera: Phlebotominae), there is lack of data on natural vectors of *Mundinia*. Biting midges (Diptera: Ceratopogonidae) most likely play an important role in their circulation, but local sand fly species cannot be ruled out. Therefore, we decided to test under laboratory conditions the vector capacity of sand flies, which are available in our insectary and share the same geographical distribution as *Mundinia;* and also to test the vector capacity of biting midges *Culicoides sonorensis,* which were available from breeding colony in England.

# 3. Publications

Sadlova, J., **Becvar, T**., Volf, P., (2022). Transmission of Enigmatic *Mundinia* parasites. *Journal of Infection Diseases and Therapy*, 10:507.

**Becvar, T**., Siriyasatien, P., Bates, P., Volf, P., & Sádlová, J. (2020). Development of *Leishmania* (*Mundinia*) in guinea pigs. *Parasites & vectors*, *13*(1), 1-6.

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.

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

**Becvar, T**., Vojtkova, B., Siriyasatien, P., Votypka, J., Modry, D., Jahn, P., ... & Sadlova, J. (2021). Experimental transmission of *Leishmania* (*Mundinia*) parasites by biting midges (Diptera: Ceratopogonidae). *PLoS Pathogens*, *17*(6), e1009654.

# Transmission of Enigmatic *Mundinia* parasites

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# Transmission of Enigmatic *Mundinia* Parasites

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**Abstract**

*Mundinia*, the recently established *Leishmania* subgenus, includes five species, three of which are pathogenic to humans. Although *Mundinia* represents the oldest branch of the genus *Leishmania*, most species have escaped the attention of scientists and have only been discovered in the last 20 years. Their transmission ecology is enigmatic, with unknown identity of reservoir hosts and insect vectors. This mini-review summarizes the history of *Mundinia* discovery and the current knowledge about the reservoirs and vectors with emphasis on the role of biting midges (Diptera: Ceratopogonidae) in their transmission.

**Keywords:** *Mundinia*; *L. macropodum*; *L. enriettii*; *L. orientalis*; *L. martiniquensis*; *Culicoides sonorensis*

## Introduction

*Leishmania* (Kinetoplastida: Trypanosomatidae) are digenetic parasites circulating between mammal or reptile vertebrate hosts and insect vectors. They are currently divided into four subgenera. The most important and abundant human parasites belong to subgenera *Viannia* and *Leishmania*, the third subgenus *Sauroleishmania* includes reptile parasites [1]. The fourth subgenus *Mundinia* was established in 2016 by Espinosa, et al. for five species previously known as the *L. enriettii* complex. It is the most ancient group, diverging at the base of a phylogenetic tree of the *Leishmania* genus [1].

## Literature Review

*Mundinia* is an exceptional and enigmatic subgenus in several aspects:

### Worldwide geographical distribution

The five species are distributed on all continents except Antarctica (Figure 1): *L. enriettii* occurs in Brazil, *L.* sp. strain GH5, not yet formally described, in Africa (Ghana), *L. orientalis* in southeast Asia and *L. macropodum* in Australia, representing the only *Leishmania* species known from that continent. The fifth species, *L. martiniquensis*, has been reported from areas as distant as the island of Martinique, Florida, central Europe and southeast Asia. This wide distribution of the subgenus has been attributed to its ancient origin and is explained by the formation of individual species from a common ancestor after the breakup of Gondwana [2]. However, in the case of *L. martiniquensis*, the recent introduction and anthropogenic spread must be considered, as the infection is only known from humans and domestic animals.

**Figure 1:** Geographical distribution of five *L.* (*Mundinia*) species and schematic representation of hosts from which they were isolated. **Note:** (●) *L.* (*Mundinia*) *enriettii* (●) *L.* (*Mundinia*) *martiniquensis* (●) *L.* (*Mundinia*) sp. from Ghana (●) *L.* (*Mundinia*) *orientalis* (●) *L.* (*Mundinia*) *macropodum*

### The hidden way of life

Despite their ancient origins, three species (*L. macropodum*, *L. orientalis* and the unnamed African species) have escaped scientific knowledge and have only been discovered in the last 20 years. The remaining two species have been known for a long time, but were reported very sporadically. This is particularly true for the Brazilian *L. enriettii*. It was first isolated in 1948 from domestic guinea pigs, then detected 20 years later in 1967 and after another long gap again in 1994, each time in domestic guinea pigs [3,4]. However, as a species non-pathogenic to humans, *L. enriettii* has become a popular model organism for experimental studies. *Leishmania martiniquensis* was first described in 1995 in an HIV-infected man in Martinique and was initially misidentified as a monoxenous "lower" trypanosomatid. Two years later, the same parasite was also reported in Martinique in an immunocompetent patient [5,6]. In 2014, Pothirat, et al. showed that autochthonous infections in cattle or horses sporadically reported from Florida, Switzerland and Germany and occasionally causing human infections in south-east Asia, belong to the same *Mundinia* species [7-11]. In the same year the name *L. martiniquensis* was established [12]. In Thailand, *L. martiniquensis* is not the only species of the subgenus *Mundinia*, the other being *L. orientalis*, formally described in 2018 [13]. In older literature, all Thai isolates were confused and referred to as "*L. siamensis*". This name was first used by Sukmee, et al. for the sequence of their isolate submitted in GenBank and then introduced into the scientific literature by Müller, et al. [9,14]. As the name "*L. siamensis*" has never been formally described, it is not a valid name and should no longer be used.

In horses and cattle, *L. martiniquensis* causes skin lesion while in humans, a wide spectrum of manifestations ranging from cutaneous and diffuse cutaneous to visceral forms has been reported [5,6,8-10,14,15]. Similarly, *L. orientalis* is also responsible for both diffuse cutaneous and visceral leishmaniasis [16]. The African *Mundinia* species was hidden among human cutaneous leishmaniasis cases in the Volta region of Ghana caused by *L. major*. However, in 2009 the parasite was identified as a new *Leishmania* species, in 2015 the strain was isolated and according to phylogenetic analysis placed into the subgenus *Mundinia* [17,18]. Australia was traditionally considered a continent where *Leishmania* species were not

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endemic until 2004, when a case of autochthonous cutaneous leishmaniasis was described in red kangaroos (*Marcopus rufus*) in Australia's Northern Territory [19].

### Unknown reservoirs

No reservoir wild hosts have been identified for any species of the subgenus *Mundinia*. *Leishmania enriettii* has only been found in domestic guinea pigs, but experimental infections of wild guinea pigs (and several other species of wild mammals) have failed [3]. In the case of *L. macropodum*, all Australian causes of CL have been diagnosed in captive animals - red kangaroos (*Macropus rufus*), the northern wallaroo *(M. robustus woodwardi)*, the black wallaroo (*M. bernardus*) and the agile wallaby (*M. agilis agilis*) [19,20]. All records have been restricted to the rural Darwin area in the Northern Territory with a humid tropical climate, where only agile wallabies are native, but further epidemiological studies are needed to determine their possible role as reservoirs of *L. macropodum* [20]. The autochthonous cases of *L. martiniquensis* in horses and cattle in central Europe and Florida are truly enigmatic, the animal reservoirs are completely unknown and have yet to be identified, and the same is true for human-infecting species in Thailand and Ghana. In Thailand, *Leishmania* DNA has been found in *Rattus rattus*, making this rodent species suspected of serving as a reservoir, but the capacity of black rats to harbour the parasite long-term and their infectiousness to sand flies has not yet been proven [21]. Experimental studies on the genera *Arvicanthis* and *Mastomys* have been done but did not demonstrate a reservoir role of these rodents for the Ghanaian *Mundinia* species [22]. *Mundinia* have probably evolved a well-balanced relationship with their reservoir hosts over a long evolutionary history and cause only asymptomatic unapparent infections, which are thus difficult to detect.

### Unique vectors

Although species identity for vectors of any *Mundinia* species is not confirmed yet, the findings so far are fascinating. For many years, there was a widely accepted paradigm that *Leishmania* species pathogenic to humans are transmitted exclusively by phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World [23,24]. But most *Leishmania* of the subgenus *Mundinia* have never been found in sand flies, with exception of PCR detection of *Mundinia* DNA in Thai *Sergentomyia* (*Neophlebotomus*) *gemmea* and *S. iyengari* [25,26]. However, molecular findings cannot be considered as a proof of the vector identification. Without microscopy, it is not possible to distinguish late mature infections from early ones which are non-specific and may be lost with defecation in refractory vectors [27]. The paradigm of exclusive transmission of *Leishmania* by sand flies has been seriously challenged by discoveries related to the subgenus *Mundinia* published in the last decade.

In areas of Australian *L. macropodum* distribution, the extensive field survey revealed DNA of the parasites in 6% females of three species of biting midges of the genus *Forcipomyia* (Diptera: Ceratopogonidae) while no *Leishmania*-positive specimens were detected among nearly 2000 sand fly females collected. Importantly, heavy late stage infections and presence of metacyclic forms in biting midges were confirmed also microscopically [28]. Consequent laboratory experiments have revealed susceptibility of North American *C. sonorensis* to infection with *L. enriettii, L. macropodum* and *L. orientalis* [29,30].

Altogether, these studies had showed that biting midges satisfy most of Killick-Kendrick's criteria of vector competence [31]. However, the most important criterion was still missing, namely the demonstrations of transmission by the vector bite. This has changed with the recent publication of Becvar, et al [32]. These authors compared the development of all 5 *Mundinia* species in biting midges and sand flies and demonstrated experimental transmissions of three *Mundinia* species, *L. martiniquensis, L. orientalis* and *L.* sp. from Ghana by biting midges *C. sonorensis* to BALB/c mice. The parallel experiments with the same parasite lines and epidemiologically relevant sand fly species resulted in limited infections and no transmission to mice. This study therefore provides the strong evidence that biting midges may play a role in the transmission of *Mundinia* parasites.

## Discussion and Conclusion

Further field-based studies are necessary to identify particular vector species in areas of pathogen transmission. Besides Australian *Forcipomyia* species, another good candidate emerged in 2021, when *L. martiniquensis* DNA was detected in wild caught *C. mahasarakhamense* near the home of a leishmaniasis patient in Lamphun province, northern Thailand [33].

Finally, the reduced repertoire of enzymes modifying LPG side chains, a molecule important for *Leishmania* and *Viannia* development in the sand fly, is also indicative of non-standard *Mundinia* vectors [34]. The identification of vectors and reservoir hosts of *Mundinia* parasites is a significant challenge and is also of importance in the context of the unique geographical distribution of *L. martiniquensis*, which is the only species of the *Leishmania* genus occurring in Central Europe.

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# Development of *Mundinia* in guinea pigs

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Parasites & Vectors

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# Development of *Leishmania* (*Mundinia*) in guinea pigs

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**Abstract**

**Background:** Leishmaniasis is a human and animal disease caused by parasites of the genus *Leishmania*, which is now divided into four subgenera, *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia*. Subgenus *Mundinia*, established in 2016, is geographically widely dispersed, its distribution covers all continents, except Antarctica. It consists of 5 species; *L. enriettii* and *L. macropodum* are parasites of wild mammals while *L. martiniquensis*, *L. orientalis* and an unnamed *Leishmania* sp. from Ghana are infectious to humans. There is very little information on natural reservoir hosts and vectors for any *Mundinia* species.

**Methods:** Experimental infections of guinea pigs with all five *Mundinia* species were performed. Animals were injected intradermally with $10^7$ culture-derived promastigotes into both ear pinnae. The courses of infections were monitored weekly; xenodiagnoses were performed at weeks 4 and 8 post-infection using *Lutzomyia migonei*. The distribution of parasites in different tissues was determined *post-mortem* by conventional PCR.

**Results:** No significant differences in weight were observed between infected animals and the control group. Animals infected with *L. enriettii* developed temporary lesions at the site of inoculation and were infectious to *Lu. migonei* in xenodiagnoses. Animals infected with *L. martiniquensis* and *L. orientalis* developed temporary erythema and dry lesions at the site of inoculation, respectively, but were not infectious to sand flies. Guinea pigs infected by *L. macropodum* and *Leishmania* sp. from Ghana showed no signs of infection during experiments, were not infectious to sand flies and leishmanial DNA was not detected in their tissue samples at the end of experiments at week 12 post-inoculation.

**Conclusions:** According to our results, guinea pigs are not an appropriate model organism for studying *Mundinia* species other than *L. enriettii*. We suggest that for better understanding of *L.* (*Mundinia*) biology it is necessary to focus on other model organisms.

**Keywords:** *Leishmania*, *Mundinia*, Guinea pig, *Leishmania enriettii*, *Leishmania martiniquensis*, *Leishmania orientalis*, *Leishmania macropodum*, Animal model

## Background

Leishmaniases are vector-borne diseases whose etiological agents are protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). Known previously as the *L. enriettii* complex, the subgenus *Mundinia* was established recently and currently contains 5 species: *L. enriettii*, *L. macropodum*, *L. orientalis*, *L. martiniquensis* and an unnamed *Leishmania* sp. from Ghana [1–3]. According to phylogenetic analyses, this subgenus is the first to branch from the other *Leishmania* subgenera, indicating that species of this subgenus are likely to represent the most ancient and divergent group of species within the *Leishmania* [2, 4]. The geographical distribution of *Mundinia* species covers all continents except Antarctica, which can be

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explained by the formation of individual species from their common ancestor after the breakup of Gondwana [2].

Many important details of the biology of these parasites are unknown. The identity of the insect vectors responsible for transmission of *L.* (*Mundinia*) has not been confirmed for any species yet. It has been assumed that these parasites, similar to other *Leishmania*, would be transmitted by sand flies of the genus *Phlebotomus* and/or *Sergentomyia* in the Old World and *Lutzomyia* in the New World (Diptera: Phlebotominae), and this may be the case. Recently, however, *Forcipomyia* (*Lasiohelea*) biting midges (Diptera: Ceratopogonidae) were reported as likely vectors of *L. macropodum* in Australia [5], and laboratory experiments have revealed a high susceptibility of *Culicoides sonorensis* to *L. enriettii* [6]. The observations raise the possibility of non-sand fly vectors for at least some of the *Mundinia*.

Similarly, there is little current information on the natural mammalian reservoir hosts for these parasites. *Leishmania enriettii*, is a parasite that has only ever been found in domestic guinea pigs (*Cavia porcellus*) in Brazil, first isolated in the 1940s [7]. The natural host of *L. enriettii* is not known although often assumed to be a wild rodent of some kind. *Leishmania macropodum* is a parasite first isolated from red kangaroos in Australia, but from a game park in a region where these animals are not found [5]. There is evidence of *L. macropodum* infection in three other species of Australian macropods, which are more likely to be the true host(s) of this parasite [7]. On the other hand, human cases have been described with *L. martiniquensis*, *L. orientalis* and *Leishmania* sp. from Ghana. *Leishmania martiniquensis* was first isolated from a HIV positive man on Martinique Island in 1992 [8]. According to recent findings, this *Leishmania* species has a worldwide distribution with single or multiple cases reported from various continents where these parasites were isolated from various hosts such as horses, cows and humans [9–12]. *Leishmania orientalis* was formally described in 2018 [13]; in the past it was reported as "*L. siamensis*" [9, 14] but this name is a *nomen nudum* and should not be used anymore. *Leishmania* sp. from Ghana is a species causing cutaneous leishmaniasis in the Volta region in Ghana [4]. The last two species were not isolated from any mammalian species, except humans, and the identity of their reservoir hosts remains enigmatic.

Since very little is known about biology of these neglected parasites, the aim of our study was the establishment of model host organisms, which would enables testing their behaviour and properties in a mammalian host. Here we present results of experimental infections in guinea pigs with all five known *L.* (*Mundinia*) species.

## Methods

### Parasites and guinea pigs

*Leishmania enriettii* (MCAV/BR/45/LV90), *L. macropodum* (MMAC/AU/2004/AM-2004), *Leishmania* sp. from Ghana (MHOM/GH/2012/GH5), *L. orientalis* (MHOM/TH/2014/LSCM4) and two strains of *L. martiniquensis* (MHOM/MQ/1992/MAR1 and MHOM/TH/2011/CU1) were used. Parasites were maintained at 23 °C in M199 medium supplemented with 10% fetal calf serum (Gibco, Prague, Czech Republic), 1% BME vitamins (Sigma-Aldrich, Prague, Czech Republic), 2% sterile urine and 250 µg/ml amikacin (Amikin, Bristol-Myers Squibb, Prague, Czech Republic). In our laboratory both strains were maintained in a cryobank with 2–3 sub-passages *in vitro* before experimental infections of guinea pigs and no passages in animals were performed. Before experimental infection, parasites were washed by centrifugation (6000 × *g* for 5 min) and resuspended in saline solution.

Female guinea pigs (Dunkin-Hartley) originating from AnLab (Prague, Czech Republic) were maintained in groups of 2 specimens in T4 boxes (58 × 37 × 20 cm); Velaz (Prague, Czech Republic) equipped with bedding (German Horse Span; Pferde, Prague, Czech Republic), breeding material (Woodwool) and hay (Krmne smesi Kvidera, Spalene Porici, Czech Republic), provided with a feed mixture V2233 Ms-H Guinea Pig maintenance (AnLab) and water *ad libitum*, with a 12 h light/12 h dark photoperiod, temperature of 22–25 °C and relative humidity of 40–60%. At the beginning of experiments the average weight of animals was 499 g and average age was 7 weeks.

### Infection and xenodiagnoses of guinea pigs

Eighteen guinea pigs (*Cavia porcellus*) anaesthetized with ketamin/xylazin (37.5 mg/kg and 1.5 mg/kg, respectively) were injected with $10^7$ stationary-stage promastigotes in 5 µl of sterile saline intradermally into the ear pinnae of both ears. The course of infection was recorded weekly. Three animals inoculated with the same volume of saline solution were used as a control for external signs of infection.

Xenodiagnoses were performed at weeks 4 and 8 post-infection (pi) using the permissive vector *Lutzomyia migonei* [15]. Five to six-day-old *Lu. migonei* were placed into plastic vials covered by fine nylon mesh and allowed to feed on the ear pinnae of anaesthetized animals. Engorged individuals were maintained for two days at 25 °C and then stored in tissue lysis buffer (Roche, Prague, Czech Republic) at -20 °C in pools of 5 females for subsequent PCR. Altogether 192 sand fly pools were tested.

At the end of the experiments, 12 weeks post-infection (pi), the hosts were euthanized, dissected and tissues

from ears, paws, ear-draining lymph nodes, spleens, livers and blood were stored at −20 °C for subsequent PCR.

### Conventional PCR

DNA extraction from vectors and animal tissues was performed using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. The total DNA was used as a template for PCR amplification with the primers for a 246 bp long ITS1 sequence (forward primer 5′-AGA TTA TGG AGC TGT GCG ACA A-3′ and reverse primer 5′-TAG TTC GTC TTG GTG CGG TC-3′). Reactions were performed using EmeraldAmp® GT PCR Master Mix and cycling conditions were as follows: step 1, 94 °C for 3 min 30 s; step 2, 94 °C for 30 s; step 3, 60 °C for 30 s; step 4, 72 °C for 20 s; step 5, 72 °C for 7 min; followed by cooling at 12 °C. Steps 2–4 were repeated 35 times. Samples were analysed using 1% agarose gels.

### Statistical analysis

Statistical analyses were carried out using R software ((http://cran.r-project.org/). Correlation of animal weight in different groups (infected and non-infected) and time was tested by co-variance analysis.

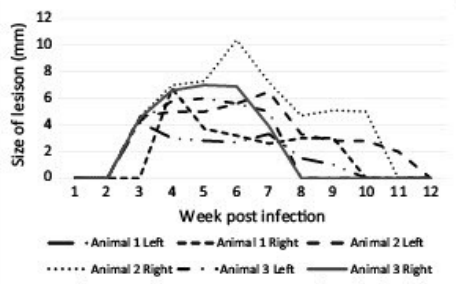


**Fig. 1** Development of lesions in guinea pigs infected with *L. enriettii*

## Results

No significant differences in weight were observed between infected animals and the control group (*L. macropodum*, $P=0.70$; *L. enriettii*, $P=0.12$; *L. martiniquensis* MAR1, $P=0.77$; *L. martiniquensis* CU1, $P=0.12$; *Leishmania* sp. from Ghana, $P=0.20$; *L. orientalis*, $P=0.11$; see Additional file 1: Table S1).

Development of dry lesions was observed in animals infected with *L. enriettii*. The lesions appeared on ear pinnae (the site of inoculation) by week 2–3 pi, increased in size through to week 5–6 pi and then healed, completely disappearing between weeks 8–12 pi (Figs. 1, 2a). Animals were efficiently infectious to sand flies on week 4 pi (9/16 positive pools) while infectiousness was reduced by week 8 pi (1/16 positive pools).

In animals infected with *L. martiniquensis* (MAR1), temporary erythema was observed at the site of inoculation between weeks 4–8 pi, but no lesions developed and animals were not infectious to sand flies (0/32 positive pools).

Erythema at the site of inoculation was also observed in guinea pigs infected with *L. orientalis*. The erythematic spot appeared by week 3–4 pi. The spot nodulated, and the nodules subsequently transformed into dry lesions surrounded by a purple skin macula (Fig. 2b, Additional file 2: Table S2). The lesions healed by week 7–8 pi. However, animals were not infectious to sand flies (0/32 positive pools) and no leishmanial DNA was detected in any of the tested tissue samples.

Guinea pigs infected with *L. macropodum*, *Leishmania* sp. from Ghana and *L. martiniquensis* (CU1) did not show any external signs of infection during the whole experiment, animals were not infectious to sand flies (0/96 positive pools consisting of 5 blood-fed females

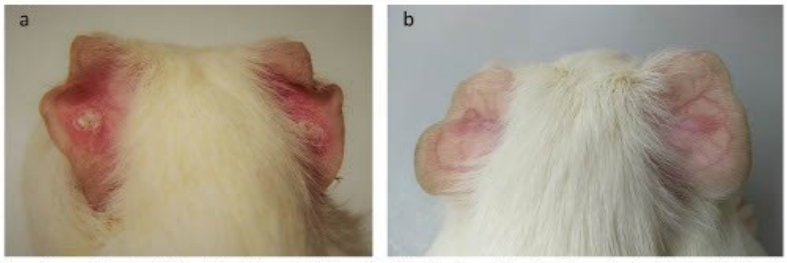


**Fig. 2** Dry lesions observed in animals infected with *L. enriettii* (**a**) and nodules transforming to dry lesions in animals infected by *L. orientalis* (**b**); both at week 5 pi

each), and leishmanial DNA was not detected in any of tissue samples collected by the end of experiment on week 12 pi.

## Discussion

Leishmaniases of men and animals caused by *L.* (*Mundinia*) species are emerging all over the world. The diseases in humans are characterized by symptoms varying from self-healing skin lesions [2, 12] to visceral forms. The latter prevail in HIV-positive patients [8, 16] but was also observed in immunocompetent humans [12, 17]. Very little is known about the life-cycle of these ancient and neglected species and an appropriate animal model is necessary for closer understanding of their biology.

Guinea pigs were chosen for the experimental model in our study as they are the only known non-human mammalian hosts of *L.* (*Mundinia*) species, except for kangaroos, cows and horses [6, 7, 9, 10, 18], which are not practicable for most laboratory investigations. *Leishmania enriettii* was repeatedly isolated from domestic guinea pigs from various localities in Brazil [6, 19]. Interestingly, individual cases were separated by long time periods, which does not agree with the fact that guinea pigs are popular pets, and according to several studies, they are very susceptible to infection [5, 20, 21]. We suggest that this rare incidence may have two different explanations. First, the prevalence of infection is actually much higher, but the owners of infected guinea pigs do not take them for veterinary checks, therefore, parasites are not isolated. Alternatively, guinea pigs are only incidental hosts and the primary reservoir hosts (and primary insect vectors) are not present in close vicinity to households. In this case, secondary vectors and/or reservoirs may be temporarily involved in transmission to domestic localities and domestic guinea pigs.

Our experiments confirmed the susceptibility of guinea pigs to *L. enriettii*. All infected animals showed development of typical ear lesions and the animals were infectious to sand flies. The numbers of positive sand flies were significantly higher at week 4 pi than at the later time interval, week 8 pi. This decrease of infectivity was also observed previously by Seblova et al. [6]. At week 12 pi, the animals did not show any more external signs of infection and *Leishmania* DNA was not detected in any of the examined tissue samples. Spontaneous healing of lesions was observed also by Paranaiba et al. [22]. In their experiments initiated by intradermal inoculation of $10^5$ promastigotes, a different virulence between the two strains used was observed. The Cobaia strain did not develop any lesions, while strain L88 developed lesions that were growing by weeks 4–6 pi, and then diminution of lesions was observed until the end of the experiment. The authors also described development of larger lesions in groups where sand fly salivary glands were added to the inoculum.

However, the virulence of the *L. enriettii* parasite strain and the presence of sand fly salivary glands are not the sole factors influencing the degree of pathogenicity for guinea pigs. The outcome of infections is also dependent on the method of their initiation, i.e. on parasite numbers and stages (amastigotes *vs* promastigotes) used as an inoculum. Thomaz-Soccol et al. [23] described the development of serious symptoms of disease, such as the dissemination of parasites and subsequent death of all tested animals, when the inoculum consisted of amastigotes of a strain identical to L88 (according to isoenzyme analyses). Wide dissemination of *L. enriettii* in animals was also observed by Paraense et al. [20], who infected guinea pigs with amastigotes from lesion homogenates, and by Seblova et al. [6] who infected animals with $10^7$ culture derived promastigotes.

Development of *L. enriettii* has also been tested in hamsters (*Mesocricetus auratus*), where infections were characterized by the development of temporary lesions at the site of inoculation and their subsequent healing [5, 24]. In experiments with wild guinea pigs (*Cavia aperea*), rhesus macaques and dogs [7], no animals showed any signs of infection, so domestic guinea pigs remain the best laboratory model for *L. enriettii* at present.

Here, we compared the susceptibility of guinea pigs to four other *L.* (*Mundinia*) species. The infections were lost in animals infected with *L. macropodum*, *Leishmania* sp. from Ghana and *L. martiniquensis* strain CU1. Animals infected with a second *L. martiniquensis* strain, MAR1, and with *L. orientalis* developed only temporary changes on the ears and the animals were not infectious to sand flies. However, PCR analysis showed no presence of leishmanial DNA by week 12 pi in any of the tested samples. We suggest that *L. martiniquensis* and *L. orientalis* are capable of temporary survival at the site of inoculation, but they cannot disseminate to other tissues of guinea pigs.

We suggest that for a better understanding of *L.* (*Mundinia*) biology it is necessary to focus on other model host organisms. The first choice could be BALB/c mice or hamsters as the most common animal models for research used with many *L.* (*Leishmania*) and *L.* (*Viannia*) species. Infections of these standard laboratory animals with their controlled genetic background may bring valuable information. Alternatively, genetically polymorphic models like wild rodents mimicking natural hosts could be used. These less common models can allow a better understanding of the dynamics of infection and host-parasite relationships related more closely to the situation in the wild [24]. On the other hand, when infected with *L. enriettii* and *L. orientalis*, guinea pigs

could serve as a potential model for spontaneous healing, which could be informative for the design of vaccines.

## Conclusions

Experimental infections showed that guinea pigs are not a good animal model for the subgenus *Mundinia*, with exception of *L. enriettii*. All other *Mundinia* species studied, *L. orientalis*, *L. martiniquensis*, *L. macropodum* and *L.* (*Mundinia*) sp. from Ghana, were not able to develop infections transmissible to sand flies.

## Supplementary information

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13071-020-04039-9.

**Additional file 1: Table S1.** Weight gain of guinea pigs during the experiment.

**Additional file 2: Table S2.** External signs of infection on ears of infected guinea pigs during the experiment. *Abbreviations*: E, erythema; N, nodulus; DL, dry lesion.

### Abbreviations

PCR: polymerase chain reaction; pi: post-infection.

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### Authors' contributions

TB and JS carried out the experimental infections of animals and xenodiagnostic experiments. Molecular analysis was done by TB. JS and PV substantially contributed to conception and design of experiments. Article was drafted by TB and JS. Parasites were provided and manuscript was revised by PS and PB. All authors read and approved the final manuscript.

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### Availability of data and materials

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

### Ethics approval and consent to participate

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of Charles University in Prague and were performed under permission no. MSMT-1373/2016-5 of the Ministry of the Environment of the Czech Republic. Investigators are certificated for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

# 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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# 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 $5 \times 10^6$ 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 $3.6 \times 10^4$ with LV 110 strain (35% metacyclic forms) and ranged between $3.5 \times 10^4$–$7 \times 10^4$ parasites/rodent with FVI strain (23–69% metacyclic forms) and $4.1 \times 10^4$–$5.4 \times 10^4$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).

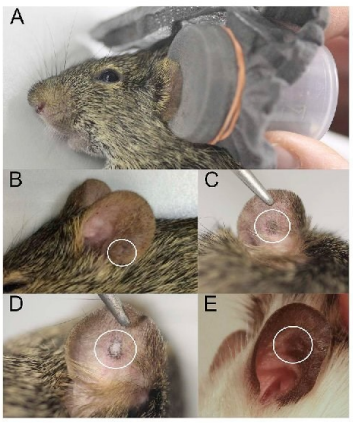


**Fig. 1. Xenodiagnosis and external manifestation of *L. major* in rodents.** Direct xenodiagnosis with *P. duboscqi* in plastic tubes covered with fine mesh held on the ear of the anaesthetized *A. niloticus* (A) and external manifestation of *L. major* LV109 in ear pinnae (site of inoculation) of *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 hyper-pigmentation 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 | 25 |
| C1* | | | 2 | 3 | 4 | X | X | X | X | X | X | X | X |
| C2 | | | | | 1 | 2.5 | 3.5 | 3.5 | 4 | 4 | 4 | 4 | 4 |
| C3 | | | | | 1 | 2.5 | 3.5 | 3.5 | 4 | 4 | 4 | 4 | 4 |
| C4 | | | 1 | 1 | 1 | 1 | 3.5 | 3.5 | 4 | 4 | 4 | 3 | 3 |
| C5 | | | | 1 | 1 | 2.5 | 3.5 | 4 | 4 | 4 | 4 | 3 | 3 |
| A1 | | | | | 1 | 2 | 2 | 2.5 | 3 | 3 | 3 | 3 | 3 |
| A2 | | | 1 | 1 | 2 | 4 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| A3 | | | | 1 | 1 | 4 | 4 | 2 | 1 | 1 | 1 | 1 | 1 |
| A4 | | | | | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | 1 |
| A5 | | | 1 | 1 | 2.5 | 2.5 | 3 | 1.5 | 1.5 | 1.5 | 1 | 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 | 25 |
| C1* | | | | 1 | 1 | 1 | 1 | 1 | 5.8 | X | 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 | X |
| C5* | | | | 1 | 1 | 2 | 3.3 | 3.8 | 4 | X | 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 | 1.6 |
| A3 | | | | | | | | 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%) | $\chi2 = 17.015$, | 12/161 (7.4%) | $\chi2 = 0.118$, | 26/76 (34.2%) | $\chi2 = 0.119$, |
| | BALB/c mouse | 95 (47.5%) | $P < 0.0001$ | 6/95 (6.3%) | $P = 0.472$ | 24/76 (31.6%) | $P = 0.432$ |
| *Arvicanthis* vs. *Mastomys* | *Arvicanthis* | 94 (47.0%) | $\chi2 = 0.129$, $P = 0.719$ | 25/94 (26.6%) | $\chi2 = 0.007$, | 20/28 (71.4%) | $\chi2 = 0.012$, |
| | *Mastomys* | 81 (40.5%) | | 22/81 (27.2%) | $P = 0.534$ | 14/20 (70.0%) | $P = 0.582$ |
| *Mastomys* vs. BALB/c mouse | *Mastomys* | 134 (67.0%) | $\chi2 = 0.055$, $P = 0.808$ | 6/100 (6.0%) | $\chi2 = 0.787$, | 18/20 (90.0%) | $\chi2 = 0.784$, |
| | BALB/c mouse | 135 (67.5%) | | 10/200 (5.0%) | $P = 0.132$ | 16/20 (80.0%) | $P = 0.661$ |

**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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# Steppe lemmings and Chinese hamsters as new potential animal models for the study of the leishmania subgenus *Mundinia* (Kinetoplastida: Trypanosomatidae)

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# Steppe lemmings and Chinese hamsters as new potential animal models for the study of the leishmania subgenus *Mundinia* (Kinetoplastida: Trypanosomatidae)

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## Abstract

*Leishmania*, the dixenous trypanosomatid parasites, are the causative agents of leishmaniasis currently divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania,* and the recently described *Mundinia*, consisting of six species distributed sporadically all over the world infecting humans and/or animals. These parasites infect various mammalian species and also cause serious human diseases, but their reservoirs are unknown. Thus, adequate laboratory models are needed to enable proper research of *Mundinia* parasites. In this complex study, we compared experimental infections of five *Mundinia* species (*L. enriettii*, *L. macropodum*, *L. chancei*, *L. orientalis,* and four strains of *L. martiniquensis*) in three rodent species: BALB/c mouse, Chinese hamster (*Cricetulus griseus)* and Steppe lemming (*Lagurus lagurus*). Culture-derived parasites were inoculated intradermally into the ear pinnae and progress of infection was monitored for 20 weeks, when the tissues and organs of animals were screened for the presence and quantity of leishmania. Xenodiagnoses with *Phlebotomus duboscqi* were performed at weeks 5, 10, 15 and 20 post-infection to test the infectiousness of the animals throughout the experiment. BALB/c mice showed no signs of infection and were not infectious to sand flies, while Chinese hamsters and Steppe lemmings proved susceptible to all five species of *Mundinia* tested, showing a wide spectrum of disease signs ranging from asymptomatic to visceral. *Mundinia* induced significantly higher infection rates in Steppe lemmings compared to Chinese hamsters, and consequently Steppe lemmings were more infectious to sand flies: In all groups tested, they were infectious from the 5th to the 20th week post infection. In conclusion, we identified two rodent species, Chinese hamster (*Cricetulus griseus)* and Steppe lemming (*Lagurus lagurus*), as candidates for laboratory models for *Mundinia* allowing detailed studies of these enigmatic parasites. Furthermore, the long-term survival of all *Mundinia* species in Steppe lemmings and their infectiousness to vectors support the hypothesis that some rodents have the potential to serve as reservoir hosts for *Mundinia*.

Keywords: leishmania; model animal; *Mundinia*; rodent; murine model; leishmaniasis

## Author Summary

*Leishmania* parasites of the subgenus *Mundinia* are an emerging health and veterinary problem that should not be ignored. Being the most recent of all *Leishmania* described, many aspects of *Mundinia* biology are enigmatic. We have very scarce data on their life cycles and biology, thus proper laboratory research must be done to enable their better understanding. One of the most crucial parts of the life cycle of *Leishmania* is the development in the mammalian host. In the past, we worked on establishment of other laboratory models for the subgenus, but either *Arvicanthis*, *Mastomys* or Guinea pigs did not prove to be a good choice. Other authors performed experiments with BALB/c mice using various inoculation techniques, but they also failed. Here we describe the establishment of two new potential laboratory model species, Chinese hamsters and Steppe lemmings, which proved to be susceptible to *Mundinia* and such findings will enable other scientists to continue in research of these parasites.

## 1. Introduction

Leishmaniases are a group of diseases caused by digenetic parasites of the genus *Leishmania that* naturally occur in tropical and subtropical regions around the world. These parasites are currently divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania,* and *Mundinia* (1). With more than one million human cases a year, leishmaniases are among the world's most important tropical diseases caused by protozoa. There are three main clinical manifestations of the disease: cutaneous, mucocutaneous, and visceral, with symptoms depending on many endogenous and exogenous factors, such as parasite species and virulence, infectious dose, the host immune response, and other biological, socio-economic and environmental factors (2–4).

*Mundinia* is the most recently described *Leishmania* subgenus consisting of six species, all of them reservoir hosts are unknown (5,6). Biting midges (Diptera: Ceratopogonidae) are the most probable vectors of *Mundinia,* changing the established dogma that leishmania are transmitted solely by phlebotomine sand flies (Diptera: Psychodidae) (7–10). Three species of *Mundinia* do not infect humans*; Leishmania enriettii* was found in domestic Guinea pigs in Brazil, and even though nearly 80 years passed since its discovery, it remains one of the most enigmatic species of leishmania (Muniz & Medina, 1948). *Leishmania macropodum,* which causes cutaneous and mucocutaneous symptoms in kangaroos, is the only representative species of the *Leishmania* genus in Australia (12,13) and *L. procaviensis have* recently been described from hyrax (*Procavia capensis*) isolates in Namibia (6,14).

Other three species of *Mundinia* infect humans; the symptoms of the disease ranged from a single cutaneous lesion to general visceral infection (15–17). *Leishmania martiniquensis* is the most widespread species of all known leishmania, present in Central and North America, Europe and Asia (17-20), while *L. orientalis* is endemic to Southeast Asia (16,21). In Thailand, a high prevalence of *L. martiniquensis* and several cases of *L. orientalis* have been described in immunosuppressed people (22), but cases of immunocompetent people showing signs of cutaneous or visceral leishmaniasis have also been reported (23). The last member of the subgenus is recently described *L. chancei* (6) which causes cuta-

neous leishmaniasis in humans, mainly children and young adults in Ghana (15). Based on the low number of human cases, the zoonotic circulation of this parasite is predicted (24).

Several mammalian species are currently incriminated for their potential involvement in *Mundinia* circulation. Black rats (*Rattus rattus*), cows, and horses are suggested candidates, since *L. martiniquensis* DNA was detected and viable parasites were cultured from these hosts (18–20,25). However, it should be noted that detection of parasites or their DNA does not imply the reservoir potential of the host, since parasites can be present in the organism incidentally, as a "sink" of infection, not as a "source", transmittable to other hosts (26). Finding a suitable animal model can help to clarify issues of pathology and immune response to *Mundinia* infections and can be used in life cycle research and for control, prevention, and treatment.

In this study, we decided to test both a classic model laboratory animal, the BALB/c mouse, and two less established model rodent species, Steppe lemmings *Lagurus lagurus* and Chinese hamster *Cricetulus griseus*. Laboratory mice (*Mus musculus*) have become one of the most widely used biological models in many areas of biomedical research, including the study of cutaneous and visceral leishmaniasis, mainly due to the wide range of inbred strains and transgenic animals. In contrast, *Lagurus lagurus* and *Cricetulus griseus* are genetically polymorphic wild rodents. They were chosen because they are commercially available and easy to maintain and several old publications (27–30) as well as more recent ones (31,32), have demonstrated their high susceptibility to various *Leishmania* parasites.

## 2. Materials and Methods

### 2.1. Parasites and rodents

*Leishmania enriettii* (MCAV/BR/45/LV90), *L. macropodum* (MMAC/AU/2004/AM-2004), *L. chancei* (MHOM/GH/2012/GH5), *L. orientalis* (MHOM/TH/2014/LSCM4) and four strains of *L. martiniquensis* (MHOM/MQ/1992/MAR1; MHOM/TH/2011/Cu1R1; MHOM/TH/2019/Cu2 and MEQU/CZ/2019/Aig1) were used.

Parasites were maintained at 28 °C in M199 medium supplemented with 20% fetal calf serum (Gibco, Prague, Czech Republic), 1% BME vitamins (Sigma-Aldrich, Prague, Czech Republic), 2% sterile urine and 250 μg/ml amikacin (Amikin, Bristol-Myers Squibb, Prague, Czech Republic). All strains were kept in a cryobank with 2–3 subpassages *in vitro* prior to experimental infections of rodents. Before experimental infection, the parasites were washed by centrifugation (1400×g/5 min) and resuspended in saline solution.

BALB/c mice (Velaz, Prague, Czech Republic), Steppe lemmings and Chinese hamsters (Karel Kapral s.r.o., Prague, Czech Republic) were maintained in groups of 5 specimens in T4 boxes (58 × 37 × 20 cm) (Velaz, Prague, Czech Republic), equipped with bedding (SubliCZ, Sojovice, Czech Republic), breeding material (Woodwool) and hay (Krmne smesi Kvidera, Spalene Porici, Czech Republic), provided with a feed mixture ST-1 (Krmne smesi Kvidera, Spalene Porici, Czech Republic) and water *ad libitum*, with a 12 h light/12 h dark photoperiod, temperature of 22–25 °C and relative humidity of 50–60%.

## 2.2. Experimental infections and xenodiagnoses of rodents

Eighty specimens of three rodent species (*Mus musculus*, *Cricetulus griseus* and *Lagurus lagurus*) anaesthetised with ketamin/xylazin (62 mg/kg and 25 mg/kg) were injected with $10^7$ stationary-stage promastigotes (from 5-7 days old cultures, depending on the species) in 5 μl of sterile saline solution intradermally into the left ear pinnae. The course of infection was recorded weekly.

Xenodiagnoses were performed at weeks 5, 10, 15 and 20 p.i. using *Phlebotomus duboscqi*. Five to six-day-old sand flies were placed in plastic vials covered with fine nylon mesh and allowed to feed on the ear pinnae of anesthetised animals. The proven vectors of *Mundinia* are unknown, but early infections in vectors are nonspecific (33,34), so *Mundinia* can multiply before defecation of the vector, which in *P. duboscqi* is on day 4-5 PBM (35). The engorged individu-

als were maintained for two days at 25°C and then stored in tissue lysis buffer (Roche, Prague, Czech Republic) at -20°C in pools of 5 females for subsequent DNA isolation and nested PCR.

At the end of the experiments (20 weeks p.i.) the rodents were euthanised, dissected, and tissues from ears, paws, tail, ear-draining lymph nodes, spleen, and liver were stored at – 20°C for subsequent analyses of parasite load (DNA isolation, nested PCR and qPCR).

## 2.3. Morphometry of parasites

The inoculum were fixed with methanol, stained with Giemsa, and examined under the light microscope with an oil-immersion objective. One hundred and thirty randomly selected promastigotes from each infection dose were photographed using the QuickPhoto micro programme for further analysis in the ImageJ programme. The body and flagellar lengths of the parasites were measured and metacyclic forms were distinguished, based on the criteria of Walters (1993) and Cihakova and Volf (1997) (36,37): body length < 14 μm and flagellar length 2 times body length.

## 2.4. DNA isolation and nested PCR of sand fly and rodent samples

Total DNA isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions was used as a template for nested PCR amplification (detection limit $10^{-2}$ parasites per sample) with outer primers amplifying 332 bp region of 18S sequence (forward primer 18SN1F 5′- GGA TAA CAA AGG AGC AGC CTC TA3′ and reverse primer 18SN1R 5′– CTC CAC ACT TTG GTT CTT GAT TGA-3′) and inner primers amplifying the 226 bp long 18S sequence (forward primer 18SN2F 5′-AGA TTA TGG AGC TGT GCG ACA A-3′ and reverse primer 18SN2R 5′-TAG TTC GTC TTG GTG CGG TC-3′) previously used by Sadlova et al. 2022 (38). The samples were then analysed using 0,8% agarose gel. The reaction mixtures and cycling conditions were as follows:

1. Step of PCR: 3 µl of genomic DNA, 0,5 µl forward primer 18SN1F (10 µM), 0,5 µl reverse primer 18SN1R (10 µM), 10 µl of 2x EmeraldAmp® GT PCR Master Mix (Takara Bio), 6 µl of ddH2O. Step 1, 94 °C for 3 min 30 s; step 2, 94 °C for 30 s; step 3, 60 °C for 30 s; step 4, 72 °C for 25 s; step 5, 72 °C for 7 min; followed by cooling at 12 °C. Steps 2–4 were repeated 35 times.

2. Step of PCR: 1 µl of 1. Step PCR reaction, 0,5 µl forward primer 18SN2F (10 µM), 0,5 µl reverse primer 18SN2R (10 µM), 10 µl 2x EmeraldAmp® GT PCR Master Mix (Takara Bio), 8 µl ddH2O. Step 1, 94 °C for 30 s; step 2, 94 °C for 30 s; step 3, 60 °C for 30 s; step 4, 72 °C for 20 s; step 5, 72 °C for 7 min; followed by cooling at 12 °C. Steps 2–4 were repeated 35 times.

## 2.5. Quantitative PCR

Parasite quantification by quantitative PCR (qPCR) was performed in a Bio-Rad iCycler & iQ Real-Time PCR Systems using the SYBR Green detection method (SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad, Hercules, CA). Primers targeting the 226 bp long 18S sequence (forward primer 18SN2F 5′-AGA TTA TGG AGC TGT GCG ACA A-3′ and reverse primer 18SN2R 5′-TAG TTC GTC TTG GTG CGG TC-3′) were used. One microlitre of DNA was used per individual reaction. PCR amplifications were performed in duplicates using the following conditions: 98 °C for 2:30 min followed by 40 repetitive cycles: 98 °C for 10 s and 60 °C for 20 s. PCR water was used as a negative control. Detection limit of used assay is $10^3$ parasites per sample. A series of 10-fold dilutions of *L. martiniquensis* promastigote DNA, ranging from $5 \times 10^6$ to $5 \times 10^1$ parasites per PCR reaction, was used to prepare a standard curve. Quantitative results were expressed by interpolation with a standard curve. To monitor non-specific products or primer dimers, a melting analysis was performed from 70 to 95 °C at the end of each run, with a slope of 0.5 °C/c, and 6 s at each temperature. The parasite loads in tested tissues were classified into three categories: low, < 1000 parasites; moderate, 1000 – 10 000 parasites; heavy, > 10 000 parasites.

### 2.6. Statistical analysis

Differences in infection rates were analysed using Fishers Exact Tests in SPSS version 27.

## 3. Results

### 3.1. Development of *Mundinia* in BALB/c mice

Two independent trials, each with groups of 5 animals inoculated with culture-derived *Mundinia* species, were processed; in total, 10 BALB/c mice infected with each *Mundinia* species (*L. enriettii*, *L. macropodum*, *L. orientalis*, *L. chancei,* and four strains of *L. martiniquensis)* were studied. The representation of the infective metacyclic stages in the inocula ranged between 0-32 % (Table S1).

Mice did not show weight loss or external signs of infection throughout the experiment (Table S2). Xenodiagnoses were performed at weeks 5, 10, 15 and 20 p.i. on 4/10 mice from each group and revealed no positive results by PCR. Also, all examined tissue samples (i.e. ears, paws, tail, ear-draining lymph nodes, spleen and liver) were negative for the presence of leishmania DNA by PCR (Table S3). In summary, BALB/c mice were resistant to all *Mundinia* species tested.

### 3.2. Development of *Mundinia* in Chinese hamsters (*Cricetulus griseus*)

In two independent trials, 10 Chinese hamsters (*Cricetulus griseus*) were infected with each of the culture-derived *Mundinia* tested: *L. enriettii*, *L. macropodum*, *L. orientalis*, *L. chancei,* and four strains of *L. martiniquensis*. In total, 80 animals were used. The representation of infectious metacyclic stages in the inocula ranged between 0-57 % (Table S1)

Similar to BALB/c mice, Chinese hamsters did not show external signs of infection or weight loss (Table S2) during the experiment. However, hamsters infected with *L. enriettii*, *L. chancei*, *L. orientalis,* and *L. martiniquensis* were infectious to sand flies throughout the experiment from week 5 p.i. to week 15 in *L. enriettii* and to week 20 p.i. in *L. chancei*, *L. orientalis,* and *L. martiniquensis*, when the experiment was terminated. (Table 1). Nested PCR showed positivity of the inoculated ears in 6/8 tested groups at the end of the experiment, only samples from animals infected with *L. macropodum* and *L. martiniquensis* Aig1 were negative. *Leishmania enriettii* DNA was found in the inoculated ear of one animal and also in draining lymph nodes of the inoculated ear of another animal. The inoculated ears of animals infected with *L. chancei* were positive in 30% and parasites were not detected in any other tissue. In hamsters infected with *L. orientalis*, 40% of the ears were positive by nested PCR, while in one case the parasites were detected in the draining lymph nodes of the inoculated ear. Animals infected with *L. martiniquensis* MAR1 showed 20% of inoculated ears and draining lymph nodes positive for parasite DNA. In animals infected with *L. martiniquensis* Cu1R1, 30% of the inoculated ears were positive by nested PCR while no parasite DNA was detected in any other tissue. The highest proportion of infected animals was found in the group infected with *L. martiniquensis* Cu2 where 80% of the inoculated ears were positive and parasite DNA was also detected in 10% of the hind paws. Quantitative PCR showed that only a low number of parasites (<1000) was present in the all tested tissues (Table S3).

Table 1. Infectiousness of tested Chinese hamsters to sand flies during xenodiagnoses. The left numbers represent the number of PCR positive pools and the right numbers represent the total number of tested pools. Each pool consisted of 5 engorged *Phlebotomus duboscqi* females.

| | 5 weeks p.i. | 10 weeks p.i. | 15 weeks p.i. | 20 weeks p.i. |
|---|---|---|---|---|
| *L. enriettii* | 1/9 | 0/17 | 1/8 | 0/8 |

| | | | | |
|---|---|---|---|---|
| *L. macropodum* | 0/7 | 0/13 | 0/6 | 0/12 |
| *L. chancei* | 1/9 | 0/14 | 1/9 | 1/13 |
| *L. orientalis* | 6/11 | 2/16 | 0/12 | 1/12 |
| *L. martiniquensis* Mar1 | 0/7 | 0/14 | 1/18 | 0/7 |
| *L. martiniquensis* Cu1R1 | 1/9 | 0/11 | 0/5 | 0/8 |
| *L. martiniquensis* Cu2 | 1/11 | 0/16 | 1/11 | 1/13 |
| *L. martiniquensis* Aig1 | 0/11 | 0/12 | 0/7 | 0/8 |

### 3.3. Development of *Mundinia* in Steppe lemmings (*Lagurus lagurus*)

As with the previous host species, groups of 5 Steppe lemmings were inoculated with five culture derived species of *Mundinia* (*L. enriettii, L. macropodum, L. orientalis, L. chancei,* and four strains of *L. martiniquensis*) and the experiment was repeated twice; in total, 10 individuals of Steppe lemmings were infected by each parasite species. The proportion of metacyclic stages in inocula ranged between 0-25 % (Table S1)

In Steppe lemmings, most *Mundinia* species and strains produced significantly higher infection rates than in Chinese hamsters (Fig. 1). The pathological signs of infection (Fig. 2) including weight changes (Table S2) in Steppe lemmings varied from asymptomatic through cutaneous to visceral form. However, specimens in all groups were infectious to sand flies from week 5 p.i. until the end of the experiment by week 20 p.i. (Table 2).

In all animals infected with *L. enriettii,* parasite DNA was detected in the inoculated ears and also in 30% of the draining lymph nodes of the inoculated ears. Parasite DNA was also present in 10% of the forepaws, hind paws, tails, and spleens (Fig. 2). Although the number of parasites in infected ears was low or moderate (maximum 2,4x104), a very high proportion of pools of sand flies used for xenodiagnoses were positive (more than 85% from week 10 p.i.). (Table 2). All animals that were used for xenodiagnoses developed cutaneous symptoms (swelling and dry lesions) on the

ears from week 8 or 9 p.i., respectively, which presisted to the end of the experiment while other animals did not show any cutaneous symptoms. In one animal, which was not used for xenodiagnoses, we also observed hair loss in the abdomen from the 17th week p.i. (Fig. 2).

Animals infected with *L. macropodum* did not show any symptoms of infection during the experiment. We detected only low numbers of parasites (<1000) in 33% of inoculated ears (again, only in animals used for xenodiagnoses) and in the tail of one additional animal not exposed to sand flies (Fig. 2). Steppe lemmings were infectious to sand flies only in 11% by week 5 p.i. and 33% by week 20 p.i. (Table 2).

In Steppe lemmings infected with *Leishmania chancei*, one animal died 2 weeks post infection from unknown reasons. The development was observed in only one animal used for the xenodiagnosis. The lesion with swelling began to form by week 8, leading to partial destruction of the pinna by week 13 (Fig. 3c,d). The loss of hair in the abdomen appeared in two specimens between 10 and 12 weeks (Fig. 3f) and one of these animals died after 3 weeks. All other 6 animals showed no external signs of infection during the 12ntire experiment. However, parasites disseminated throughout the body and were found in all tested tissues except lymphatic nodes draining non-inoculated ears, although one of the tested non-inoculated ears tested was positive for the presence of parasites (Fig. 2). The positivity rate of inoculated ears, lymph nodes that drain them, hind paws and spleen was 37,5%. Although the positivity and infectiousness of the animals were high, only low or moderate numbers of parasites (<2500 per tested tissue) were detected by qPCR (Table S3).

Among the animals infected with *L. orientalis*, 4/10 specimens died during the experiment, by weeks 7,8, 13 and 15 p.i. One specimen that died by week 13 showed hair loss and weight loss, while no external signs of infection were recorded in others. Nested PCR revealed that 75 % of the animals were positive for leishmania DNA at the time of dissection. Parasites spread into all tested tissues except the tail (Fig. 2), most frequently they were present in the lymph nodes (75 %), forepaws (62.5 %), spleen (50 %) and liver (37.5 %). Similarly to animals infected with *L. chancei*, the

positivity and infectiousness of the animals were high, but parasite numbers were only low or moderate (<2500) (Table S3).

Large differences were observed in animals infected with various strains of *L. martiniquensis*. Although *L. martiniquensis* strains Mar1, Cu1R1, and Aig1 did not cause any pathological signs of infection, animals infected with *L. martiniquensis* Cu2 showed the most severe cutaneous signs of infection of all tested groups. All four animals that were used for xenodiagnoses developed pathological changes in the pinnae, while none of the five animals that were not used for xenodiagnoses developed lesions. After the first round of xenodiagnoses 5 weeks p.i., swelling and lesions began to appear (Fig. 3b) and continued to grow in two animals, leading to the destruction of the pinna by week 12 (Fig. 3e). In another animal, the lesion started to form by week 12 and the pinna was completely destructed by week 19, and one animal lost the entire pinna between weeks 16 to 19, while no pathological signs were observed before that (Fig. S2). One animal did not show any lesions, but weight loss was observed from week 10 and its overall health status degraded until the end of the experiment. Four other animals (that have not been xenodiagnosed) showed no external signs of infection and one animal died 6 weeks p.i. Parasites were detected by PCR in 88.9% (8/9) of the animals. The parasites often spread into all tissues tested (Fig. 2), most frequently to lymph nodes (88.9 %), forepaws (66.7%), hind paws (55.6%), and spleen (55.6%) (Fig. 2). Quantitative PCR revealed only low numbers of leishmania in most tissues tested, but higher numbers were present in 6/9 inoculated ears ($7x10^3$ – $3{,}16x10^5$) and $4x10^4$ parasites were detected in the spleen of the animal with the highest parasite load in the inoculated ear (Table S3). Interestingly, this specimen did not show signs of infection (Table S2). Animals infected with *L. martiniquensis* Mar1 were infectious to sand flies from week 5 p.i. to the end of experiment (Table 2). Parasite DNA was found in all tested tissues except of contralateral ears, most often in inoculated ears (89%), draining lymph nodes of inoculated ears (78%), spleen (66%) and forepaws (44%) (Fig. 2). Low or moderate numbers of parasites were detected in inoculated ears (maximum $3x10^4$) and only low numbers of parasites (<1000) were detected in other tested tissues (Table S3). In animals infected with *L.*

*martiniquensis* Cu1R1, parasites were detected in inoculated ears (88%), draining lymph nodes of inoculated ears (50%), draining lymph nodes of contralateral ears and tail (13%) (Fig. 2). The animals were infectious to sand flies throughout the course of experiment, but only in a low proportion compared to other strains of *L. martiniquensis* with positive pools ratio slowly increasing over time (Table 2). In Steppe lemmings infected with the Aig1 strain, parasites were found in 56% of inoculated ears and 11% of forepaws and tails (Fig. 2). Although only low numbers of parasites were detected (Table S3), the xenodiagnoses proved that animals were infectious to sand flies, even in 50% by week 20 p.i. (Table 2).

Figure 1: Infection rates in Chinese hamsters (*Cricetulus griseus*) and Steppe lemmings (*Lagurus lagurus*) infected with *Mundinia*. The numbers of animals evaluated are shown in the graph. P values indicate difference between host species; n.s., nonsignificant difference, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$.

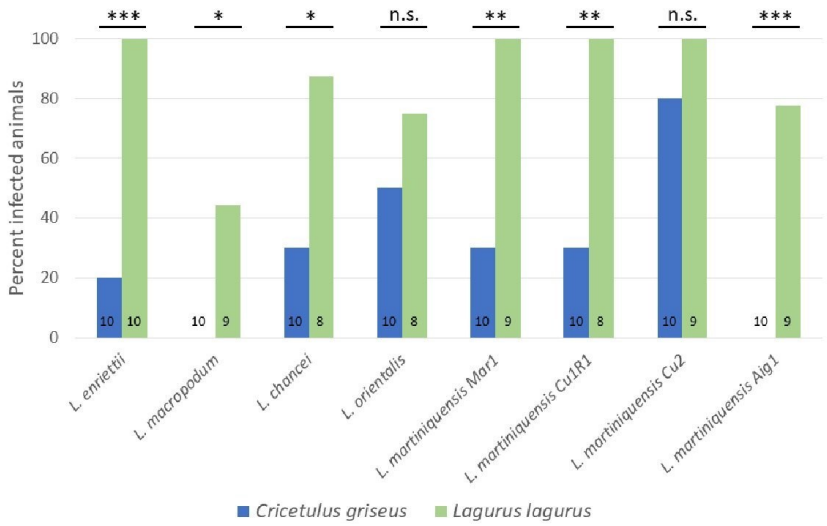

Figure 2. Anatomical distribution of parasites in rodent species determined by nested PCR. The results are presented by the balloon balloon graph where the size corresponds to the percentage of infected tissues from the total sum of tested organs of the same type. IE = inoculated ears; CE = contralateral ears; LN-IE = draining lymph nodes of inoculated ears; LN-CE = draining lymph nodes of the contralateral ears.


Figure 3. Pathological changes caused by *Mundinia* in the Steppe lemming (*Lagurus lagurus*). A) healthy ear before inoculation; B) lesion formation in animal infected with *L. martiniquensis* Cu2 12 weeks p.i.; C) dry lesion in animal infected with *L . martiniquensis* Cu2 15 weeks p.i.; D) partial pina necrosis in animal infected with *L. chancei* 13 weeks p.i.; E) destruction of the ear and dissemination of parasites around the eye in animal infected with *L. martiniquensis* Cu2 19 weeks p.i.; F) hair loss in abdomen of animal infected with *L. chancei* 12 weeks p.i. (animal on the right).

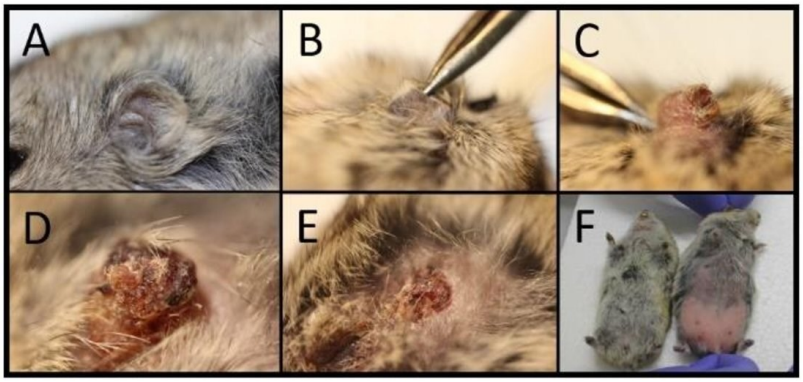


Figure 4. Graphical table showing pathological changes observed in Steppe lemmings (*Lagurus lagurus*) infected with *Mundinia*. The categories were: asymptomatic (white); swelling (yellow); dry lesion (green); partial destruction of the pinna (orange); destruction of the pinna (red); abdomen hair loss (blue); weight loss (grey); death (black). Axis Y describes week post infection, and axis X shows the number codes of examined animals (each column represents one animal). Specimens used for xenodiagnoses trials are marked with * sign.

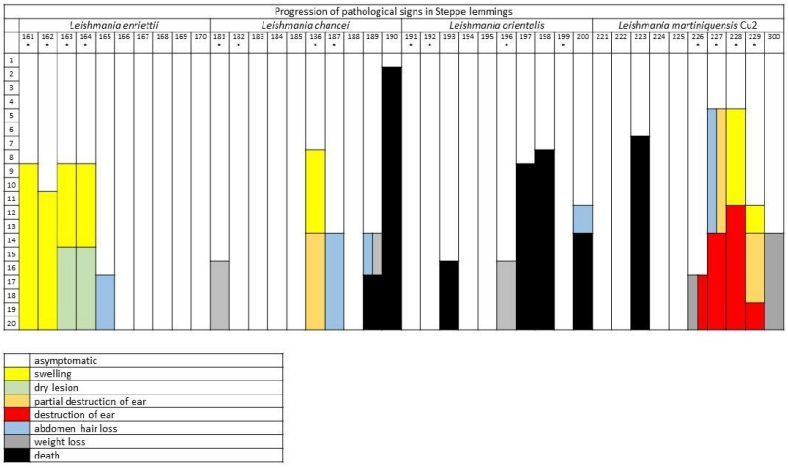

Table 2. Infectiousness of tested Steppe lemmings to sand flies during xenodiagnoses. The left numbers represent the number of PCR positive pools and the right numbers represent the total number of tested pools. Each pool consisted of 5 engorged *Phlebotomus dubosqci* females.

| | 5 weeks p.i. | 10 weeks p.i. | 15 weeks p.i. | 20 weeks p.i. |
|---|---|---|---|---|
| *L. enriettii* | 6/15 | 11/13 | 14/15 | 16/18 |
| *L. macropodum* | 1/13 | 0/13 | 0/9 | 3/9 |
| *L. chancei* | 2/12 | 0/9 | 1/10 | 2/5 |
| *L. orientalis* | 5/10 | 0/6 | 3/7 | 3/7 |
| *L. martiniquensis* Mar1 | 8/14 | 4/13 | 7/13 | 4/12 |
| *L. martiniquensis* Cu1R1 | 2/14 | 1/12 | 2/15 | 3/10 |
| *L. martiniquen¾* Cu2 | 3/4 | 2/5 | 5/8 | 5/8 |
| *L. martiniquensis* Aig1 | 2/12 | 0/14 | 9/17 | 4/8 |

## 4. Discussion

Leishmaniases caused by *Mundinia* species are an emerging health problem that cannot be ignored. While cases of animal leishmaniasis caused by *L. enriettii, L. macropodum,* and *L. procaviensis* have been described only sporadically, human disease caused by *L. martiniquensis* and *L. orientalis* is on the rise in Southeast Asia, especially in Thailand (9,39), and cutaneous leishmaniasis caused by *L. chancei* is emerging in Ghana (15,23). Since the knowledge of natural reservoirs is limited, it is necessary to develop a reliable model for experimental research on the biology of these parasites. Among the classical laboratory models, Guinea pigs (*Cavia porcellus*) were first used for *Mundinia* research. These animals have been shown to be susceptible to *L. enriettii* (9,40–43), however, infections with other *Mundinia* species were lost; only Guinea pigs infected with *L. orientalis* showed temporary pathological changes in the ear pinnae (43). In

Golden hamsters (*Mesocricetus auratus*), *L. enriettii* showed only mild symptoms with temporary lesions at the site of inoculation and their subsequent healing (9,39) while *L. martiniquensis* disseminated, causing signs of VL (44). Recently, *Leishmania chancei* was tested in *Mastomys natalensis* and *Arvicanthis niloticus* as these two rodents inhabit Ghana and could potentially serve as reservoir hosts of the parasite. However, *Leishmania* survival in both hosts was limited, making them unsuitable experimental models (45).

In the present study, we tested three rodent species: BALB/c mouse (*Mus musculus*), Chinese hamster (*C. griseus),* and Steppe lemming (*Lagurus lagurus*). BALB/c mice were chosen as the most commonly studied murine model, highly susceptible to cutaneous leishmaniasis caused by *L. major* as well as visceral leishmaniasis (reviewed by (46)). Chinese hamsters and Steppe lemmings were used as these animals have been shown to be more susceptible to *L. donovani* than Golden hamsters, the most common rodent model for visceral leishmaniasis: In experiments where the three rodent species were directly compared after intradermal inoculation of *L. donovani*, both Chinese hamsters and Steppe lemmings showed more extensive spread of parasites over the body and higher parasite loads and infectiousness to sand flies then Golden hamsters. In addition, both Chinese hamsters and Steppe lemmings were also highly susceptible to cutaneous *L. major* (31). We inoculated culture-derived parasites intradermally into rodent pinnae to simulate the natural mode of infection to the best of our knowledge. Indeed, the use of vector-derived parasites together with homogenate of salivary glands would be a more appropriate way to simulate natural mode of transmission (47), however, this experimental scheme was not applicable in the study since the natural vectors of these parasites are unknown.

BALB/c mice were proved to be resistant to infection with all five *Mundinia* species tested. At the end of the experiment, by week 20, leishmania DNA was not detected in any of the tissues tested, and the rodents were not infectious to sand flies throughout the whole experiment. Our results were consistent with previous observations: BALB/c mice infected with *L. martiniquensis* strains MAR1 and MAR2 were asymptomatic and no mortality, weight loss, or clinical

signs were observed in mice infected with these strains, regardless of the route of inoculation. But parasite dissemination and infection kinetics varied by inoculation method and inoculum size (48). Somboonpoonpol (2016) (49) described the poor development of Thai *L. martiniquensis* after subcutaneous inoculation but high parasite burdens after i.v. application of the parasite. Intakhan et al. (2020) (44) used intraperitoneal infection and detected parasite DNA only in the liver and spleen of infected animals in low numbers at 8 weeks p.i. followed by clearance at week 16 p.i. in most animals. These results suggest that BALB/c mice are not a suitable laboratory model to study the pathology of *Mundinia* infection at least after intradermal inoculation. However, there are other inbred mouse models that differ in their immune response to leishmaniasis from the BALB/c strain. It may be helpful to study the development of *Mundinia,* for example, in C57BL/6 mice, which, unlike the BALB/c strain, are resistant to *L. major* but are susceptible to *L. amazonensis* or *L. mexicana* (reviewed by (4). Another option is to study *Mundinia* in outbred mouse strains that demonstrated an intermediate phenotype between resistant and susceptible inbred strains (50).

Chinese hamsters (*C. griseus*) were demonstrated as susceptible to *L. enriettii*, *L. chancei*, *L. orientalis* and three strains of *L. martiniquensis* (MAR1, Cu1R1, and Cu2), even though no external signs of disease were observed. All hamsters were in perfect health until the end of the experiment, a low number of parasites were localized in their inoculated ears, and the animals were sporadically infectious to sand flies. Very rarely, parasites were detectable in lymph nodes draining the inoculated ear and hind paws, but also in low numbers. These data suggest that Chinese hamsters have the potential to mimic asymptomatic infection. These asymptomatic infections seem to play a major role in the circulation of *Mundinia,* as no symptomatic wild hosts have yet been found in nature (5) and most human infections in Thailand are also asymptomatic (51). On the other hand, except for *L. martiniquensis* Cu2 (80% of positive animals) and *L. orientalis* (50% of positive animals), the proportion of animals with detectable parasites was very low, which would be problematic for more robust and detailed studies.

Steppe lemmings *Lagurus lagurus* were shown to be highly susceptible to all *Mundinia* species tested, with severe signs of infection observed throughout the experiment in human infecting species. Animals were infectious to sand flies and parasites were detected in all tissues tested. Interestingly, only lemmings used for xenodiagnoses developed pathological signs on the ears. This is consistent with results previously published by Vojtková et al. (2021) (52) in BALB/c mice infected with *L. major,* showing that sand fly bites post infection enhance formation of skin lesions.

The present study demonstrated that Steppe lemmings support the survival and development of *L. enriettii*, which caused small dry skin lesions in animals exposed to sand flies, while other animals, although positive for parasites at the end of the experiment, remained asymptomatic. Interestingly, a very high percentage of positive sand fly pools (over 90%) from xenodiagnoses was observed, although we found only a low or moderate number of parasites in the inoculated ears. Also, unlike Guinea pigs, where lesions heal and reduction of infectiousness to sand flies was observed with time was observed (43), Steppe lemmings did not heal lesions over time and animals remained infectious to sand flies until the end of experiment which brings the potential to study the non-healing phenotype of cutaneous leishmaniasis and supports our hypothesis that in nature, animals infected with *Mundinia* show mild signs of infection or remain asymptomatic and may serve as reservoirs for a long term.

Up to date, it was though that *L. macropodum* is restricted to kangaroos and circulate in nature in these marsupials being transmitted by biting midges (7,12). Here we prove these parasites can infect, survive and be infectious to vectors even in rodents. Steppe lemmings were infectious to sand flies in 11% by week 5 p.i., then any of the tested pools was positive for presence of parasites for 10 weeks, but even 33% of pools were positive by week 20 p.i. By this time, parasites were present in inoculated ears and rarely in the tail. Due to these findings, we hypothesise parasites can develop well in ears for limited period of time, then they are suppressed by immune system, but they can survive, slowly overcome immunological barriers, and start to multiply again. It would be interesting to perform long term experiments and observe dynamics of the infection in several time intervals. Interestingly, only animals exposed to

sand flies remained infected, whereas no parasites were observed in other animals, which also supports the influence of sand fly bites on the course of infection (Vojtková et al. 2020) (52).

Animals infected with *Leishmania chancei* showed a mixture of cutaneous and visceral symptoms, with 7/8 animals positive in various tissues and organs. On the other hand, the xenodiagnoses were positive only sporadically.

Steppe lemmings infected with *L. orientalis* displayed no skin lesions, but 4/10 of the animals died during the experiment and one more suffered from hair loss, which is a usual symptom of visceral leishmaniasis similar to their spread widely into various body tissues and organs (38% of liver and 50% of spleen were positive by PCR). Previously, a high susceptibility of Steppe lemmings to progressive VL caused by *L. donovani* has been reported (31,32), so the present study extends the spectrum of *Leishmania* species for which Steppe lemmings may be a suitable animal model for future studies.

This study presents a unique comparison of four strains of *L. martiniquensis* originating from various continents and hosts. While the strains Cu1R1 and Cu2 came from humans living in Thailand, Mar1 originates from human in Martinique and Aig1 was isolated from a horse in the Czech Republic. *Leishmania martiniquensis* is probably the most widespread species of leishmania, which was hidden from scientists until the 1990s and causes various symptoms in different hosts. While the Cu2 strain was destructive to ear pinnae of animals and was present in moderate or high numbers, other strains did not cause any symptoms for the whole course of experiment and they were present only in low numbers in tissues. Interestingly, strains Cu1R1 and Cu2 coming from the same area developed differently in Steppe lemmings: while Mar1 and Cu2 visceralised, strains Cu1R1 and Aig1 remained mainly in inoculated ears or their draining lymph nodes. These differences point to polymorphisms between strains within the species similar to those observed in *L. major* (53).

*Mundinia* is remarkable, important and poorly described subgenus of the genus *Leishmania*. Its worldwide distribution, wide spectrum of hosts, various clinical manifestations, and probable involvement of unusual vectors add more mys-

tery to the whole story. Unravelling questions concerning vectors, reservoir hosts, etc. is a prerequisite for describing the life cycle of these parasites. The use of new animal models, such as Steppe lemmings and Chinese hamsters, can contribute significantly to this goal.

Author Contributions: TB, BV and JS carried out experimental infections of animals and xenodiagnostic experiments. Isolation of tissue and sand fly samples was done by TB, BV, BVK, LT, LP. Molecular analysis was performed by TB. JS and PV substantially contributed to the conception and design of the experiments. The article was drafted by TB and revised by JS and PV. All authors read and approved the final manuscript.

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Institutional Review Board Statement: The animals were maintained and handled in the Charles University animal facility 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 Charles University in Prague and were performed with permission no. MSMT-7831/2020-3 of the Ministry of Education, Youth, and Sports. The investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

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# Experimental transmission of *Leishmania* (*Mundinia*) parasites by biting midges (Diptera: Ceratopogonidae)

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PLOS PATHOGENS

RESEARCH ARTICLE

# Experimental transmission of *Leishmania* (*Mundinia*) parasites by biting midges (Diptera: Ceratopogonidae)

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## Abstract

*Leishmania* parasites, causative agents of leishmaniasis, are currently divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia*. The recently established subgenus *Mundinia* has a wide geographical distribution and contains five species, three of which have the potential to infect and cause disease in humans. While the other *Leishmania* subgenera are transmitted exclusively by phlebotomine sand flies (Diptera: Psychodidae), natural vectors of *Mundinia* remain uncertain. This study investigates the potential of sand flies and biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) to transmit *Leishmania* parasites of the subgenus *Mundinia*. Sand flies (*Phlebotomus argentipes*, *P. duboscqi* and *Lutzomyia migonei*) and *Culicoides* biting midges (*Culicoides sonorensis*) were exposed to five *Mundinia* species through a chicken skin membrane and dissected at specific time intervals post bloodmeal. Potentially infected insects were also allowed to feed on ear pinnae of anaesthetized BALB/c mice and the presence of *Leishmania* DNA was subsequently confirmed in the mice using polymerase chain reaction analyses. In *C. sonorensis*, all *Mundinia* species tested were able to establish infection at a high rate, successfully colonize the stomodeal valve and produce a higher proportion of metacyclic forms than in sand flies. Subsequently, three parasite species, *L. martiniquensis*, *L. orientalis* and *L.* sp. from Ghana, were transmitted to the host mouse ear by *C. sonorensis* bite. In contrast, transmission experiments entirely failed with *P. argentipes*, although colonisation of the stomodeal valve was observed for *L. orientalis* and *L. martiniquensis* and metacyclic forms of *L. orientalis* were recorded. This laboratory-based transmission of *Mundinia* species highlights that *Culicoides* are potential vectors of members of this ancestral subgenus of *Leishmania* and we suggest further studies in endemic areas to confirm their role in the lifecycles of neglected pathogens.

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## Author summary

*Leishmania* parasites are causative agents of leishmaniasis, a disease affecting millions of humans worldwide. It is widely accepted that these flagellates are transmitted exclusively by phlebotomine sand flies (Diptera: Phlebotominae). Reservoir hosts and insect vectors for the newly established *Leishmania* subgenus *Mundinia*, however, remain poorly understood. Preliminary evidence from field-based studies discovered biting midges (Diptera: Ceratopogonidae) that were naturally infected by *L.* (*Mundinia*) *macropodum* in Australia. This surprising finding led us to carry out a detailed laboratory study aimed at comparison of the development of all currently known species of the subgenus *Mundinia* in both putative vector families. We found that all five *Mundinia* species developed successfully in *C. sonorensis* and the successful transmission of three *Mundinia* species from infected insects to mice was demonstrated for the first time. This is the first detailed *in vivo* evidence that biting midges can act as competent vectors of *Leishmania* parasites of the subgenus *Mundinia* and has considerable epidemiological implications for control of these neglected pathogens.

## Introduction

Leishmaniases are a group of diseases whose etiological agents are the protozoan parasites *Leishmania* (Kinetoplastida: Trypanosomatidae). *Leishmania* circulate between a wide range of natural reservoir hosts and phlebotomine sand flies (Diptera: Phlebotominae) and most commonly cause zoonotic disease in humans, although occurrence of anthroponotic cycles has also been described [1]. The signs of the human disease range from single self-healing cutaneous lesions, diffuse cutaneous and mucocutaneous forms, to the most severe visceral leishmaniasis, which can be fatal if untreated. Over 20 human infecting *Leishmania* species have been recognized and leishmaniasis is present in more than 80 countries worldwide, with around 1 million new cases of cutaneous leishmaniasis and 50 000 to 90 000 cases of visceral leishmaniasis occurring annually. These data are likely to underestimate the true burden of the disease since cases are most common in countries with a low level of infrastructure and healthcare development, so the majority of the cases remain unreported, in addition to the potential impact of asymptomatic or mild cases of infection [2].

The genus *Leishmania* is currently divided into four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia* [3]. Subgenera *Leishmania* and *Viannia* include species most frequently detected in humans such as *L. infantum*, *L. donovani*, *L. braziliensis*, *L. major* and are transmitted by sand flies of genera *Phlebotomus* and *Lutzomyia* (Diptera: Psychodidae). The subgenus *Sauroleishmania* includes species infecting reptiles as the primary host, which are transmitted by sand flies of the genus *Sergentomyia* (Diptera: Psychodidae). Finally, the most recently described subgenus *Mundinia* [3] includes five species previously known as the *L. enriettii* complex. While *L. enriettii* [4] and *L. macropodum* [5–6] have only been detected in wildlife kept in captivity, three others, namely *L. orientalis* [7], *L. martiniquensis* [8–9] and an isolate from Ghana that is still formally undescribed [10], have been detected in humans and have the potential to cause disease.

According to phylogenetic analyses, the *Mundinia* subgenus diverges at the base of a phylogenetic tree of the *Leishmania* genus, which points to the ancestral origin of these parasites [6,10] (Fig 1A). This hypothesis is also supported by a worldwide distribution of the subgenus: *L. enriettii* is present in Brazil, *L. macropodum* in Australia, *L.* sp. strain GH5 in Africa

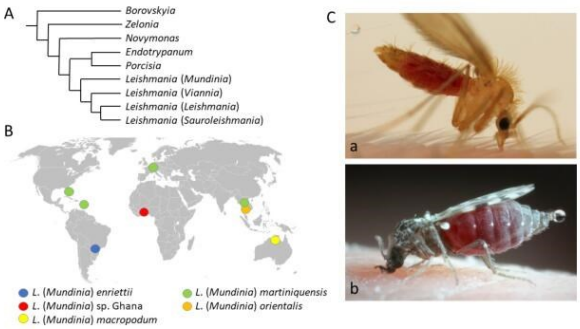


**Fig 1.** (A) Phylogenetic relationships of the four genera of the subfamily Leishmaniinae and subgenera of the genus *Leishmania*, based on [3,6,37]. (B) Geographical distribution of *L.* (*Mundinia*) species, based on [4–5,7–8,10,14–17]. The blank map source - https://commons.wikimedia.org/wiki/Atlasof the_world/. (C) Female sand fly *Phlebotomus duboscqi* feeding on the mouse demonstrating prediuresis which allows rapid concentration of proteins and restoring water and weight balance (a) and *Culicoides* biting midge feeding on a human host while also performing prediuresis (b).

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(Ghana) and *L. orientalis* in south-east Asia [11–13] (Fig 1B). *Leishmania martiniquensis* is also a widely distributed species described first from a human case in Martinique island [8], with autochthonous cattle or horse infections reported from Florida [14], Switzerland [15] and Germany [16] and frequently causing human infections in south-east Asia [17–20]. In case of *L. martiniquensis*, however, recent emergence and anthropogenic spread cannot be ruled out, as the infection is known only from humans and domestic animals.

The transmission ecology of the *Mundinia* subgenus is enigmatic, with no certain identity of the reservoir hosts and insect vectors of any species. *Leishmania enriettii* has been isolated from domestic guinea pigs, but subsequent experimental infections of wild guinea pigs as a proposed reservoir species failed [4]. *Leishmania macropodum* was described from four kangaroo species in captivity, but no reports of CL in the wild populations have been published [5,21]. In the remaining species, there is no definitive indication of reservoir or vector, an issue exacerbated by the fact that these may cause asymptomatic infections and hence detection is reliant on either random sampling or experimental studies [22]. Similarly, experimental models enabling research of *Leishmania* pathology are scarce; guinea pigs and golden hamsters were proven to be susceptible to *L. enriettii* [4,23–27] and *L. martiniquensis* was reported to widely disseminate and visceralize in BALB/c mice [28–30]. The only previous study to systematically examine susceptibility to infection for all five *Mundinia* species was carried out in guinea pigs and only *L. enriettii* demonstrated an ability to infect this host [31].

The paradigm that *Leishmania* species pathogenic to humans are transmitted exclusively by phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World has considerable weight in the literature [32–33]. However, observations published in the last decade have raised the possibility that non-sand fly vectors may contribute to the transmission of species within the *Mundinia* subgenus. *Leishmania macropodum* has been detected in biting midges of the genus *Forcipomyia* collected in areas of Australian *Leishmania* transmission [34]. While no *Leishmania*-positive specimens were detected among nearly 2000 sand fly females of four species, DNA was detected in 6% females of three species of *Forcipomyia* and, importantly, heavy late infections were confirmed microscopically [34]. More recently, laboratory experiments have revealed susceptibility of *C. sonorensis* to infection with *L. enriettii* [25] and *L. orientalis* [35].

Based on these studies, biting midges have met three of the four Killick-Kendrick´s criteria necessary to incriminate *Leishmania* vector [36]. However, the most important criterion—the demonstration of transmission by vector bite—was still lacking. Therefore, we compared the development of all 5 currently known *Mundinia* species in biting midge *C. sonorensis* and three sand fly species sharing geographical distribution with respective *Mundinia* species (Fig 1C). Importantly, we demonstrated experimental transmissions of *Leishmania* parasites by biting midges to the host for the first time.

## Results

### *Mundinia* development in sand flies

Sand fly species sharing the geographical area with four *Mundinia* species were used: Brazilian *L. migonei* were infected with *L. enriettii*, Sub-Saharan *P. duboscqi* with *L.* sp. strain GH5 from Ghana and South Asian *P. argentipes* with *L. martiniquensis* and *L. orientalis*. As Australian sand fly species have never been colonized, *L. migonei* permissive to various *Leishmania* species [38–39] was used for *L. macropodum* infections. For each experiment, *Leishmania* species known already to be transmitted by the respective sand fly species was used as the control.

### Development of *L. enriettii* and *L. macropodum* in *Lutzomyia migonei*

Both *Mundinia* species possessed heavy infections (more than 1000 parasites per gut) and high infection rates (more than 80% infected specimens) in sand flies on day 1 post bloodmeal (PBM) only. On day 5 PBM, post defecation by sand fly females, infection rates dropped radically below 9%, and parasites were present in low or moderate numbers (under 100 or 1000 parasites per gut), localized mainly in the abdominal midgut (AMG) or in the beginning of the thoracic midgut (TMG). At eight days PBM, none of the dissected females exposed to *L. enriettii* contained detectable infections and only 2 females infected with *L. macropodum* showed low or moderate infections in AMG and TMG (Fig 2). In contrast, *L. infantum* used as a positive control developed consistently in *L. migonei*, causing 77% infection rate with heavy infections and colonization of the stomodeal valve in 65% of infected females on day 8 PBM (Fig 2).

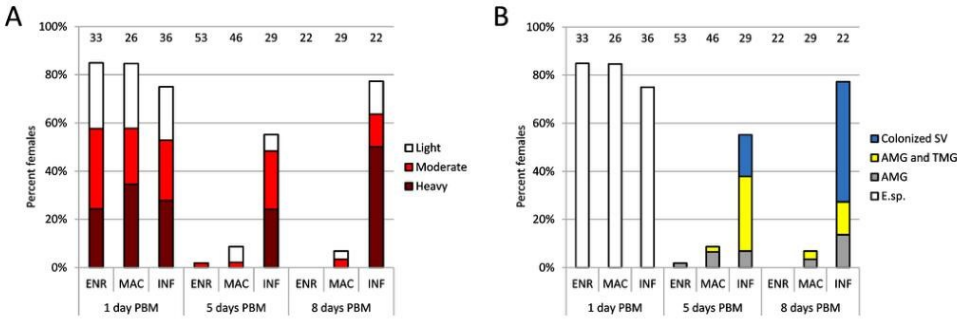


**Fig 2. *Mundinia* development in the sand fly *Lutzomyia migonei*.** Intensity (A) and localization (B) of *L. enriettii* (ENR), *L. macropodum* (MAC) and *L. infantum* (INF) infections. SV, stomodeal valve; AMG, abdominal midgut; TMG, thoracic midgut; E. sp., endoperitrophic space; PBM, post blood meal. Intensity of infection (parasite load) was categorized as light, <100 parasites per gut; moderate, 100–1000 parasites per gut and heavy, >1000 parasites per gut. Numbers of dissected females are displayed above the columns. Statistical differences in intensities of infection among *Leishmania* species were not significant on day 1 PBM ($P = 0.860$, $X^2 = 2.571$, d.f. = 6) while significant on day 5 PBM ($P < 0.0001$, $X^2 = 49.922$, d.f. = 6) and day 8 PBM ($P < 0.0001$, $X^2 = 44.950$, d.f. = 6).

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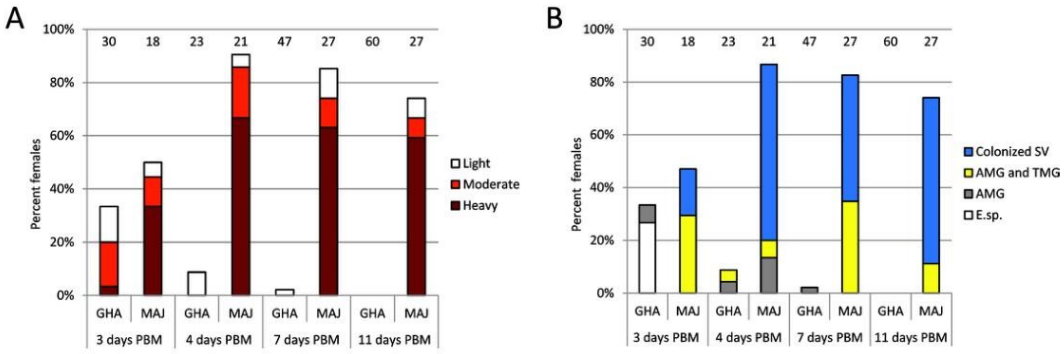


**Fig 3. *Mundinia* development in the sand fly *Phlebotomus duboscqi*.** Intensity (A) and localization (B) of *L*. sp. strain GH5 from Ghana (GHA) and *L. major* (MAJ) infections. SV, stomodeal valve; AMG, abdominal midgut; TMG, thoracic midgut; E. sp., endoperitrophic space; PBM, post blood meal. Intensity of infection (parasite load) was categorized as light, <100 parasites per gut; moderate, 100–1000 parasites per gut and heavy, >1000 parasites per gut. Numbers of dissected females are displayed above the columns. Differences among *Leishmania* species were significant and increased from day 3 PBM ($P = 0.039$, $X^2 = 8.352$, d.f. = 3) to day 4 PBM ($P < 0.0001$, $X^2 = 34.008$, d.f. = 3), 7 PBM ($P < 0.0001$, $X^2 = 54.884$, d.f. = 3) and day 11 PBM ($P < 0.0001$, $X^2 = 57.711$, d.f. = 3).

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## Development of *Leishmania* sp. strain GH5 from Ghana in *Phlebotomus duboscqi*

*Leishmania* sp. from Ghana showed low infection rates (33%) on day 3 PBM, during the bloodmeal digestion. Infection rate was reduced substantially by defecation following the bloodmeal to 9% and then was further reduced to zero over time until day 11 PBM. The few surviving parasites (less than 100 per gut) did not migrate anteriorly, remained localised in the AMG or in the beginning of the TMG (Fig 3). Under the same experimental conditions, control *L. major* developed heavy late-stage infections in 60% of *P. duboscqi* females (Fig 3).

## Development of *L. orientalis* and four *L. martiniquensis* strains in *Phlebotomus argentipes*

During early infections, on day 1 PBM, infection rates and parasite loads of *L. martiniquensis* and *L. orientalis* were comparable with the control *L. donovani*. In later time intervals PBM, *L. donovani* developed heavy infections in almost 80% of *P. argentipes* females, while infection rates of all *Mundinia* strains fell with time, less or more markedly. *Leishmania martiniquensis* MAR1 survived blood digestion and subsequent defecation in 39% of sand fly females and colonised the stomodeal valve as early as on day 4 PBM. Heavy late-stage infections (more than 1000 parasites per gut) were observed in 7% of dissected females 8 days PBM (Fig 4A and 4B). *Leishmania martiniquensis* CU1 survived defecation in 19% of females, but the infection rate further decreased to 15% on day 8 PBM. At this time point most infections were light (less than 100 parasites per gut) and did not reach the cardia region. The experimental infections of *P. argentipes* with *L. martiniquensis* CU2 showed the same trend in reductions in parasite load over time. *Leishmania martiniquensis* Aig1 did not survive defecation of blood remnants in *P. argentipes* as all the females dissected on days 4 and 8 PBM were free of visible infection. On the contrary, *L. orientalis* survived the defecation in relatively high proportion (50%) of *P. argentipes* and heavy late-stage infections with the colonization of the stomodeal valve occurred in 22% of dissected females on day 8 PBM (Fig 4A and 4B).

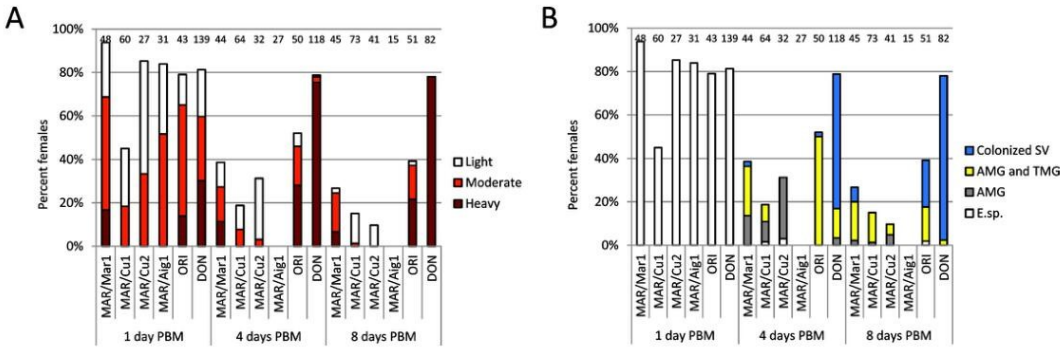


**Fig 4. *Leishmania* development in the sand fly *P. argentipes*.** Intensity (A) and localization (B) of *L. martiniquensis* (MAR; MAR1, Cu1, Cu2, Aig1), *L. orientalis* (ORI) and *L. donovani* (DON) infections assessed by light microscopy. SV, stomodeal valve; AMG, abdominal midgut; TMG, thoracic midgut; E. sp., endoperitrophic space; PBM, post blood meal. Intensity of infection (parasite load) was categorized as light, <100 parasites per gut; moderate, 100–1000 parasites per gut and heavy, >1000 parasites per gut. Numbers of dissected females are displayed above the columns. Differences among *Leishmania* species/strains were significant and increased from day 1 PBM ($P < 0.0001$, $X^2 = 97.997$, d.f. = 15) to day 4 PBM ($P < 0.0001$, $X^2 = 207.642$, d.f. = 15) and day 8 PBM ($P < 0.0001$, $X^2 = 214.778$, d.f. = 15).

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## *Mundinia* development in the biting midge *Culicoides sonorensis*

Five *Mundinia* species (8 strains) were tested for development in *C. sonorensis*: *L. enriettii*, *L. macropodum*, *L.* sp. strain GH5 from Ghana, *L. orientalis* and four strains of *L. martiniquensis* (MAR1, CU1, Cu2 and Aig1). On day 1 PBM, the control dissections (3 females per strain) revealed heavy infections and 100% infection rate in all *Leishmania* strains tested. Parasites were enclosed within the peritrophic matrix with the ingested bloodmeal. On day 3 PBM, the digestion of the engorged blood was completed and the bloodmeal remnants passed out with defecation. With the exception of *L. martiniquensis* Aig1, all *Mundinia* species and strains were at least partially successful in surviving defecation: *L. enriettii*, *L. macropodum* and *L. martiniquensis* MAR1 in about 30% of *C. sonorensis* females tested, *L. martiniquensis* Cu2 in more than 50% of females, *L.* sp. strain GH5 from Ghana in 69% and *L. orientalis* and *L. martiniquensis* Cu1 in more than 80% of females (Fig 5A). Parasites were present in the abdominal and thoracic midgut and three species, *L. orientalis*, *L. martiniquensis* Cu2 and *L.* sp. strain GH5 from Ghana, colonized the stomodeal valve in at least some individuals. Hindgut localization of parasites was not observed in any parasite strain (Fig 5B).

By day 6 PBM, the focus of infection had moved anteriorly, the quantity of parasites had increased (Fig 5C) and five (out of eight) strains had colonized the stomodeal valve in at least some *C. sonorensis* examined (Fig 5D). On the other hand, no infections were observed in midges infected with *L. martiniquensis* Aig1 and *L. enriettii*, as parasites did not resist defecation and were either lost or survived in the very small proportion of females. Finally, on day 10 PBM, heavy infections prevailed and the stomodeal valve was colonized in almost all females infected with the 7 strains (Figs 5E and 5F, 6A and 6B). Colonization of the stomodeal valve was usually associated with heavy infection (more than 1000 parasites per gut), however some females had midgut free of parasites, apart from the cardia region packed with haptomonad forms (Fig 6A and 6B). The highest proportion of females *C. sonorensis* with heavy infections including successful colonization of the stomodeal valve were found in *L. orientalis*, *L. martiniquensis* Cu2 and the Ghanaian species, comprising more than 60% of all dissected females (Fig 5E and 5F). Five *Mundinia* species developed significantly heavier late infections in *C. sonorensis* compare to sand fly vector (Table 1).

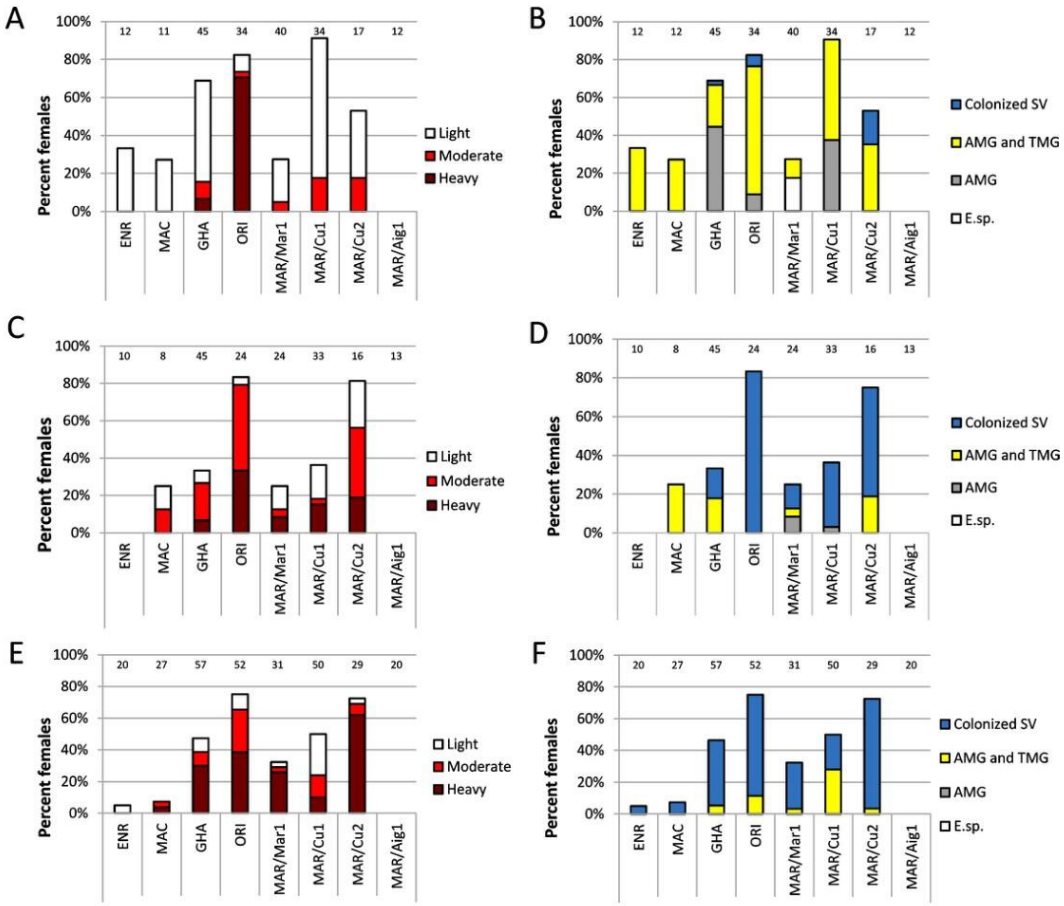


**Fig 5. *Mundinia* development in the biting midge *C. sonorensis*.** Intensity (A, C, E) and localization (B, D, F) of *L. enriettii* (ENR), *L. macropodum* (MAC), *L.* sp. strain GH5 from Ghana (GHA), *L. orientalis* (ORI) and *L. martiniquensis* (MAR/MAR1, MAR/Cu1, MAR/Cu2, MAR/Aig1) infections assessed by light microscopy. SV, stomodeal valve; AMG, abdominal midgut; TMG, thoracic midgut; E. sp., endoperitrophic space; PBM, post blood meal. Intensity of infection (parasite load) was categorized as light, <100 parasites per gut; moderate, 100–1000 parasites per gut and heavy, >1000 parasites per gut. Numbers of dissected females are written above the columns. Differences among *Leishmania* species/strains were significant on day 3 PBM ($P < 0.0001$, $X^2 = 181.173$, d.f. = 21), day 6 PBM ($P < 0.0001$, $X^2 = 70.048$, d.f. = 21) and day 10 PBM ($P < 0.0001$, $X^2 = 117.932$, d.f. = 21).

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## Metacyclic forms

Presence of infective metacyclic forms was evaluated from Giemsa-stained gut smears in 5 strains showing the high infection rate on day 10 PBM in *C. sonorensis* (*L. martiniquensis* MAR1, Cu1, Cu2, *L. orientalis* and *L.* sp. strain GH5 from Ghana) and in 2 species producing the highest infection rates on day 8 PBM in *P. argentipes* (*L. martiniquensis* MAR1 and *L. orientalis*). Infective stages were detected in *C. sonorensis* infected with *L. martiniquensis*

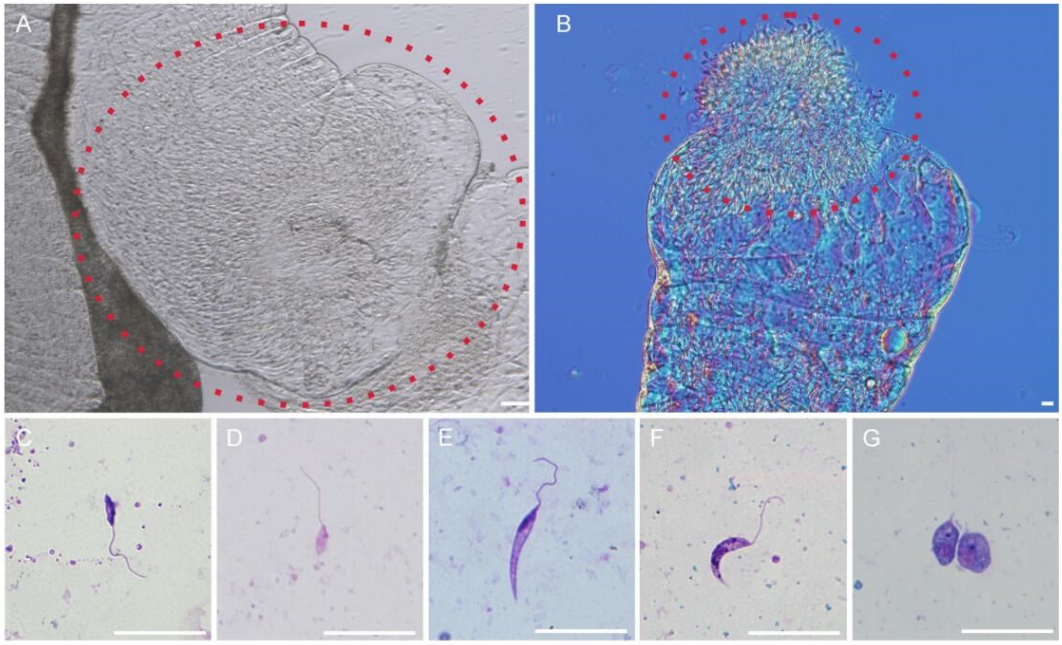


**Fig 6. Localisation and morphology of *Mundinia* in biting midge *C. sonorensis* on day 10 PBM.** A-B, colonization of the stomodeal valve with the part of the thoracic midgut filled with parasites, the region is marked by a red dotted line: (A) mature infection with *L. martiniquensis* Cu1; (B) mature infection with *L. orientalis*. C-G, various morphological forms present in the midgut on day 10 PBM: (C) metacyclic form of *L. martiniquensis* Cu2; (D) metacyclic form of *L. orientalis*; (E) nectomonad form of *L. martiniquensis* MAR1; (F) leptomonad form of *L. martiniquensis* Cu2; (G) haptomonad form of *L. martiniquensis* Cu2. Scale bar = 20μm.

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**Table 1. Comparison of intensities of late infections between *C. sonorensis* and sand fly species.**

| *Leishmania* sp. | Vector sp. | N | Statistics |
|---|---|---|---|
| *L. enriettii* | *L. migonei* | 22 | $X^2 = 1.127$, d.f. = 1, P = 0.476 |
| | *C. sonorensis* | 20 | |
| *L. macropodum* | *L. migonei* | 29 | $X^2 = 2.008$, d.f. = 3, P = 0.571 |
| | *C. sonorensis* | 27 | |
| *Leishmania* sp. from Ghana | *P. duboscqi* | 60 | $X^2 = 36.947$, d.f. = 3, P < 0.0001 |
| | *C. sonorensis* | 57 | |
| *Leishmania martiniquensis* Cu1 | *P. argentipes* | 45 | $X^2 = 8.079$, d.f. = 3, P = 0.044 |
| | *C. sonorensis* | 31 | |
| *Leishmania martiniquensis* MAR1 | *P. argentipes* | 73 | $X^2 = 22.099$, d.f. = 3, P < 0.0001 |
| | *C. sonorensis* | 50 | |
| *Leishmania martiniquensis* Cu2 | *P. argentipes* | 41 | $X^2 = 39.595$, d.f. = 3, P < 0.0001 |
| | *C. sonorensis* | 29 | |
| *Leishmania martiniquensis* Aig1 | *P. argentipes* | 15 | Statistics not applicable |
| | *C. sonorensis* | 20 | |
| *Leishmania orientalis* | *P. argentipes* | 51 | $X^2 = 14.271$, d.f. = 3, P = 0.003 |
| | *C. sonorensis* | 52 | |

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MAR1 and Cu2, *L. orientalis* and *L.* sp. from Ghana (Table 2), but not with *L. martiniquensis* Cu1 infected midges. The highest representation of metacyclics (5% and 10% respectively) was observed in *C. sonorensis* infected with *L. orientalis* and *L. martiniquensis* Cu2 (Fig 6C and 6D), i.e., the strains which developed heavy late-stage infections in the highest percentage of *Culicoides* females (Fig 5E). The spectrum of other morphological forms produced by *Mundinia* species in *C. sonorensis* is showed in Fig 6E–6G.

In contrast, a lower proportion of metacyclic forms was observed in *Mundinia* infections in sand flies. Two species producing heavy late-stage infections in *P. argentipes* (*L. orientalis* and *L. martiniquensis* MAR1) were analysed. The metacyclics comprised 3% from *L. orientalis* promastigotes (N = 132) and no metacyclic stages were detected in gut smears from females infected with MAR1 (N = 130) 8 days PBM.

### Transmission experiments

Transmission experiments were done with four species of *Leishmania* showing heavy late-stage infections in *C. sonorensis*: *L. orientalis*, *L. martiniquensis*, *L. macropodum* and *L.* sp. strain GH5 from Ghana, and with two species: *L. orientalis* and *L. martiniquensis*, producing heavy late-stage infections in *P. argentipes*. In total, 71 midges and 107 sand flies were allowed to feed on the ear pinnae of anaesthetized BALB/c mice 8–11 days post infective blood meal. Immediately post exposure, mice were sacrificed, their ears stored for PCR analysis, and vector infections were confirmed by microscopical observation. Presence of *Leishmania* infection in engorged vectors was confirmed microscopically in all the experimental groups except in *C. sonorensis* infected with *L. macropodum* (Table 3). Table 3 also illustrates unfed females with mature infections characterized by colonisation of the stomodeal valve, as these females may also contribute at least theoretically to transmission by probing, even without taking a blood meal.

A polymerase chain reaction (PCR) assay with primers flanking a 116 bp segment of the minicircle kinetoplast DNA (kDNA) was used to detect *Leishmania* in mice ear tissues. PCR amplification showed the presence of *Leishmania* minicircle kDNA in mice exposed to *C. sonorensis* infected with two strains of *L. martiniquensis* (Cu1, Cu2), *L. orientalis* and *L.* sp. strain GH5 from Ghana. The samples gathered from transmission experiments performed with *P. argentipes* were negative for both *Mundinia* species in two independent experiments. On the other hand, positive transmission was achieved for control *L. donovani* (S1 Fig and Table 3).

## Discussion

The paradigm that *Leishmania* parasites are transmitted solely by sand flies has been undermined in recent years by the apparent transmission of *L. macropodum* by biting midges of the subgenus *Forcipomyia* (*Lasiohelea*) [34]. Later, successful development of *L. orientalis*, *L. enriettii* and *L. macropodum* was also observed in the biting midge *C. sonorensis* in laboratory

**Table 2. Proportion of metacyclic forms developing in *C. sonorensis* guts 10 days PBM.**

| ***Leishmania* strain** | **Number of measured cells** | **Percent of metacyclic forms** |
|---|---|---|
| *Leishmania martiniquensis* MAR1 | 168 | 0.6 |
| *Leishmania martiniquensis* Cu1 | 214 | 0 |
| *Leishmania martiniquensis* Cu2 | 134 | 10 |
| *Leishmania orientalis* LSCM4 | 175 | 5 |
| *Leishmania* sp. from Ghana GH5 | 180 | 0.6 |

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**Table 3. Microscopical examination of *C. sonorensis* and *P. argentipes* females exposed to BALB/c mice for transmission of parasites and result of PCR detection of *Leishmania* minicircle kDNA in mouse ears.**

| Vector species | *Leishmania* strain | Day PBM | No. of females exposed to the mouse | No. of infected engorged females | No. of infected unfed females | No. of unfed females with colonization of the SV | Transmission confirmed by PCR |
|---|---|---|---|---|---|---|---|
| *C. sonorensis* | *L. martiniquensis* MAR1 | 11 | 6 | 1/3 | 2/3 | 2 | No |
| | *L. martiniquensis* Cu1 | 10 | 19 | 2/7 | 5/12 | 5 | Yes |
| | *L. martiniquensis* Cu2 | 11 | 10 | 2/3 | 6/7 | 6 | Yes |
| | *L. orientalis* LSCM4 | 10 | 15 | 7/11 | 4/4 | 4 | Yes |
| | *L.* sp. from Ghana GH5 | 10 | 14 | 4/5 | 7/9 | 7 | Yes |
| | *L. macropodum* | 11 | 7 | 0/3 | 0/4 | 0 | No |
| *P. argentipes* | *L. orientalis* LSCM4 | 8 | 17 | 2/3 | 10/14 | 5 | No |
| | | 8 | 30 | 2/18 | 2/12 | 1 | No |
| | *L. martiniquensis* MAR1 | 8 | 11 | 1/1 | 7/10 | 0 | No |
| | | 8 | 34 | 7/8 | 19/26 | 4 | No |
| | *L. donovani* CUK3 | 8 | 15 | 2/8 | 4/7 | 2 | Yes |

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conditions [25,35]. Among sand flies, *Leishmania* of the subgenus *Mundinia* were detected only in Thailand where *L. orientalis* DNA was PCR-detected in *Sergentomyia* (*Neophlebotomus*) *gemmea* and *S. iyengari* [12,40]. However, without microscopy, it is not possible to distinguish late mature infections from early ones which are non-specific and may be lost with defecation in refractory vectors. Therefore, these molecular findings cannot be considered as a proof of the vector identification [41].

In the current study we have convincingly demonstrated successful infection, propagation and transmission of *Leishmania* species of the subgenus *Mundinia* in *C. sonorensis* under laboratory conditions. In parallel, infection and transmission experiments with epidemiologically relevant sand fly species conducted under the same laboratory conditions and using the same *Leishmania* lines provided only limited evidence of infection and propagation and no evidence of transmission using the *in vivo* mouse model. This study therefore provides the strong underpinning evidence that biting midges may play a role in the transmission of *Leishmania* strains within the subgenus *Mundinia* and highlights the importance of further field-based studies to define this role in areas of pathogen transmission. This is particularly required in the context of the unique geographical distribution of *Leishmania martiniquensis* which is the only *Leishmania* species that occurs in Central or Eastern Europe (the isolate Aig1 used in this study originated from a horse infected either in the Czech Republic or in Ukraine).

*Culicoides sonorensis* is a member of the monophyletic subgenus *Monoculicoides*, which has 24 identified species worldwide and in Europe is represented by five species [42]. A key limitation in research on the *Culicoides* genus as a whole is the lack of availability of epidemiologically relevant colony lines and *C. sonorensis*, which transmits a range of arboviruses in North America, is currently the only major vector species available [43–44]. This limitation is exacerbated by a lack of knowledge regarding the major anthropophilic *Culicoides* species in regions where transmission of *Leishmania* strains occurs, particularly given that these are likely to differ significantly from those adapted for feeding on livestock, both in biology and ecology. The evidence presented in the current paper provides a fundamental reason for studies aimed at

both providing resources for laboratory experimentation and defining transmission ecology of the subgenus *Mundinia*.

Within the studies conducted, *L. enriettii* originating from Brazil was tested in *L. migonei*, a sand fly species widespread in South America [45–46] and known to support development of *L. infantum*, *L. amazonensis* and *L. braziliensis* [38–39]. Our results demonstrated that *L. migonei* possessed limited susceptibility to *L. enriettii*, since none of dissected females developed mature infections. In a majority of females, parasites were defecated with blood remnants and in case parasites survived defecation, they were present in very low numbers in abdominal or thoracic midgut with a very low probability of transmission to the mammalian host. Similar failure of the development was described for *L. enriettii* in *L. longipalpis* [25], the proven vector of *L. infantum* and the most important permissive vector in Latin America [47]. On the other hand, *L. enriettii* colonized the stomodeal valve in 5% of *C. sonorensis*, which corresponds to previously published results [25].

The Ghanaian *Mundinia* species shares the distribution of *P. duboscqi* [48], a proven vector of *L. major* [33]. Our experimental results showed that this member of the *Mundinia* subgenus is not adapted to survive in this sand fly since parasites were lost with defecation of bloodmeal remnants. Nevertheless, this species developed heavy infections and colonized the stomodeal valve in 40% of *C. sonorensis* and were transmitted to mice via feeding on the ear, leading to the potential that this species may also be adapted to biting midge infection and transmission.

*Leishmania macropodum* from Australia did not generate mature infection in *L. migonei* in this study, although it was previously reported to develop in *L. longipalpis* more successfully than *L. enriettii*, showing colonization of the SV in 6% of females [25]. Vector competence of both *Lutzomyia* species tested may be different from those *Sergentomyia* and *Phlebotomus* species that are native to Australia [49], but not available in captivity [50]. In *C. sonorensis*, however, *L. macropodum* colonized the SV in just 7% of females and no infection was detected in females exposed to mice in transmission experiment. However, the transmission may be substantially more effective in biting midges of the subgenus *Lasiohelea*, which were found to be naturally infected in Australia, but which have not been colonised to date [34].

Development of *L. martiniquensis* and *L. orientalis* was assessed in the sand fly species *P. argentipes*, a proven vector of *L. donovani* [51], with a distribution from the Indian peninsula to south-east Asia including Sri-Lanka [52]. We performed experimental infections with *L. orientalis* originating from Thailand and with 4 strains of *L. martiniquensis*—MAR1 originating from the human case in the Martinique island, Cu1 and Cu2 isolated from humans in Thailand and strain Aig1 isolated in the Czech Republic from the four-year-old grey Akchal teke mare horse, imported to the Czech Republic two years ago from Ukraine. Both Thai strains Cu1 and Cu2 survived poorly in *P. argentipes*, failing to develop mature infections, but colonized the SV in 22% and 69% of *C. sonorensis* females, respectively. The strain from the Martinique Island MAR1 generated heavy mature infections in both vector types—colonization of the SV was observed in 7% of *P. argentipes* and 29% of *C. sonorensis* females. Thus, interestingly, the geographically distant isolate of *L. martiniquensis* developed better in *P. argentipes* than sympatric isolates. Importantly, three human *L. martiniquensis* isolates developed late-stage infections in *C. sonorensis* and Thai isolates Cu1 and Cu2 were transmitted to the mouse by *C. sonorensis* bite, while the Aig1 isolate failed to develop.

*Leishmania orientalis* developed heavy late-stage infections with colonization of the SV in 20% of *P. argentipes*, although the same *L. orientalis* strain was reported to be unable to establish infection in *Lu. longipalpis* [35]. In *C. sonorensis*, the SV was colonized even in 63% of females, a much higher rate than previously reported [35]. Metacyclic stages were present in both vector groups, but transmission by bite was demonstrated only for *C. sonorensis*. Thus, the involvement of biting midges in *L. orientalis* transmission is highly likely, although the role

of *Phlebotomus* species was not convincingly excluded. Generally, transmission of *Mundinia* by sand flies must be still considered as various sand fly species present in endemic localities cannot be included into the laboratory study being never colonized, particularly South American and South Asian members of the genus *Sergentomyia*. The only species of the genus *Sergentomyia* where vector competence has been directly tested by experimental infections, *S. schwetzi*, was demonstrated to be refractory to *Leishmania* parasites [53–54]. However, the vector competence of other species of this large genus of sand flies may differ (reviewed by [55]).

One of the basic traits characterizing *Leishmania* subgenera is the mode of their development in the vector. Members of the *Viannia* subgenus undergo peripylarian development, (the hindgut infection is followed by anterior migration of the parasites to the midgut and foregut) while the suprapylarian development in the *Leishmania* subgenus takes place only in the midgut and foregut. Both suprapylarian and peripylarian modes result in transmission by bite contrary to the hypopylarian development in the *Sauroleishmania* subgenus, restricted to the hindgut and resulting in contaminative transmission [56]. In this study, development of all *Mundinia* species tested was purely suprapylarian in both biting midges and sand flies; we did not observe attachment of haptomonad stages in the hindgut, typical for peripylarian development in any sand fly species, nor in *C. sonorensis*. According to the basal position of the subgenus *Mundinia* on the phylogenetic tree of the genus [6], the suprapylarian development may thus represent the ancestral type. However, this finding must be confirmed after identification and colonization of natural vectors of the respective *Mundinia* species.

Within this study, the highest proportion of infective metacyclic forms in mature infections were 10% in *C. sonorensis* infected with *L. martiniquensis* Cu2, 5% of *C. sonorensis* infected with *L. orientalis* and 3% in *P. argentipes* infected with *L. orientalis*. Although these frequencies might look low, similar proportions were reported for mature infections of *L. donovani* in *P. argentipes*; 3–5% of metacyclics were apparently sufficient to transmit the infections to mice [57]. As reported recently, population of metacyclic forms may increase with second and further bloodmeals in natural infections, resulting in greater potential to transmit parasites [58]. Besides metacyclics, other morphological forms are equally important for successful transmission—haptomonads attached to the chitin surface of the stomodeal valve and leptomonads producing promastigote secretory gel create the blocked fly, which forces infected female to regurgitate parasites into the skin (reviewed in [59]).

Taken together, our results strongly suggest potential involvement of biting midges in transmission of *Mundinia* parasites. All five tested species developed better in *C. sonorensis* than in a range of sand fly species, based on survival of vector defecation, the higher rate of the stomodeal valve colonisation and the successful production of metacyclic stages. Most importantly, the transmission to an *in vivo* mouse model was achieved using *C. sonorensis* infected with *L. orientalis*, *L. martiniquensis* and *L.* sp. strain GH5 from Ghana and failed entirely with the sand fly lines used. Adaptation of *Mundinia* to biting midges is most probably specific to this subgenus, as human pathogens *L. donovani*, *L. major* and *L. infantum* of the subgenus *Leishmania* do not establish mature infections in *C. sonorensis* or *C. nubeculosus* [25,60]. Although the results presented here support the significant role of biting midges in *Mundinia* transmission, many aspects of this vector—parasite interaction remain to be resolved.

## Methods

### Ethics statement

BALB/c mice 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 permission no. MSMT-7831/2020-3 of the Ministry of Education, Youth and Sports. Investigators are certificated for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

## Sand flies, biting midges and *Mundinia* parasites

Sand fly colonies (*Lutzomyia migonei*, originating from Brazil, *Phlebotomus duboscqi*, originating from Senegal and *Phlebotomus argentipes*, originating from India) were maintained in the Insectary of the Department of Parasitology, Charles University, under the standard conditions (26˚C, humidity in the insectary 60–70%, photoperiod 14 h light/ 10 h dark and fed with 50% sucrose) as described previously [61]. *Culicoides sonorensis* (subgenus *Monoculicoides*) were sent to Charles University from the Pirbright Institute, UK and kept at 25˚C before exposure to feeding. All insects were given free access to 50% sucrose.

*Leishmania enriettii* (MCAV/BR/45/LV90), *L. macropodum* (MMAC/AU/2004/AM-2004), *L.* sp. from Ghana (MHOM/GH/2012/GH5), *L. orientalis* (MHOM/TH/2014/LSCM4), four strains of *L. martiniquensis* (MHOM/MQ/1992/MAR1; MHOM/TH/2011/CU1; MHOM/TH/2019/Cu2 and MEQU/CZ/2019/Aig1), *L. major* (MARV/SN/XX/RV24; LV109) and *L. donovani* s. lat. *(L. infantum*/*donovani* hybrid (ITOB/TR/2005/TOB2) [62] and *L. infantum* (MHOM/TR/2000/OG-VL*)* were used. Parasites were maintained at 28˚C in M199 medium supplemented with 20% foetal calf serum (Gibco, Prague, Czech Republic), 1% BME vitamins (Sigma-Aldrich, Prague, Czech Republic), 2% sterile urine and 250 μg/ml amikacin (Amikin, Bristol-Myers Squibb, Prague, Czech Republic). Before experimental infection, parasites were washed by centrifugation (2400 x g for 5 min), resuspended in saline solution and counted using haemocytometer (Bürker chamber).

## Experimental infections of insects

Female biting midges and sand flies (3–5 days old) were infected by feeding through a chick-skin membrane with promastigotes from log-phase cultures resuspended 1:10 in a heat-inactivated rabbit blood (LabMediaServis) at a final concentration of $1 \times 10^6$ promastigotes/ml. Engorged females were separated and maintained in the same conditions as the colony for subsequent dissections at various time intervals. Intensity and localisation of infection were evaluated under the light microscope; the infections were scored as light (<100 parasites per gut), moderate (100–1000 parasites per gut) or heavy (>1000 parasites per gut) [63]. Differences in intensities of infections were tested by Chi-Square test using the software SPSS version 23. Morphology of parasites from insect guts was evaluated from gut smears fixed with methanol and stained with Giemsa. Promastigotes were examined by light microscopy with an oil immersion objective and photographed using Olympus DP70 camera. Body length and flagellar length of parasites were measured using Image J software. Promastigotes were scored as metacyclic forms when flagellar length $\geq$ 2 times body length and body length $<$ 14 μm, leptomonad forms when body length $<$ 14 μm and flagellar length $>$ 2 μm and $<$ 2 times body length; nectomonads when body length $>$ 14 μm and haptomonads when flagellum $\leq$ 2 μm, according to [64].

## Transmission experiments

Experimentally infected insects were maintained for 10 days at 25˚C and then allowed to feed on the naive BALB/c mouse. Animals were anaesthetized with the mixture of ketamin and

xylazine (62 mg/kg and 25 mg/kg). Insect females were placed into small plastic tubes covered with fine mesh and the tubes were held on the ear pinnae of the anaesthetized mouse for one hour. Mice were euthanized immediately post experiment by cervical dislocation under anesthesia. The ear pinnae (the place of biting) were dissected and stored at -20˚C. Insects were dissected immediately post bloodmeal and checked for the presence of *Leishmania* under the light microscope.

### Polymerase chain reaction (PCR) assay

DNA extraction from ear pinnae was performed using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. The total DNA was used as a template for conventional PCR targeting *Leishmania* minicircle kDNA.

Conventional PCR targeting *Leishmania* minicircle kDNA was performed using EmeraldAmp GT PCR Master Mix (TaKaRa) and cycling conditions were as follows: step 1, 94˚C for 3 min; step 2, 94˚C for 20 s; step 3, 63˚C for 20 s; step 4, 72˚C for 5 s; step 5, 72˚C for 5 min; followed by cooling at 12˚C. Steps 2–4 were repeated 40 times. Product length was 116 bp and primers sequences were: Forward- 5´- AGA TTA TGG AGC TGT GCG ACA A- 3´ and Reverse- 5´- TAG TTC GTC TTG GTG CGG TC- 3´ [65]. Samples were analysed using 2% agarose gels.

## Supporting information

**S1 Fig. Amplification of a 116 bp *Leishmania* minicircle kDNA fragment.** 1, Positive control from cultured parasites; 2, Negative control; 3–8, Mouse ear exposed to biting midges (*Culicoides sonorensis*) infected with *L. martiniquensis* Mar1 (3), *L. martiniquensis* Cu1 (4), *L. martiniquensis* Cu2 (5), *L. orientalis* (6), *L.* sp. from Ghana (7), *L.* macropodum (8); 9–13, Mouse ear exposed to *P. argentipes* infected with *L. orientalis* (9, 10), *L. martiniquensis* Mar1 (11, 12) and *L. donovani* (13).
(TIF)

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# 4. Summary and Discussion

The experiments and results in these theses summarise data from independent projects leading to one common goal: to deepen our knowledge of *Mundinia* biology. Over the past few years, I have focused on this enigmatic subgenus and have tried to develop strategies to increase their understanding. Together with other team members, we have performed a number of experimental infections of animals and insects in the hope of describing proper mammalian and insect laboratory models for research on these parasites.

In mammalian infections, we decided to use intradermal inoculation of culture-derived parasites, which most closely resembles the natural mode of transmission, because the parasites are exposed to immune cells localised in skin immediately after inoculation. It would be optimal to inoculate mammals with a low dose of parasites derived from sand flies and to co-inoculate homogenate of salivary glands of vectors [73], [74], [102], but since the vectors are unknown, we decided to keep the classical and simple method of infection (inoculation of culture-derived parasites). We used a higher infectious dose ($10^7$ parasites/rodent) compared to the natural situation because there was a lower percentage of infectious metacyclic stages in culture compared to the thoracic guts of the vectors and also because infection-enhancing factors present in the midgut and salivary glands of sand flies were not added [13], [103], [104]. After inoculation, we followed similar experimental schemes for all species tested. We divided the animals into two groups; the first was used for xenodiagnostic experiments to test animal infectiousness to sand flies at regular time intervals, and the second group was left without any interventions. Throughout the experiment, the animals were checked for external signs of infection. At the end of the experiment, the animals were killed, dissected and the parasite load and distribution in the tissues were evaluated by PCR and qPCR.

In the first study, we tested the susceptibility of guinea pigs to all currently known *Mundinia*. They were chosen because, along with golden hamsters, they were the only known non-human mammalian hosts suitable for laboratory experiments with *Mundinia*. Previous studies and case reports from endemic localities have shown that *L. enriettii* infection in guinea pigs results in cutaneous lesions at the site of inoculation, which may heal spontaneously, or, when infected with more virulent strains, dissemination throughout the body may occur and symptoms of visceral leishmaniasis may appear [65], [66], [81], [105]. In our experiments, we aimed to confirm the susceptibility of guinea pigs to *L. enriettii* and to extend the spectrum of parasite species that can be studied in these mammals. We used three guinea pigs for each species tested, *L. enriettii*, *L. macropodum*, *L. chancei*, *L. orientalis,* and two strains of *L. martiniquensis* (MAR1 and Cu1R1), and

inoculated parasites into both ears to minimize the number of animals used with maximum throughput.

In all guinea pigs infected with *L. enriettii,* dry lesions appeared at the site of inoculation between the 2nd and 3rd week p.i., were enlarged by the 5th-6th week p.i. and then healed spontaneously by the 8th-12th week p.i. In accordance with the results previously published by Seblova et al. [81], animals were significantly more infectious in xenodiagnoses performed at week 4 p.i. (9/16 positive pools) than by week 8 p.i. (1/16 positive pools). At week 12, at the end of the experiment, no pathological signs of infection were observed on the ears and no parasite DNA was detected in any of the tissues tested. This course of infection shows, that parasites are capable of infection and temporary development in guinea pigs, but they are suppressed and destroyed by immune system after certain period of time. On the other hand Thomaz-Soccol [67], observed non-healing phenotype in naturally infected animals where all specimen died in the end. These data illustrate different pathogenicity and behaviour of various strains and also influence of experimental scheme on the output of experiment. Temporary erythema was observed in animals infected with *L. martiniquensis* (MAR1) between weeks 4 and 8 weeks p.i., but no further development of skin changes or infectiousness to sand flies was observed. The last parasite species that was capable to cause pathological changes in the ears was *L. orientalis*, which at 3-4 weeks p.i. caused a erythematous spot in the ears similar to *L. martiniquensis*, but unlike *L. martiniquensis,* this spot nodulated and later turned into a dry lesion surrounded by a purple skin macula. These pathological changes healed spontaneously at 7-8 weeks p.i. Interestingly, no positive sand flies were observed at any time point during xenodiagnoses and no parasite DNA was present in the *post mortem* PCR. Guinea pigs infected with other parasite species (*L. macropodum*, *L. chancei,* and *L. martiniquensis* Cu1R1) showed no signs of infection throughout the experiment and no parasite DNA was observed in the tissues.

In summary, we have demonstrated that guinea pigs are susceptible to *L. enriettii* under laboratory conditions and that *L. martiniquensis* and *L. orientalis* can temporarily survive and develop skin changes at the inoculation site, but cannot disseminate through the body or be a source of infection for vectors.

In the second study, we tested the susceptibility of *Arvicanthis niloticus* and *Mastomys natalensis* to *Leishmania chancei.* These wild mammals have a good potential to be reservoirs of *L. major* [74], and since they live in high population numbers in endemic localities, they were suspected as potential reservoir hosts also for *L. chancei.* We used the same experimental design as for guinea pigs with a difference in duration of the experiment (20-25 weeks instead of 12 weeks). Xenodiagnoses were performed every five weeks, but no positive sand flies were observed. At the end of experiment, all *A. niloticus* samples were negative for the presence of parasite DNA.

The animals also showed no external signs of infection during the experiment. In *M. natalensis*, although all xenodiagnoses were negative, low numbers of parasites were found in the inoculated ear and its draining lymph nodes, the contralateral ear and the forepaws of one animal, suggesting dissemination through the skin, but no pathological cutaneous symptoms were observed in this or any other animal. Additionally, the weight gain of the animals was significantly lower compared to the control group. These findings support the hypothesis that *A. niloticus* is not involved in circulation of *L. chancei,* but we can't exclude the possible involvement of *M. natalensis*. We also need to consider the possibility of different parasite development in these mammals when infected with other strains, the mode of inoculation or the possibility that parasites simply need a longer time to overcome immune barriers and successfully multiply in the host.

After experiments with guinea pigs and wild rodents, we decided to test the susceptibility of the most used laboratory animal in biological and biochemical research, BALB/c mice, which are a well-described model for, for example, *L. major* [106]. We also decided to test two of less established model rodent species, steppe lemmings (*Lagurus lagurus*) and Chinese hamsters (*Cricetulus griseus*), mainly because they have been previously found highly susceptible to *L. major* and *L. donovani* [73]. Unlike inbred BALB/c mice, these wild rodents are genetically polymorphic and they better mimic a true situation in nature. We used the same experimental scheme and intradermally inoculated 10 individuals of each rodent species with $10^7$ culture-derived parasites of 8 *Mundinia* strains covering 5 species: *L. enriettii*, *L. macropodum*, *L. chancei*, *L. orientalis,* and 4 strains of *L. martiniquensis* (one isolate from a horse from the Czech Republic (Aig1) and three human isolates from Martinique (MAR1) and Thailand (Cu1R1, Cu2), respectively. The experiment lasted 20 weeks and xenodiagnoses were performed every 5 weeks.

While, BALB/c mice showed no pathological signs of infection, were not infectious to sand flies, and parasites were not present in any of animals tested, Chinese hamsters and steppe lemmings appeared susceptible to infection. Chinese hamsters showed no external signs of infection but were infectious to sand flies throughout the experiment by *L. chancei*, *L. orientalis* and *L. martiniquensis* (MAR1, Cu1R1 and Cu2) and in weeks 5-15 p.i. for *L. enriettii*. Animals in these groups were also tested positive for parasite DNA by the end of the experiment, although only low number of parasites (<1000 per organ) was detected in the tissues. Parasites were present mainly in the inoculated ears, but rarely in draining lymph nodes of inoculated ears and hind paws. Animals infected with *L. macropodum* and *L. martiniquensis* Aig1 were negative for parasites. The highest proportion of inoculated ears was positive in *L. martiniquensis* Cu2 (80%), followed by *L. orientalis* (40%), *L. chancei* and *L. martiniquensis* Cu1R1 (30%), *L. martiniquensis* MAR1 (20%) and *L. enriettii* (10%). These data suggest that in some *Mundinia* species, Chinese hamsters can be used as a model for asymptomatic infection. No pathology seems to be prevail in hosts infected by *Mundinia*

in the wild as no symptomatic wild animals have been ever described, and also most human infections in Thailand are asymptomatic, even in HIV-positive community [86].

Steppe lemmings were found to be highly susceptible to all tested *Mundinia* species and infections were symptomatic in some individuals. Rodents were often infectious to sand flies throughout the experiment, parasites disseminated through the body of the animals and heavy infections (over 10,000 parasites/organ) were described. Symptoms of infection varied from asymptomatic to visceral, both between groups and within the same group. Interestingly, only animals used for xenodiagnoses developed skin lesions, which supports the hypothesis published by Vojtkova et al. [64] that sand fly bites enhance the progression of infection.

All steppe lemmings infected with *L. enriettii* were positive at the end of experiment (100% of inoculated ears, 30% of draining lymph nodes of the inoculated ear, 10% of forepaws, hind paws, and spleens) and even though only low or moderate loads were detected (maximum $2{,}4x10^4$ parasites/organ), a high proportion of positive pools of xenodiagnoses were found (more than 85% from week 10 p.i.). As already mentioned, animals that were not used for xenodiagnoses remained asymptomatic. In the other rodents, swelling and dry lesions on the ears appeared from week 8-9 p.i. and persisted until the end of experiment. Steppe lemming are, after guinea pigs and golden hamsters, the third species that have been shown to be susceptible to this enigmatic *Leishmania*. Based on these results, we support hypothesis that *L. enriettii* circulates in nature in wild rodents and domestic guinea pigs are only incidental hosts [107].

*Leishmania macropodum* did not cause any signs of infection even in the xenodiagnosed group, but parasites were found in 33% of inoculated ears (xenodiagnosed group only) and in the tail of one animal (non-xenodiagnosed group), and steppe lemmings were infectious to sand flies at week 5 p.i. (11%) and at week 20 p.i. (33%). Even though infection rates were low, steppe lemmings are the first animals, other than kangaroos, that have been shown to be susceptible to *L. macropodum.* This species was thought to be restricted to marsupials, but field studies targeting natural reservoirs should obviously include rodents as well. The dynamics of xenodiagnoses positivity suggest that parasites are capable to infecting the host, then are temporarily suppressed by the immune system, but later overcome immunological barriers and start to progress and multiply. Thus, experiments of longer duration would be needed to show the true dynamics and potential of *L. macropodum* development in rodents.

Steppe lemmings infected with *L. chancei* showed a mixture of cutaneous and visceral symptoms. One animal developed a lesion leading to partial destruction of the inoculated ear from 8 weeks p.i. to 13 weeks p.i., and two other animals were observed to have loss of hair on the abdomen at weeks 10-12 p.i., one of which died 3 weeks after the appearance of these symptoms. All other animals remained asymptomatic. In contrast to *L. enriettii* and *L. macropodum*, these parasites

often spread through the body of hosts, but the number of parasites detected were low or moderate (less than 2500 per organ). Steppe lemmings are the first documented animals susceptible to *L. chancei*, as there has been very limited scientific work on this parasite species. Even though steppe lemmings may help in research of *L. chancei*, they cannot serve as natural reservoirs, because their geographical distribution do not overlap.

*Leishmania orientalis* did not cause any pathological changes on the ears, but it was shown to cause severe visceral leishmaniasis in steppe lemmings, when 4/10 animals died during the experiment and one more suffered from hair loss. The parasites were widely distributed throughout body tissues and inner organs (75% of positive inoculated ears and their draining lymph nodes, 63% of forepaws, 50% of spleens and 38% of livers and hind paws). They were also very infectious to sand flies at all time points except 10 weeks p.i.

Last but not least, four strains of *L. martiniquensis* were tested in steppe lemmings. When comparing strains from different hosts and geographical areas, we found high intraspecific variation. Equine isolate Aig1, which was not infectious to BALB/c mice or Chinese hamsters, and the human isolate Cu1R1 did not cause any pathological changes, but they were infectious to sand flies and parasites were detected mainly in inoculated ears (56% for Aig1 and 88% for Cu1R1, respectively). On the other hand, human isolates MAR1 and Cu2, from Martinique and Thailand, respectively, often visceralised and the Cu2 strain caused serious cutaneous lesions on the ears leading to their destruction. All strains were infectious to sand flies and were detectable in a high proportion of animals. The tissues and organs most affected by strain MAR1 and Cu2 were inoculated ears (89%), draining lymph nodes from inoculated ears (78% and 89%), forepaws (44% and 67%) and spleens (66% and 56%). *Leishmania martiniquensis* is the main pathological agents of visceral leishmaniasis in Thailand [108], and steppe lemmings may contribute to the discovery of ways to control and treat of leishmaniasis in Southeast Asia. The intraspecific variation in disease manifestation described here has also been previously observed in *L. major* infections [74], highlighting the importance of proper selection of the parasite - rodent combination for laboratory studies.

For experimental infections of insects, we used a standard experimental scheme used in our laboratory for many years. Culture-derived promastigotes were washed and resuspended in heat-inactivated rabbit blood at a final concentration of $10^6$ parasites/ml. This suspension was applied to a glass feeder and female sand flies or biting midges were infected by feeding through a chicken skin membrane. Engorged females were separated and kept under the same conditions as the colony for subsequent dissections at various time intervals. We tried to choose sand fly species whose geographic distribution overlaps with the respective parasite species. Therefore, *Lu. migonei*, a permissive vector of *Leishmania* from South America [109], was infected with *L. enriettii*; *P.*

*duboscqi*, a natural vector of *L. major* in Africa [110], was infected with *L. chancei* and *P. argentipes*, a natural vector of *L. donovani* in South Asia [111] [112], was infected with *L. orientalis* and various strains of *L. martiniquensis*. We could not use a species from Australia to test *L. macropodum*, as the local sand flies are not yet colonized, so we used the permissive New World species *Lu. migonei*.

*L. enriettii*, *L. macropodum, or L. chancei* did not survive in sand flies up to late-stage infections. After a successful early infection, when parasites were present in sand fly guts during bloodmeal digestion, we observed a rapid decline of parasites with defecation of blood remnants that usually led to their complete disappearance from the guts. If parasites were present, they were in low numbers and localized in the hindgut only.

Experiments with *P. argentipes* infected with *L. orientalis* and *L. martiniquensis* were more successful. These two parasite species were able to survive defecation, and to colonize the stomodeal valve of sand flies in 20% and 7% of sand flies infected with *L. martiniquensis* MAR1 and *L. orientalis,* respectively. Interestingly, in *L. martiniquensis*, a strain originating from a different part of the world (Martinique) infected female *P. argentipes* and developed late-stage infections, whereas strains originating from the same area as the sand flies (Thailand) did not. In subsequent experiments, we tested the ability of *P. argentipes* to transmit parasites to a naïve host by bite 8-11 days post infective blood meal, sand flies were allowed to feed on anesthetized naïve mice. The mice were immediately killed and PCR was performed to detect the presence of parasite DNA in sampled tissues. In the sand fly experiments, all attempts failed, although the parasites were present in the stomodeal valve.

The paradigm that sand flies are the sole vectors of leishmania has been taken as a dogma for decades, but the last few years have shown that biting midges can also serve as vectors of these parasites. First, Dougall et al. [3] found late-stage *L. macropodum* infections in biting midges of the genus Forcipomya (Lasiohelea) and then Seblova et al. [81] succeeded in infecting *C. sonorensis* with *L. enriettii* and *L. macropodum*. More recently, these midges were also shown to promote the development of *L. orientalis* [100]. Therefore, we decided to test susceptibility of *C. sonorensis* to other *Mundinia* species and to verify their vector potential by transmission experiments to a naïve host. The possible involvement of biting midges is particularly important mainly in the case of *L. martiniquensis* which is also found in central Europe [93], [94] where sand flies occur sporadically or not at all [113].

In our experiments, we observed successful late stage infection of *C. sonorensis* with all tested species of the genus *Mundinia*, although *L. enriettii* and *L. macropodum* were present only in a very low percentage of bloodfed females (5% and 7%, respectively). In experiments with the other three *Mundinia* species, we found heavy late stage infection in relatively high proportions (69% in *L. martiniquensis* Cu2, 63% in *L. orientalis* and 40% in *L. chancei*), associated with the presence

of infective metacyclic stages. This, together with successful transmission to the mouse, strongly supporting the hypothesis of the involvement of biting midges in the circulation of these parasites.

In a future project, we would like to perform experimental transmission from biting midges to steppe lemmings to see if the transmitted parasites cause disease in naïve hosts and if these hosts are infective to the vectors. If our findings are confirmed by field research, this may in the end lead to a redefinition of the *Leishmania* genus where sand flies are considered the sole vectors or to reclassification of the subgenus *Mundinia* to a separate genus [76].

In summary, our work led to the enrichment of the spectrum of model animals useful for *Mundinia* research; two rodent species, Chinese hamsters and steppe lemmings, have been proven to be susceptible to these parasites. These experiments have also shown that all *Mundinia* species can successfully develop in rodents and be infectious to vectors, shedding some light on the question of natural reservoirs, since until now the identity of the animals maintaining their circulation has been a complete mystery. Rodents are among the prime interest, as well as being one of the most common hosts of other leishmania subgenera.

We have also shown that biting midges can transmit parasites to the naïve hosts, which strongly support hypothesis of their major role in spread and circulation of *Mundinia* parasites.

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# 6. Appendix

## 6.1 Curriculum vitae

### Education

| | |
|---|---|
| 2014-2017 | Bachelor´s degree program in Molecular biology and biochemistry of organisms, Department of Parasitology, Faculty of Science, Charles University, Prague<br>Thesis: Biology of *Leishmania enriettii* species complex.<br>Supervisor: RNDr. Jovana Sádlová, PhD. |
| 2017-2019 | Master´s degree in Parasitology, Department of Parasitology, Faculty of Science, Charles University, Prague<br>Thesis: *Leishmania* of the subgenus *Mundinia*: genetical analysis and experimental infections of rodents and vectors.<br>Supervisor: RNDr. Jovana Sádlová, PhD. |
| 2019 – present | PhD. study – Department of Parasitology, Faculty of Science, Charles University, Prague<br>Title – Experimental animal models and vectors of *Leishmania* (*Mundinia*)<br>Supervisor: doc. RNDr. Jovana Sádlová, PhD. |

### Internships

| | |
|---|---|
| 2017 | Sir William Dunn School of Pathology, University of Oxford, Laboratory of prof. Eva Gluenz<br>Duration: 3 weeks<br>Topic: Preparation of chitinase (LmxM.16.0790) knock-out *Leishmania mexicana* cell lines using CRISPR-Cas9 technology<br><br>Laboratory of Trypanosomatid Biology, University of Ostrava<br>Duration: 1 week<br>Topic: Whole genome sequencing training |
| 2018 | Sir William Dunn School of Pathology, University of Oxford, Laboratory of prof. Eva Gluenz<br>Duration: 5 weeks<br>Topic: qPCR assay preparation and double knock out of *L. mexicana* cell lines using CRISPR-Cas9 technology |

| | |
|---|---|
| 2019 | Sir William Dunn School of Pathology, University of Oxford,<br>Laboratory of prof. Eva Gluenz<br>Duration: 2 weeks<br>Topic: Fluorescent tagging of *L. major* |
| 2022 | Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunity and Inflammation, College of Medical Veterinary and Life Sciences, University of Glasgow<br>Duration: 2 months<br>Topic: Addback generation of double knock out *L. mexicana* cell line and cloning training |
| 2023 | Department of Protozoology, Institute for Tropical Medicine in Antwerp, Belgium<br>Duration: 1 month<br>Topic: SureSellect and Spliced-Leader sequencing training |

## Courses

| | |
|---|---|
| 2020 | Certificate of professional competence to design experiments and experimental projects under Section 15d (3) of Act No. 246/1992 Coll., on the protection of Animals against Cruelty, number: CZ03965 |

## Teaching

| | |
|---|---|
| 2019 – present | teacher, Subject: Microscopic techniques MB160C45<br>Charles University, Prague |
| 2023 | teacher, Subject: Field Course in fish parasitology II, MB160T69<br>Charles University, Prague |

## Scientific projects

| | |
|---|---|
| 2021-2023 | Investigator in the project GAČR 21-15700S *Leishmania*-sand fly interaction: new approaches to answer old questions |
| 2023-2025 | Investigator in the project GAČR 23-06299S Transmission cycles of *Leishmania major* in natural reservoir hosts: local analyses with global implications |
| 2021-2026 | Investigator in the project Wellcome Trust 221944/Z/20/Z Defining the molecular determinants required for *Leishmania* life cycle progression and virulence |

| | |
|---|---|
| 2022-2025 | Investigator in the project HORIZON Europe 101057690 CLIMOS - Climate Monitoring and Decision Support Framework for Sand Fly-borne Diseases Detection and Mitigation with Cost-benefit and Climate-policy Measures |
| 2018-2022 | Investigator in the project Medical Research Council MRC: MR/R014973/1 Development of a human challenge model of *Leishmania major* infection as a tool for assessing vaccines against leishmaniasis |
| 2020-2022 | Investigator in the project GAUK 180220 Development of *Sauroleishmania* in sand flies and geckos |
| 2017-2019 | Investigator in the project GAUK 288217 Comparison of different rodent species as hosts of leishmania |
| 2021-2023 | Principal investigator in the project START/SCI/083 Development of *Mundinia* in vectors and hosts: comparison with other *Leishmania* subgenera |

## International conferences

Leishmaniasis 2018, 1st International Caparica Congress on Leishmaniasis – 29th – 31st October 2018, Caparica, Portugal
Shotgun presentation and poster: **Tomáš Bečvář** , Jovana Sádlová, Barbora Vojtková, Paul Bates and Petr Volf: Development of *Leishmania* of the subgenus *Mundinia* in sand flies and guinea pigs

ISOPS X, 10th International Symposium On Phlebotomine Sandflies – 15th – 19th July 2019 San Cristobal – Galapagos
Poster: **Tomáš Bečvář**, Jovana Sádlová, Barbora Vojtková, Paul Bates, Padet Siriyasatien and Petr Volf: Development of *Leishmania* of the subgenus *Mundinia* in sand flies and guinea pigs

Worldleish 7, 7th World Congress on Leishmaniasis, 1st – 6th August 2022, Cartagena, Colombia
Poster: **Tomáš Bečvář**, Barbora Vojtková, Barbora Vomáčková-Kykalová, Lenka Pacáková, Lucie Tichá, Petr Volf and Jovana Sádlová: Experimental infections of rodents with *Mundinia*

Leishmaniasis 2022, 3rd International Caparica Congress on Leishmaniasis – 24th – 26th October 2022, Caparica, Portugal
Poster and shotgun presentation: **Tomáš Bečvář**, Barbora Vojtková, Padet Siriyasatien, Jan Votýpka, David Modrý, Paul Bates, Simon Carpenter, Petr Volf and Jovana Sádlová: Biting midges: New important players in the field of leishmaniasis

## Publications


Sadlova, J., Vojtkova, B., Hrncirova, K., Lestinova, T., Spitzova, T., **Becvar, T.**, ... & Volf, P. (2019). Host competence of African rodents *Arvicanthis neumanni*, *A. niloticus* and *Mastomys natalensis* for *Leishmania major*. *International Journal for Parasitology: Parasites and Wildlife*, *8*, 118-126.

Beneke, T., Demay, F., Hookway, E., Ashman, N., Jeffery, H., Smith, J., Valli, J., **Becvar, T.**,... & Gluenz, E. (2019). Genetic dissection of a *Leishmania* flagellar proteome demonstrates requirement for directional motility in sand fly infections. *PLoS pathogens*, *15*(6), e1007828.

Butenko, A., Kostygov, A. Y., Sádlová, J., Kleschenko, Y., **Bečvář, T.**, Podešvová, L., ... & Yurchenko, V. (2019). Comparative genomics of *Leishmania* (*Mundinia*). *BMC genomics*, *20*, 1-12.

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.

**Becvar, T.**, Siriyasatien, P., Bates, P., Volf, P., & Sádlová, J. (2020). Development of *Leishmania* (*Mundinia*) in guinea pigs. *Parasites & vectors*, *13*(1), 1-6.

Sádlová, J., Podešvová, L., **Bečvář, T.**, Bianchi, C., Gerasimov, E. S., Saura, A., ... & Kraeva, N. (2021). Catalase impairs *Leishmania mexicana* development and virulence. *Virulence*, *12*(1), 852-867.

Ashwin, H., Sadlova, J., Vojtkova, B., **Becvar, T.**, Lypaczewski, P., Schwartz, E., ... & Kaye, P. M. (2021). Characterization of a new *Leishmania major* strain for use in a controlled human infection model. *Nature Communications*, *12*(1), 215.

**Becvar, T.**, Vojtkova, B., Siriyasatien, P., Votypka, J., Modry, D., Jahn, P., ... & Sadlova, J. (2021). Experimental transmission of *Leishmania* (*Mundinia*) parasites by biting midges (Diptera: Ceratopogonidae). *PLoS Pathogens*, *17*(6), e1009654.

Zakharova, A., Albanaz, A. T., Opperdoes, F. R., Škodová-Sveráková, I., Zagirova, D., Saura, A., Chmelová, L., Gerasymov E.S., Leštinová, T., **Bečvář, T.,** ... & Yurchenko, V. (2022). Leishmania guyanensis M4147 as a new LRV1-bearing model parasite: phosphatidate phosphatase 2-like protein controls cell cycle progression and intracellular lipid content. *PLoS Neglected Tropical Diseases*, *16*(6), e0010510.

Sadlova, J., Bacikova, D., **Becvar, T.**, Vojtkova, B., England, M., Shaw, J., & Volf, P. (2022). *Porcisia* transmission by prediuresis of sand flies. *Frontiers in Cellular and Infection Microbiology*, *12*, 981071.

Sadlova, J., Vojtkova, B., Lestinova, T., **Becvar, T.**, Frynta, D., Benallal, K. E., ... & Volf, P. (2023). Infectiousness of Asymptomatic *Meriones shawi*, Reservoir Host of *Leishmania major*. *Pathogens*, *12*(4), 614.

**Bečvář, T.**, Vojtková, B., Pacáková, L., Vomackova Kykalova, B., Tichá, L., Volf, P., & Sádlová, J. (2024). Steppe lemmings and Chinese hamsters as new potential animal models for the study of the leishmania subgenus *Mundinia* (Kinetoplastida: Trypanosomatidae). *bioRxiv*, 2024-01.