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

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ABSTRACT

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

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

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

1. Introduction

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

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

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

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

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

2. Material and methods

2.1. Sand flies, parasites and rodents

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

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

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

2.2. Experimental infection of sand flies

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

2.3. Infections of rodents

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

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

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

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

2.4. Xenodiagnosis

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

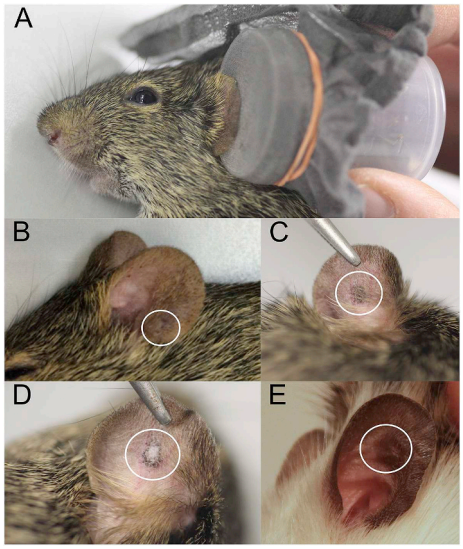

Fig. 1. Xenodiagnosis and external manifestation of *L. major* in rodents. Direct xenodiagnosis with *P. duboscqi* in plastic tubes covered with fine mesh held on the ear of the anaesthetized *A. niloticus* (A) and external manifestation of *L. major* LV109 in ear pinnae (site of inoculation) of *A.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

2.5. Tissue sampling and quantitative PCR

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

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

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

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

2.7. Statistical analysis

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

2.8. Animal experimentation guidelines

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

3. Results

3.1. Experimental infections and xenodiagnosis with A. neumanni

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

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

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

3.2. Experimental infections and xenodiagnosis with A. niloticus

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

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

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

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

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

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

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

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

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

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

3.3. Experimental infections and xenodiagnosis with *M. natalensis*

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

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

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

Animals	Weeks post infection												
	2	4	6	8	10	12	14	16	18	20	22	24	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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