

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

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15

16 **Abstract**

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

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

31 Three different *L. major* strains used significantly differed in infectivity: the Middle
32 Eastern strain Friedlin infected a low proportion of rodents, while two Sub-Saharan isolates
33 from Senegal (LV109, LV110) infected a high percentage of animals and LV 110 also
34 produced higher parasite loads in all host species. All three rodent species maintained
35 parasites of the LV109 strain for 20-25 weeks and were able to infect *P. duboscqi* without
36 apparent health complications: infected animals showed only temporary swellings or
37 changes of pigmentation on the site of inoculation. However, the higher infection rates,
38 more generalized distribution of parasites and longer infectiousness period to sand flies in

39 *M. natalensis* suggest that this species plays the more important reservoir role in the life
40 cycle of *L. major* in Sub-Saharan Africa. *Arvicanthis* species may serve as potential reservoirs
41 in seasons/periods of low abundance of *Mastomys*.

42 **Key words:** wild reservoir, xenodiagnosis, Grass Rats, Multimammate Mice,
43 leishmaniasis, *Arvicanthis*, *Mastomys*

44 **1. Introduction**

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

53 CL caused by *L. major* is a typical zoonosis maintained in reservoir rodent hosts.
54 Humans are infected incidentally; lesions appear at the site of insect bite and cure without
55 treatment after about three months. The short duration of the disease precludes survival of
56 the parasite in humans through any non-transmission season (Ashford 2000). Proven
57 reservoir hosts are the Fat Sand-Rat *Psammomys obesus* and gerbils of the genus *Meriones*
58 in North Africa and the Middle East, and the Great Gerbil *Rhombomys opimus* in Central
59 Asia. On the other hand, reservoir rodent species in Sub-Saharan Africa remains to be
60 confirmed. *Leishmania major* has been isolated from several rodent species in this region;
61 most isolates have been made from Grass Rats *Arvicanthis* spp. and Multimammate Mice
62 *Mastomys* spp. which live in immediate vicinity of humans, and are the most dominant
63 rodents in many Sub-Saharan African endemic localities of CL (reviewed by Ashford 1996,
64 Ashford 2000, Desjeux 1996). *Arvicanthis* and *Mastomys* belong to the same large subfamily
65 Murinae, but are separated in different tribes – Arvicanthini and Praomyini, respectively
66 (Lecompte et al. 2008). The origin of both tribes was estimated to about 10. 2 Mya. Recently,

67 the genus *Arvicanthis* was reported to include seven species and the genus *Mastomys* eight
68 species (Granjon and Ducroz 2013, Leirs 2013).

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

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

90 xenodiagnoses. Feeding rates of *P. duboscqi* on these rodents were tested by host-choice
91 experiments.

92

93 **2. Material and Methods**

94 **2.1. Sand flies, parasites and rodents.**

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

98 Three *L. major* strains were used: MHOM/IL/81/Friedlin, a human isolate from
99 Israel, and two strains isolated in Senegal by Ranque - MARV/SN/XX/RV24;LV109 and
100 MHOM/SN/XX/BO-DK;LV110. The identity of the Senegalese strains was confirmed by
101 sequencing of the RPL23a intergenic sequence (Dougall et al., 2013). Promastigotes were
102 cultured in M199 medium (Sigma) containing 10% heat-inactivated fetal bovine calf serum
103 (FBS, Gibco) supplemented with 1% BME vitamins (Basal Medium Eagle, Sigma), 2% sterile
104 urine and 250 µg/mL amikacin (Amikin, Bristol-Myers Squibb).

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

113

114 **2.2. Experimental infection of sand flies.**

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

120

121 **2.3. Infections of rodents**

122 Two methods of rodent infections were used – infections initiated with sand fly-derived
123 *Leishmania* according to Sadlova et al. (2015) and infections initiated with culture-derived
124 promastigotes. For the first method, *P. duboscqi* females experimentally infected with *L.*
125 *major* (for details see above) were dissected on day 10 or 12 post bloodmeal (PBM); their
126 midguts were checked microscopically for the presence of promastigotes, and thoracic
127 midguts (the site of accumulation of metacyclic forms) with a good density of parasites were
128 pooled in sterile saline. Pools of 100 freshly dissected thoracic midguts were homogenized in
129 50 μ l of saline.

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

133 Dissected salivary glands of *P. duboscqi* females (SG) were pooled in sterile saline (10 glands
134 per 5 μ l of saline) and stored at -20°C . Prior to mice inoculation, SG were disintegrated by 3
135 successive immersions into liquid nitrogen and added to the parasite suspension.

136 Rodents anaesthetized with ketamin/xylazine (33 mg and 13 mg/kg in *A. neumanni*,
137 62 mg and 7 mg/kg in *A. niloticus*, 50 mg and 20 mg/kg in *M. natalensis*, 62 mg and 25
138 mg/kg in mice, respectively) were injected with 5.5 µl of the mixed parasite and SG
139 suspension intradermally into the ear pinnae. Therefore, the inoculum dose per one animal
140 with culture-derived promastigotes comprised 10^7 parasites. Exact numbers of sand fly –
141 derived parasites stages were calculated using a Burker apparatus, and the proportions of
142 metacyclic forms were identified on Giemsa stained smears based on morphological criteria
143 described previously (Sadlova et al. 2010). The inoculum dose in sand fly-derived parasites
144 was 3.6×10^4 with LV 110 strain (35% of metacyclic forms) and ranged between 3.5×10^4 - $7 \times$
145 10^4 parasites/rodent with FVI strain (23-69% of metacyclic forms) and 4.1×10^4 - 5.4×10^4 with
146 LV109 strain (43 – 68% of metacyclic forms). Animals were checked weekly for external signs
147 of the disease until week 20-35 post infection (p.i.) when they were sacrificed.

148

149 **2.4. Xenodiagnosis**

150 Five to seven-day-old *P. duboscqi* females were allowed to feed on the site of inoculation of
151 *L. major* (ear pinnae) of anaesthetized rodents (using ketamin/xylazine) between weeks 2
152 and 25 p.i. Smaller size rodents *M. natalensis* and *A. neumanni* were covered with the cotton
153 bag, so that only the left ear pinnae were accessible to sand flies, placed into a small cage
154 (20 x 20 x 20 cm) and 40-70 sand fly females were allowed to feed for one hour. In the larger
155 sized *A. niloticus*, the xenodiagnoses were made using small plastic tubes with 30 sand fly
156 females covered with fine mesh. The tubes were held on the ear of the anaesthetized animal
157 for one hour (Fig 1A). Fed sand fly females were separated and maintained at 26°C on 50%
158 sucrose. On day 7-10 PBM, females were dissected and their guts examined under the light

159 microscope. Intensities and locations of infections were evaluated as described previously
160 (Sadlova et al. 2010).

161

162 **2.5. Tissue sampling and quantitative PCR**

163 Rodents were sacrificed at different weeks p.i by injecting them with an overdose of
164 ketamin/xylazine anesthesia. Both ears (inoculated and contralateral), both ear-draining
165 lymph nodes, spleen, liver, paws and tail were stored at -20°C for qPCR. Extraction of total
166 DNA from rodent tissues and sand flies was performed using a DNA tissue isolation kit
167 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.
168 Quantitative PCR (Q-PCR) for detection and quantification of *Leishmania* parasites was
169 performed in a Bio-Rad iCycler & iQ Real-Time PCR Systems using the SYBR Green detection
170 method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA) as described (Sadlova et al. 2010).

171

172 **2.6. Host choice experiments and assessment of mortality and** 173 **fecundity of sand flies fed on different hosts.**

174 Pair-wise comparisons between two types of host were performed using a row of three
175 connected small cages (20 x 20 cm). *P. duboscqi* females (200 specimens) were placed into
176 the central cage and left for habituation for 20 minutes. Anaesthetized animals were placed
177 in each of the lateral cages and partitions with the central cage were opened. After one
178 hour, the cages were separated and closed, host animals removed and the numbers of
179 blood-fed sand flies in each host cage was counted. *Arvicanthis neumanni* and *M. natalensis*
180 are species of comparable size (60-80 g) and therefore one animal each was placed in cages.
181 For comparison between mice and *Arvicanthis* or *Mastomys*, two mice were used against

182 one *Arvicanthis* or *Mastomys* to counterbalance weight differences between these host
183 types. Each pair of different hosts was tested four times, with the hosts alternated between
184 lateral cages in each repeat. Experiments were conducted in darkness at 24-26°C.

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

193

194 **2.8. Animal experimentation guidelines**

195 Animals were maintained and handled in the animal facility of Charles University in Prague in
196 accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and
197 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which
198 complies with all relevant European Union and international guidelines for experimental
199 animals. All the experiments were approved by the Committee on the Ethics of Laboratory
200 Experiments of the Charles University in Prague and were performed under permission no.
201 MSMT-10270/2015-5 of the Ministry of the Environment of the Czech Republic. Investigators
202 are certificated for experimentation with animals by the Ministry of Agriculture of the Czech
203 Republic.

204

205 **3. Results**

206 **3.1. Experimental infections and xenodiagnosis with *A. neumanni*.**

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

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

220 The second Sub-Saharan strain LV109 originating from Senegal was inoculated into
221 15 *A. neumanni* (Table 1); 12 with sand fly-derived *Leishmania* (experimental groups A and
222 B) and 3 with culture-derived promastigotes (experimental group C). Wet skin lesions did not
223 develop, but hyper-pigmentations of left ear pinnae (site of inoculation) were observed in 3
224 animals, two from the group A and one from the group C (Fig 1B). PCR showed presence of
225 parasites in 7 from 15 animals. They were localized mostly in the left ear (site of inoculation)
226 and once in the blood. Interestingly, the numbers of detected *Leishmania* were higher
227 (hundreds to thousands) in 3 animals, two of which also showed hyper-pigmentation of the

228 ear. All three animals with hyperpigmentation were infective to sand flies, two by week 5
229 and the third by week 10 p.i. In total, 0.4 % of 748 *P. duboscqi* females tested were positive
230 (Table 2).

231

232 **3.2. Experimental infections and xenodiagnosis with *A. niloticus*.**

233 Twelve *A. niloticus* of both sexes were inoculated with the strain LV109 originating from
234 Senegal. Six *A. niloticus* (3 males and 3 females) were infected with sand fly-derived
235 *Leishmania* (experimental group A) and the same numbers of animals were infected with
236 culture-derived promastigotes (experimental group C), but one animal from group C died
237 early during the experiment and thus was not evaluated. In both groups, the first external
238 signs of the disease appeared on inoculated ear pinnae on week 6 p.i. The affected area was
239 characterized by mild flaking of the skin and hyper-pigmentation (Fig 1C). The pigmentation
240 was lost in the centre while the borders remained hyper-pigmented in a part of animals (Fig
241 1D, Tab. 3). These dry lesions increased to 3-4 mm by weeks 12-14 p.i; then, in 3 animals the
242 lesion size remained constant until the end of the experiment by week 25 p.i., while in the
243 others lesions decreased or completely disappeared (Tab. 3).

244 PCR confirmed the presence of *Leishmania* in 4 of 11 animals, with localization in
245 ears, forepaws, hindpaws and tail (Table 1). The numbers of detected parasites were higher
246 (hundreds to thousands) in the animal killed by week 12 p.i., while no parasites or only low
247 numbers (around one hundred) were present in organs dissected by week 25p.i. (by the end
248 of the experiment). This fact corresponds with results of xenodiagnoses: similarly to *A.*
249 *neumanni*, the period of infectiousness of *A. niloticus* to *P. duboscqi* was restricted to weeks
250 5 and 10 p.i. (4.1 % and 10.0 % of sand fly females became infected, respectively) while no
251 females developed *Leishmania* infection in feeding experiments in weeks 15-25 p.i.(Table 2).

252

253 **3.3. Experimental infections and xenodiagnosis with *M. natalensis*.**

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

262 Ten *M. natalensis* were experimentally infected with the LV109 strain (Table 1), 5
263 with sand fly-derived *Leishmania* (experimental group A) and 5 with culture-derived
264 promastigotes (experimental group C). Skin swellings developed at the site of inoculation
265 (left ear pinnae) in animals of both experimental groups approximately 10 weeks p. i. (Table
266 4, Fig 1E)). Prior to the swelling the affected site usually reddened, which was observed more
267 often in specimens of the group C. The size of the swelling increased gradually to 6-8 mm,
268 then decreased and finally disappeared. Hyper-pigmentation often accompanied healing of
269 the swellings (Table 4) and it mostly persisted until the end of the experiments.

270 Parasites were detected by Q-PCR in all tested animals and they disseminated to
271 draining lymph nodes, forepaws, hindpaws and tail in several animals and also to the spleen
272 in one specimen (Table 1). Infectiousness to sand flies was tested at weeks 15 and 25 p.i.:
273 0.7% of females from group A became infected after feeding at week 15 p.i., while 3.3% and

274 4.1% of females from the group A and C, respectively, became *Leishmania* positive at week
275 25 p.i. (Table 2).

276

277 **3.4. Host choice experiments with *P. duboscqi*.**

278 Two host types were offered to *P. duboscqi* females in each pair-wise comparison.

279 Preliminary experiments showed that *P. duboscqi* did not distinguish between males and
280 females of *A. neumanni* and both species of the genus *Arvicanthis* (smaller *A. neumanni* and
281 bigger *A. niloticus*). Then, different host genera (represented by *A. neumanni*, *M. natalensis*
282 and BALB/c mice) were compared: each host combination was tested twice with hosts
283 alternated between lateral cages. Sand fly females showed a high feeding rate on all tested
284 rodents: 40.5 - 80.5 % of females took bloodmeals during experiments (Table 5). The only
285 significant preference was observed when *Arvicanthis* was compared with BALB/c mice -
286 *Arvicanthis* was significantly preferred over mice. On the other hand, no difference was
287 observed between *Mastomys* and *Arvicanthis* or *Mastomys* and BALB/c mice.

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

294

295 **5. Discussion**

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

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

310 Frequent field findings of *L. major* in *Arvicanthis* and *Mastomys* have been reported,
311 and the eco-etiological and physiological characteristics of these rodents match the
312 requirements essential for reservoirs: they live in colonies with high population numbers in
313 the vicinity of humans in endemic localities, and they have sufficient longevity. These
314 characteristics encouraged us to perform laboratory experiments which can help to confirm
315 or exclude their reservoir role. The results revealed the importance of the *L. major* strain
316 used for the experiments. Substantial differences were observed in the infectivity of *L. major*
317 strains isolated from the Middle East and Sub-Saharan Africa. The Sub-Saharan strain LV109
318 persisted in all three tested rodent species for several months and, importantly, the
319 parasites were accessible and infective to *P. duboscqi* females. On the other hand, the

320 Middle Eastern strain FV1 produced only poor infections in *A. neumanni* and *M. natalensis*,
321 parasites were present in low numbers and the animals were not infectious to sand flies.
322 These differences correspond with results of the study of Elfari et al. (2005) testing cross-
323 infectivity of three *L. major* strains differing in geographical origin in three rodent species –
324 *Psammomys obesus*, *Rhombomys opimus* and *Meriones libycus*. No infections were detected
325 in *R. opimus* when infected with the African or Middle Eastern strains and no signs of disease
326 were seen in any *P. obesus* infected with a Central Asian strain (Elfari et al. 2005).

327 Important methodological points influencing results of experimental infections are
328 the size and nature of the inocula and the infection route (reviewed by Loria-Cervera and
329 Andrade-Narvaez 2014). It has been shown repeatedly that the number of parasites
330 transmitted by sand flies to the host is highly variable but it does not exceeded 10^5 parasites
331 inoculated per bite (Kimblin et al. 2008, Maia et al. 2011, Secundino et al. 2012). Here we
332 used an intradermal route of inoculation which is close to the natural mode of transmission,
333 since parasites are exposed to the localized immune responses in the skin (Belkaid et al.
334 1998, 2002). Infections were initiated with either $3-7 \times 10^4$ of sand fly-derived parasites or
335 with 10^7 of parasites derived from stationary-phase promastigote cultures. The former
336 inocula comprised mainly metacyclic stages present in thoracic regions of sand fly midguts
337 during the late stage infections. Rodent infections initiated in our study by natural numbers
338 of sand fly derived *Leishmania* showed the same outcome as those initiated with an
339 unnaturally large inoculum from the culture. Dissemination of parasites in the host's body as
340 well as infectiousness to sand flies was very similar with both types of infection.

341 Infection rates, the percentage of sand flies that became infected while biting on
342 experimental animals, ranged between 0 -1.2% in *A. neumanni*, 0 - 10% in *A. niloticus* and 0 –
343 4.1% in *M. natalensis*. Similarly low infection rates were detected previously: 0 – 7% in *P.*

344 *sergenti* feeding on rats (*Rattus rattus*) experimentally infected with *L. tropica* (Svobodová et
345 al. 2013), 0 – 5% in *Lu. youngi* feeding on *Proechimys semispinosus* experimentally infected
346 with *L. panamensis* (Travi et al. 2002) or 0 – 11% in *P. perniciosus* feeding on hares (*Lepus*
347 *granatensis*) naturally infected with *L. infantum* (Molina et al. 2012). Higher infection rates
348 have been reported more rarely, for example 19% of *P. orientalis* feeding on BALB/c mice
349 experimentally infected with *L. donovani* (Sadlova et al. 2015) or up to 27- 28 % of *L.*
350 *longipalpis* feeding on symptomatic dogs infected with *L. infantum* in Brazil (Michalsky et al.
351 2007, Courtenay et al. 2002).

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

367 One of the important prerequisites of the involvement of any rodent species in the
368 life-cycle of *Leishmania* parasites is its attractiveness to sand flies. It is also known from
369 laboratory colonies that some sand fly species are opportunistic and readily feed on mice,
370 while the others, like species in the subgenera *Larroussius* and *Adlerius* , prefer hamsters or
371 rabbits (Volf and Volfova 2011). Since the blood of vertebrate species varies in several
372 properties influencing its nutritive value (Harrington et al. 2001), host choice affects the
373 fitness of fed females as was repeatedly demonstrated in mosquitoes (Lyimo and Ferguson
374 2009). In the neotropical sand fly *Lutzomyia longipalpis* significant differences in the
375 numbers of eggs laid among flies fed on various hosts were reported (Macedo - Silva 2014),
376 and in fleas significant differences in the energetic cost of blood digestion was found even at
377 the level of two rodent species from the same family (Sarfati et al. 2005). On the other hand,
378 studies on the Old World sand fly species *P. papatasi* and *P. haleyensis* revealed no
379 appreciable differences between the fecundity of females fed on human blood and different
380 animal blood sources (Hare et al. 2001, Sadlova et al. 2003). In our experiments, *P. duboscqi*
381 females manifested as opportunistic feeders which were ready to feed on all offered rodent
382 species, although they preferred *Arvicanthis* over laboratory mice. Mortality and fecundity of
383 *P. duboscqi* females was comparable post feeding on all rodents tested. This is in accordance
384 with a study from Kenya where *P. duboscqi* also showed opportunistic behavior, being
385 attracted to wild rats, chickens, mongooses, dogs and goats (Mutinga et al. 1985).

386 The definition of reservoir hosts in leishmaniasis has changed in recent years.
387 Ashford (1996, 1997) originally distinguished primary reservoirs (species ensuring long-term
388 persistence of the parasite) and secondary reservoir hosts (species acting as liaison between
389 primary reservoirs and incidental hosts), but this division was assessed to be arbitrary by
390 Chaves et al. (2007), as hosts may vary locally and seasonally with the dynamics of

391 transmission. According to the widely accepted ecological concept of Pulliam (1988),
392 populations generally exhibit source – sink dynamics, where sources sustain exponential
393 growth and are characterized by emigration while sinks operating under worse conditions
394 demonstrate positive immigration. Chaves et al. (2007) applied this concept on reservoirs
395 for leishmaniasis and proposed to recognize reservoirs (sources) as species which have a
396 dynamic feedback to the hosts through pathogen transmission by the vector. Incidental
397 hosts (sinks) lack such a dynamic feedback and cannot transmit the pathogen to new hosts.
398 In this light, our results suggest that both *Mastomys* and *Arvicanthis* can be assessed as
399 promising reservoirs (sources of the parasite) as both are able to maintain parasites for
400 several months and infect the vector without apparent health complications. However, the
401 higher infection rates, more generalized distribution of parasites and longer infectiousness
402 period to sand flies in *M. natalensis* suggest that this species plays the more important
403 reservoir role in the life cycle of this parasite in Sub-Saharan Africa. *Arvicanthis* species may
404 serve as potential reservoirs in seasons/periods of low abundance of *Mastomys*.

405 Both *Arvicanthis* and *Mastomys* are known to undergo enormous abundance
406 fluctuations: they are able to breed very rapidly and their population numbers may become
407 very large when environmental conditions are favorable but with deteriorating conditions
408 the numbers decline very rapidly (Granjon and Ducroz 2013, Leirs 2013). In the same locality,
409 the Paloich district in Sudan, numbers of *Arvicanthis* and *Mastomys* alternated in two
410 consecutive years (Hoogstraal and Dietlein 1964). Therefore, the scenario that these species
411 maintain the parasite alternatively is highly likely: in localities/seasons with a low abundance
412 of *Mastomys* then *Arvicanthis* could serve as source of the parasite and vice versa. A similar
413 scenario, alteration of *L. major* between two host species *P. obesus* and *M. shawi*, was
414 proposed in Central Tunisia (Ghawar et al. 2011). Involvement of another rodent species in

415 maintenance of *L. major* in Sub-Saharan region is also not excluded - it was suggested in
416 Kenya where *Tatera robusta* possessed higher infection rates of *L. major* than *A. niloticus*
417 and *M. natalensis* (Githure et al. 1996). Moreover, a high prevalence of *L. major* in invasive
418 *Rattus rattus* was recently described in the southern part of Senegal (Cassan et al. 2018).

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

425

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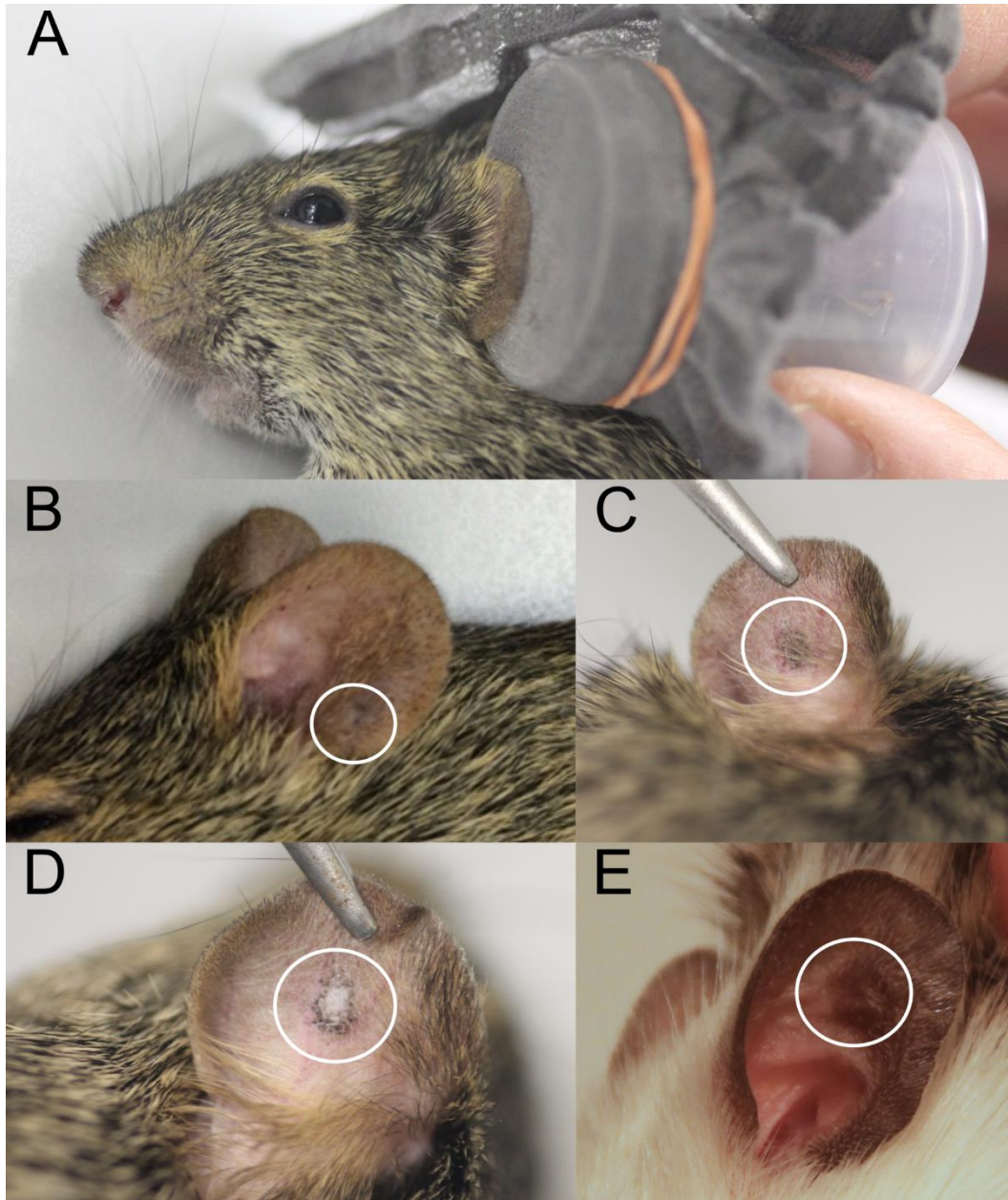
558

559 LEGENDS TO FIGURES:

560 **Figure 1. Xenodiagnosis and external manifestation of *L. major* in rodents.** Direct
561 xenodiagnosis with *P. duboscqi* in plastic tubes covered with fine mesh held on the ear of the
562 anaesthetized *A. niloticus* (A) and external manifestation of *L. major* LV109 in ear pinnae

563 (site of inoculation) of *A. neumanni* by week 10 p.i., (B); *A. niloticus* by week 30 p.i. (C, D) and
564 *M. natalensis* by week 19 p.i. (E).

565



566

567 **TABLES:**

Rodent species	<i>L. major</i> 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
<i>A. neumanni</i>	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	

<i>A. niloticus</i>	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	
<i>M. natalensis</i>	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	
	LV109	Group A	20	2	1	IE*, CE*	No	not tested	
			35	1	0	IE*, CE*	No	not tested	
						-	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 %)

568

569 Table 1. Presence of *L. major* DNA in *A. neumanni*, *A. niloticus* and *M. natalensis* and their infectiousness to *P. duboscqi*. Group A, rodent
570 infections initiated with sand fly-derived *Leishmania* and animals exposed to sand flies; Group B, rodent infections initiated with sand fly-
571 derived *Leishmania* and animals not exposed to sand flies; Group C, rodent infections initiated with culture-derived promastigotes and animals
572 exposed to sand flies. IE, inoculated ear; CE, contralateral ear; DN-IE, draining lymph nodes of the inoculated ear; DN-CE, draining lymph nodes
573 of the contralateral ear; FP, forepaws; HP, hindpaws; T, tail; L, liver; S, spleen; B, blood; *, <100 parasites; **, 100 – 1000 parasites; ***, > 1000
574 parasites; H-Pi, hyper-pigmentation. A1-A6 and C1-C5 - individual marks of animals referring to tables 3 and 4. ^a9 tested animals, ^b 10 tested
575 animals.

Rodent species	<i>L. major</i> strain	Experimental group	Week p.i.	No of animals exposed	No of dissected sand flies	No and (%) of positive sand flies	
<i>A. neumanni</i>	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				
	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				
	<i>A. niloticus</i>	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)
<i>M. natalensis</i>	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)

576

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

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

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				

579

580 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
581 infected with culture-derived promastigotes (Group C), animals A1-A5 were infected with sand fly-derived *Leishmania* (Group A). Black colour –
582 hyper-pigmentation, grey colour – depigmentation in the centre surrounded with hyper-pigmented borders. The numbers are the length of the
583 affected area in mm. *, animal died by week 10 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					

584

585 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
586 infected with culture-derived promastigotes (Group C), animals A1-A5 were infected with sand fly-derived *Leishmania* (Group A). Light grey
587 colour – red macula, dark grey colour – swelling, black colour – hyper-pigmentation of the site where swelling had healed. The numbers are the
588 length of the swelling area in mm. *, animals killed by week 15 p.i.

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
<i>Arvicanthis</i> vs. BALB/c mouse	<i>Arvicanthis</i>	161 (80.5%)	$\chi^2 = 17.015,$	12/161 (7.4%)	$\chi^2 = 0.118,$	26/76 (34.2%)	$\chi^2 = 0.119,$
	BALB/c mouse	95 (47.5%)	$P < 0.0001$	6/95 (6.3%)	$P = 0.472$	24/76 (31.6%)	$P = 0.432$
<i>Arvicanthis</i> vs. <i>Mastomys</i>	<i>Arvicanthis</i>	94 (47.0%)	$\chi^2 = 0.129,$	25/94 (26.6%)	$\chi^2 = 0.007,$	20/28 (71.4%)	$\chi^2 = 0.012,$
	<i>Mastomys</i>	81 (40.5%)	$P = 0.719$	22/81 (27.2%)	$P = 0.534$	14/20 (70.0%)	$P = 0.582$
<i>Mastomys</i> vs. BALB/c mouse	<i>Mastomys</i>	134 (67.0%)	$\chi^2 = 0.055,$	6/100 (6.0 %)	$\chi^2 = 0.787,$	18/20 (90.0 %)	$\chi^2 = 0.784 ,$
	BALB/c mouse	135 (67.5%)	$P = 0.808$	10/200 (5.0 %)	$P = 0.132$	16/20(80.0 %)	$P = 0.661$

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

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

591

592 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.