

1 **Feeding behavior and activity of *Phlebotomus pedifer* and potential reservoir**  
2 **hosts of *Leishmania aethiopica* in southwestern Ethiopia.**

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4 Myrthe Pareyn<sup>1+\*</sup> and Abena Kochora<sup>2+</sup>, Luca Van Rooy<sup>1</sup>, Nigatu Eligo<sup>2</sup>, Bram Vanden Broecke<sup>1</sup>, Nigatu Girma<sup>2</sup>,  
5 Behailu Merdekios<sup>3</sup>, Teklu Wegayehu<sup>2</sup>, Louis Maes<sup>4</sup>, Guy Caljon<sup>4</sup>, Bernt Lindtjørn<sup>5</sup>, Herwig Leirs<sup>1</sup>, Fekadu  
6 Massebo<sup>2\*</sup>

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8 + shared contribution

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10 <sup>1</sup> Evolutionary Ecology Group, University of Antwerp, Belgium

11 <sup>2</sup> Biology Department, Arba Minch University, Ethiopia

12 <sup>3</sup> Public Health Department, Arba Minch University, Ethiopia

13 <sup>4</sup> Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium

14 <sup>5</sup> Centre for International Health, University of Bergen, Norway

15

16 \*corresponding authors:

17 [myrthe.pareyn@uantwerpen.be](mailto:myrthe.pareyn@uantwerpen.be), [fekadu.massebo@amu.edu.et](mailto:fekadu.massebo@amu.edu.et)

18

## 19 Abstract

### 20 Background

21 Cutaneous leishmaniasis (CL) is a major public health concern in Ethiopia. However, knowledge  
22 about the complex zoonotic transmission cycle is limited, hampering implementation of control  
23 strategies. We explored the feeding behavior and activity of the vector (*Phlebotomus pedifer*) and  
24 established the role of livestock in CL transmission.

### 25 Methods

26 Blood meal origins of engorged sand flies were determined by sequencing host DNA. A host choice  
27 experiment was performed to assess the feeding preference of *P. pedifer* when humans and hyraxes  
28 are equally accessible. Ear and nose biopsies from goats and bovines were screened for the presence  
29 of *Leishmania* parasites. Sand flies were captured indoor and outdoor with human landing catches  
30 (HLC) and CDC light traps to determine at which time and where *P. pedifer* is mostly active.

### 31 Principal findings

32 A total of 180 sand flies were found to bite hosts of 12 genera. Humans were the predominant blood  
33 meal source (59.4%,  $p < 0.001$ ) in all habitats, even in caves, where hyraxes are abundant. Only  
34 10.6% of the sand flies fed on hyraxes based on the blood meal analysis, but the host choice  
35 experiment revealed that sand flies have a significant preference for feeding on hyraxes over  
36 humans ( $p = 0.009$ ) when hosts are equally accessible. Only a single goat nose biopsy from 412  
37 animal samples was found with *Leishmania* RNA. We found that *P. pedifer* is predominantly  
38 endophagic ( $p = 0.003$ ), but occurs both indoors and outdoors. A substantial amount of sand flies  
39 was active in the early evening, which increased over time reaching its maximum around midnight.

### 40 Conclusion

41 We indicate that *Leishmania* transmission in southwestern Ethiopia is, in contrast to earlier  
42 suggestions, likely mainly anthroponotic and that livestock does not play a role in transmission.

43 Combined indoor and outdoor vector control measures at night are required for efficient vector  
44 control.

## 45 Author summary

46 Cutaneous leishmaniasis is a major public health problem in Ethiopia. It is caused by  
47 *Leishmania aethiopica* protozoa that are transmitted when female sand flies take a blood  
48 meal. Hyraxes are assigned as the reservoirs of the infection, because many were found  
49 infected with *Leishmania*. There is very limited knowledge about the behavior of sand flies  
50 and other potential hosts of the infection. However, this information is a prerequisite for  
51 disease control, which is currently hampered. In this study, we found that humans are likely  
52 the main source of the infection and that the role of hyraxes in disease transmission needs  
53 further investigation to decide whether they should be included in control programs.  
54 Livestock appears not play a role in transmission, even though sand flies like to feed on  
55 them. We also show that sand flies are active indoors and outdoors, but have a preference  
56 for feeding inside human dwellings and that they are mostly active around midnight.  
57 Overall, we conclude that disease prevention and control should emphasize on human  
58 protection by applying vector control indoors, at night.

## 59 Introduction

60 Cutaneous leishmaniasis (CL) is a vector born disease, caused by *Leishmania* protozoa and  
61 transmitted by female phlebotomine sand flies. It is characterized by nodules or ulcerative skin  
62 lesions on people's faces and extremities, which result in disfiguring scars after healing [1,2].  
63 CL is a major public health concern in Ethiopia, affecting approximately 20,000 to 50,000 people  
64 annually [3], in which *Leishmania aethiopica* is responsible for the majority of the infections [4–6].  
65 Ochollo, our study site, is a village in the mid-highlands of southwestern Ethiopia, where CL is  
66 endemic and is mainly affecting young children [7,8]. A recent study identified 4% of the primary  
67 school children with active lesions, 1.5% with lesions and scars and 59.8% with scars [8]. Adults are

68 very seldom found with active lesions, because they already recovered from a childhood *Leishmania*  
69 infection, thereby becoming resistant to the development of clinical infection [9]. There are  
70 currently no control programs for CL in southern Ethiopia, mainly because of the complexity of the  
71 zoonotic transmission cycle and the limited understanding of the vector's behavior.

72 Previous researchers described *Phlebotomus pedifer* as the only vector in Ochollo [10–13], showing a  
73 3.5% infection rate [13]. A study in Kenya found a good vectorial capacity of *P. pedifer* when feeding  
74 on active human CL lesions caused by *L. aethiopica*, implying that it is an efficient vector [14]. The  
75 species has been found indoors, around household compounds, in tree holes, rocky areas and inside  
76 caves [12,13]. A study in Ochollo in 1973 showed that 11 *P. pedifer* sand flies from indoors and five  
77 from caves were solely feeding on humans and hyraxes respectively [12]. However, until now  
78 relatively little is known about its biting behavior. Sand flies are generally known to be active  
79 between dusk and dawn and females feed on a wide variety of vertebrate hosts. However, the peak  
80 activity and host preference differs among sand fly species, so species specific entomological data  
81 are crucial to obtain a clear image of the transmission cycle [15–17].

82 Besides the vector, the reservoirs of the infection should be well documented. Hyraxes (*Heterohyrax*  
83 *brucei* and *Procavia capensis*) have been described as the reservoir of the zoonotic transmission of CL  
84 in Ethiopia [4–6,13]. *H. brucei* is abundant in Ochollo and a large proportion has been found infected  
85 with *L. aethiopica*. They live near human settlements, in caves and rock crevices, where sand flies  
86 and other potential hosts are abundant [12,13]. Rodents were found most probably not to play a  
87 role in transmission in Ochollo [13], but other animals have so far not been investigated yet as  
88 carriers of *L. aethiopica*. Given that bovines are commonly bitten by the main CL vector in Ethiopia,  
89 *P. longipes*, they could potentially serve as a reservoir as well [12,18,19].

90 Successful disease control requires profound understanding of the transmission cycle. Knowledge  
91 about the blood meal preference of sand flies is crucial to demonstrate which vertebrates might  
92 contribute to disease transmission and should be included in control programs. Moreover,

93 information on where and at what time sand flies are biting is a prerequisite to decide which vector  
94 control methods should be applied.

95 In this study, we aimed to gather knowledge on (i) the blood meal sources of *P. pedifer* in different  
96 habitats and its feeding preference when hosts are equally available, (ii) the role of domestic animals  
97 in CL transmission, and (iii) the indoor and outdoor activity pattern of *P. pedifer*. This information  
98 will shed light on the natural transmission cycle of CL in southwestern Ethiopia and help in  
99 instructing control efforts in the area.

## 100 **Methods**

### 101 **Ethics Statement**

102 This study was reviewed, approved and monitored by the Institutional Ethics Review Board (IRB) of  
103 Arba Minch University (cmhs/1203482/111 and cmhs/120017/111). Healthy adults (> 18 years) with  
104 obvious scar formation, who have been living in Ochollo their whole life, were selected as subjects  
105 for the human landing collections and host choice experiment. Written informed consent was  
106 obtained from all human volunteers who participated. All animal handlings were carried out  
107 according to the 2016 Guidelines of the American Society of Mammologists for use of mammals in  
108 research and education and in agreement with the appropriate institutional authorities.

### 109 **Study area**

110 Ochollo is located in southwestern Ethiopia (6°11'N, 37° 41' E), about 20 km North of Arba Minch  
111 (Fig 1). It is a rocky area with steep slopes and basalt cliffs with caves, situated at an altitude ranging  
112 between 1600 m and 2200 m. The area has a modest climate with an average yearly temperature  
113 around 20°C and a high humidity from May until October [13]. The village covers approximately  
114 1100 hectares and is divided into eight sub-villages, namely Shole-Kokuma, Zuza, Keya, Denkera,  
115 Casha-Afilaketsa, Kancho, Abale-Mowale and Oddo. Ochollo is densely inhabited by approximately  
116 5000 people, which are mainly clustered on the tops of hills and steep slopes. People ranch cattle  
117 and goats, and some households have dogs. Hyraxes are abundant and live in caves and rocky areas  
118 near human residences, while rodents mainly occupy stone fences and human and animal dwellings.

119 Houses are mainly made of mud, wood and grass, leaving many openings for sand fly entry and  
120 resting places.

121 **Fig 1: Map of the location of the study site, Ochollo, in Ethiopia [20,21].** SNNPR: Southern Nations,  
122 Nationalities and People's Region.

### 123 Host identification

124 **Sand fly collection** Sand flies were collected indoors and outdoors from 72 households (nine  
125 households in each of the eight sub-villages) between February and May 2018. Additionally, ten  
126 caves were selected in the village for monthly sand fly collections from March to June 2018.

127 Trapping was performed once per month at each sampling site with a particular entomological  
128 approach. Indoors and in caves, one CDC miniature light trap (John W. Hock Company, Florida, USA)  
129 and five sticky traps (ST, A4 format white papers attached to card board, covered with plastic  
130 impregnated with sesame oil on both sides) were placed at the bed end and wall cracks indoors, and  
131 inside caves. Collection with the two methods was performed on separate days. Only ST were  
132 utilized outdoors (N = 5 per collection site), which were placed on wall cracks of the houses and  
133 surrounding potential sand fly breeding or resting sites. Traps were set at 18h and collected again  
134 the next morning at sunrise. Blood fed female sand flies were sorted out, and the thorax and  
135 abdomen were dissected and stored in 97% ethanol at -20°C until further analysis. No distinction  
136 was made among different stages of blood digestion in sand flies.

137 **Blood meal analysis** DNA isolation the blood fed specimens was performed with a NucleoSpin Tissue  
138 kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Finally, the DNA  
139 was eluted in 50µl nuclease free water. Unfed sand flies and sand flies fed on laboratory mice  
140 (*Lutzomyia longipalpis*, acquired from the Laboratory of Microbiology, Parasitology and Hygiene,  
141 University of Antwerp, Belgium) were respectively used as negative and positive extraction controls.  
142 DNA extracts were subjected to a PCR targeting a fragment of the Cytochrome B gene (*Cyt B*, 359  
143 bp) as described by Steuber *et al.* (2005) and Carvalho *et al.* (2017) [22,23]. In short, the 15µl  
144 reaction mixture consisted of 1X Green GoTaq Flexi buffer (Promega, Leiden, Netherlands), 1.5mM

145 MgCl<sub>2</sub> (Promega, Leiden, Netherlands), 0.5μM of both primers Cyt1 (5'-CCA TTC AAC ATC TCA GCA  
146 TGA TGA AA-3') and Cyt2 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3')(Life Technologies,  
147 Merelbeke, Belgium), 0.2 mM dNTPs (GE Healthcare Lifescience, Diegem, Belgium), 1U GoTaq G2  
148 Flexi DNA Polymerase (Promega, Leiden, Netherlands) and 1.5μl DNA template. Amplification was  
149 carried out with an initial activation step of two minutes at 95°C, followed by 40 cycles of 30 seconds  
150 at 94°C, 30 seconds at 52°C and one minute at 72°C, and a final extension step of five minutes at  
151 72°C. The PCR was performed on Biometra T professional gradient Thermocycler (Biometra,  
152 Westburg, Netherlands). Positive and negative PCR controls and the above-mentioned extraction  
153 controls were included in each of the PCR reactions. PCR results were visualized on a 1.5% gel. After  
154 the PCR analyses were carried out at Arba Minch University in Ethiopia, the amplicons were sent to  
155 the Vlaams Instituut voor Biotechnologie (VIB) at the University of Antwerp in Belgium for  
156 sequencing. The obtained *Cyt B* sequences were aligned in GenBank using BLAST to determine the  
157 host species that served as a blood source. Results were only included when both query coverage  
158 and identity exceeded 95%.

159 **Sand fly species identification** If the blood meal of a specimen was successfully identified, the sand  
160 fly species was determined with a PCR targeting a 700 bp fragment of the *cytochrome c oxidase*  
161 *subunit I (COI)* gene, as described by Kumar *et al.* (2012) and Pareyn *et al.* (2019) [13,24].

162 **Livestock sample collection** Ear and nose biopsy samples from livestock (bovines and goats) were  
163 collected between January and April 2019. Samples originated either from animals that were  
164 slaughtered for human consumption or live animals. For the latter collection method, Xylocaine 2%  
165 gel (Astra Zeneca, Dilbeek, Belgium) was applied on the nose and ear for local anesthesia. Samples  
166 were collected using a 3 mm Biopsy puncher (Henry Schein, Vilvoorde, Belgium) and stored in 97%  
167 ethanol at -20°C until further analysis. To stop the bleeding, the incised skin wound was ligated with  
168 skin glue. Between ear and nose biopsy collections of each animal, the puncher was cleansed with  
169 1% bleach and rinsed in distilled water, and a new puncher was used for each animal.

170 *Leishmania* detection in livestock Nose and ear biopsies were screened for the presence of  
171 *Leishmania* nucleic acids at Arba Minch University (Ethiopia). Samples of each animal were subjected  
172 to a reverse transcriptase real-time PCR (RT-qPCR) targeting Spliced-Leader (SL-)RNA. Additionally, a  
173 selection of the samples (216/412) was also screened for the presence of kinetoplast DNA (kDNA) to  
174 confirm the results of the first assay. Nose and ear biopsy samples of each animal were pooled  
175 before extraction. Both RNA and DNA were isolated from the selection of samples that were tested  
176 by the two assays using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and NucleoSpin  
177 RNA/DNA buffer set (Macherey Nagel, Düren, Germany) following the manufacturer's instructions.  
178 For the remaining samples, only RNA isolation was carried out using solely the first method. A naive  
179 and *L. major* infected mouse ear from an experimental infection (Laboratory of Microbiology,  
180 Parasitology and Hygiene, University of Antwerp, Belgium; ethical approval UA ECD 2017-80) were  
181 respectively used as negative and positive extraction controls. The SL-RNA and kDNA qPCR assays  
182 were carried out as described previously by Eberhardt *et al.* and Pareyn *et al.* respectively [13,25].  
183 Extracts were 1:10 diluted before addition to the reaction mixture to avoid inhibition. Each PCR run  
184 included a no-template (negative) control and an infected (positive) control (a *L. aethiopica* infected  
185 hyrax nose biopsy DNA/RNA extract). For positive animals, ear and nose tissue samples were  
186 subjected to a separate extraction, followed by a PCR to determine the parasite RNA/DNA presence  
187 in the different tissues.

## 188 Sand fly activity pattern

189 **Human landing catches** To assess the indoor and outdoor human biting rhythms of *P. pedifer*,  
190 human landing catches (HLC) were conducted in and around four different household compounds  
191 between March and August 2018. HLC were done once per month at each sampling site, indoors and  
192 outdoors on the same day. Four collectors were used for each collection night, each working for six  
193 hours. In the first part of the night (between 18h and 24h), one collector performed the HLC indoors  
194 and one outdoors. In the second part of the night (24h until 6h), two other collectors carried out the  
195 same activities. The person sitting outdoors was positioned at least 10 m from the house. The



196 collectors sat on chairs with only their legs exposed and sand flies that landed on their legs were  
197 collected using a mouth aspirator. For each iteration of the experiment, there was an exchange in  
198 pre- and post-midnight and indoor-outdoor shifts to compensate for individual differences in  
199 attractiveness and collection skills. The collected specimens were cleared in Nesbitt's solution,  
200 washed with 70% ethanol and mounted in Hoyer's medium. Sand flies were determined up to  
201 species level with morphological identification keys [11,26].

202 **CDC light trap captures** Sand fly activity was also studied with CDC miniature light traps (John W.  
203 Hock Company, Florida, USA) from January to March 2019. Sand flies were captured during eight  
204 trapping sessions in a cave, which was previously determined as a hotspot of *P. pedifer* [13]. Traps  
205 were placed between 18h and 18h30 and collections were performed with an interval of 75 minutes  
206 until about 1h, eventually resulting in five collections per night. Sand flies were collected with a  
207 mouth aspirator and the number of male and female sand flies was recorded to establish the hourly  
208 activity of the sand flies. Captured sand flies were later used for the host choice experiment.

#### 209 **Host choice experiment**

210 A host choice experiment was carried out using the sand flies that were captured with CDC light  
211 traps for the sand fly activity assessment. Additional sand flies were trapped in other surrounding  
212 caves in Ochollo with CDC miniature light traps (John W. Hock Company, Florida, USA) in order to  
213 increase the sample size. The experiment was performed at 2h, immediately after the hourly CDC  
214 light trap captures.

215 The experimental set-up consisted of three connected cages (Fig 2). Female non-fed sand flies were  
216 placed in the middle cage. In each of the lateral cages, a particular host served as a blood meal  
217 source: a human volunteer's hand and a hyrax. Hyraxes were trapped by local people using  
218 traditional trapping methods. They were sedated during the experiment with ketamine (10mg/kg  
219 intramuscularly; Verve Human Care Laboratories, Uttarakhand, India) and placed inside the cage  
220 exposing the nose, ears and forepaws to the sand flies. Their eyes were protected from sand fly bites  
221 with a napkin. Human volunteers were positioned with their head nearby the cage for CO<sub>2</sub>

222 attractiveness towards the sand flies. The experimental set-up was covered by a plastic canvas to  
223 avoid interference of wind and other potential feeding sources in the surroundings.

224 **Fig 2: Host choice experiment set-up.** Female non-fed sand flies captured from caves were transferred to the  
225 middle cage, where they were left for 30 minutes to adapt. Then, the connecting tubes to the lateral cages,  
226 where a hyrax and human hand were exposed, were opened to allow sand flies to obtain their preferred blood  
227 meal during four hours. The hosts themselves and their places were changed for each iteration of the  
228 experiment.

229 After 30 minutes adaptation in the middle cage, the connecting tubes to the two lateral cages were  
230 opened for four hours to allow sand flies to bite their preferred host. Blood fed sand flies were  
231 collected using a mouth aspirator and stored in 97% ethanol at -20°C until further analysis. The  
232 experiment was conducted eight times and for each iteration, the position of the hosts was changed  
233 and new subjects were used. Hyraxes were released at their trapping site after the experiment. The  
234 blood meal sources and sand fly species were determined by sequencing a fragment of the *Cyt B* and  
235 *COI* gene respectively, according to the methods described above (blood meal analysis and sand fly  
236 identification).

### 237 Data analysis

238 All statistical analyses were carried out in R version 3.5.0, using packages “lme4” and “lmerTest”  
239 [27,28]. P-values < 0.05 were assumed statistically significant.

240 **Sand fly blood meal sources** To assess which host group served as an important blood meal source  
241 for sand flies, a generalized linear mixed model (GLMM) with Poisson error distribution was used.  
242 The number of sand flies that fed on a particular host, within a specific habitat, during a certain  
243 month was included as the response variable. The habitat where sand flies were captured (indoors,  
244 outdoors, cave), the host group they acquired their blood meal from and the interaction between  
245 habitat and host type were included as fixed effects. In order to correct for monthly variation in sand  
246 fly presence, we incorporated the collection as a random effect in the model. A post hoc test,  
247 specified as Tukey test, was applied to compare the hosts groups with each other [29].

248 After the previous general model, GLMMs were made similarly for each habitat separately to  
249 determine the important blood meal sources in each habitat. The model was constructed as  
250 described above, but only the host group was included as a fixed variable.

251 **Sand fly activity** The sand fly human biting rhythms indoors and outdoors were measured with HLC.

252 We had to transform our data to a binomial distribution (0 = no sand flies were caught within an  
253 hourly time interval, 1 = one or more sand flies were captured within a time interval), because the  
254 hourly counts were low. A GLMM was used with the HLC at each time interval as the dependent  
255 variable with a binomial error distribution. The location where sand flies were trapped  
256 (indoor/outdoor) was included as a fixed effect in order to assess if sand flies bite significantly more  
257 indoors compared to outdoors. Time interval was included as a fixed effect to compare at which  
258 moment sand flies were mostly active. We used the time interval as a categorical variable instead of  
259 continuous, because our preliminary analyses showed that there was a non-linear correlation. The  
260 trapping month and sampling site were incorporated as random effects in the model.

261 Sand fly activity was based on the number of captured sand flies with a CDC light trap. A GLMM with  
262 Poisson error distribution was used to estimate the sand fly activity. Due to technical difficulties, we  
263 were not able to include the fifth trapping night in the final dataset. The number of male and female  
264 sand flies at a certain time interval was used as the dependent variable. Sex was implemented as a  
265 fixed effect in the model to assess whether there were more males or females. Time interval was  
266 included as a categorical fixed effect to determine during which period most individuals were  
267 present. The experiment day was incorporated as a random effect to correct for potential  
268 differences between the sampling days.

269 **Host choice experiment** A GLMM with binomial distribution was used to determine the preferred  
270 blood meal source of sand flies when both humans and hyraxes are available. The proportion of  
271 sand flies that either fed on a hyrax or human during a single experiment was used as the response  
272 variable. The proportions were weighed by the total amount of sand flies that took a blood meal  
273 within a single experiment, since this varied between the different experiments. The type of host

274 and its position in the experiment were included as fixed effects to establish which host was  
275 preferred for a blood meal while correcting for potential personal and environmental bias. The  
276 experiment day was included as a random effect to correct for variation between days.

## 277 Results

### 278 Sand fly blood meal sources

279 A total of 368 blood fed female sand flies were collected, which underwent procedures for blood  
280 meal origin identification. 92 (25.0%) of these samples were excluded from the analysis, as negative  
281 extraction controls tested positive, indicating contamination during the DNA isolation procedure.  
282 The *Cyt B* gene could not be amplified for 11 samples (3.0%) and the sequence identity of 83  
283 samples (22.6%) could not be determined using the previously set cut-off requirements for the  
284 BLAST analysis. The overall analysis resulted in successful blood meal identification for 182 (49.5%)  
285 specimens. All of these specimens, except for two, turned out to be *P. pedifer*. The other two  
286 matched with several *Phlebotomus Laroussius* sand fly species in GenBank with low query coverage  
287 and identity, but could not be identified up to species level. One sand fly acquired its blood meal  
288 from a human and the other one from a bush hyrax.

289 A total of 180 *P. pedifer* sand flies fed on 12 different hosts, presented in Table 1. Overall, humans  
290 were the most important blood meal source ( $p < 0.001$ ), accounting for 59.4% of the identified  
291 origins, followed by bovines (13.9%), bush hyraxes (10.6%), goats (7.2%) and rodents (5.0%).  
292 Residual blood meals were acquired from a wide variety of vertebrates, together covering 4.0% of  
293 the determined sources. From the sand flies that fed on humans, five out of 137 (two collected from  
294 caves and three from indoors) were positive for *Leishmania* kDNA.

295 **Table 1: Blood meal analysis of *Phlebotomus pedifer* in Ochollo between February and May 2018.** Scientific  
296 and common name of blood meal sources grouped into categories for further analysis, and the number and  
297 percentage of sand flies that fed on each host.

Host category	Scientific name	Common name	Number	Percentage
Human	<i>Homo sapiens</i>	Human	107	59.4%

<b>Livestock</b>	<i>Bos taurus</i>	Bovine	25	13.9%
	<i>Capra hircus</i>	Goat	13	7.2%
<b>Hyrax</b>	<i>Heterohyrax brucei</i>	Bush hyrax	19	10.6%
<b>Rodent</b>	<i>Acomys spp.</i>	Spiny mouse	6	3.3%
	<i>Grammomys sp.</i>	Thicket rat	2	1.1%
	<i>Arvicanthis sp.</i>	Grass rat	1	0.6%
<b>Other</b>	<i>Myonycteris angolensis</i>	Bat	2	1.1%
	<i>Canis lupus familiaris</i>	Dog	2	1.1%
	<i>Gallus gallus</i>	Chicken	1	0.6%
	<i>Felis catus</i>	Cat	1	0.6%
	<i>Tragelaphus sp.</i>	Bushbuck	1	0.6%
<b>Total identified</b>			180	

298

299 Indoors, 129 blood fed sand flies were collected (Fig 3A). Significantly more sand flies (65.9%,  $p <$   
 300 0.001) had fed on humans (S1 Table) compared to 23.3% that fed on livestock (16.3% on bovines and  
 301 7.0% on goats) and 5.4% on rodents. Three of the sand flies that were captured indoors had  
 302 acquired their blood meal from hyraxes.

303 **Fig 3: Blood meal sources of *Phlebotomus pedifer* captured in different habitats in Ochollo 2018.** Blood meal  
 304 sources of sand flies captured (A) indoors (B) outdoors and (C) in caves. The category livestock includes  
 305 bovines and goats and the category rodents consists of *Acomys spp.*, *Grammomys sp.* and *Arvicanthis sp.*. The  
 306 'other' host group includes all other vertebrates that sand flies fed on (Table 1).

307 No significant difference in blood meal sources could be determined from the 18 sand flies that were  
 308 captured outdoors (Fig 3B, S1 Table), but the most important origins were again humans (38.9%) and  
 309 livestock (33.3%).

310 In caves, 33 sand flies were blood fed, which mainly acquired their blood meal more from humans  
 311 (45.5%) and hyraxes (42.4%), although the numbers were not significantly different from the other  
 312 three groups (Fig 3C, S1 Table).

### 313 *Leishmania* in livestock

314 A total of 412 ear and nose samples, of which 209 from bovines and 203 from goats, were collected.  
 315 Of the 412 samples, 17 were collected from slaughtered bovines and 395 from live animals. The

316 selection of the samples that were subjected to both kDNA and SL-RNA assays were all negative. The  
317 pooled sample of one live goat was positive for SL-RNA. After separate tissue extractions of this  
318 goat, the nose sample appeared positive for kDNA and SL-RNA, with a Ct value of approximately 28  
319 in both assays.

#### 320 Sand fly activity

321 Human landing catches A total of 161 sand flies were captured with HLC, of which 93% were  
322 identified as *P. pedifer*, while the remaining 7% belonged to the subgenus *Sergentomyia*, which were  
323 removed from further analysis.

324 *P. pedifer* was found to bite humans both indoors and outdoors, but the overall the probability of  
325 indoor biting was significantly higher ( $p = 0.003$ , Fig 4A).

326 **Fig 4: Indoor and outdoor human biting rhythms of *Phlebotomus pedifer* by human landing catches.** (A)  
327 Average probability (%) of sand fly biting indoors and outdoors. (B) Average probability of temporal sand fly  
328 biting (%) indoors (dark grey bars) and outdoors (light grey bars). Error bars represent the standard error of  
329 the response variable.

330 Sand fly collections showed a similar temporal biting pattern indoors and outdoors ( $p = 0.912$ , Fig  
331 4B). There was a substantial probability of sand fly biting in the early evening, which increased  
332 during the night, reaching a its maximum around midnight. After that, a drop was observed, with the  
333 lowest biting probability just before sunrise. Although Fig 4B shows a clear pattern in the activity,  
334 comparison of the biting activity at the different time intervals provided no significant differences  
335 between neighboring intervals (S2 Table).

336 CDC light trap captures A total of 821 sand flies were captured with CDC light traps during seven  
337 trapping nights, of which 711 were female and 110 male. The hourly activity pattern of female and  
338 male sand flies is depicted in Fig 5. Significantly more female than male sand flies were captured ( $p <$   
339  $0.001$ ). The activity of female sand flies between 19h-20h was significantly lower compared to the  
340 other hours, except for 24h-1h (S3 Table). Other time intervals were not significantly different from  
341 each other. Overall, the activity pattern of female sand flies shows that there was considerable

342 activity in the early evening, which increased over time, reaching its maximum at 22h-23h. For male  
343 sand flies, no clear trend could be distinguished.

344 **Fig 5: *Phlebotomus pedifer* activity pattern based on CDC light trap captures.** The left, dark grey and the right,  
345 light grey bars are respectively the average number of female and male sand flies at a certain time interval.  
346 Error bars represent the standard error of the response variable.

### 347 Host preference

348 A total of 716 female *P. pedifer* sand flies were used in the host choice experiment, of which in total  
349 65 sand flies were found blood fed over the eight repeats of the experiment (S4 Table).

350 The *Cyt B* fragment was successfully amplified and sequenced for all freshly engorged sand flies. All  
351 sand flies included in the experiment were *P. pedifer*. Fig 6 shows that sand flies were biting both  
352 hosts, but significantly more sand flies fed on hyraxes (61.5%) than on humans (38.5%,  $p = 0.009$ ).  
353 The position of the host had no effect on the host choice ( $p = 0.776$ ).

354 **Fig 6: Host choice preference of *Phlebotomus pedifer* in an experimental set-up.** Average percentage of sand  
355 flies that fed on a human or hyrax host during the host choice experiment. Error bars represent the standard  
356 error of the response variable.

### 357 Discussion

358 We gathered novel insights in the biting behavior and activity of *P. pedifer* and the role of livestock  
359 in transmission of CL in southwestern Ethiopia, which can be used as a guidance in disease control  
360 programs.

361 We identified the blood meal sources of sand flies in Ochollo indoors, outdoors and in caves. Sand  
362 flies acquired their blood meals from hosts of 12 different genera, which is a wider variety compared  
363 to the results of Ashford *et al.* (1973) from Ochollo, who found only hyraxes and humans as blood  
364 meal sources in caves and indoors [12]. This may be linked to our larger sample size and the  
365 availability of more sophisticated analysis methods.

366 Overall, the majority of sand flies fed on humans and 3.6% of these sand flies were *Leishmania* DNA  
367 positive. Additionally, previous research showed that there is a high infection prevalence in humans

368 in Ochollo and that *L. aethiopica* transmission from a human CL lesion to *P. pedifer* is very efficient  
369 [8,14]. These combined data suggest that humans are probably more than just an accidental host in  
370 the transmission cycle [8,14,19]. On the contrary, only 10.6% of the sand fly blood meals were  
371 derived from hyraxes and none of these sand flies were found kDNA positive. This is an interesting  
372 result, since we recently documented that 20% (5/25) of the hyraxes captured in Ochollo were  
373 *Leishmania* DNA positive [13]. Although CL in Ethiopia has only been reported as zoonotic with  
374 hyraxes serving as the only reservoir host [5,6,19,30], this study demonstrates that there is very  
375 likely also anthroponotic transmission in southwestern Ethiopia. Hence, control should focus on  
376 humans, whether or not with additional reservoir control. Notably, the kDNA positive blood fed sand  
377 flies should be interpreted with care, because sand flies could have been infected before the current  
378 blood meal was acquired.

379 Some sand flies fed on rodents, in particular on different spiny mouse species (*Acomys spp.*), thicket  
380 rat (*Grammomys sp.*) and grass rat (*Arvicanthis sp.*). Several researchers have focused already on  
381 rodents as potential reservoirs of CL in Ethiopia. In a previous study that we carried out in Ochollo  
382 (2019), only a single African pigmy mouse (*Mus mahomet*) out of 192 rodents of eight different  
383 species was found kDNA positive. Despite the large trapping effort in that study, *Acomys* and  
384 *Grammomys spp.* were not captured [13]. In another study carried out all over Ethiopia by Kassahun  
385 and his colleagues (2015), 141 *Acomys spp.* were collected, of which 14 (9.9%) were found kDNA  
386 positive and three of these could be further identified as *L. tropica* infections [31]. A giant rat  
387 (*Cricetomys sp.*) and a ground squirrel (*Xerus rutilus*) have been found naturally infected with *L.*  
388 *aethiopica*. The latter was found in Aba Roba (1200 m), a visceral leishmaniasis (VL) endemic area in  
389 Ethiopia, where human CL cases have never been reported [32,33]. Except these observations, *L.*  
390 *aethiopica* has to our knowledge never been found in rodents before, despite the various sampling  
391 efforts that have been undertaken previously [5,12,33,34]. This suggests that rodents are probably  
392 not a reservoir for *L. aethiopica* and hence do not play an important role in the transmission  
393 dynamics.



394 *Acomys spp.* in Ethiopia are known to inhabit rocky slopes and rock crevices, but in our study, five  
395 out of six sand flies that fed on this species were found indoors [35]. Likewise, three sand flies that  
396 were captured indoors had fed on hyraxes. This result suggests that sand flies might rest indoors  
397 after have taken their blood meal elsewhere, which could be further investigated with i.e.  
398 fluorescent powder on sand fly wings to demonstrate their dispersal [36,37].

399 Remarkably, the blood meal analysis from cave collected sand flies demonstrated that sand flies  
400 feed on humans as much as on hyraxes, while hyraxes are abundant and live inside the caves. This  
401 could be interpreted as an increased preference for biting humans. We tested this by a host choice  
402 experiment, in which human and hyrax were both available. Surprisingly, while sand flies do feed on  
403 both hosts, there was a significant preference for hyraxes, which contradicts the previous  
404 hypothesis. The result that humans are equally dominant as hyraxes as sand fly host meals in caves  
405 is probably not due to blood meal preference, but potentially to an increased availability of humans  
406 during the peak sand fly activity hours. Previously, Ashford *et al.* recommended complete hyrax  
407 elimination by shooting or biological control, such as release of predators [38]. Other researchers  
408 suggested hyrax elimination near human settlements (about 1 km) as a possible intervention against  
409 *L. aethiopica* transmission [30,39]. This rises the concern that *P. pedifer's* preferred blood meal host  
410 would not be available anymore, resulting in a shift towards biting humans, thereby increasing their  
411 exposure to sand fly bites and accordingly their risk of infection. A study of Svobodova *et al.* (2006)  
412 showed that asymptotically infected hyraxes were infectious to *P. arabicus*, but with a low  
413 success rate [40]. Additional research remains necessary to establish the transmission efficiency of  
414 parasites from infected hyraxes to the current vector to deliberate whether elimination of hyraxes  
415 should be included in control programs. The fact that that sand flies captured from caves obtained a  
416 similar proportion of blood meals from humans as from hyraxes implies that humans are accessible  
417 as blood source in proximity to the hyrax habitats.

418 Many specimens in the blood meal analysis did not provide a successful PCR or sequence according  
419 to the previously set requirements, while host sequences could be determined from all freshly fed

420 sand flies in the host choice experiment. It has been shown that the success rate of host DNA  
421 analysis is negatively correlated with the time-course after the blood meal was taken [15,41–43]. We  
422 did not record the estimated days post-feeding, but sand flies with partially digested blood were  
423 included in the blood meal analysis, which explains the success rate of the blood meal analysis.

424 To unravel the complex CL cycle in southwestern Ethiopia, it is important to assess all players of  
425 transmission. This study demonstrates that livestock accounts for 21.1% of the blood meal sources  
426 of *P. pedifer*, but in ear and nose biopsies from goats and bovines, we found only a single goat nose  
427 biopsy positive for kDNA and SL-RNA with a high Ct value. This points to a relatively low, but viable  
428 parasitemia, although persistence and transmission of the parasites are not guaranteed [25].

429 Overall, it should be considered that some animals in the current study might have had parasites in  
430 their skin, which remained undetected due to the collection of only a small tissue biopsy [44].

431 Studies have already found DNA or antibodies indicating the presence of VL parasites in livestock,  
432 also in northern Ethiopia [45–48]. Research investigating the role of livestock in CL transmission is  
433 rather scarce. A study conducted during a CL outbreak in a non-endemic village in Venezuela found  
434 suspected active CL lesions in seven out of 29 (24%) donkeys in hairless areas (ear, tail, etc.), of  
435 which six lesion samples contained *Leishmania* parasites [49]. In a similar research conducted in a CL  
436 endemic area in Kenya, one goat was found with lesions and detectable levels of *L. aethiopica* DNA  
437 in the skin and other organs [50].

438 Based on our results, gathered from a large sample size collected from areas with different  
439 ecological features and screened with highly sensitive assays, we conclude that domestic animals in  
440 similar ecological areas in southwestern Ethiopia are likely not to play a considerable role in  
441 transmission. However, many sand flies acquired their blood meal from these animals and it was  
442 observed that livestock is living close to or even inside human settlements in Ochollo. It has been  
443 suggested to keep livestock close to human settlements to divert vector biting from humans  
444 (zoonophylaxis) or to use them as baits for vector attraction to insecticide-treated livestock [51–  
445 54]. In contrast, other researchers assert that this could increase the vector population near humans

446 (zoopotential) or augment the vector infectivity if blood meal sources are readily available  
447 [46,52,54,55]. More research is necessary to determine whether domestic animals could serve as  
448 protection against contraction of leishmaniasis.

449 Understanding the vector's biting behavior gives an indication about when and where *Leishmania*  
450 transmission occurs, and at which time and place control strategies would be most effective. Both  
451 activity experiments showed that sand flies are predominantly active around midnight and the  
452 majority of the sand flies were captured indoors with HLC. Therefore, insecticide-treated bed nets or  
453 indoor residual spraying are potentially effective control strategies to manage the peak transmission  
454 at night [56–59]. Considerable activity was also observed in the early evening with about 30% of the  
455 sand flies captured outdoors by HLC. During the fieldwork, children were collecting water near caves  
456 and rock crevices and adults were performing outdoor activities in the early evening (e.g. dinner  
457 preparation and washing), thereby increasing their risk of exposure to potentially infectious sand fly  
458 bites. This was also shown in a study by Sang *et al.* in a CL endemic area in Kenya, where almost all  
459 CL cases admitted that they often visit caves [60]. Hence, improvement of community knowledge  
460 and attempts to decrease the vector population densities near places of outdoor activity could  
461 contribute to a reduction of residual transmission [61].

462 The activity of the CL vectors in Ethiopia has never been studied so far, but similar studies were  
463 carried out on *P. orientalis* in different VL foci in northern Ethiopia and Sudan [62–67]. These studies  
464 found various activity patterns for this vector species, indicating that the activity of a single species  
465 can differ between regions. Research on sand fly behavior in each ecologically different setting is  
466 accordingly necessary to accomplish efficient vector control.

467 This study shows that sand flies in Ochollo mainly feed on humans and that there is likely also  
468 anthroponotic transmission of *L. aethiopica*. Hyraxes are the preferred blood meal source when  
469 hosts are equally accessible, so the efficiency of parasite transmission from *H. brucei* to *P. pedifer*  
470 should be investigated before including them in control programs. Livestock appears an important  
471 blood meal source for sand flies, but does probably not play a significant role in transmission of CL in

472 southwestern Ethiopia. *P. pedifer* is mainly active at night indoors, but there is also considerable  
473 outdoor activity, suggesting that combined measures are required for efficient disease control.

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## 480 References

- 481 1. World Health Organization. Leishmaniasis [Internet]. 2018 [cited 2018 Aug 29]. Available  
482 from: <http://www.who.int/leishmaniasis/disease/en/>
- 483 2. Reithinger R, Dujardin J, Louzir H, Pirmez C, Alexander B, Brooker S, et al. Cutaneous  
484 leishmaniasis. *Lancet*. 2007;7(9):581–96.
- 485 3. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and  
486 global estimates of its incidence. *PLoS One*. 2012;7(5).
- 487 4. Bray RS, Ashford RW, Bray MA. The parasite causing cutaneous leishmaniasis in Ethiopia.  
488 *Trans R Soc Trop Med Hyg*. 1973;67(3):345–8.
- 489 5. Lemma W, Erenso G, Gadisa E, Balkew M, Gebre-michael T, Hailu A. A zoonotic focus of  
490 cutaneous leishmaniasis in Addis Ababa, Ethiopia. *Parasit Vectors*. 2009;2(60).
- 491 6. Lemma W. Zoonotic leishmaniasis and control in Ethiopia. *Asian Pac J Trop Med*.  
492 2018;11(5):313–9.
- 493 7. Mengistu G, Laskay T, Gemetchu T, Humber D, Ersamo M, Eva D, et al. Cutaneous  
494 leishmaniasis in south-western Ethiopia: Ocholo revisited. *Trans R Soc Trop Med Hyg*.  
495 1992;86:149–53.
- 496 8. Bugssa G. The Current Status of Cutaneous Leishmaniasis and the Pattern of Lesions in  
497 Ochollo Primary School Students, Ochollo, Southwestern Ethiopia. *Sci J Clin Med*.

- 498 2014;3(6):111.
- 499 9. Nagill R, Kaur S. Vaccine candidates for leishmaniasis: A review. *Int Immunopharmacol.*
- 500 2011;11(10):1464–88.
- 501 10. Gemetchu T, Laskay T, Frommel D. Phlebotomine sandflies (Diptera: Psychodidae,
- 502 Phlebotominae) of Ochollo, southwestern Ethiopia: species composition and natural infection
- 503 of *Phlebotomus pedifer* with *Leishmania aethiopica*. *Ethiop J Sci.* 1990;13(1):43–50.
- 504 11. Gebre-Michael T, Lane RP. A new sandfly species, *Phlebotomus (Larrousius) ashfordi*
- 505 (Diptera, Psychodidae) from Ethiopia, previously confused with *P. (L.) aculeatus*. *Ann Trop*
- 506 *Med Parasitol.* 1996;90(5):523–31.
- 507 12. Ashford W, Bray M, Hutchinson P, Bray S. The epidemiology of cutaneous leishmaniasis in
- 508 Ethiopia. *Trans R Soc Trop Med Hyg.* 1973;67(4).
- 509 13. Pareyn M, Van Den Bosch E, Girma N, Houtte N Van, Van Dongen S, Van Der Auwera G, et al.
- 510 Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in
- 511 Ochollo, a hotspot in southern Ethiopia. *PLoS Negl Trop Dis.* 2019;13(8).
- 512 14. Mutinga MJ, Odhiambo TR. Cutaneous leishmaniasis in Kenya 2. Studies on vector potential
- 513 of *Phlebotomus pedifer* (Diptera, Phlebotomidae) in Kenya. *Insect Sci its Appl.* 1986;7(2):171–
- 514 4.
- 515 15. Gaudêncio K, Costa PL, Carla R, Morais S De, Otranto D, Brandão-filho SP, et al. Identification
- 516 of phlebotomine sand fly blood meals by real-time PCR. *Parasit Vectors.* 2015;8(230).
- 517 16. Guy MW, Killick-Kendrick R, Gill GS, Rioux J, Bray RS. Ecology of leishmaniasis in the South of
- 518 France. *Ann Parasitol Hum Comp.* 1984;59(5):449–58.
- 519 17. Bongiorno G, Habluetzel A, Khoury C, Maroli M. Host preferences of phlebotomine sand flies
- 520 at a hypoendemic focus of canine leishmaniasis in central Italy. *Acta Trop.* 2003;88:109–16.
- 521 18. Mutinga MJ. *Phlebotomus longipes*, a vector of cutaneous leishmaniasis in Kenya. *Trans R Soc*
- 522 *Trop Med Hyg.* 1970;56(1):106.
- 523 19. Henten S Van, Adriaensen W, Fikre H, Akuffo H, Diro E, Hailu A, et al. Cutaneous

- 524 Leishmaniasis Due to *Leishmania aethiopica*. *EClinicalMedicine*. 2018;65(1):106.
- 525 20. OpenAFRICA: Ethiopia shapefiles [Internet]. 2016 [cited 2019 Aug 8]. Available from:  
526 <https://africaopendata.org/dataset/ethiopia-shapefiles>
- 527 21. QGIS Development team. QGIS Geographic Information System. Open Source Geospatial  
528 Foundation Project; 2019.
- 529 22. Steuber S, Abdel-Rady A, Clausen P. PCR-RFLP analysis: a promising technique for host species  
530 identification of blood meals from tsetse flies (Diptera: Glossinidae). *Parasitol Res*.  
531 2005;97(3):247–54.
- 532 23. Carvalho G, Rego F, Tanure A, Silva A, Dias T, Paz G, et al. Bloodmeal identification in field-  
533 collected sand flies from Casa Branca, Brazil, using the cytochrome b PCR Method. *J Med*  
534 *Entomol*. 2017;54(4):1049–54.
- 535 24. Kumar NP, Srinivasan R, Jambulingam P. DNA barcoding for identification of sand flies  
536 (Diptera: Psychodidae) in India. *Mol Ecol*. 2012;12:414–20.
- 537 25. Eberhardt E, Kerkhof M Van Den, Bulté D, Mabilie D, Bockstal L Van, Monnerat S, et al.  
538 Evaluation of a pan-*Leishmania* Spliced-Leader RNA detection method in human blood and  
539 experimentally infected syrian golden hamsters. *J Mol Diagnostics*. 2018;20(2).
- 540 26. Killick-Kendrick R, Tang Y, Killick-Kendrick M, RN J, Ngumbi P, Sang D, et al. Phlebotomine  
541 sandflies of Kenya (Diptera: Psychodidae). III. The identification and distribution of species of  
542 the subgenus *Larrousius*. *Ann Trop Med Parasitol*. 1994;88(2):183–96.
- 543 27. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J*  
544 *Stat Softw*. 2015;67:1–48.
- 545 28. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects  
546 Models. *J Stat Softw*. 2017;82(13):26.
- 547 29. Russel VL. Least-Squares Means: The R package lsmeans. *J Stat Softw*. 2016;69(1):1–33.
- 548 30. Gebremichael D. Zoonotic impact and epidemiological changes of leishmaniasis in Ethiopia.  
549 *Open Vet J*. 2018;8(4):432–40.

- 550 31. Kassahun A, Sadlova J, Dvorak V, Kostalova T, Rohousova I, Frynta D, et al. Detection of  
551 *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents. *Acta Trop.* 2015;145:39–44.
- 552 32. Mutinga MJ. The animal reservoir of cutaneous leishmaniasis on Mount Elgon, Kenya. *East*  
553 *Afr Med J.* 1975;52(3):142–51.
- 554 33. Abebe A, Evans D, Gemetchu T. The isolation of *Leishmania aethiopica* from the ground  
555 squirrel *Xerus rutilus*. *Trans R Soc Trop Med Hyg.* 1990;2:907.
- 556 34. Lemma A, Foster W, Gemetchu T, Preston P, Bryceson A, Minter D. Studies on leishmaniasis  
557 in Ethiopia. I. Preliminary investigation into the epidemiology of cutaneous leishmaniasis in  
558 the highlands. *Ann Trop Med Parasitol.* 1969;63:455–72.
- 559 35. Kingdon J. *Mammals of Africa: Volume III: Rodents, Hares and Rabbits.* Bloomsbury Publishing  
560 PLC; 2013.
- 561 36. Silva N, De Melo S, Massafera R, Rossi R, Silveira T, Teodoro U. Dispersal and Memory of Sand  
562 Flies in an Endemic Area of Cutaneous Leishmaniasis, Southern Brazil. *J Med Entomol.*  
563 2013;50(5):986–93.
- 564 37. Casanova C, Costa AIP, Natal D. Dispersal pattern of the sand fly *Lutzomyia neivai* (Diptera:  
565 Psychodidae) in a cutaneous leishmaniasis endemic rural area in Southeastern Brazil.  
566 2005;100(7):719–24.
- 567 38. Ashford W. Leishmaniasis Reservoirs and Their Significance in Control. *Clin Dermatology.*  
568 1996;14(5):523–32.
- 569 39. World Health Organization/Expert committee on the Control of Leishmaniases. Control of the  
570 leishmaniases. 2010.
- 571 40. Svobodová M, Volf P, Votýpka J. Experimental transmission of *Leishmania tropica* to hyraxes  
572 (*Procavia capensis*) by the bite of *Phlebotomus arabicus*. *Microbes Infect.* 2006;8(7):1691–4.
- 573 41. Sant MR V, Jones NG, Hindley JA, Mendes-sousa AF, Dillon RJ, Cavalcante RR, et al. Blood  
574 meal identification and parasite detection in laboratory-fed and field-captured *Lutzomyia*  
575 *longipalpis* by PCR using FTA databasing paper. *Acta Trop.* 2008;107:230–7.

- 576 42. Abbasi I, Cunio R, Warburg A. Identification of Blood Meals Imbided by Phlebotomine Sand  
577 Flies Using Cytochrome b PCR and Reverse Line Blotting. Vector-borne zoonotic Dis.  
578 2009;9(1):79–86.
- 579 43. Haouas N, Pesson B, Boudabous R, Dedet J, Babba H, Ravel C. Development of a Molecular  
580 Tool for the Identification of *Leishmania* Reservoir Hosts by Blood Meal Analysis in the Insect  
581 Vectors. Am J Med Hyg. 2007;77(6):1054–9.
- 582 44. Doehl JSP, Bright Z, Dey S, Davies H, Magson J, Brown N, et al. Skin parasite landscape  
583 determines host infectiousness in visceral leishmaniasis. Nat Commun. 2017;8(57).
- 584 45. Singh N, Mishra J, Singh R, Singh S. Animal reservoirs of visceral leishmaniasis in India. J  
585 Parasitol. 2013;99(1):64–7.
- 586 46. Bhattarai NR, Auwera G Van Der, Rijal S, Picado A, Speybroeck N, Khanal B, et al. Domestic  
587 animals and epidemiology of visceral leishmaniasis, Nepal. Emerg Infect Dis. 2010;16(2):231–  
588 7.
- 589 47. Alam MS, Ghosh D, Khan GM, Islam MF. Survey of domestic cattle for anti-*Leishmania*  
590 antibodies and *Leishmania* DNA in a visceral leishmaniasis endemic area of Bangladesh. BMC  
591 Vet Res. 2011;27(7).
- 592 48. Rohousova I, Talmi-Frank D, Kostalova T, Polanska N, Lestinova T, Kassahun A, et al. Exposure  
593 to *Leishmania spp.* and sand flies in domestic animals in northwestern Ethiopia. Parasites and  
594 Vectors. 2015;8(1).
- 595 49. Aguilar CM, Fernandez E, De Fernandez R, Deane LM. Study of an outbreak of cutaneous  
596 leishmaniasis in Venezuela. The role of domestic animals. Mem Inst Oswaldo Cruz.  
597 1984;79(2):181–95.
- 598 50. Williams AO, Mutinga J, Rodgers M. Leishmaniasis in a domestic goat in Kenya. Mol Cell  
599 Probes. 1991;5:319–25.
- 600 51. Franco AO, Gomes MGM, Rowland M, Coleman PG, Davies CR. Controlling Malaria Using  
601 Livestock-Based Interventions: A One Health Approach. PLoS One. 2014;9(7).



- 602 52. Bern C, Courtenay O, Alvar J. Of Cattle, sand flies and men: A systematic review of risk factor  
603 analyses for South Asian visceral leishmaniasis and implications for elimination. PLoS Med.  
604 2010;4(2).
- 605 53. Gebresilassie A, Abbasi I, Aklilu E, Yared S, Kirstein OD, Moncaz A, et al. Host-feeding  
606 preference of *Phlebotomus orientalis* (Diptera: Psychodidae) in an endemic focus of visceral  
607 leishmaniasis in northern Ethiopia. Parasit Vectors. 2015;8(270).
- 608 54. Yared S, Gebresilassie A, Abbasi I, Aklilu E, Kirstein OD, Balkew M, et al. A molecular analysis  
609 of sand fly blood meals in a visceral leishmaniasis endemic region of northwestern Ethiopia  
610 reveals a complex host-vector system. Heliyon. 2019;5.
- 611 55. Serafim TD, Coutinho-abreu I V, Oliveira F, Meneses C, Kamhawi S, Valenzuela JG. Sequential  
612 blood meals promote *Leishmania* replication and reverse metacyclogenesis augmenting  
613 vector infectivity. Nat Microbiol. 2018;3(5):148–55.
- 614 56. Mondal D, Huda MM, Karmoker MK, Ghosh D, Matlashewski G, Nabi SG, et al. Reducing  
615 Visceral Leishmaniasis by Insecticide Impregnation Bangladesh. Emerg Infect Dis.  
616 2013;19(7):1131–3.
- 617 57. Rowland T. Efficacy of Permethrin Treated Bed Nets Against *Leishmania Major* Infected Sand  
618 Flies. US Army Med Dep J. 2015;10–5.
- 619 58. Coulibaly CA, Traore B, Dicko A, Samake S, Sissoko I, Anderson JM, et al. Impact of insecticide-  
620 treated bednets and indoor residual spraying in controlling populations of *Phlebotomus*  
621 *duboscqi*, the vector of *Leishmania major* in Central Mali. Parasit Vectors. 2018;11(345).
- 622 59. Kayedi MH, Rassi Y, Chegeni-sharafi A, Rafizadeh S, Abdali N. Control of Cutaneous  
623 Leishmaniasis Using Deltamethrin Treated Nets in Comparison to Indoors Residual Spraying in  
624 a Rural Area of Iran. Iran J Public Heal. 2017;46(6):835–42.
- 625 60. Sang DK, Okelo GBA, Chance ML. Cutaneous leishmaniasis due to *Leishmania aethiopica*, on  
626 Mount Elgon, Kenya. Ann Trop Med Parasitol. 1993;87(4):349–57.
- 627 61. Kebede N, Worke A, Ali A, Anmut A, Negash Y, Gebreyes W, et al. Community knowledge,

- 628 attitude and practice towards cutaneous leishmaniasis endemic area Ochello, Gamo Gofa  
629 Zone, South Ethiopia. *Asian Pac J Trop Biomed.* 2017;7(12):1129–50.
- 630 62. Aklilu E, Gebresilassie A, Yared S, Kindu M, Tekie H, Balkew M, et al. Comparative study on  
631 the nocturnal activity of phlebotomine sand flies in a highland and lowland foci of visceral  
632 leishmaniasis in north-western Ethiopia with special reference to *Phlebotomus orientalis*.  
633 *Parasit Vectors.* 2017;10(393).
- 634 63. Lemma W, Tekie H, Abassi I, Balkew M, Gebre-michael T, Warburg A, et al. Nocturnal  
635 activities and host preferences of *Phlebotomus orientalis* in extra-domestic habitats of Kafta-  
636 Humera lowlands, Kala-azar endemic, Northwest Ethiopia. *Parasit Vectors.* 2014;7(594).
- 637 64. Elnaiem DA, Hassan HK, Ward RD. Phlebotomine sandflies in a focus of visceral leishmaniasis  
638 in a border area of eastern Sudan. *Ann Trop Med Parasitol.* 2016;91(3):307–18.
- 639 65. Gebremeskel SY, Balkew M, Warburg A, Hailu A. Nocturnal activity of *Phlebotomus* species  
640 (Diptera: Psychodidae) in a visceral leishmaniasis endemic area of northwest Ethiopia. *J Biol*  
641 *Agric Healthc.* 2015;5(5):185–94.
- 642 66. Quate LW. *Phlebotomus* sand flies of the Paloich area in the Sudan (Diptera: Psychodidae). *J*  
643 *Med Entomol.* 1964;1:213–68.
- 644 67. Ashford RW, Hutchinson P, Bray RS. Kala-azar in Ethiopia: Epidemiological studies in a  
645 highland valley. *Ethiop Med J.* 1973;11(4):259–64.

## 646 Supporting information Captions

647 **S1 Table: Comparison of different blood meal sources of *Phlebotomus pedifer* overall and in each habitat**  
648 **(indoor, outdoor, cave) separately.**

649 **S2 Table: Comparisons of hourly differences in human biting behavior of *Phlebotomus pedifer* sand flies**  
650 **indoors and outdoors by human landing catches.**

651 **S3 Table: Comparisons of hourly differences in activity of male and female *Phlebotomus pedifer* sand flies by**  
652 **means of CDC light trap captures.**

653 **S4 Table: Overview of the sand fly blood meal sources in the host choice experiment.** For each of the eight  
654 iterations of the experiment, the number (%) of sand flies that were used for the experiment, that eventually  
655 took a blood meal and which host they were found to feed on are presented.

656 **S1 Data: Dataset of blood meal sources of *Phlebotomus pedifer*, captured in different months and different**  
657 **habitats.**

658 **S2 Data: Binomial dataset of sand fly activity indoors and outdoor based on human landing catches (HLC).**

659 **S3 Data: Dataset of activity of cave collected *Phlebotomus pedifer* sand flies based on CDC light trap**  
660 **captures.**

661 **S4 Data: Dataset of host choice experiment.**

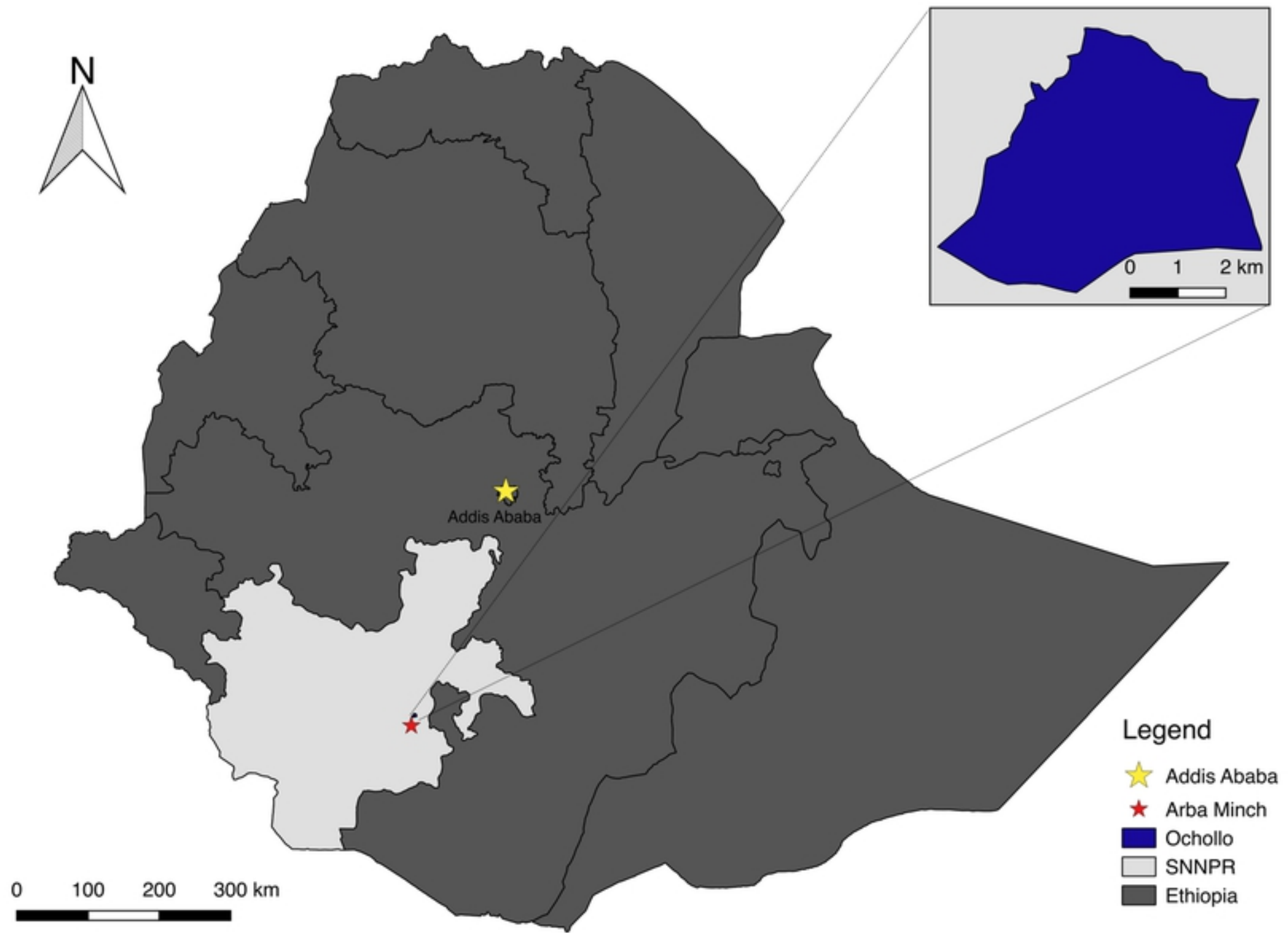


Fig 1

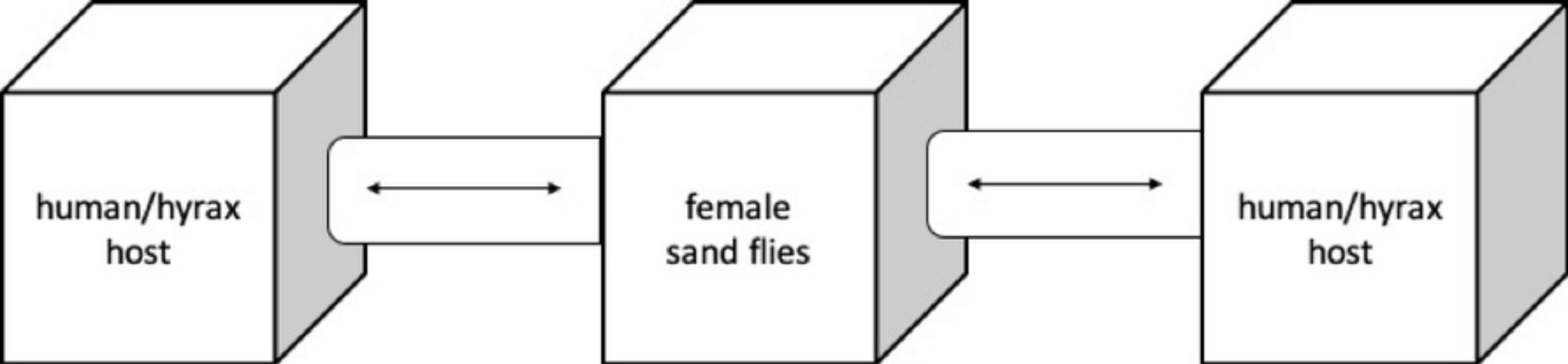


Fig 2

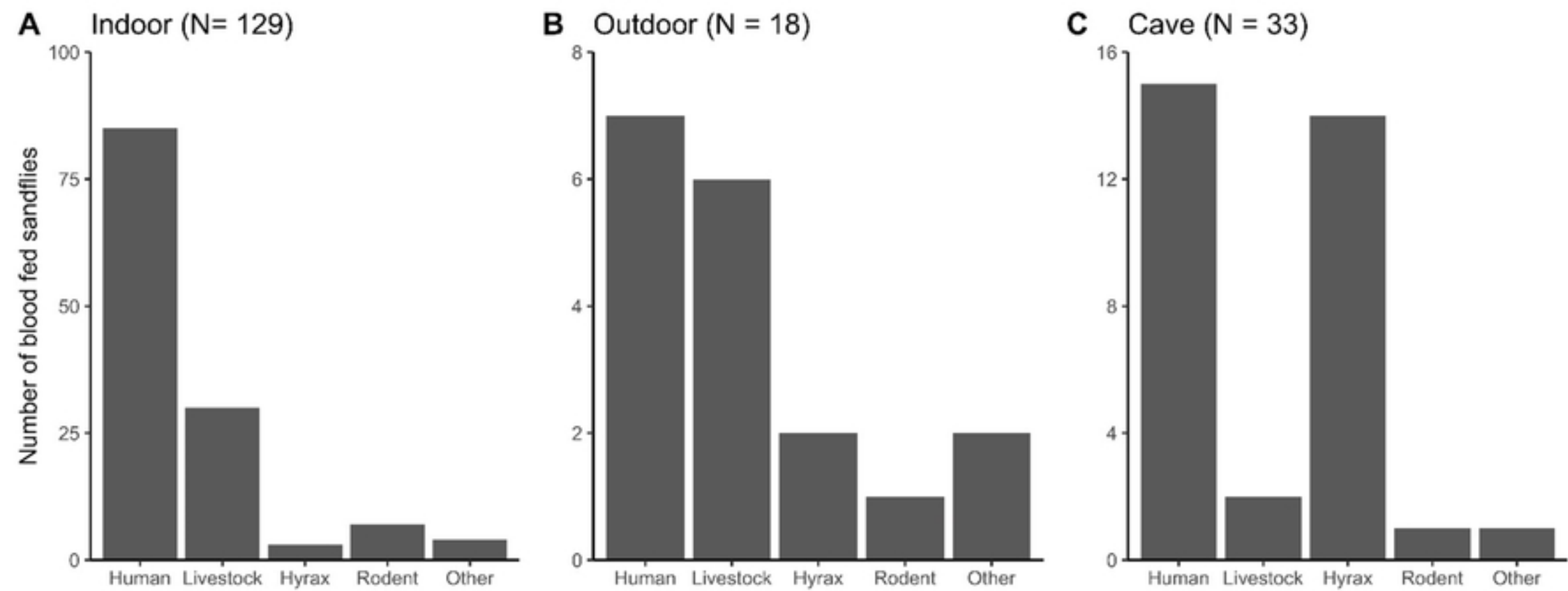


Fig 3

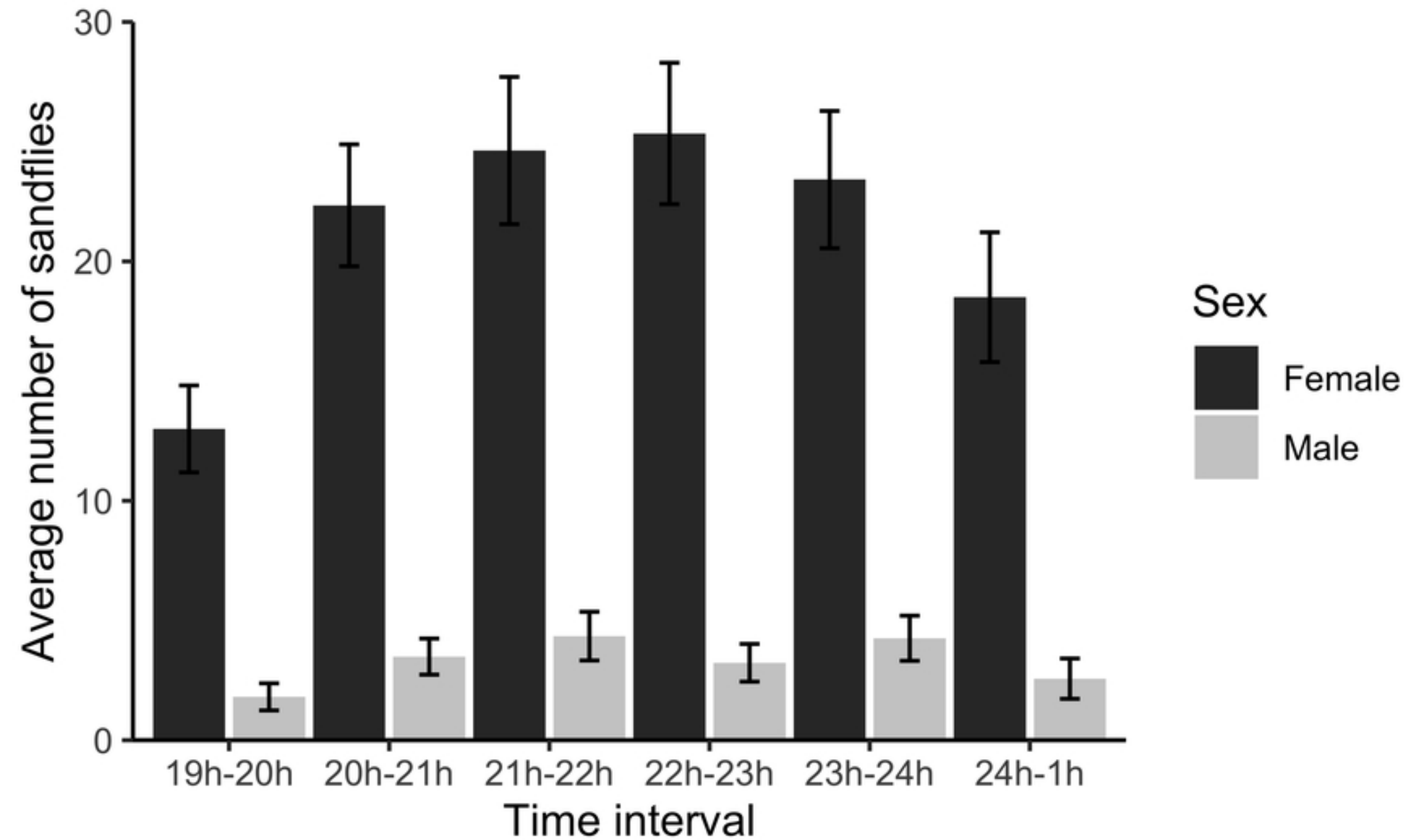


Fig 5

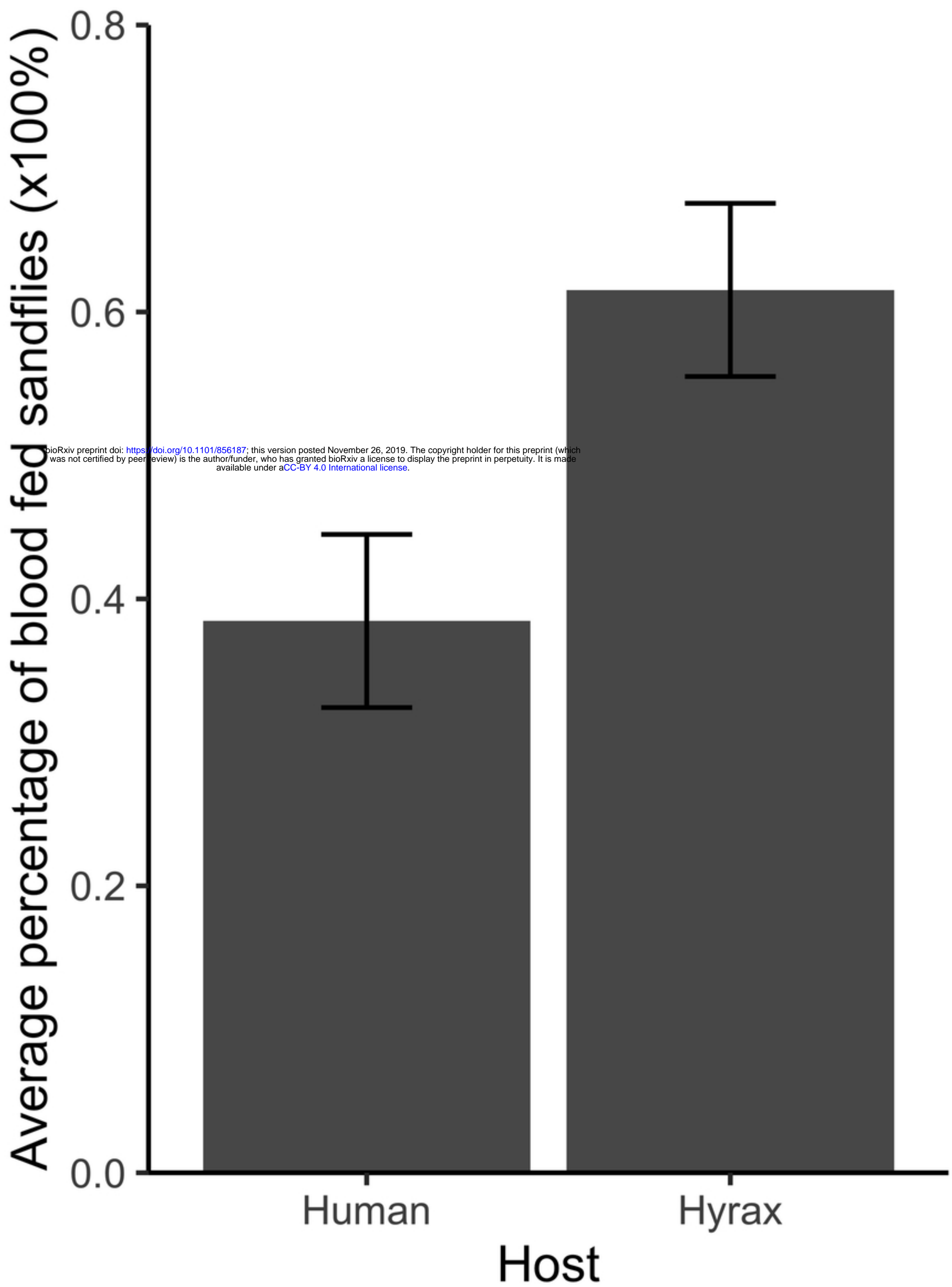


Fig 6



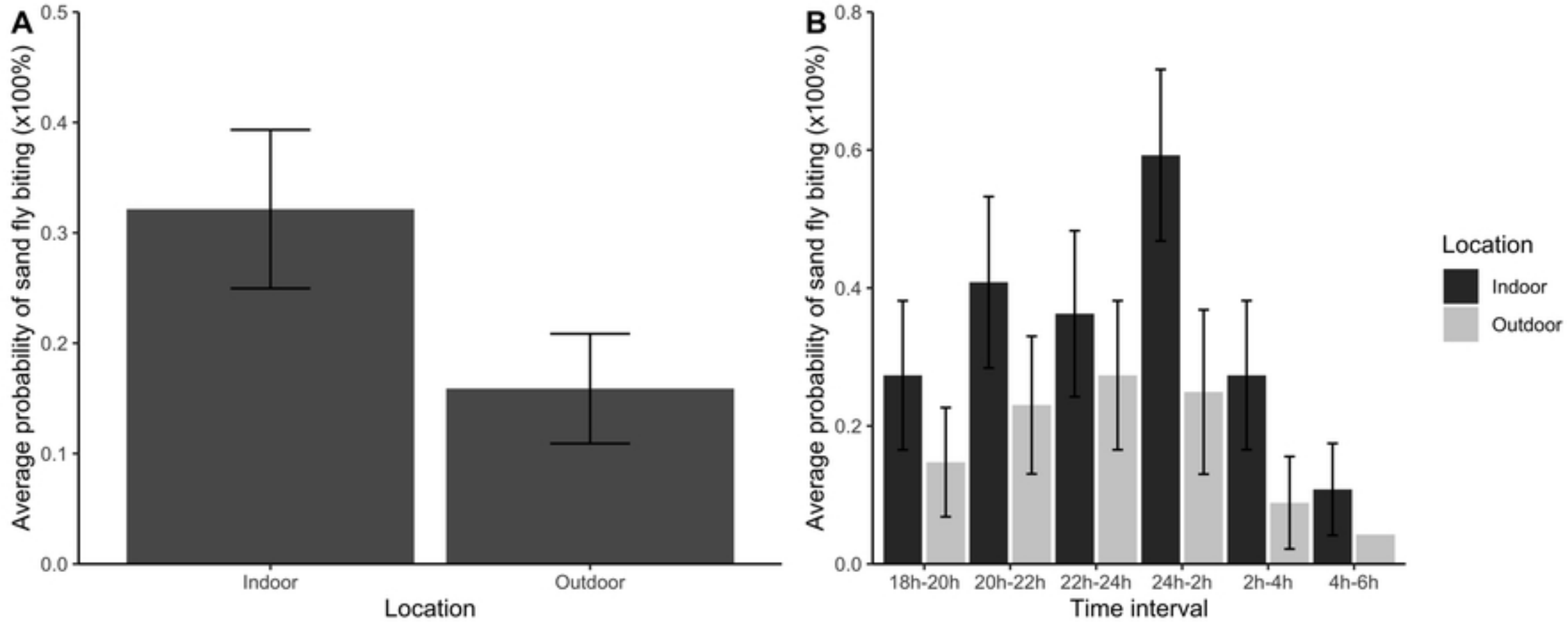


Fig 4