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

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Abstract

Background

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

Methods

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

Principal findings

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

Conclusion

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

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

Author summary

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

Introduction

Cutaneous leishmaniasis (CL) is a vector born disease, caused by *Leishmania* protozoa and transmitted by female phlebotomine sand flies. It is characterized by nodules or ulcerative skin lesions on people's faces and extremities, which result in disfiguring scars after healing [1,2].

CL is a major public health concern in Ethiopia, affecting approximately 20,000 to 50,000 people annually [3], in which *Leishmania aethiopica* is responsible for the majority of the infections [4–6]. Ochollo, our study site, is a village in the mid-highlands of southwestern Ethiopia, where CL is endemic and is mainly affecting young children [7,8]. A recent study identified 4% of the primary school children with active lesions, 1.5% with lesions and scars and 59.8% with scars [8]. Adults are

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

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

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

Successful disease control requires profound understanding of the transmission cycle. Knowledge about the blood meal preference of sand flies is crucial to demonstrate which vertebrates might 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.

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

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

Host identification

Sand fly collection Sand flies were collected indoors and outdoors from 72 households (nine households in each of the eight sub-villages) between February and May 2018. Additionally, ten caves were selected in the village for monthly sand fly collections from March to June 2018. Trapping was performed once per month at each sampling site with a particular entomological approach. Indoors and in caves, one CDC miniature light trap (John W. Hock Company, Florida, USA) and five sticky traps (ST, A4 format white papers attached to card board, covered with plastic impregnated with sesame oil on both sides) were placed at the bed end and wall cracks indoors, and inside caves. Collection with the two methods was performed on separate days. Only ST were utilized outdoors (N = 5 per collection site), which were placed on wall cracks of the houses and surrounding potential sand fly breeding or resting sites. Traps were set at 18h and collected again the next morning at sunrise. Blood fed female sand flies were sorted out, and the thorax and abdomen were dissected and stored in 97% ethanol at -20°C until further analysis. No distinction was made among different stages of blood digestion in sand flies.

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

145 $MgCl_2$ (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.

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

Sand fly activity pattern

Human landing catches To assess the indoor and outdoor human biting rhythms of *P. pedifer*, human landing catches (HLC) were conducted in and around four different household compounds between March and August 2018. HLC were done once per month at each sampling site, indoors and outdoors on the same day. Four collectors were used for each collection night, each working for six hours. In the first part of the night (between 18h and 24h), one collector performed the HLC indoors and one outdoors. In the second part of the night (24h until 6h), two other collectors carried out the 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_2

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

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

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

Data analysis

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

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

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

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

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

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

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

Results

Sand fly blood meal sources

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

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

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

Host category	Scientific name	Common name	Number	Percentage
Human	*Homo sapiens*	Human	107	59.4%

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

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

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

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

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

Leishmania in livestock

A total of 412 ear and nose samples, of which 209 from bovines and 203 from goats, were collected. 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

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

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

Host preference

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

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

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

Discussion

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

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

Overall, the majority of sand flies fed on humans and 3.6% of these sand flies were *Leishmania* DNA 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 asymptomatically 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

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

To unravel the complex CL cycle in southwestern Ethiopia, it is important to assess all players of transmission. This study demonstrates that livestock accounts for 21.1% of the blood meal sources of *P. pedifer*, but in ear and nose biopsies from goats and bovines, we found only a single goat nose biopsy positive for kDNA and SL-RNA with a high Ct value. This points to a relatively low, but viable parasitemia, although persistence and transmission of the parasites are not guaranteed [25]. Overall, it should be considered that some animals in the current study might have had parasites in their skin, which remained undetected due to the collection of only a small tissue biopsy [44].

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

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

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

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

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

This study shows that sand flies in Ochollo mainly feed on humans and that there is likely also anthroponotic transmission of *L. aethiopica*. Hyraxes are the preferred blood meal source when hosts are equally accessible, so the efficiency of parasite transmission from *H. brucei* to *P. pedifer* should be investigated before including them in control programs. Livestock appears an important 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.

2014;3(6):111.

9. Nagill R, Kaur S. Vaccine candidates for leishmaniasis: A review. Int Immunopharmacol. 2011;11(10):1464–88.

10. Gemetchu T, Laskay T, Frommel D. Phlebotomine sandflies (Diptera: Psychodidae, Phlebotominae) of Ochollo, southwestern Ethiopia: species composition and natural infection of *Phlebotomus pedifer* with *Leishmania aethiopica*. Ethiop J Sci. 1990;13(1):43–50.

11. Gebre-Michael T, Lane RP. A new sandfly species, *Phlebotomus (Larroussius) ashfordi* (Diptera, Psychodidae) from Ethiopia, previously confused with *P. (L.) aculeatus*. Ann Trop Med Parasitol. 1996;90(5):523–31.

12. Ashford W, Bray M, Hutchinson P, Bray S. The epidemiology of cutaneous leishmaniasis in Ethiopia. Trans R Soc Trop Med Hyg. 1973;67(4).

13. Pareyn M, Van Den Bosch E, Girma N, Houtte N Van, Van Dongen S, Van Der Auwera G, et al. Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochollo, a hotspot in southern Ethiopia. PLoS Negl Trop Dis. 2019;13(8).

14. Mutinga MJ, Odhiambo TR. Cutaneous leishmaniasis in Kenya 2. Studies on vector potential of *Phlebotomus pedifer* (Diptera, Phlebotomidae) in Kenya. Insect Sci its Appl. 1986;7(2):171–4.

15. Gaudêncio K, Costa PL, Carla R, Morais S De, Otranto D, Brandão-filho SP, et al. Identification of phlebotomine sand fly blood meals by real-time PCR. Parasit Vectors. 2015;8(230).

16. Guy MW, Killick-Kendrick R, Gill GS, Rioux J, Bray RS. Ecology of leishmaniasis in the South of France. Ann Parasitol Hum Comp. 1984;59(5):449–58.

17. Bongiorno G, Habluetzel A, Khoury C, Maroli M. Host preferences of phlebotomine sand flies at a hypoendemic focus of canine leishmaniasis in central Italy. Acta Trop. 2003;88:109–16.

18. Mutinga MJ. *Phlebotomus longipes*, a vector of cutaneous leishmaniasis in Kenya. Trans R Soc Trop Med Hyg. 1970;56(1):106.

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, Mabille 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 *Larroussius*. 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 Imbibed 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, Animut A, Negash Y, Gebreyes W, et al. Community knowledge,

attitude and practice towards cutaneous leishmaniasis endemic area Ochello, Gamo Gofa Zone, South Ethiopia. Asian Pac J Trop Biomed. 2017;7(12):1129–50.

62. Aklilu E, Gebresilassie A, Yared S, Kindu M, Tekie H, Balkew M, et al. Comparative study on the nocturnal activity of phlebotomine sand flies in a highland and lowland foci of visceral leishmaniasis in north-western Ethiopia with special reference to *Phlebotomus orientalis*. Parasit Vectors. 2017;10(393).

63. Lemma W, Tekie H, Abassi I, Balkew M, Gebre-michael T, Warburg A, et al. Nocturnal activities and host preferences of *Phlebotomus orientalis* in extra-domestic habitats of Kafta-Humera lowlands, Kala-azar endemic, Northwest Ethiopia. Parasit Vectors. 2014;7(594).

64. Elnaiem DA, Hassan HK, Ward RD. Phlebotomine sandflies in a focus of visceral leishmaniasis in a border area of eastern Sudan. Ann Trop Med Parasitol. 2016;91(3):307–18.

65. Gebremeskel SY, Balkew M, Warburg A, Hailu A. Nocturnal activity of *Phlebotomus* species (Diptera: Psychodidae) in a visceral leishmaniasis endemic area of northwest Ethiopia. J Biol Agric Healthc. 2015;5(5):185–94.

66. Quate LW. *Phlebotomus* sand flies of the Paloich area in the Sudan (Diptera: Psychodidae). J Med Entomol. 1964;1:213–68.

67. Ashford RW, Hutchinson P, Bray RS. Kala-azar in Ethiopia: Epidemiological studies in a highland valley. Ethiop Med J. 1973;11(4):259–64.

Supporting information Captions

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

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

S3 Table: Comparisons of hourly differences in activity of male and female *Phlebotomus pedifer* sand flies by 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.**

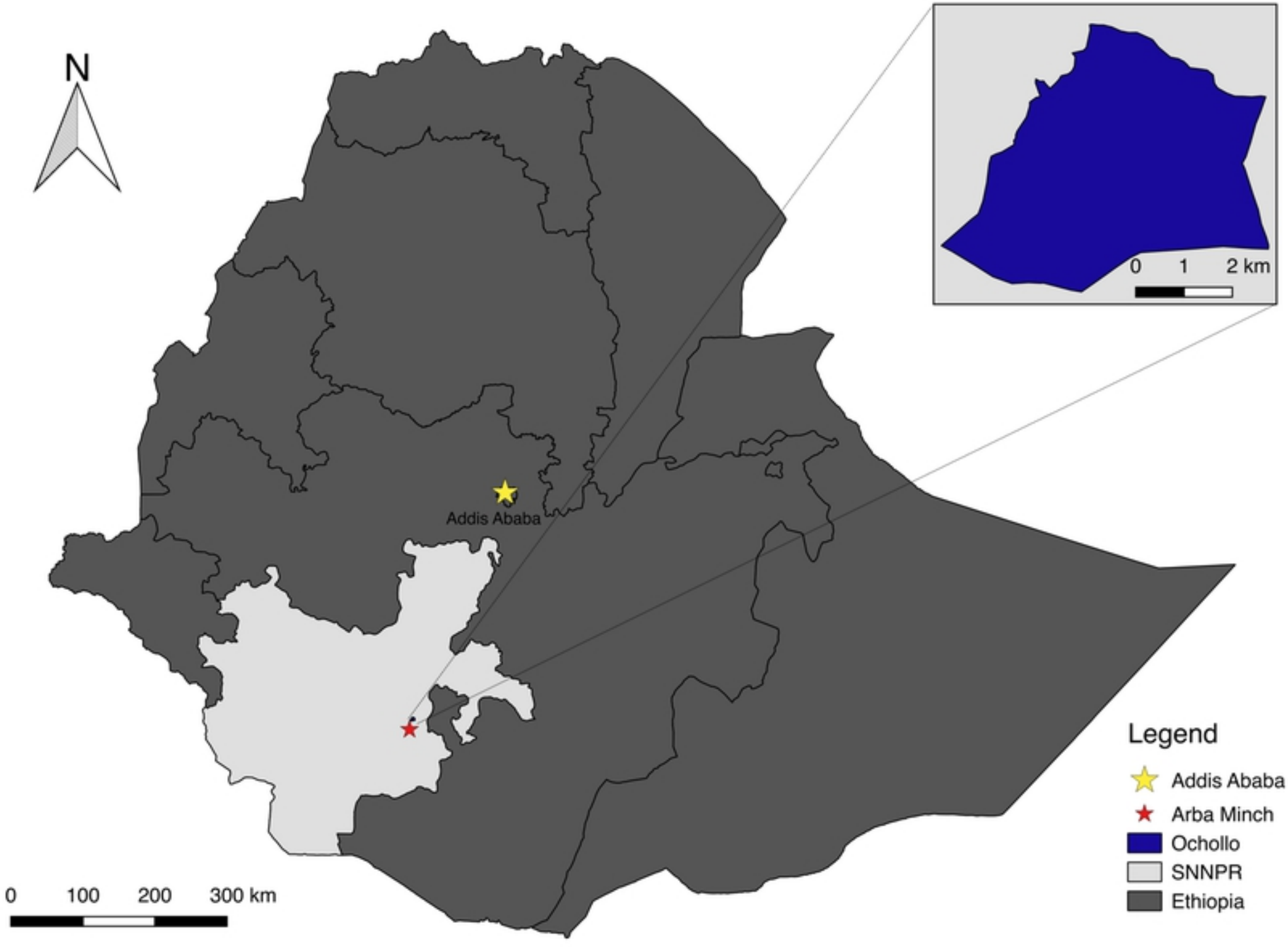
N
Addis Ababa
0 1 2 km
0 100 200 300 km
Legend
Addis Ababa
Arba Minch
Ochollo
SNNPR
Ethiopia

Fig 1

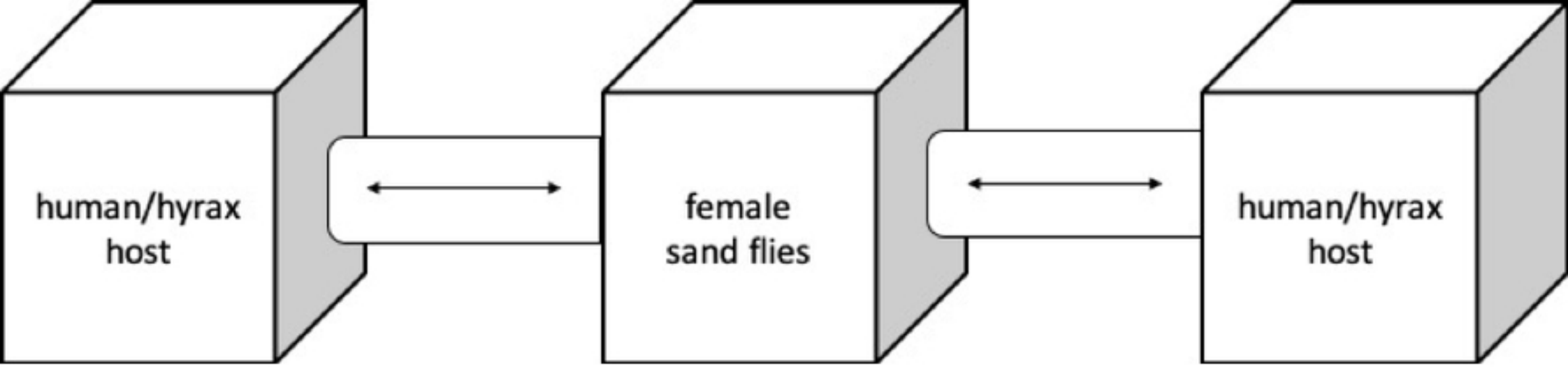

Fig 2

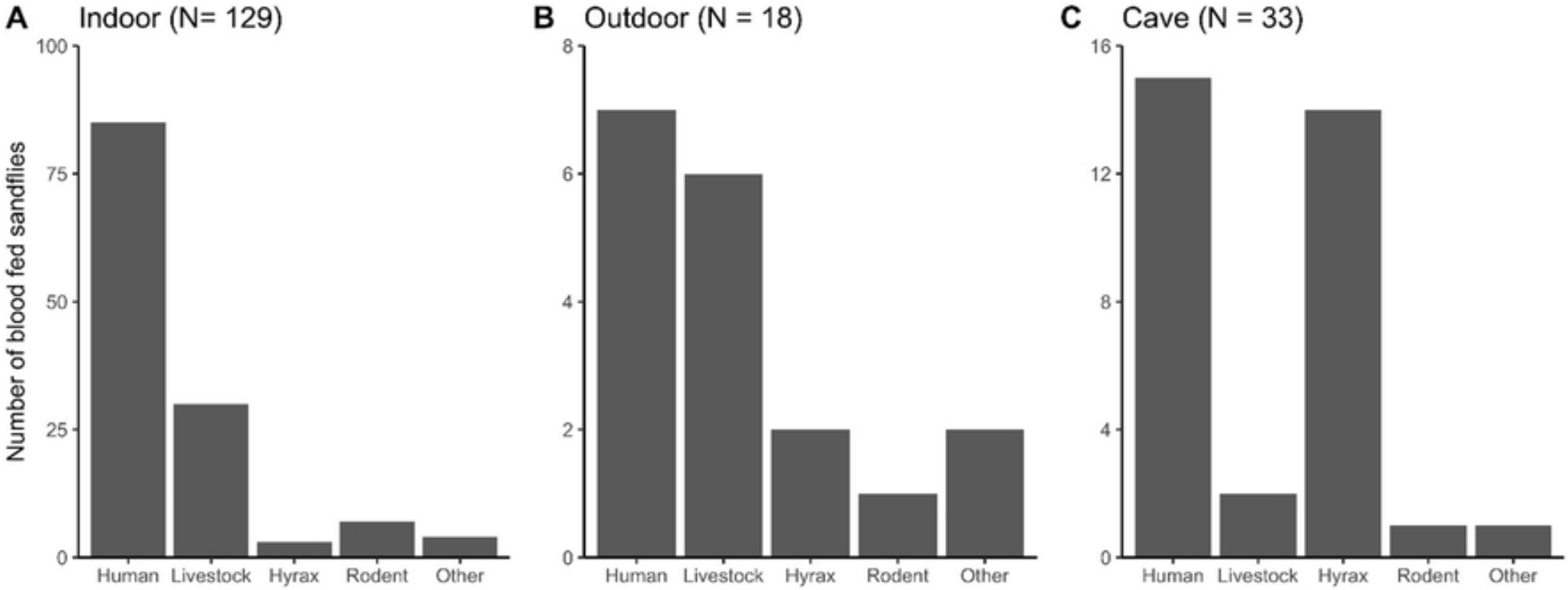
A
Indoor (N= 129)
B
Outdoor (N = 18)
C
Cave (N = 33)
Number of blood fed sandflies
100
75
50
25
0
8
6
4
2
0
16
12
8
4
0
Human
Livestock
Hyrax
Rodent
Other

Fig 3

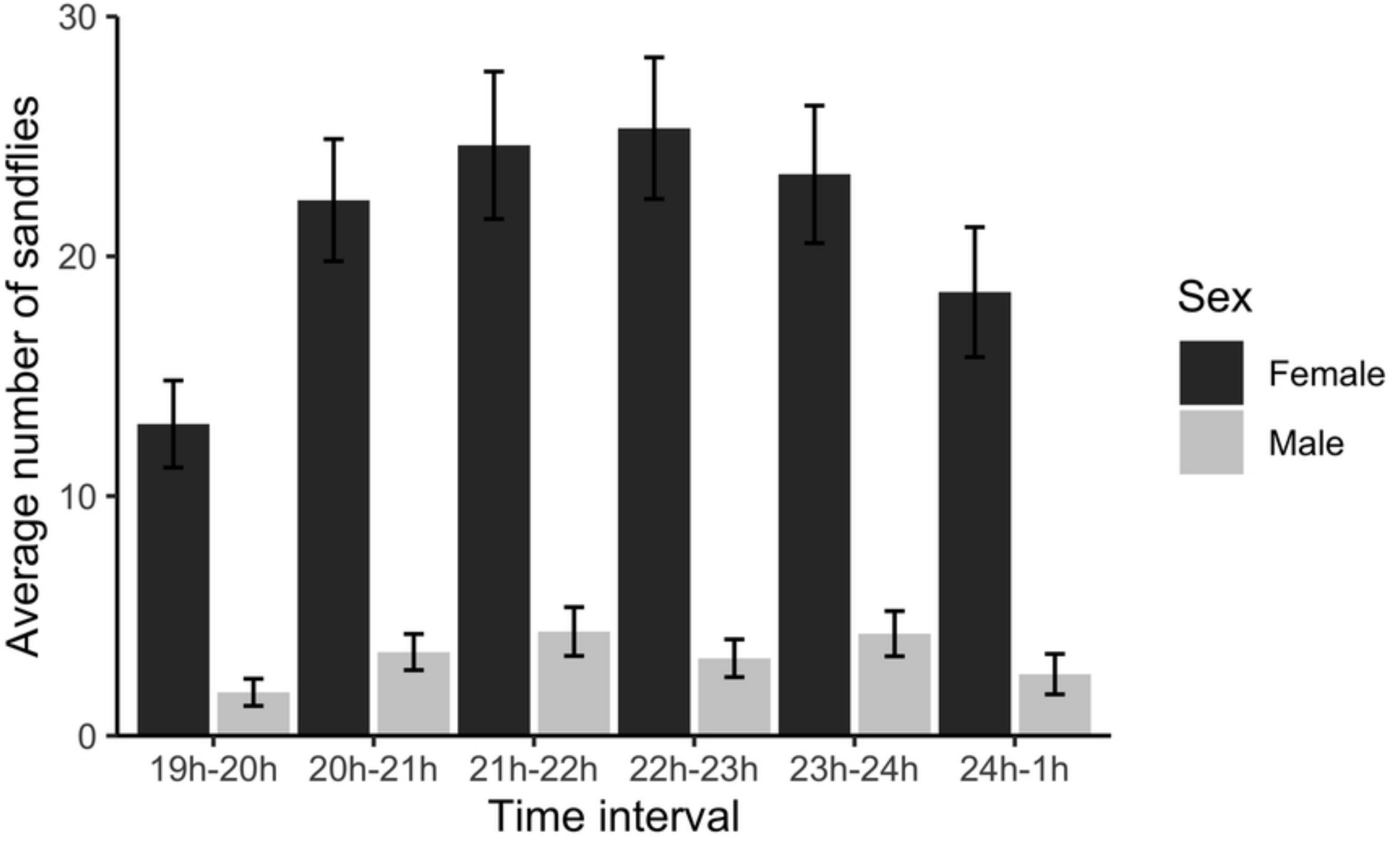
30
20
10
0
Average number of sandflies
19h-20h
20h-21h
21h-22h
22h-23h
23h-24h
24h-1h
Time interval
Sex
Female
Male

Fig 5

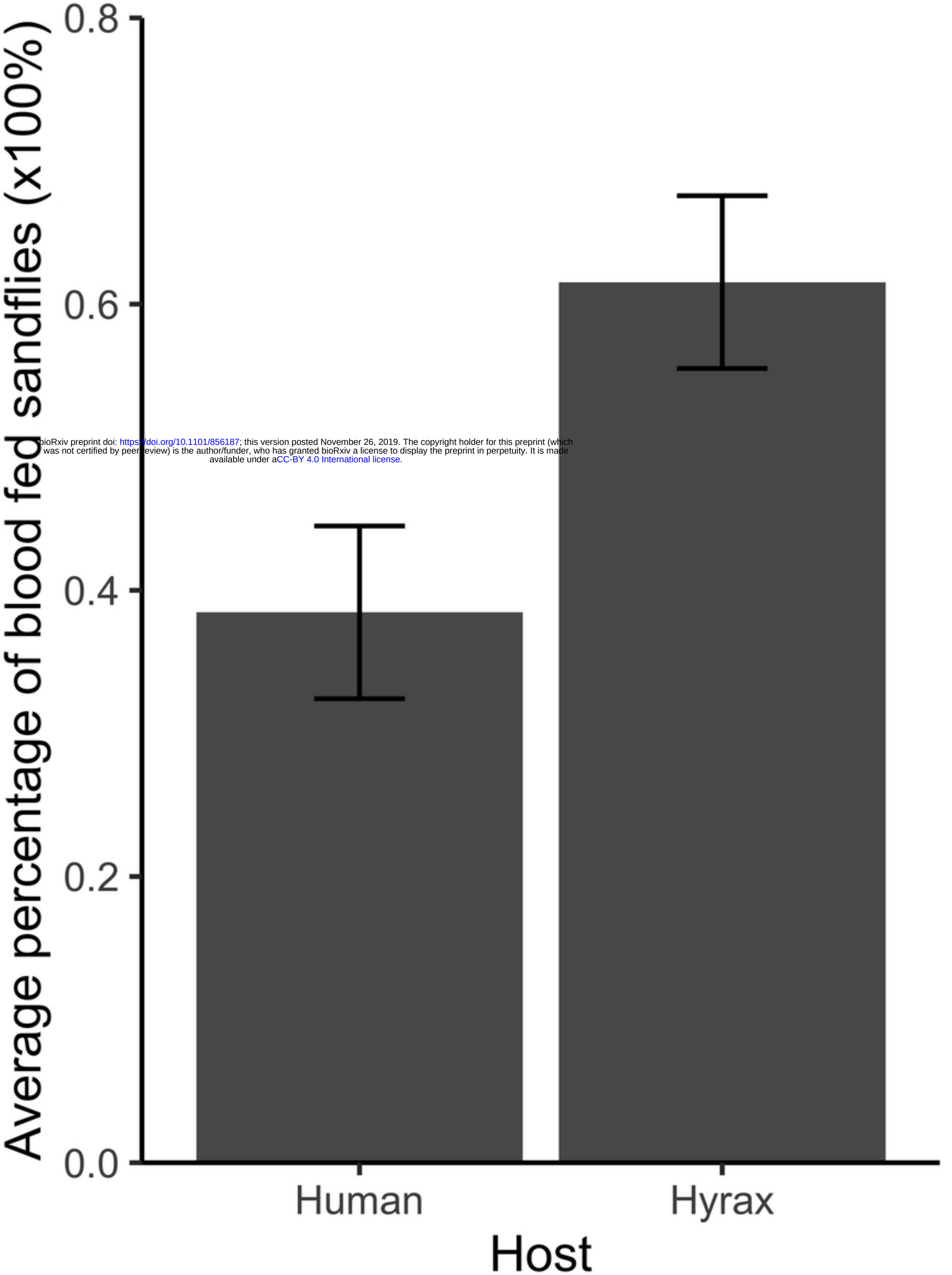

Fig 6

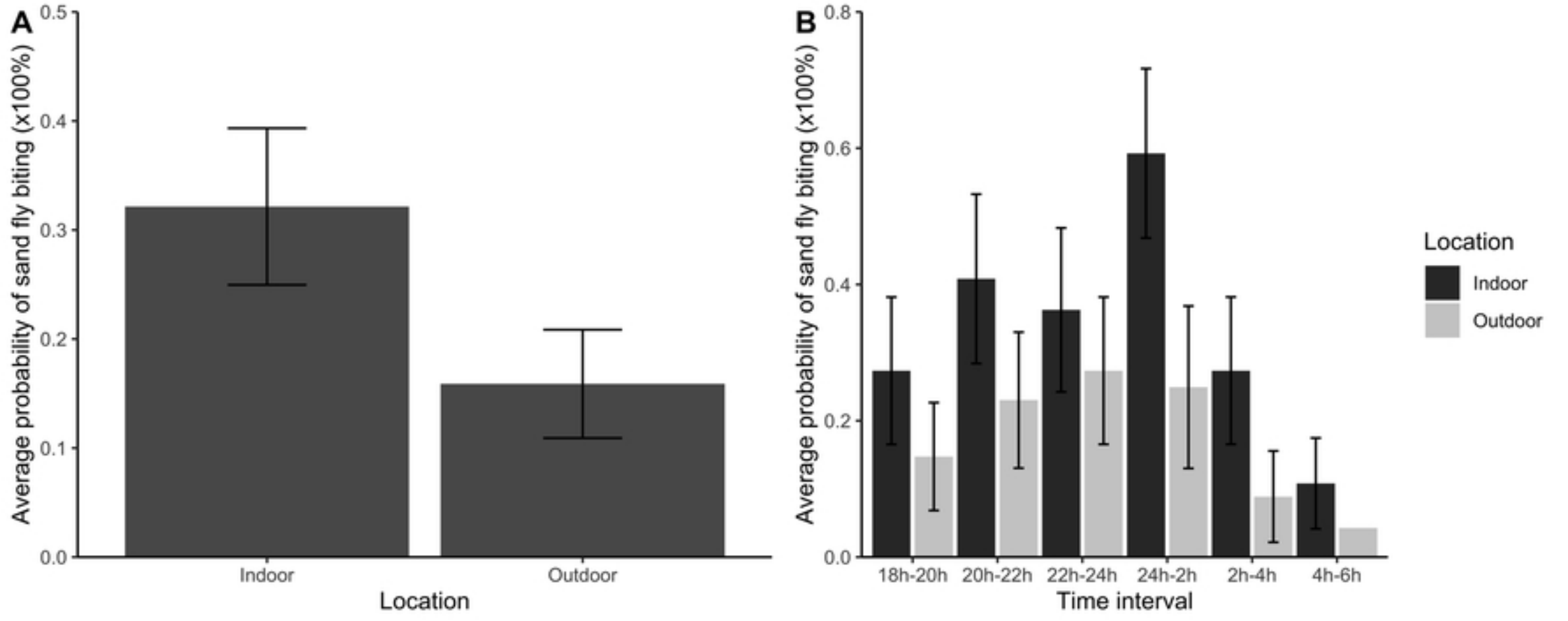
A
Average probability of sand fly biting (x100%)
0.5
0.4
0.3
0.2
0.1
0.0
Indoor
Outdoor
Location
B
Average probability of sand fly biting (x100%)
0.8
0.6
0.4
0.2
0.0
18h-20h
20h-22h
22h-24h
24h-2h
2h-4h
4h-6h
Time interval
Location
Indoor
Outdoor

Fig 4