

Hourly Activity and Natural Infection of Sandflies (Diptera: Psychodidae) Captured from the Aphotic Zone of a Cave, Minas Gerais State, Brazil

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Abstract

Sandflies are holometabolous insects that are of great epidemiological importance in the neotropical region as vectors of leishmaniasis. Caves are ecotopes that significantly differ from external environments and, among the insects that live or visit their internal area and adjacent environment, sandflies are commonly found. Based on this context, the objective of this work was to examine the period of activity of sandflies in the cave environment in the aphotic zone. Thus, four sandfly captures were conducted, one in each season of the year, in a cave where studies on the bioecological aspects of sandfly fauna have been conducted since 2008. In this same study, we have also noticed the presence of flagellates in some captured females. Catches were carried out for 24 hours using a Shannon trap, light bait, and cave walls were actively searched. We collected a total of 638 sandflies, representing 11 species. The most abundant species and with more intense period of activity were, in descending order: *Lu. cavernicola* (62%), *Ev. spelunca* (16%) and *Ev. sallesi* (14%). A total of 69 females were dissected to check for natural infection, and in five specimens we found living flagellated forms: two *Ev. spelunca*, two *Ev. sallesi* and one *Sc. sordellii*. This study shows that the activity of some species caught in the aphotic zone of the cave, especially *Lu. cavernicola*, differs from what has already been reported in previous sandfly captures, which are almost always conducted at night and during twilight. The existence of sandflies that were naturally infected with flagellates and the lack of awareness regarding the behaviour of sandflies in cave environments are strong indicators of the need for further study on this group of insects in this ecotope, as a safety measure to protect the visitors of such environment.

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Introduction

Leishmaniasis is a complex infectious disease caused by protozoan parasites of the morphologically homologous genus *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatidae), all with varying degrees of specificity to invertebrate host [1]. The main form of parasite transmission to humans and other mammalian hosts is through the bite of female flies of the family Psychodidae, subfamily Phlebotominae, popularly known as sandflies. The occurrence of this parasite in a given area depends primarily on the putative presence of the vector and a host/reservoir [2].

Caves are natural cavities, often found in limestone regions, in which the composition favors the dissolution of rocks by acidic water. The cave environment is characterized by small daily temperature changes, which are always similar to the mean temperature of the external environment; the relative humidity tends to range from 95% to 100% [3].

Thus, the caves are considered stable and peculiar environments that are important for the balance of ecosystems, since

inside, the microclimate shows little variation of these factors. Several Brazilian caves stand out on the international scene for their size and rarity. Caves are relatively more isolated than others ecosystems, with specific physical and constant environment, thus, their fauna and flora present peculiar aspects. With more than 600 species that have already been classified, the cave fauna of Brazil is the richest in South America [4].

Among the insects that live in or visit the caves and their surrounding environments, the sandflies deserve special attention, not only because several species are vectors of diseases like arboviruses, bartonellosis and leishmaniasis [5,6], but also because they cause discomfort to visitors, because of their painful bite, often causing allergic reactions.

There is today an important scientific question, and an urgent need to better study the cave environment. Many caves are open to visitors without having gone through any type of scientific examination or study.

The number of sandflies found is increasing rapidly, with descriptions of new species every year, including species associated with caves [7,8,9]. However, with regard to biology, there is still a large gap in the understanding of these insects. Sandflies are considered insects with crepuscular and nocturnal habits. Male and female sandflies during the day remain in protected locations with rarely changing environments. However, there are only a few reports on the biological behaviour of sandflies found in caves.

Accordingly, the goal of our work was to analyze the period of activity of sandflies in the cave environment, specifically in the aphotic zone, where there is no light throughout the day, as well as to observe the natural infection of captured females.

Materials and Methods

Ethics Statement

Catches were made in a cave situated on a private farm. A term of consent was established to run the captures in this cave.

Study Area

This study was conducted in the municipality of Lassance, located in northern Minas Gerais state, in the microregion of Pirapora. The city covers an area of 3,204,213 km² and has an estimated population of 6,484 inhabitants [10]. The region has nine caves recorded in the National Register of Caves of Brazil (CNC) [11]. The cave chosen for this study has not yet been cataloged and is situated at about 20 km from the city, located on a private farm and belonging to Speleological Province of Bambuí, which is situated partly in the Southeastern region of Tocantins, central east and Southeast of Goiás, central West and North West of Minas Gerais and west of Bahia. Other studies that address the biocological aspects of sandflies caught in this cave have been carried out since 2008 (unpublished data). This is a limestone cave with a horizontal extension of approximately 100 meters that is located at an altitude of approximately 700 meters, with the following coordinates: $-17^{\circ}59'40.01''\text{S}$, $-44^{\circ}39'3.23''\text{W}$.

Sampling of sandflies

Catches were made with a manual aspirator, at the end of cave (aphotic zone, 60 meters from the entrance), using a Shannon trap [12] (Figure 1), a light bait, and cave walls were actively searched. The samples were standardized as follows: two people conducting the collections, which lasted ten minutes in every hour, over 24 hours. Captures were performed in August 2010 (winter), November 2010 (spring), February 2011 (summer) and May 2011 (autumn). The sandflies were separated into plastic pots, according



Figure 1. Shannon trap inside the cave, Lassance/MG.
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to their time of capture. The classification used for the identification of sandflies was that proposed by Galati [13].

Search of natural infection

The females were separated and kept alive in the pots, for us to verify natural infection using the digestive tract dissection technique. Only the living specimens were used for this study.

When flagellated forms were found, a suspension containing PBS 1 with antibiotics (streptomycin and penicillin) and intestinal contents of insects were inoculated in hamsters and in culture medium NNN-Schneider, in an attempt to isolate the parasite.

Data statistical analysis

The Graph Pad InStat software was used for statistical analyses. Chi-square test $df = 5$ was used to compare species distribution on day shifts (daylight and darkness) in the overall analysis. Pairwise analyses of species distribution was performed using Fisher's exact test, two sided. Comparison of species distributions across all seasons were performed using the Chi-square test $df = 12$. Pairwise analysis of species distributions in the seasons was performed using the Chi-square $df = 4$.

Results

Hourly activity

Considering all catches together, winter (August 2010), spring (November 2010), summer (February 2011) and autumn (May 2011), a total of 638 sandflies were collected, 358 (56%) males and 280 (44%) females, representing 11 species, where the most abundant species were, in descending order: *Lutzomyia cavernicola* (Costa Lima, 1932) (62%), *Exandromyia spelunca* Carvalho, Sanguinette, Brazil & Andrade Filho, 2011 (16%) and *Exandromyia sallesi* (Galvão & Coutinho, 1939) (14%) (Figure 2).

Figure 3 shows the distribution of species over 24 hours, in the four seasons when captures took place. The following parameters were taken into account: a photoperiod with a light/dark cycle (LD) 12:12, observed in the external environment, and peak activity of sandflies in all seasons during scotophase (18:00–5:00). However, it has been noted that sandfly activity across all seasons also occurred during periods of photophase (6:00–17:00).

Data analysis in the four seasons (Figure 4) shows, however, that when considering the species caught, a statistically significant difference ($p\text{-value} < 0.0001$) occurs.

The distribution of total sandflies captured (across all four trials) over the LD cycle was represented by 50% for each phase of the cycle. However, this distribution does not take into account the comparison per species in the two periods (scotophase/photophase) and in this case this same distribution shows a statistically significant difference (chi-square test: $p\text{-value} < 0.0001$) (Figure 5). Thus, the purpose of this analysis was to verify whether the faunal composition varied between the two periods (photophase/scotophase). Figure 5 draws attention to the most abundant species (*Lu. cavernicola*, *Ev. spelunca* and *Ev. sallesi*) during photophase, and greater amounts of the first and last species were captured during this phase.

Compared to the most abundant species, with emphasis over its distribution across the same 24 hours of collection, one can note that they were all captured during the entire LD cycle, which is, recorded in virtually all hours of capture. *Lu. cavernicola* was undoubtedly, the species that showed the most regular distribution throughout the 24 hours (Figure 6).

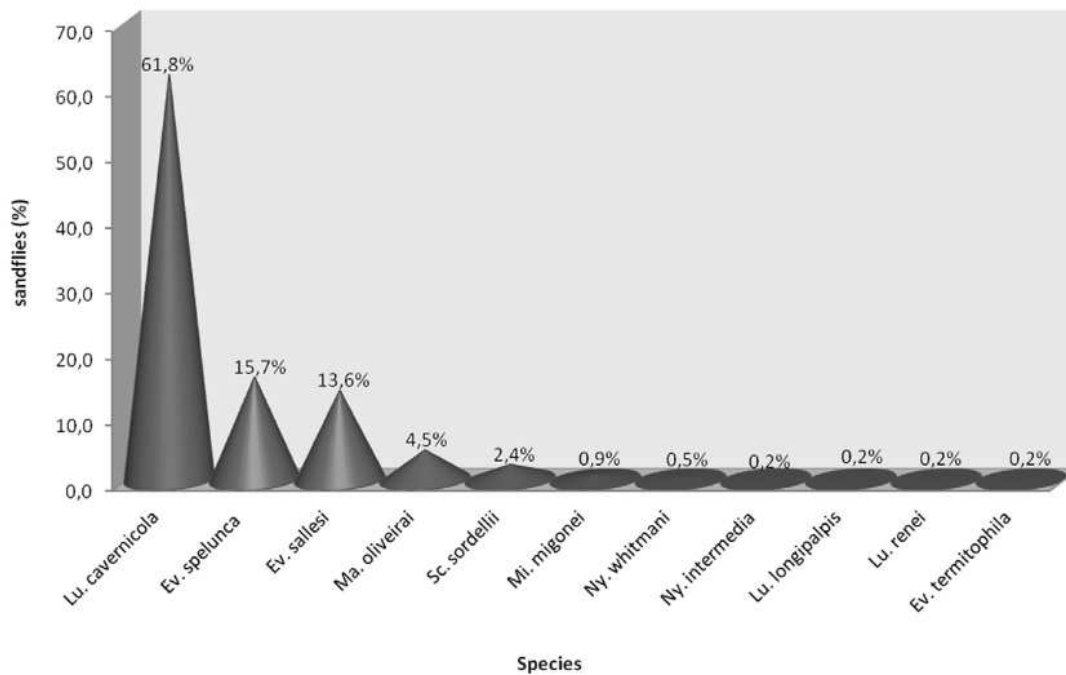


Figure 2. Total of sandflies captured with Shannon trap in winter, spring, summer and autumn, in the aphotic zone of the cave - Lassance/MG - Brazil.

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Natural infection research

Surprisingly in a single capture, conducted in August 2010, five specimens were found through the technique of dissection to be naturally infected with flagellated forms: two *Ev. spelunca*, two *Ev. sallesi* and one *Sciopemyia sordellii* (Shannon & Del Pont, 1927). A suspension containing PBS 1 with antibiotics (streptomycin and penicillin) and intestinal contents of insects was inoculated in hamsters and in culture medium NNN-Schneider. No growth of protozoa in culture was found, and all of them were contaminated with bacteria and/or fungi that could have prevented cultures from becoming established. No clinical signs were observed in hamsters with inoculated flagellate suspensions. We examined the presence of *Leishmania* DNA in sacrificed hamsters using ITS1-PCR [14], however, when using this technique we were unable to identify the parasites found. The slides with infected specimens were photographed and filmed and after the isolation procedures they were stained.

Discussion

Caves are considered stable environments when compared to epigeal habitats and are also characterized by a permanent lack of light far from entrances [3,15]. Fauna adapted to these conditions can be classified according to their level of adaptation: troglobites (animals that present unique modifications to cave environments), troglóphiles (adapted animals, but devoid of modifications that can also develop in the external environment) and troglóxenos (animals that use caves for shelter or refuge) [16].

Although caves are apparently inhospitable environments for sandflies, some studies have shown that caves may harbor sandflies [7,17,18,19]. This shows the importance of these breeding sites for such insects. Most sandflies have troglóxenos habits, almost always using caves as a refuge or shelter. However, little is known about the behaviour of sandflies in cave environments. Recently, a new

species of sandfly, *Lutzomyia maruaga* (Alves, Freitas & Barret 2008) was found in a cave in the Amazon region, which shows that it is at least facultatively parthenogenetic and autogenic, as previously observed for *Lutzomyia mamedei* by Brazil and Oliveira [20], and therefore regarded as a kind of troglóbite. Other studies have also described new species of sandflies reported from cave environments [8,9]. *Evandromyia spelunca* is a new phlebotomine species of the *cortelezzii* complex described from this cave [9]. This species might show evidence of adaptation to the cave environment since it was captured in this study, in all months, was the third most abundant species and demonstrated a consistent distribution over the LD cycle.

This work has shown that in fact the phlebotomine fauna of the cave environment may present some peculiarities in regards to their behaviour. Of the total 11 species captured in this study, all of them can also be found in the external environment (unpublished data), agreeing with other findings where the diversity and density of sandflies captured in caves can be equal to or greater than surrounding environments [21,22].

Regarding sandfly period of activity, there are few evaluative studies on this behaviour. Such studies focus on species of vectoral importance in different environments, but none in cave environment. One of these studies has addressed the behaviour of *Nyssomyia intermedia* (Lutz & Neiva, 1912) and *Nyssomyia neivai* (Pinto, 1926), which are species incriminated in the transmission of cutaneous leishmaniasis in the peridomestic area of Iporanga, state of São Paulo, where 70% of the specimens of both species were collected between 18:00 and 24:00 [23]. In another study the activity period of sandflies, also *Nyssomyia neivai*, in the endemic zone of cutaneous leishmaniasis, in Tucuman, Argentina, also showed that the hourly activity of this species was mainly nocturnal [24].

Most man-biters feed at dusk and during the evening but *Psychodopygus wellcomei* (Fraiha, Shaw and Lainson, 1971), *Psycho-*

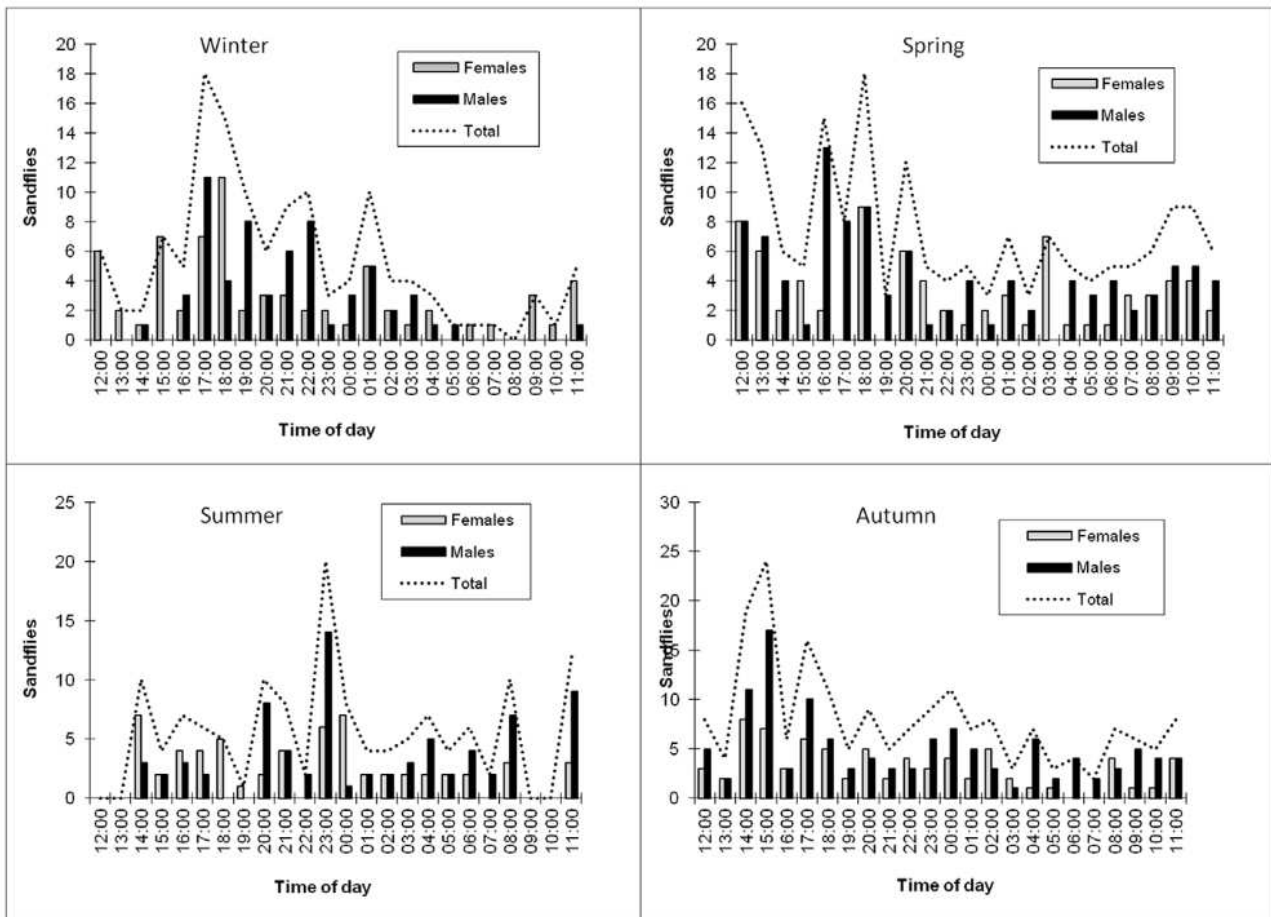
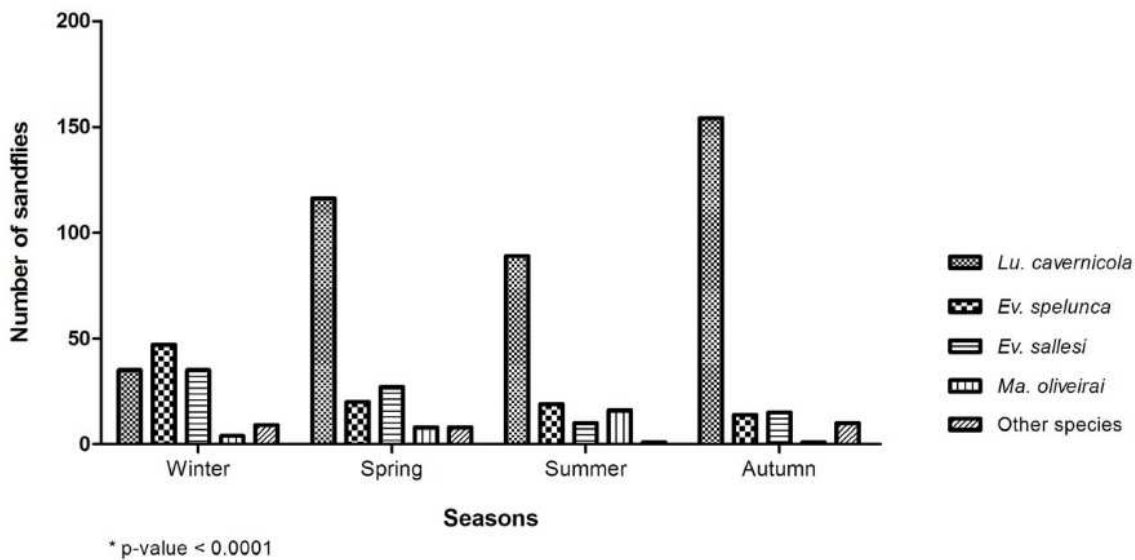


Figure 3. Dispersion of sandflies over the 24 hours, in the four seasons of capture, inside the cave, Lassance/MG.
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* p-value < 0.0001

Figure 4. Sandfly species captured with Shannon trap in the four seasons: winter, spring, summer, autumn, inside the cave, Lassance/MG.
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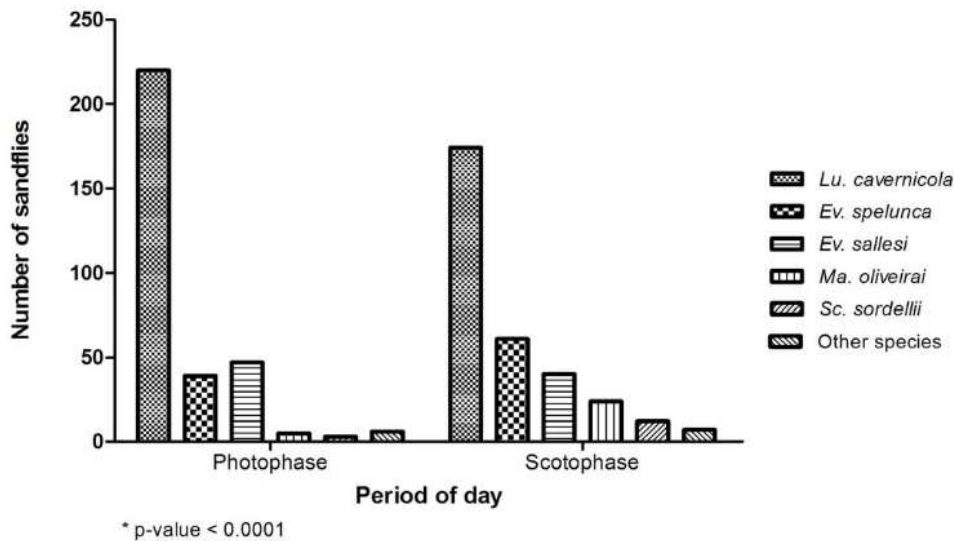


Figure 5. Comparison of the proportions of specimens per species of sandflies captured in winter, spring, summer and autumn, in the aphotic zone of the cave, over the LD cycle (photophase/scotophase) - Lassance/MG - Brazil.
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dopygus carrerai (Barreto, 1946), and others will attempt to feed in the daytime as well. Windless or nearly windless conditions, along with other optimal conditions, may suddenly induce greater-than-expected numbers of sandflies to seek their hosts. Bloodfeeding females may also release aggregation pheromones that attract other females to the host [25].

Unlike almost always observed in studies of sandfly activity outside the cave environment, where a general habit of these insects is crepuscular/nocturnal, our study revealed that the cave environment presents a fauna with different behaviour. The results found by the sum of four catches for 24 hours, show 50% of the sandflies captured inside the cave, during the photophase (6:00–

17:00), a finding that deserves attention because it differs from most reports of the behaviour of these insects.

Through graph 3, it was important to observe that in the winter and spring the peaks of occurrence of sandflies occur after dusk (during the twilight), contrary to what was observed in the summer, when the peaks were observed more frequently at night (20:00 to 0:00) and in the fall when the peaks were started in the early afternoon and ended in the early evening. However, we do not have enough data to infer hypotheses about these differences found. But, on all four catches, sandflies were found in the period of photophase.

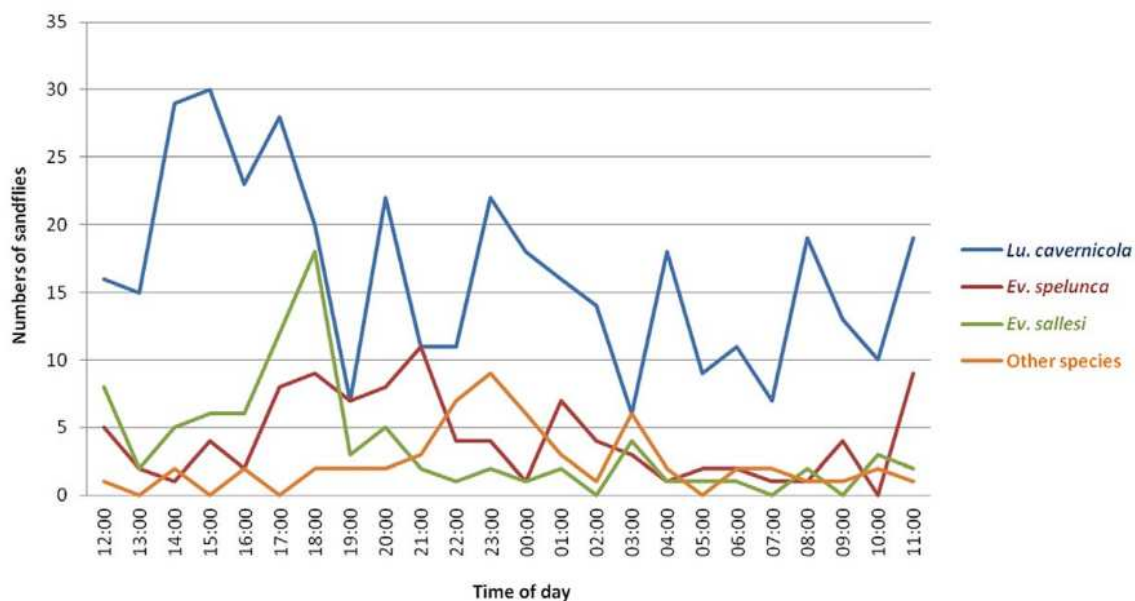


Figure 6. Hourly activity of the most abundant species captured with Shannon trap in winter, spring, summer and autumn, in the aphotic zone of the cave - Lassance/MG - Brazil.
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These data led us to examine whether all species caught inside the cave had the same distribution over 24 hours. From this analysis we found that the most abundant species, mainly *Lu. cavernicola*, were responsible for the large number of individuals captured during the photophase. This brings us to the hypothesis that these species may have a troglone behaviour, but in a way that is suited to this type of environment, maybe presenting a circadian rhythm different from that observed for other species of sandflies.

This result draws attention to the vectoral capacity of these insects, coupled with the existence of vertebrates, also troglone, inside the caves, increasing the chances of a parasite transmission cycle occurring in this type of environment and the chances of visitors being infected if bitten by sandflies occasionally in daylight hours. These species probably have altered their biological behaviour in this environment, becoming more opportunistic and increasing their chances of transmission in this biotope.

The finding of flagellated forms in five sandflies, namely: two *Ev. spelunca*, two *Ev. sallesi* and one *Sc. sordellii*, draws attention to a possible cycle inside the cave at the time of capture. In a previous study also conducted in Lassance [26], promastigotes were found in the pyloric region and in the abdominal midgut of *Ny. neivai* and *Ev. sallesi*. Insects found by microscopic examination were macerated in saline solution and inoculated into hamsters. Subsequent analysis by polymerase chain reaction-restriction fragment length polymorphism revealed both isolates to belong to the species *Leishmania infantum chagasi*.

All samples showed no trace of blood in the digestive contents, thus increasing the actual possibility that the parasite had developed in the digestive tract of these species. However, the techniques used for the isolation of parasites were not successful in

their identification and characterization. The stained forms will be submitted to other methodologies in an attempt to identify the flagellates.

The report of species incriminated in the transmission of leishmaniasis, such as *Migonomys migonei* (França, 1920), *Nyssomyia whitmani* (Antunes & Coutinho, 1939), *Ny. intermedia* and *Lutzomyia longipalpis* (Lutz & Neiva, 1912), should be taken into consideration. Although the findings of natural infection have not permitted the identification of the *Leishmania* sp. parasite, the presence of these species inside the cave can be crucial for the existence of a cycle in this environment. Though found in small percentages in these catches, these species showed that they may occasionally visit the cave environment and opportunistically could come to disseminate the parasites to hosts/reservoirs that are also present in this habit.

Further studies on the biological behaviour and natural infection of sandflies caught in caves are important to the development of new hypotheses or to the finding of new cycles of disease in this ecotope, which is an environment with significant ecological and biological aspects and that has relevant unique characteristics. Furthermore, our findings have shown the relevance of studies that will account for the assessment of this type of environment, since there is a high demand for it for many different types of activities.

Author Contributions

Conceived and designed the experiments: GMLC JDAF RPB. Performed the experiments: GMLC JDAF RPB PCLSM CCS HAB LS PFQ APLAZ MCNFR. Analyzed the data: GMLC LS JDAF. Contributed reagents/materials/analysis tools: PFQ APLAZ MCNFR PCLSM CCS HAB. Wrote the paper: GMLC JDAF RPB.

References

- Chance ML (1985) The biochemical and immunotaxonomy of *Leishmania*. In: Leishmaniasis 1st ed. Chang MP, Bray RS. Els. Sci. Publ. Comp., Inc. USA.
- Gontijo CME, Melo MN (2004) Leishmaniose Visceral no Brasil: quadro atual, desafios e perspectivas. Rev Bras Epidemiol 7: 338–349.
- Poulson TL, White WB (1969) The cave environment. Limestone caves provide unique natural laboratories for studying biological and geological processes. Science 165, 3897: 971–981.
- Lino CF (2007) Províncias Espeleológicas Brasileiras. Available: <http://www.mre.gov.br/cdbrasil/itamaraty/web/port/mcioamb/ecossist/caverna/pspeleo/index.htm> Accessed 2008 May 5.
- Forattini OP (1953) Nota sobre criadouros naturais de flebotomos em dependências peridomiliares, no Estado de São Paulo. Arq Fac Hig S Pub Univ São Paulo 7: 158–167.
- Cipa Group, Bermudes H, Dedet JP, Falcão AL, et al. (1991) Proposition of a standard description for Phlebotomine sand flies. Parasitologia 33: 127–135.
- Alves VR, Freitas RA, Barrett TV (2008) *Lutzomyia maruaga* (Diptera: Psychodidae), a new bat-cave sand fly from Amazonas, Brazil. Mem Inst Oswaldo Cruz 103: 251–253.
- Carvalho GML, Brazil RP, Sanguinette CC, Andrade-Filho JD (2010) Description of a new phlebotomine species, *Martinsomyia reginae* sp. nov. (Diptera: Psychodidae: Phlebotominae) from a cave in the state of Tocantins Brazil. Mem Inst Oswaldo Cruz 105: 336–340.
- Carvalho GML, Brazil RP, Sanguinette CC, Andrade-Filho JD (2011) Description of *Evandromyia spelunca*, a new phlebotomine species of the *coelestii* complex, from a cave in Minas Gerais State, Brazil (Diptera: Psychodidae: Phlebotominae). Parasit Vectors 4: 158.
- Instituto Brasileiro de Geografia e Estatística (IBGE). Censos Demográficos, Brasil. Available: <http://www.ibge.gov.br/cidadesat/default.php>. Accessed in 4th February 2010.
- SBE (Sociedade Brasileira de Espeleologia). Available: <http://www.sbe.com.br/>. Accessed in 5th May 2007.
- Shannon RC (1939) Methods for collecting and feeding mosquitoes in jungle yellow fever studies.
- Galati EAB (2003) Morfologia e taxonomia. Classificação de Phlebotominae. In EF Rangel, R. Lainson (org.), 1st ed., *Flebotomíneos do Brasil*, Fiocruz, Rio de Janeiro, p. 23–51.
- Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, et al. (2003) PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis 47: 349–358.
- Culver DC (1982) Cave Life. Evolution and Ecology. Harvard University Press, Cambridge, Massachusetts and London, England, 189pp.
- Holsinger JR, Culver DC (1988) The invertebrate cave fauna of Virginia USA and a part of eastern Tennessee zoogeography and ecology. Brimleyana 14: 1–162.
- Galati EAB, Andrade-Filho JD, Silva ACL, Falcão AL (2003a). Description of a new genus and new species of New World Phlebotominae (Diptera, Psychodidae). Rev Bras Entomol 47: 63–70.
- Galati EAB, Marassá AM, Andrade RMG (2003b) *Microphomyia (Sauromyia) felizi*, a new species of Phlebotominae (Diptera: Psychodidae) from Vale do Ribeira, São Paulo state, Brazil. Rev Bras Entomol 47: 455–459.
- Alves VR, Freitas RA, Santos FL, Barrett TV (2011) Diversity of sandflies (Psychodidae: Phlebotominae) captured in sandstone caves from Central Amazonia, Brazil. Mem Inst Oswaldo Cruz 106: 353–359.
- Brazil RP, Oliveira SM (1999) Parthenogenesis in the sandfly *Lutzomyia mamedei* (Diptera: Psychodidae). Med Vet Entomol 13: 463–464.
- Galati EAB, Nunes VLB, Boggiani PC, Dorval MEC, Cristaldo G, et al. (2003c) Phlebotomines (Diptera: Psychodidae) in caves of the Serra da Bodoquena, Mato Grosso do Sul State, Brazil. Rev Bras Entomol 47: 283–296.
- Galati EAB, Nunes VLB, Boggiani PC, Dorval MEC, Cristaldo G, Rocha HC, et al. (2006) Phlebotomines (Diptera: Psychodidae) in forested areas of the Serra da Bodoquena, state of Mato Grosso do Sul, Brazil. Mem Inst Oswaldo Cruz 101: 175–193.
- Galati EAB, Marassá AM, Andrade RMG, Bueno EFM, Paiva BR, et al. (2010) *Nyssomyia intermedia* (Lutz & Neiva) and *Nyssomyia neivai* (Pinto) (Diptera, Psychodidae, Phlebotominae) in a sympatric area: seasonal and nocturnal hourly rhythm in black and white modified Shannon traps. Rev Bras Entomol 54: 677–686.
- Fuenzalida AD, Quintana MG, Salomón OD, Grosso MSL (2011) Hourly activity of *Lutzomyia neivai* in the endemic zone of cutaneous leishmaniasis in Tucumán, Argentina: preliminary results. Mem Inst Oswaldo Cruz 106: 635–638.
- Young DG, Duncan MA (1994) Guide to the Identification and Geographic Distribution of *Lutzomyia* Sand Flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Mem Am Entomol Inst 54: 1–381.
- Saraiva L, Carvalho GML, Quaresma PF, Lima ACVMR, Falcão AL, et al. (2009) Natural infection of *Nyssomyia neivai* (Pinto, 1926) and *Evandromyia sallesi* (Galvão & Coutinho, 1939) (Diptera: Psychodidae) by *Leishmania infantum chagasi* Cunha and Chagas, 1937 in Minas Gerais, Brazil. J Med Entomol 46: 1159–1163.