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RESEARCH FOR *SARCOCYSTIS* SPP., *LEISHMANIA* SPP. AND *TRYPANOSOMA CRUZI* IN OPOSSUMS AND SMALL WILD RODENTS FROM NATIVE FORESTS IN A REGION IN THE URBAN AREA OF LONDRINA, PARANÁ

(PESQUISA DE *SARCOCYSTIS* SPP., *LEISHMANIA* SPP. E *TRYPANOSOMA CRUZI* EM GAMBÁS E PEQUENOS ROEDORES SILVESTRES ORIUNDOS DE MATAS NATIVAS EM UMA REGIÃO NA ÁREA URBANA DE LONDRINA, PARANÁ)

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RESUMO

O estudo teve como objetivo identificar a presença de *Sarcocystis* spp., *Leishmania* spp. e *Trypanosoma cruzi* em gambás e roedores silvestres capturados em matas localizadas no município de Londrina, Paraná, avaliando a frequência desses parasitos e os fatores associados à infecção. Entre os meses de outubro de 2019 e fevereiro de 2020, foram capturados, na região Oeste do município, em seis pontos de coleta diferentes, quatro gambás (*Didelphis albiventris*) e dois roedores (*Rattus rattus*), os quais foram submetidos à eutanásia e autópsia. Para a detecção de *Sarcocystis* spp., coletaram-se fragmentos de tecido cardíaco, macerados em solução salina estéril, e observadas ao microscópio óptico (10X e 40X) e, também, filtrado em gaze, observados em lupa. Para a detecção de parasitos do gênero *Leishmania*, foi realizada cultura em meio BHI-ágar sangue e PCR para amplificação do gene 18S. Para a identificação de *T. cruzi*, foi realizada coleta de sangue na veia marginal e as amostras foram processadas pelo método de Strout. Nenhuma das amostras foi positiva para os parasitos pesquisados. Sugere-se que maiores estudos sejam realizados para elucidar a epidemiologia, principalmente de *Leishmania* spp. e *T cruzi*, nesses animais sinantrópicos, no município de Londrina, Paraná.

Palavras-chave: ambiente; *Didelphis albiventris*; *Rattus rattus*; sinantrópico; triatomíneos

ABSTRACT

The main of this study was to identify the presence of *Sarcocystis* spp., *Leishmania* spp. and *Trypanosoma cruzi* in opossums and wild rodents captured in woods located in Londrina, Paraná, evaluating the frequency of these parasites and the factors for infection. Between October 2019 and February 2020, four opossums (*Didelphis albiventris*) and two rodents (*Rattus rattus*) were captured in the western region of the city of Londrina, at six different points. These animals were euthanized and autopsied. To detect *Sarcocystis* spp., fragments of cardiac tissue were collected, macerated in sterile saline solution, and observed under an optical microscope (10X and 40X) and filtered through gauze, observed under a magnifying glass. For the detection of parasites of the genus *Leishmania*, culture was performed on BHI-blood agar and PCR for gene 18S amplification. To identify *T. cruzi*, blood was collected from the marginal vein and samples were processed using the Strout method. None of the samples was positive for the parasites studied. None of the samples tested positive for the researched parasites. It is suggested that further studies be conducted to elucidate the epidemiology, particularly of *Leishmania* spp. and *T. cruzi*, in these synanthropic animals in the city of Londrina, Paraná.

Keywords: environment; *Didelphis albiventris*; *Rattus rattus*; synanthropic; triatomines

INTRODUÇÃO

Londrina is a municipality situated in the northern part of Paraná state, Brazil. The rapid expansion of urban areas, coupled with intensive agriculture and livestock farming, has led to a significant reduction in native vegetation. Nowadays, small forest fragments, known as valley bottoms, are the only remaining remnants of the original forest cover in the urban area. These valley bottoms, classified as Permanent Preservation Areas (APP) by Federal Law number 6,766/79, provide habitat for a variety of small wild rodents and other synanthropic animals, including opossums and various species of rodents. (MENDONÇA et al., 2002).

Opossums (*Didelphis* spp.) are marsupials with nocturnal and crepuscular habits (REIS et al., 2006) and are frugivorous-omnivorous (FONSECA et al., 2012). These animals play a crucial ecological role as seed dispersers, contributing to forest regeneration (CÁCERES, 2002). However, opossums can also serve as definitive hosts for *Sarcocystis* spp.(DUBEY et al., 2000, 2001a), are known as the main reservoirs of *T. cruzi* (JANSEN et al., 2015). Rodents are mammals characterized by their continuously growing incisor teeth, which they use for gnawing (LEGENDRE, 2003). These animals can pose significant health

risks and cause damage to agricultural crops, also have been implicated in the epidemiology of various parasites, including *Sarcocystis* spp., *Leishmania* spp. e *T. cruzi* (JANSEN et al., 2015).

Sarcocystis spp. are protozoa of great relevance in veterinary medicine as they affect wild, domestic and human animals (NAZIR et al., 2018), which may cause economic losses to producers and slaughterhouses due to reduced animal productivity, condemnation of carcasses, and increased veterinary costs (HATSUE et al., 2008). They are facultative and/or obligatory coccidia (PESCADOR et al., 2007). Carnivores and omnivores serve as definitive hosts, while herbivores act as intermediate hosts. When in intermediate hosts, sarcocysts develop in various tissues, such as muscle, heart, and brain. These tissue cysts can be ingested by predators through carnivorism. When in definitive hosts, sporulated oocysts are shed into the environment, completing the biological cycle (HATSUE et al., 2008).

Leishmaniasis is a globally distributed disease caused by obligate intracellular protozoa of the genus *Leishmania*. These parasites are transmitted to humans and animals by the bite of female hematophagous sandflies, primarily of the genus *Lutzomyia*. Leishmaniasis can cause a variety of clinical manifestations in humans, including cutaneous, visceral, and mucocutaneous leishmaniasis. In animals, leishmaniasis can lead to skin lesions, fever, weight loss, and other symptoms (BRASIL, 2017). In the vertebrate host, *Leishmania* parasites in the amastigote form infect the phagocytic cells of the mononuclear system. Depending on the *Leishmania* species, this infection can lead to the development of cutaneous, mucocutaneous, or visceral leishmaniasis. Visceral leishmaniasis (VL) primarily affects organs such as the spleen, liver, lymph nodes, and bone marrow and can be fatal. In the state of Paraná, VL is reported in the extreme West. Cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML) vary in the presentation of lesions and can be localized or diffuse. These forms are widely distributed throughout the state of Paraná. (RODRIGUES and MELO, 1942a).

Trypanosoma cruzi is a digenetic zoonotic parasite that causes trypanosomiasis, popularly known as “Chagas disease”, in humans (DEANE et al., 1984). It is transmitted by hematophagous triatomines of the order Hemiptera, in which permanent infection is established and is highly capable of infecting all mammalian cells, favoring the maintenance mechanism in nature (BRIGADA et al., 2010).

Opossums and wild rodents are animals that easily adapt to urban areas, a process known as synurbization (LUNIAK, 2004). Additionally, the close proximity of these potential hosts to humans likely facilitates the transmission and maintenance of parasites in wild, rural,

and urban environments. Therefore, the objectives of this study were to identify the occurrence of *Sarcocystis* spp., *Leishmania* spp. and *T. cruzi* in opossums and wild rodents from native forests in an urban area of Londrina, Paraná.

MATERIAL AND METHODS

The research was carried out between the months of October 2019 and February 2020, with approval from the Ethics Committee on the Use of Animals of Londrina State University (CEUA/UEL) on June 21, 2016 (number 124/2016) and the National Biodiversity System (SISBIO) under number 55429-4.

Opossums and wild rodents were captured using cage-type mousetraps, Sherman and Tomahawk models, with their trigger mechanism activated by bait placed inside: sardines, peanuts, bananas, cornmeal and mortadella. The traps were assembled and installed in the late afternoon in valley bottom regions with dense forest, near bodies of water and human settlements, in the western region of Londrina municipality, Paraná. They were inspected the following morning. Epidemiological questionnaires were completed in order to obtain data on collection sites, animal condition, species classification and autopsy information. Furthermore, the collection sites were identified and mapped using the Global Positioning System (GPS).

The opossums and rodents were anesthetized with intraperitoneal Xylazine and Ketamine, following the dosage according to the species captured: *Mus musculus*: 50 – 200 mg/kg of Ketamine Hydrochloride and 05 - 10 mg/kg Xylazine; *Rattus rattus* and *Rattus norvegicus*: 40 – 90 mg/kg of Ketamine Hydrochloride and 05 mg/kg Xylazine and *Didelphis albiventris*: 20 – 30 mg/kg of Ketamine Hydrochloride and 05 mg/kg of Xylazine. Euthanasia was performed through exsanguination by cardiac puncture after general anesthesia, following the CONCEA Euthanasia Practice Guidelines (2013).

To detect sarcocysts, the adapted technique Minuzzi et al. (MINUZZI et al., 2019) was used. Cardiac tissue samples from the animals were macerated with sterile saline to prepare slides, which were then examined under an optical microscope using 10X and 40X objectives. Additionally, the macerate was filtered through gauze under a sterile petri dish and observed using an Opton® electronic magnifying glass (ANATOMIC™, Cotia, São Paulo, Brazil).

To isolate *Leishmania* parasites, fragments of spleen and mesenteric lymph nodes were aseptically collected and immediately transferred to a saline solution containing antibiotics (penicillin 25,000 IU + streptomycin 100 μg/mL). The fragments were washed again in a tube containing antibiotics under a laminar flow hood and seeded in 15 mL conical

tubes containing rabbit blood agar as a solid medium and BHI (Brain Heart Infusion) as a liquid medium. After preparation, the tubes were stored in a BOD (Biochemical Oxygen Demand) incubator at 24 °C. The first observation under an optical microscope was conducted after 3 days, followed by subsequent observations every seven days until four weeks had passed.

To detect Leishmania spp. DNA, samples of liver, spleen, lymph nodes, bone marrow, and skin from captured animals were subjected to DNA extraction using the Phenol-Chloroform-Isoamyl Alcohol method (SAMBROOK and RUSSEL, 1989). The extracted DNA was eluted in 50 µL of ultrapure water and stored at -20°C for 48 hours until quantification. L-QUANT (Loccus Biotechnology) was used to quantify the extracted DNA, and only samples with a concentration above 20 ng/µL were sent for PCR. For each extraction, DNA extracted from a *L. braziliensis* culture (MHOM/BR/1987/M11272) served as a positive control, while ultrapure water was used as a negative control. PCR was performed with oligonucleotide primers 332 and 221 in the first reaction and primers 333 and 222 in the nested-PCR, generating a fragment of 393 base pairs (VAN et al., 1992).

To investigate *T. cruzi*, each animal was subjected to a puncture of the caudal marginal vein, and the blood was collected in a dry tube and processed using the Strout method modified by Flores et al. (1966). Ten milliliters of blood were collected from opossums, while up to 2 milliliters were collected from rodents. The samples were left to rest for five hours, after which the serum was separated and centrifuged at 165 x g for 10 minutes. Approximately 10 µl of serum was transferred to a slide covered with a 24x32 mm coverslip, and 50 fields were examined under a microscope at 400X magnification.

RESULTS

Traps were installed at ten different collection points, resulting in the capture of four opossums (*D. albiventris*) and two rodents (*R. rattus*) at six of these points. This represents a success rate of 60% (6/10). The placement locations of the traps where the animals were captured are illustrated in Figure 1.

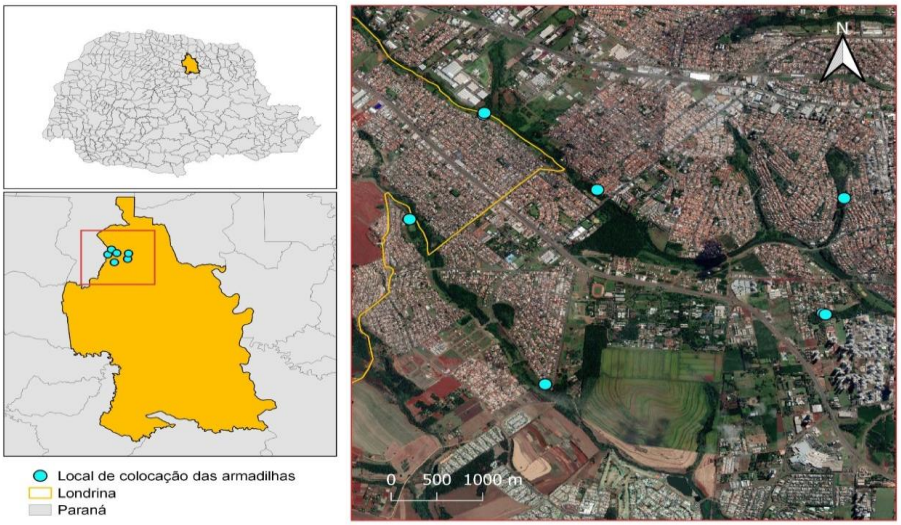

Figure 1. Distribution of opossum and wild rodents captured in Londrina, Paraná, 2019-2020.

Regarding seasonality, 50% (2/4) of the opossums were captured in the spring and the other 50% (2/4) in the summer, while both rodents were captured during the summer. As for the landscape, only one opossum was captured near the livestock farm, where horses were present. No animals were found near agricultural areas.

In the microscopic analysis, none of the cardiac tissue samples were found to contain sarcocysts. Both, the macerates examined under an optical microscope, and the filtrates viewed under a magnifying glass appeared normal, showing no signs of parasitism. Furthermore, none of the animals tested positive for *T. cruzi* using the Strout method. Regarding the parasite *Leishmania* spp., the presence of the parasite was not detected in any of the samples submitted for isolation, and no *Leishmania* DNA was identified in the PCR reactions.

DISCUSSION

Animals were collected from March 2019 to June 2020, a period of less than a year, which precluded a comprehensive analysis of seasonal effects on capture rates. Captures were less frequent on rainy days, likely due to animals seeking refuge in sheltered areas. The higher

abundance of opossums compared to rodents is likely attributable to the use of fruit-based baits, which are known to be more attractive to this species. Although both species are omnivorous, R. rattus exhibits a preference for grains, fruits, and vegetables (BRASIL, 2002) foods rarely used as bait in traps. Another hypothesis is the fact that this species of marsupial is considered synanthropic in Paraná. This phenomenon is likely attributed to the global expansion of urbanization, which has compelled these animals to adapt to new environments and conditions (LUNIAK, 2004). Additionally, these animals exhibit a preference for habitats near urban areas, utilizing forests as corridors for movement and resource acquisition (Cáceres, 2002).

Only one opossum was captured near the livestock farm, where horses were present. The remaining animals were found in areas with no livestock and agriculture. It is possible that the places where the animals were captured had a sufficient food supply to meet their needs, not forcing them to live close to domestic animal farming or crops. Another important point is that *D. albiventris* is one of the definitive hosts for *Sarcocystis neurona* (DUBEY et al., 2001a), responsible for the neurological disease equine protozoal myeloencephalitis (STELMANN e AMORIM, 2010), which makes the fact that possums are captured near horses relevant.

Didelphis spp. are definitive hosts of many *Sarcocystis* species, as already evidenced by prior studies: *D. marsupialis* parasited by *Sarcocystis speeri* (DUBEY et al., 2000), *D. marsupialis* parasited by *Sarcocystis falcatula* (DUBEY et al., 2001b), *D. albiventris* parasited by *S. neurona* (DUBEY et al., 2001a). While there are limited reports in the scientific literature of opossums serving as intermediate hosts for *Sarcocystis:* Mandour (1965) identified cysts of *Sarcocystis garnhami* in skeletal muscle of *D. marsupialis*, in British Honduras, now Belize. This finding may explain the absence of sarcocysts in the cardiac tissue of opossums in the present study.

There are few reports of *R. novergicus* harboring *Sarcocystis* spp. cysts in their muscles. Grikienienė et al. (2001) identified the presence of this parasitic genus in this rodent species in Lithuania, and Prakas et al. (2019) described a new species, *Sarcocystis ratti*, in the skeletal muscles of the same rodent species. In contrast, *Sarcocystis cymruensis* was reported in China, in two rodent species, *Rattus flavipectus* and *R*attus *norvegicus*, with the latter serving as both an intermediate host and, in cases of cannibalism, as a definitive host for this parasite (HU et al., 2011). Thus, while opossums are well-established intermediate hosts for *Sarcocystis* spp., the role of *R. norvegicus* as an intermediate host appears to be less common.

The most abundant *Leishmania* species in the state of Paraná is *Leishmania (Viannia) braziliensis* (SVS, 2018; OLIVEIRA et al., 2010), which has been reported in rodents such as *Oryzomys concolor*, *Oryzomys capito*, *Oryzomys nigripes*, and *Rattus rattus*, as well as in marsupials like *Didelphis marsupialis*, in Brazil (LAINSON, 2010). The first isolation of *L. braziliensis* from liver and spleen samples of *R. rattus* was conducted in the southeastern region of Brazil, in the state of Minas Gerais, by Pereira et al. (2017). Roque and Jansen (2014) suggest that opossums of the genus *Didelphis* may act as reservoirs of this parasite, as they have been reported to harbor *L. (V.) braziliensis*, *L. (Leishmania) amazonensis*, and *L. (Leishmania) infantum.*

In terms of molecular detection, Caldart et al. (2017) reported, for the first time, the detection of *Leishmania (Leishmania) amazonenses*, a species involved in American cutaneous leishmaniasis (ACL), in the state of Paraná, in *Rattus rattus* captured at recycling centers and scrap yards in the municipality of Londrina. In an environmental protection area in the municipality of Campinas, 2.4% (2/82) of *D. albiventris* individuals were found to be positive for *Leishmania* via molecular methods, and after genetic sequencing, one of the samples was identified as *L. (L.) infantum* (PAIZ et al., 2016). These findings highlight the dispersion of the parasite in urbanized environments, using hosts that are well-adapted to both urban and wild habitats, and underscore the associated public health risks (CALDART et al., 2017; PAIZ et al., 2016).

The findings from other studies support the hypothesis that these animal species play a role in the parasite's life cycle. However, the limited number of animals evaluated in the present study likely contributed to the absence of positive cases, despite the state of Paraná being considered an endemic region for ACL (SVS, 2018).

Regarding *Trypanosoma cruzi*, previous studies have documented the presence of triatomines near urban areas and suggested that forest fragments contribute to maintaining the enzootic cycle of the protozoan (FÉ et al., 2009; FORATTINI et al., 1970). In Belém (PA), Rodrigues and Melo (1942b) reported high infection rates by *T. cruzi* in opossums, with 91.7% positivity, while in Rio de Janeiro (RJ), Guimarães and Jansen (1943) found a 35.7% positivity rate in these animals. In the present study, however, despite Paraná being considered an endemic region for the etiological agent (CAMARGO et al., 1984), no *D. albiventris* tested positive in the Strout test. One possible explanation for the absence of positive results could be the limited contact these opossums have with wild triatomines, as they inhabit urban areas. This finding aligns with the study by Fernandes et al. (1989), who conducted research in Bambuí (MG) and observed 7.7% infection by *T. cruzi* in opossums in

peridomestic urban areas, compared to 34.9% in wild environments through direct examination.

Concerning the species *R. rattus*, none of the samples tested positive for *T. cruzi*. A study by Hodo et al. (2017) suggests that this species may currently have limited importance as a wild reservoir for the parasite, as none of the 145 samples analyzed using PCR were positive. However, other studies conducted in endemic regions of Latin America have reported a prevalence ranging from 5% to 57% using PCR, microscopy, or culture methods (GALUPPO et al., 2009; HERRERA e URDANETA-MORALES, 1997; RAMSEY et al., 2012). These results show significant variability, emphasizing the need for further studies in the researched region.

CONCLUSION AND FINAL CONSIDERATIONS

In the present study, none of the samples tested positive for *Sarcocystis* spp., *Leishmania* spp., or *T. cruzi*. Due to the small sample size, it is not possible to conclude that these etiological agents do not utilize *D. albiventris* and *R. rattus* as hosts in the study region. Further research involving these species is recommended to clarify their role as potential hosts in the epidemiology, especially of leishmaniasis and trypanosomiasis, in the municipality of Londrina, Paraná.

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