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Núcleo de Medicina Tropical

TAUANA DE SOUSA FERREIRA

Identificação de *Leishmania* spp. e fontes alimentares em flebotomíneos (Diptera: Psychodidae) capturados no município de Rio Verde de Mato Grosso - MS, Brasil

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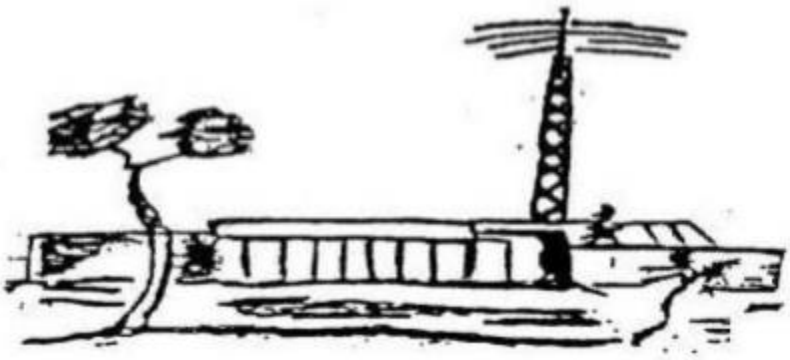
Orientador: Profº Dr. Rodrigo Gurgel Gonçalves

Co-orientadora: Prof. Drª. Nadjar Nitz



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BANCA EXAMINADORA

Dr. Rodrigo Gurgel Gonçalves (Orientador)

Universidade de Brasília

Drª. Elisa Neves Vianna (Titular)

Universidade de Brasília

Dr. Marcos T. Obara (Titular)

Universidade de Brasília

Drª. Mariana Machado Hecht (Suplente)

Universidade de Brasília

Brasília

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DEDICATÓRIA

Aos meus pais, Raimundo Ferreira e Mere Ruth Ferreira.

Aos meus irmãos, Taumaturgo e Tássio

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LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIAÇÕES

BLASTn: Basic Local Alignment Search Tool (nucleotide)

DNA: Deoxyribonucleic acid (Ácido desoxirribonucleico)

ITS: Internal Transcribed Spacer (Região espaçadora do gene de RNA Ribossomal)

kDNA: Kinetoplast DNA (DNA do Cinetoplato)

LT: Leishmaniose Tegumentar

LV: Leishmaniose Visceral

MS: Mato Grosso do Sul

nDNA: DNA nuclear

pb: pares de base

PCR: Polymerase Chain Reaction (Reação de Polimerização em Cadeia)

pg: pico grama

qPCR: PCR em tempo real ou PCR quantitativa

TIM: Taxa de Infecção Mínima

UFs: Unidades Federativas

WHO: World Health Organization (Organização Mundial de Saúde)

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ÍNDICE

RESUMO

O Estado do Mato Grosso do Sul é endêmico para as leishmanioses, onde *Leishmania infantum* tem sido detectado em humanos, cães, gatos e flebotomíneos. O monitoramento da ocorrência de flebotomíneos sinantrópicos é fundamental para avaliação das atividades de vigilância e controle das leishmanioses. Nosso objetivo foi verificar a taxa de infecção natural por *Leishmania* e identificar as fontes alimentares em flebotomíneos capturados em áreas de transmissão de leishmaniose do município de Rio Verde do Mato Grosso, Mato Grosso do Sul, Brasil nos anos de 2014 e 2016. Seis bairros do município foram selecionados devido a presença de casos de Leishmaniose Visceral e/ou Tegumentar. Em uma casa de cada bairro foram instaladas duas armadilhas do tipo CDC no peridomicílio. A identificação de *Leishmania* nas amostras foi realizada por kDNA-qPCR e por sequenciamento com alvo ITS. A detecção da fonte alimentar das fêmeas ingurgitadas foi realizada por meio de qPCR-cytb após análise High Resolution Melting (HRM-cyt b-qPCR). O esforço amostral total foi de 420 armadilhas CDC, das quais 380 foram positivas para flebotomíneos (sucesso de captura = 90,5%) com a captura de 24.989 flebotomíneos. Foram identificados 3.088 flebotomíneos distribuídos em 12 espécies. *Lutzomyia longipalpis* foi mais abundante em todos os pontos de coleta (n= 2.775), seguido de *Nyssomyia whitmani* (n=297). Foram analisadas 1261 fêmeas, agrupadas em 159 *pools*, dos quais 99 foram positivos na kDNA-qPCR de *Leishmania*, 92 de *Lu. longipalpis* (Taxa de infecção mínima – TIM = 8%) e 7 de *Ny. whitmani* (TIM = 7%). A maioria dos *pools* positivos de *Lu. longipalpis* foi detectada no ano de 2016 nos meses chuvosos. O sequenciamento revelou *L. infantum* nas amostras de *Lu. longipalpis*. DNA de galinha foi detectado em 57 flebotomíneos (98,3%) e apenas em uma fêmea foi detectado DNA humano (1,7%), a qual estava negativa para *Leishmania* na kDNA-qPCR. A taxa de infecção natural das fêmeas com DNA de galinha no conteúdo estomacal foi de 64,9% (37/57). Conclui-se que o risco de transmissão de *L. infantum* para humanos permanece na área estudada com alta frequência de flebotomíneos infectados no ambiente peridomiciliar.

Palavras-chave: *Lutzomyia longipalpis*; *Nyssomyia whitmani*; *Leishmania infantum*; qPCR; infecção; fontes alimentares; Rio Verde de Mato Grosso.

ABSTRACT

Human leishmaniases are endemic in the state of Mato Grosso do Sul (MS), Brazil, where *Leishmania infantum* has been detected in humans, dogs, cats, and phlebotomine sandfly vectors. Monitoring synanthropic vector populations is a critical component of leishmaniasis control-surveillance systems. Here, we used a suite of molecular approaches to assess *Leishmania* infection frequency and identify blood-meal sources in a large sample of sandflies collected in 2014 and 2016 in anthropic environments of a *Leishmania*-transmission area in MS (Rio Verde do Mato Grosso municipality). We sampled vectors in one peri-domestic site within each of six neighborhoods with recent records of human visceral and/or tegumentary leishmaniasis. We used kDNA-qPCR plus rDNA ITS sequencing to detect and identify *Leishmania* in pooled female sandflies. Individual blood-fed females (n = 58) were used for blood-meal analyses using qPCR plus High-Resolution Melting (HRM) of the mtDNA cytb gene. Overall, 90,5% of 420 CDC trap-nights yielded vectors, for a total of 24,989 sandflies. We identified 3088 sandflies in 12 species, including 2775 *Lutzomyia longipalpis* (the most abundant species at all sampling points) and 297 *Nyssomyia whitmani*. We tested 1261 female sandflies in 159 pools, of which 92 *Lu. longipalpis* (minimum infection rate [MIR] 8%) and 7 *Ny. whitmani* pools (MIR 7%) were *Leishmania* kDNA-qPCR-positive. Most positive *Lu. longipalpis* were collected in the 2016 rainy season. Sequencing confirmed *L. infantum* in *Lu.* l*ongipalpis* samples. HRM identified chicken DNA in 57 sandflies (98.3%), 37 of which were *Leishmania* DNA-positive; human blood was found in just one (*Leishmania*-negative) female (1.7%). Our data show ongoing risk of *L. infantum* transmission to humans in the study area, where *Leishmania*-infected sandfly vectors are common (and heavily rely on chicken blood) in the peri-domestic environment.

Keywords: *Lutzomyia longipalpis*; *Nyssomyia whitmani*; *Leishmania infantum*; qPCR; infection; blood-meal sources; Rio Verde de Mato Grosso.

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Apêndice: Manuscrito submetido para o periódico Acta Tropica

Molecular identification of *Leishmania* spp. and blood-meal sources in sandfly vectors (Phlebotominae) from a *Leishmania*-transmission area in Mato Grosso do Sul, Brazil

Tauana de Sousa Ferreira[1], Renata Velozo Timbó[1], Thaís TC Minuzzi-Souza[1], Douglas de Almeida Rocha [1], Mariana Neiva[1], Joana Ribeiro de Albuquerque[1], Paulo Silva de Almeida[2], Mariana Hecht[3], Nadjar Nitz[3], Rodrigo Gurgel-Gonçalves[1*]

[1]Laboratório de Parasitologia Médica e Biologia de Vetores, Faculdade de Medicina, Universidade de Brasília, Brasil.

[2]Laboratório Regional de Entomologia, Núcleo Regional de Saúde da Secretaria Estadual de Saúde, Dourados, Mato Grosso do Sul, Brasil

[3]Laboratório Interdisciplinar de Biociências, Faculdade de Medicina, Universidade de Brasília, Brasil.

*Corresponding author

Address: Laboratório de Parasitologia Médica e Biologia de Vetores – UnB, Área de Patologia, Faculdade de Medicina, Universidade de Brasília, Campus Universitário Darcy Ribeiro Asa Norte, Brasília-DF, CEP: 70904-970, Caixa Postal: 4569 ++ 55 61 3107 1786. E-mail: gurgelrg@hotmail.com

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Abstract

Human leishmaniases are endemic in the state of Mato Grosso do Sul (MS), Brazil, where *Leishmania infantum* has been detected in humans, dogs, cats, and phlebotomine sandfly vectors. Monitoring synanthropic vector populations is critical for leishmaniasis control-surveillance in such transmission-prone areas. Here, we used a suite of molecular approaches to assess *Leishmania* infection frequency and to identify blood-meal sources in a large sample of sandflies collected in anthropic environments of a *Leishmania*-transmission area in MS (Rio Verde do Mato Grosso municipality). We sampled vectors monthly (January-June 2014 and 2016) in one peri-domestic site within each of six neighborhoods with recent records of human visceral and/or tegumentary leishmaniasis. We used kDNA-qPCR plus rDNA ITS-sequencing to detect and identify, *Leishmania* in pooled female sandflies. Individual blood-fed females (n = 58) were used for blood-meal analyses through qPCR plus High-Resolution Melting (HRM) of the mtDNA cytb gene. Overall, 83.3% of 420 CDC trap-nights yielded vectors, for a total catch of 24,989 sandflies. We sub-sampled and identified 3088 sandflies in 12 species, including 2775 *Lutzomyia longipalpis* (the most abundant species at all sampling sites) and 297 *Nyssomyia whitmani*. We tested 1261 female sandflies in 159 pools, of which 92 *Lu. longipalpis* (minimum infection rate [MIR] 8%) and 7 *Ny. whitmani* pools (MIR 7%) were *Leishmania* kDNA-positive. Most positive *Lu. longipalpis* were collected in the 2016 rainy season. Sequencing confirmed *L. infantum* in *Lu. longipalpis* samples. HRM identified chicken DNA in 57 sandflies (98.3%), 37 of which were *Leishmania* DNA-positive; human blood was found in just one (*Leishmania*-negative) female. Our data show ongoing risk of *L. infantum* transmission to humans in the study area, where *Leishmania*-infected sandfly vectors are common (and heavily rely on chicken blood) in the peri-domestic environment.

Keywords: *Lutzomyia longipalpis*; *Nyssomyia whitmani*; *Leishmania infantum*; qPCR; infection; blood-meal

1. Introduction.

Parasites of the genus *Leishmania* are transmitted through the bites of female phlebotomine sandflies that acquired the infection with a blood-meal. *Leishmania* infection can result in visceral (VL) or tegumentary leishmaniasis (TL) depending on parasite species and on complex host-parasite interactions. The disease is endemic in 98 countries and afflicts about 350 million people worldwide (WHO 2017). In the Americas, most cases occur in Brazil (Alvar et al. 2012), where disease incidence has increased over the last decades. This worrisome trend owes much to the rise of urban VL brought about by the combined effects of massive rural-urban migration, a growing population of chronically infected dogs, and the adaptation of sandfly vectors to anthropic environments including urban/suburban peri-domestic habitats (Harhay et al. 2011).

The central-west region of Brazil has so far produced records of approximately 130 sandfly species (Almeida et al. 2015, Rapello et al. 2018). In the state of Mato Grosso do Sul (MS), where VL caused at least 267 deaths in 2000-2016 (MS/SVS 2017), *Lutzomyia longipalpis* is the main vector of *Leishmania infantum*; *Lu. cruzi* also vector *L. infantum* and, potentially, *L. amazonensis* in some areas of the state (Santos et al. 1998, Cunha et al. 2014, Oliveira et al. 2015, Falcão de Oliveira et al. 2017). *Lu. longipalpis* occupies preferentially the drier, less vegetated regions of MS (Andrade et al. 2014), suggesting a possible association with anthropic (deforested) landscapes. Further, the geographic distributions of *Lu. longipalpis* and VL clearly overlap in the state (Almeida et al. 2013). Molecular studies have reported *Leishmania* infection rates of about 2% in *Lu. longipalpis* from MS (Paiva et al. 2008, Silva et al. 2008). The MS state health department stratifies VL transmission risk at the municipal level, with transmission classified as intense or moderate in municipalities where *L. infantum* has well-established domestic/peri-domestic cycles involving humans, dogs, cats, and synanthropic sandfly populations (Souza et al. 2013, Cunha et al. 2014, Castro et al. 2016, Metzdorf et al. 2017). In such scenarios, identifying *Leishmania* parasites and the blood-meal sources of female

sandflies (Baum et al. 2015, Guimarães-e-Silva et al. 2017) can help clarify the complex vector-host-parasite interactions underlying human VL transmission.

Several recent studies have shed light on the incidence of autochthonous leishmaniasis and on the geographic distribution and potential spread of phlebotomine sandflies in the state of MS. However, to develop a more robust understanding of local transmission scenarios we need insight about vector-host-parasite interactions at a much finer scale – insight, for example, about how sandfly infection frequency may relate to the blood-meal sources of the vectors. Aiming to fill this gap, we investigated infection by *Leishmania* spp. and identified blood-meal sources in sandflies collected in 2014 and 2016 in neighborhoods reporting human leishmaniasis cases in the municipality of Rio Verde do Mato Grosso, MS, Brazil.

2. Material and methods

2.1. Study area

The municipality of Rio Verde de Mato Grosso (18º55'05" S, 54º50'39" W, ~330 m.a.s.l.) is located in the Pantanal Sul Matogrossense region, ~200 km north of Campo Grande, the state's capital city. The municipality extends over ~8152 km^2 and has about 19,500 inhabitants (IBGE 2015). Mean temperature is ~25°C, with the lowest mean monthly values (~22°C) recorded in July; mean annual rainfall is just below 1500 mm, with monthly values ranging from less than 30 mm in July to 250 mm in January (Climate-date.org). The western part of the municipality is within the Pantanal floodplains, whereas drier Cerrado savannahs dominate to the east. The municipality health department recently recorded VL and/or TL cases in six neighborhoods of Rio Verde do Mato Grosso. We chose one dwelling in each of those six neighborhoods for sandfly collection; we intentionally selected dwellings in which animal shelters or enclosures (chicken coops plus kennels or corrals) and fruit trees were present in the peri-domestic area – i.e., dwellings with traits that may favor sandfly presence and reproduction, thus leading to increased vector abundance.

2.2. Sandfly collection and identification

We sampled vectors for 3 consecutive nights each month over January to June in 2014 and in 2016. Each night (5:00 pm-6:00 am), we set 2 CDC light traps in the peri-domestic area of each sampling dwelling. All insects caught in each trap-night were separately stored in 70% ethanol and shipped to the University of Brasília. We sub-sampled each of those samples to retain up to 10 male and 15 female phlebotomine sandflies per trap-night. For each trap-nigh sample, we did this by evenly spreading sandflies on a Petri dish with a square-grid drawn on its bottom and then picking the sandfly closest to the center of each square for further study. We separately sub-sampled male and female sandflies – using, respectively, 10- and 15-square grids. We identified recently blood-fed females (with visible blood inside the digestive tract) in each sub-sample and stored them individually in phosphate-buffered saline (PBS) 1X pH 7,4 (3.2 mM Na2PO4, 0.5mM KH2PO4, 1.3mM KCl and 1.35 mM NaCl). Females were dissected to separate the head and the last three (distal) abdominal segments from the rest of the body. Whole males and female heads and distal abdomen segments were mounted following Forattini (1973); we then used the keys by Galati (2016) and the Lutzodex app (Rocha et al. 2016) to identify all specimens to species level. Female thoraces and proximal abdominal segments were stored in PBS 1× at −20°C for DNA extraction.

2.3 *DNA extraction and quality assessment*

For non-blood-fed females, we used pools of 2 to 10 thorax/proximal abdomen samples for DNA extraction, with each pool containing sandflies of the same species and caught in the same trap-night (i.e., from the same site and date of capture). For blood-fed females, each thorax/abdomen sample was processed individually. We extracted and purified DNA with the Biopur Kit Extraction Mini Spin Plus (Biometrix). The samples remained in proteinase K at 37 ° C overnight and other steps followed the manufacturer's instructions. We quantified DNA with a NanoVue™ Plus Spectrophotometer (GE Healthcare) at 220–330nm wavelength. Sample purity was evaluated using absorbance (A260/280 ratio) values. We stored samples at −20°C until use for PCR.

To confirm that good-quality DNA was present in the samples, we amplified a 200-base pair fragment of the IVS6 region of the sandfly cacophony gene using primers 5'-GTGGC-CGAACATAATGTTAG-3' and 5'-CCACGAACAAGT-TCAACATC-3' (Lins et al. 2002) and the methods described by Machado et al. (2017).

2.4 *Detection of* Leishmania *spp.* DNA

We used qPCR with primers targeting the conserved sequences of the *Leishmania* spp. kinetoplast minicircle DNA (5'-GGC CCA CTA TAT TAC ACC AAC CCC-3' and 5'-GGG GTA GGG GCG TTC TGC GAA-3') (Pita-Pereira et al. 2012). The kDNA-qPCR reaction mix contained 1× Power SYBR Green Master Mix (Applied Biosystems, CA, USA), 0.2 μM of each primer, 40 ng of template DNA, and ultra-pure distilled water to a final volume of 20 μL. Reactions were run in triplicate on 96-well optical reaction plates (MicroAmpR) in the QuantStudio 3 Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: initial incubation for 12 min at 94°C plus 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. All PCRs included positive (*L. infantum* DNA) and negative (DNA from male sandfly pools) controls. Samples were considered positive when they presented CT less than or equal to 35 cycles. During the standardization of the kDNA-qPCR it was possible to amplify culture samples of *L. (L.) infantum* with up to 5pg of DNA, the limit of detection of our reaction.

Selected kDNA-qPCR-positive samples were submitted to a second PCR to identify *Leishmania* species. We amplified a 265-288-base pair region of the first internal transcribed spacer (ITS) of the ribosomal DNA 18S gene using primers ITS-219 F (5′-AGC TGG ATC ATT TTC CGA TG-3′) and ITS-219 R (5′-ATC GCG ACA CGT TAT GTG AG-3′) (Talmi-Frank et al. 2010). The kDNA-qPCR reaction mix contained 1× Power SYBR Green Master Mix (Applied Biosystems, CA, USA), 0.4 μM of each primer, 15 ng of template DNA, and ultra-pure distilled water to a final volume of 20 μL. Reactions were run in QuantStudio 3 Real-Time PCR System as described above with the following conditions: 50ºC for 2 min, 95ºC for 10 min and 40 cycles of 15 sec at 95ºC, 40 sec at

53°C, and 08 sec at 72°C. Amplified fragments were purified using the Illustra GFX PCR DNA &Gel Band Purification kit (GE Healthcare, New York, USA) and sent out for sequencing at the University of São Paulo's *Centro de Pesquisa sobre o Genoma Humano e Células-Tronco*. Sequences were edited with Geneious (Biomatters, New Zealand) and compared with *Leishmania* spp. sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) at the US National Center for Biotechnology Information (NCBI). We considered parasites as confidently assigned to species when their sequences were ≥99% identical to species-labeled GenBank sequences with e-value > 10^{-3}.

2.5 *Blood-meal identification*

We first prepared positive controls including 7 potential hosts: human (*Homo sapiens*), dog (*Canis familiaris*), cat (*Felis catus*), sheep (*Ovis aries*), chicken (*Gallus gallus*), and two native small mammals – a marsupial (*Gracilianus agilis*) and a rodent (*Rhipidomys macrurus*). We extracted and purified DNA from blood samples using the Biopur Kit Extraction Mini Spin Plus and following the manufacturer's recommendations; DNA was quantified with the NanoVue™ Plus Spectrophotometer (GE). To identify blood-meal sources in female blood-fed sandflies we used High-Resolution Melting (HRM) of a qPCR-amplified, 383-base pair fragment of the vertebrate mitochondrial cytochrome b gene; for this, we used primers cyt b-F (5'-CCC CCT AGA ATG ATA TTT GTC CTC A-3') and cyt b-R (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') (Boakye et al. 1999). Amplification reactions were run in a final volume of 20 µl including 1× MeltDoctor™ HRM Master Mix (Applied Biosystems), 0.5 µM of each primer, and 20 ng of template DNA. We used 96-well plates (Optical 96-Well Reaction Plate, MicroAmpR) and the QuantStudio 3 Real-Time PCR System (Applied Biosystems) thermal cycler to run HRM reactions under the following conditions: 95ºC for 10 min plus 40 cycles of 95ºC for 15 sec, 55ºC for 45 sec, and 72ºC for 10 sec. For DNA amplification, we submitted samples to 95°C for 10 sec, 60°C for 1 min, 95°C for 1 min, and 60°C for 15 sec; we determined 'melting curve' patterns by progressively heating the samples from 60ºC to 95ºC with

steps of 0.1ºC sec^{-1}. Positive controls included the seven vertebrate hosts mentioned above, and negative controls contained DNA from male sandflies. We analyzed results with the High Resolution Melt Software 3.1 program (Applied Biosystems, CA, USA) and the following reference melting-curve patterns: human, 79.3-84.3ºC; dog, 75.5-80.3ºC; cat, 77.1-81.3ºC; sheep, 76.9-80.3ºC; chicken, 80.9-85.3ºC; marsupial, 76.2-80.7ºC; and rodent, 76.1-80.2ºC.

2.6 *Statistical analysis*

Our dependent variables were (a) the proportion of sandfly-positive CDC trap-nights, (b) the number of sandflies trapped, and (c) sandfly infection rates. Independent variables included (a) time of collection – year (2014 vs. 2016) or rainy (January-March) vs. dry (April-June) season, (b) sampling sites (dwellings/neighborhoods), and (c) blood-meal sources identified through HRM. We used chi-squared or Fisher's exact tests as implemented in Graphpad to compare the proportions of sandfly-positive trap-nights and of *Leishmania*-positive pools across time (years, seasons) and space (dwellings). As a conservative index of the frequency of vector infection with *Leishmania*, we calculated the species-specific sandfly Minimum Infection Rate (MIR) by dividing, for each species, the number of positive pools by the total number of female individuals (across all pools) investigated.

3. Results

Total sampling effort amounted to 420 CDC trap-nights over the 12 months of fieldwork. Of those, 380 trap-nights were positive for sandflies, for an overall capture success rate of 90.5%. The proportions of positive trap-nights were similar in the drier (April-June, 88%) and rainier months (January-March, 93%; chi-square 2.3, p = 0.12), as well as between years (92% in 2014 vs. 89% in 2016; chi-squared test statistic 0.65, p = 0.42). Across neighborhoods, trap-night positivity varied

between 79% (in one neighborhood) and 100% (at two sites); pairwise comparisons yielded $p < 0.01$ when comparing those extreme values.

We caught 24,989 sandflies over the 12 months of sampling. Of those, 68.5% were collected in 2016, when captures were most productive in January (52.6% of specimens) and least productive in May (5.6%). In 2014, 25.5% of sandflies were caught in February, vs. just 6.5% in June (Fig. 1). The overall male to female ratio in our sample was 3.72 to 1. After sub-sampling (see Methods), we identified 1750 female sandflies in 10 species. The most abundant at all sampling sites was *Lutzomyia longipalpis* (*n* = 1570), followed by *Nyssomyia whitmani* (*n* = 167), *Evandromyia sallesi* (*n* = 4), *Psathyromyia shannoni* (*n* = 2), *Evandromyia lenti* (*n* = 2), *Sciopemyia sordellii* (*n* = 1), *Micropygomyia peresi* (*n* = 1), *Micropygomyia acantopharynx* (*n* = 1), *Micropygomyia carrerai carrerai* (*n* = 1), and 1 *Nyssomyia intermedia* (*n* = 1). Sub-sampling yielded 1338 male sandflies, which were identified as *Lu. longipalpis* (*n* = 1205), *Ny. whitmani* (*n* = 130), *Nyssomyia delsionatali* (*n* = 1), *Evandromyia cortelezzii* (*n* = 1), and *Ev. lenti* (*n* = 1).

We screened 1261 female sandflies, grouped into 159 pools, for infection with *Leishmania* spp. – 145 *Lu. longipalpis* pools (*n* = 1166 flies) and 14 *Ny. whitmani* pools (*n* = 95). We successfully amplified a fragment of the sandfly cacophony gene in 154 pools, thus confirming that good-quality DNA was available for most (96.9%) of our samples. Ninety-nine pools were *Leishmania* kDNA-qPCR positive – 92 *Lu. longipalpis* pools (MIR = 8%) and 7 *Ny. whitmani* pools (MIR = 7%). Across collection sites (i.e., dwellings within neighborhoods), MIR values ranged from 6 to 13% for *Lu. longipalpis* and from 0 to 10% for *Ny. whitmani* (Table 1).

We found significant between-year variation in the proportion of *Leishmania*-positive *Lu. longipalpis* pools (chi-square 24.4, $p < 0.01$); most positive pools were detected in 2016, when the overall MIR was 9% (Table 1). We also detected differences between the rainy (when 66% of pools were positive) and dry seasons (44%; chi-square 6.5, $p < 0.01$) for *Lu. longipalpis*. Eighty-six of our kDNA-qPCR positive samples were confirmed as positive for *Leishmania* by ITS amplification via

PCR; sequencing identified *L. infantum* in the subset of *Lu. longipalpis* samples that contained larger amounts of PCR-amplified ITS DNA.

Fifty-eight of the 1570 female sandflies we sub-sampled had visible blood in their digestive tract and were classified as (recently) blood-fed. We extracted good-quality DNA from all these blood-fed flies, and found, using HRM, chicken blood in 57 (98.3%) and human blood in 1 (1.7%) of them. Using kDNA-qPCR, we found that *Leishmania* infection was as high as 63.8% in this subset of blood-fed females; *Leishmania* kDNA-qPCR was negative in the only human-fed female, so that infection among chicken-fed flies was 64.9%.

4. Discussion

The sand fly fauna found in our study was composed of 12 species and included known vectors of leishmaniasis. We detected high frequency of positive traps for sandflies (90%) when compared to other studies in Brazil using similar light traps in Palmas, Tocantins (30%) and the Federal District (18%) (Machado et al., 2017, Rapello et al., 2018). The trapping success obtained in the present study evidences the high vector population density in the peri-domestic areas of the municipality of Rio Verde de Mato Grosso. The clear predominance of *Lu. longipalpis* in our samples is consistent with previous reports from MS state (Oliveira et al. 2006, 2008, Nascimento et al. 2007, Araújo e Silva et al. 2007, Figueiredo et al. 2016). This confirms the synanthropic behavior of *Lu. longipalpis*, and suggests that local peri-domestic environments offer suitable foraging conditions (and, probably, breeding microhabitats) for the species. Peri-domestic chickens and fruit trees may favor sandfly occurrence (Alexander et al. 2002, Santini et al. 2012); both were present in our sampling dwellings, which we selected to maximize sandfly catch, but were also very common in other, nearby dwellings. On the other hand, and based on previous reports from central-western Brazil with comparable CDC-trapping effort, we expected to find a larger number of sandfly species in our study area; for example, Oliveira et al. (2006) recorded 20 species, and Missawa et al. (2007) 22 species, in MS state. As in our survey, *Lu. longipalpis* was the most common species in those studies (Oliveira et al. 2006,

Missawa et al. 2007). Of the 12 species captured in the present study, *Ny. delsionatali* had not been recorded in Mato Grosso do Sul (Almeida et al., 2015).

Our finding that sandflies, and in particular *Lu. longipalpis*, occurred with higher frequency and were more abundant in dwellings during rainier months is also consistent with previous reports from MS state (Araújo e Silva et al. 2007, Oliveira et al. 2008). We also observed that the highest occurrence of sandflies was observed in the rainy months of 2016. When comparing the rainfall data, it was observed that in 2016 the precipitation in the month of January (300 mm) was much higher than in 2014 (130 mm), this could explain the greater abundance of sand flies in 2016.

The rates of natural infection with *Leishmania* spp. in *Lu. longipalpis* (minimum infection rate of up to 13% for non-blood-fed females and infection rate of 65% for blood-fed females) and *Ny. whitmani* (minimum infection rate of up to 10% for non-blood-fed females) we report are higher than previously found in MS state (Paiva et al. 2008, Silva et al. 2008, Oliveira et al. 2008, 2015) and elsewhere in Brazil (Rodrigues et al. 2014, Fonteles et al. 2017, Saraiva et al. 2017). This may be due to a higher sensitivity of our qPCR-based approach (Bezerra-Vasconcelos et al 2011, Pita-Pereira et al 2012), to a truly higher prevalence of infection among vectors in our study area (where leishmaniasis is known to be endemic and transmission is likely active), or both. Sequencing confirmed *L. infantum* as the parasite infecting *Lu. longipalpis* in some of our samples, thus providing further evidence for the association of those two species in peri-domestic environments.

We found that an overwhelming majority of recently blood-fed sandflies had taken their blood-meals from chickens; this was expected, if anything else because all our sampling dwellings had peri-domestic chicken coops. Although chickens are important blood sources for sandflies, they do not sustain *Leishmania* infections (Alexander et al. 2002). Somewhat unexpected, then, were the very high rates of *Leishmania* spp. infection in chicken-fed *Lu. longipalpis* and *Ny. whitmani*. Chicken blood, however, provides adequate nutrition to the sandflies and does not inhibit *Leishmania* development in the vectors' digestive tract (Sant'anna et al. 2010). Further, even if *Lu. longipalpis*

feeds often on chickens, it is an eclectic, opportunistic species (Macedo-Silva et al. 2014, Guimarães-e-Silva et al. 2017, Fonteles et al. 2017) that may feed (and must have fed in our study area and sites) on *Leishmania*-susceptible mammals, thus maintaining transmission cycles. One of the female sandflies in our sample had indeed taken a recent human blood-meal.

Our results strongly underscore the need for a close monitoring of *Leishmania* transmission in the study area; dwellers and their dogs should be periodically tested for infection to (1) determine whether such transmission is ongoing and (2) adopt therapeutic and preventative measures as required. Joint evaluations involving entomological, epidemiological, and environmental surveillance staff could assist in the development of integrated disease-prevention strategies that combine insecticide spraying with longer-term environmental management interventions – potentially including, e.g., improved poultry husbandry practices or the physical protection of houses with insect screens. In this latter respect, investigating the use of indoor environments by synanthropic sandflies (including their choice of blood-meal sources) would help refine our understanding of *Leishmania* spp. transmission risk in our study region and likely across many comparable settings in South America.

5. **Conclusion**

Our study revealed high rates of natural infection with *Leishmania* spp. in *Lu. longipalpis* and *Ny. whitmani* collected in peri-domestic environments – where the flies appear to feed mostly on chickens but may also take blood-meals from human hosts. Sequencing confirmed *L. infantum* DNA in *Lu. longipalpis* samples. These findings, combined with the high frequency (and abundance) at which *Lu. longipalpis* occurred around houses (especially during the rainy season, and particularly in January 2016), indicate ongoing risk of *L. infantum* transmission to dwellers in the neighborhoods of Rio Verde do Mato Grosso we studied.

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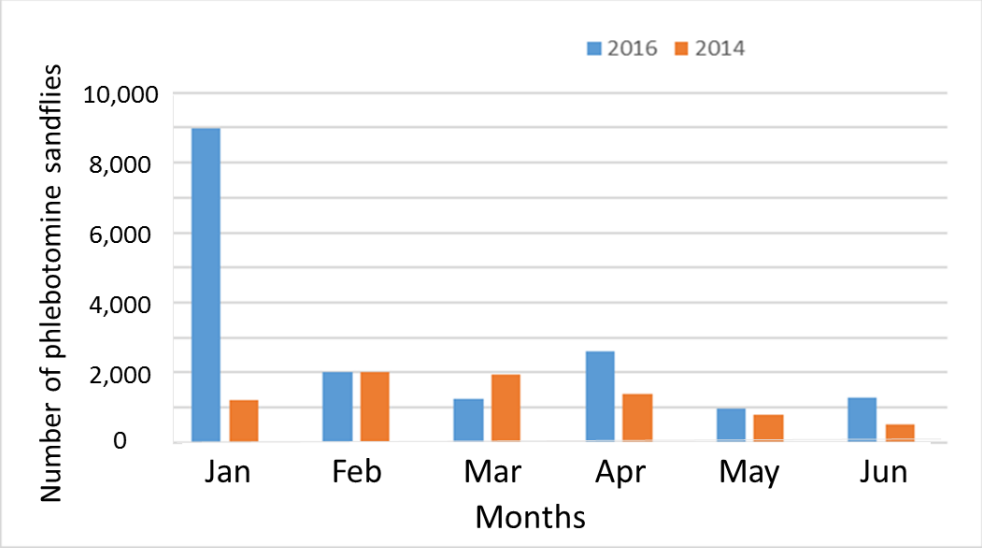

Figure 1. Number of phlebotomine sandflies captured in each month of sampling in six dwellings of Rio Verde do Mato Grosso municipality, Mato Grosso do Sul, Brazil, in 2014 and 2016

Table 1. Number of female sandfly pools tested with kDNA-qPCR and found positive for *Leishmania* spp. plus minimum infection rates (MIR) among phlebotomine sandflies captured in dwellings of six neighborhoods with recent *Leishmania* transmission in Rio Verde do Mato Grosso municipality, Mato Grosso, Brazil, in 2014 and 2016

Sandfly species	Dwelling*	Year				MIR (%)
		2014 pools		2016 pools		
		Tested	qPCR +	Tested	qPCR +	
Lutzomyia longipalpis	1	3	2	17	11	7
	2	16	7	4	3	6
	3	15	7	16	13	8
	4	16	5	18	15	6
	5	11	7	9	8	11
	6	12	4	8	10	13
Nyssomyia whitmani	1	0	-	0	-	-
	2	0	-	1	0	0
	3	1	0	1	1	10
	4	0	-	0	-	-
	5	2	1	0	-	7
	6	8	5	1	0	9

*One sampling dwelling in each of six neighborhoods with recent records of human leishmaniases.

MIR, minimum infection rate (see Methods)