

Original citation:

Pereira-Fonseca, D. C. M., Oliveira-Rovai, F. M., Rodas, L. A. C., Beloti, C. A. C., Torrecilha, R. B. P., Ito, P. K. R. K., Avanço, S. V., Cipriano, R. S., Utsunomiya, Y. T., Hiramoto, R. M., Calvo-Bado, Leo A., Courtenay, Orin, Machado, G. F., Lima, V. M. F. and Nunes, C. M.. (2017) Dog skin parasite load, TLR-2, IL-10 and TNF- α expression and infectiousness. *Parasite Immunology*, 39 (11). e12493.

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Dog skin parasite load, TLR-2, IL-10 and TNF- α expression and infectiousness

Journal:	<i>Parasite Immunology</i>
Manuscript ID	PIM-2016-0142.R3
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
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Key Words:	Leishmaniasis < Disease, Canine < Host species, Cytokine < Immunological terms, Leishmania spp < Parasite, Gene expression < Tools and techniques

1
2 1 **Effect of skin parasite load on TLR-2, IL-10 and TNF- α expression and infectiousness to**
3 2 **sandflies during canine leishmaniosis**
4 3

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19 18

20 19 **DISCLOSURES**

21 20 None.
22 21

23 22
24 23 **ABSTRACT**
25 24

26 25 Visceral leishmaniosis is a zoonotic disease that is transmitted by *Lutzomyia longipalpis* sandflies.
27 26 Dogs are the main peri-urban reservoir of the disease, and progression of canine leishmaniosis is
28 27 dependent on the type of immune response elaborated against the parasite. Type 1 immunity is
29 28 characterized by effective cellular response, with production of pro-inflammatory cytokines such as
30 29 tumor necrosis factor alpha (TNF- α). In contrast, Type 2 immunity is predominantly humoral,
31 30 associated with progression of the disease, and mediated by anti-inflammatory cytokines such as
32 31 interleukin 10 (IL-10). Although seemingly important in the dynamics of leishmaniosis, other gene
33 32 products such as toll-like receptor 2 (TLR-2) and inducible nitric oxide synthase (iNOS) exert unclear
34 33 roles in the determination of the type of immune response. Given that the dog skin serves as a micro-
35 34 environment for the multiplication of *Leishmania spp.*, we investigated the parasite load and the
36 35 expression of TLR-2, iNOS, IL-10 and TNF- α in the skin of 29 infected and eight control dogs. We
37 36 found that increased parasite load leads to up-regulation of TLR-2, IL-10 and TNF- α , indicating that
38 37 abundance of these transcripts is associated with infection. We also performed a xenodiagnosis to
39 38 demonstrate that increased parasitism is a risk factor for infectiousness to sandflies.
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INTRODUCTION

The domestic dog (*Canis lupus familiaris*) is the main peri-urban reservoir of leishmaniosis in Brazil¹. This zoonotic disease is caused by *Leishmania infantum* (Syn. *chagasi*), a protozoon transmitted to vertebrate hosts by female sandflies from the *Lutzomyia longipalpis* species². The progression and severity of canine leishmaniosis (CanL) has been argued to relate both to the infectivity of the parasite and the immunogenetic profile of the host³. Dogs that do not develop clinical disease usually present an effective cellular response characterized by Type 1 immunity, which is presumably mediated by pro-inflammatory cytokines such as interleukin 2 (IL-2), interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α)^{4,5}. On the other hand, sick dogs often exhibit predominance of a Type 2 profile, with expression of transforming growth factor beta (TGF- β) and interleukin 10 (IL-10), as well as an exacerbated humoral immune response due to B lymphocyte proliferation⁶.

The activation of Toll-like receptors has recently emerged as an important step in determining the type of immune response^{7,8,9} and therefore may have an impact on the development of the disease^{10,11}. Toll-like receptors are capable of recognizing lipophosphoglycan molecules present in the surface of the flagellated promastigote form of the protozoon¹², resulting in intracellular reactions responsible for the production of pro-inflammatory and anti-inflammatory cytokines^{13,14,15}. In particular, increased expression of Toll-like receptor 2 (TLR-2) has been related to a lower parasite load in the skin of naturally infected dogs, as well as to reduced infectiousness to the vector¹⁶. Of note, TLR-2 can affect the production of the inducible nitric oxide synthase (iNOS) enzyme, which catalyzes nitric oxide (NO) production^{17,18,19,20,21}. Conversely, TLR-2-deficient mice infected with *Leishmania donovani* showed enhanced macrophage antileishmanial activity with no changes in the liver expression of iNOS as compared to wild type mice²².

Considering the importance of the skin in the transmission of parasites of the *Leishmania* genus, we aimed at evaluating the expression of the immune response genes TLR-2, iNOS, TNF- α and IL-10 in the skin of dogs naturally affected by CanL and relate them to the skin parasite load (PL). We also investigated the relationship between skin PL and infectiousness to sandflies in a xenodiagnosis.

MATERIAL AND METHODS

Ethics statement

This research was approved by the local Ethics and Animal Welfare Committee (CEUA-FOA/FMVZ, process 01984-2012).

Study area

Araçatuba (latitude 21°2'3"S; longitude 50°5'58"W) is located in the Northwestern region of the state of São Paulo (Brazil), and it is an endemic area for leishmaniosis since 1999. The majority of the cases take the visceral form, whereas cutaneous leishmaniosis is sporadic in this area. A seroepidemiological survey is conducted annually by the Municipal Center for Zoonosis Control (CZC), and dogs that present positive serological diagnosis or amastigotes in a lymph node biopsy are recruited and culled after the consent of owners to comply with the local legislation.

Animals

Mongrel dogs from the CZC aging from 1 to 10 years were initially divided into two categories: (i) the control group (C), comprising eight dogs from an adoption program that were negative for both the serological test and the parasite quantification in blood and lymph node aspirates by quantitative polymerase chain reaction (qPCR); and the parasited group (P), including 29 naturally infected dogs

with positive serological and skin qPCR results. All dogs in group P presented clinical alterations compatible with CanL, including: onychogryphosis (n=25); dermatopathies as dermatitis, alopecia, hyperkeratosis or ulcerated lesions (n=24); lymphadenopathy (n=22); ocular lesions such as conjunctivitis, keratoconjunctivitis or uveitis (n=18); and cachexia (n=3). These animals were further classified as stage I, II or III according to Solano-Galego and collaborators²³.

Diagnosis of CanL

Anti-Leishmania antibodies were evaluated in serum samples by the TR-DPP® rapid test and confirmed by EIE-ELISA® (Bio-Manguinhos/Fiocruz-Rio de Janeiro). All samples were processed in duplicates following the manufacturer's instructions. Ear tip biopsies of uninjured skin of positive and negative dogs were taken with a 3 mm punch to assess the parasite load and the gene expression of TLR-2, iNOS, TNF- α and IL-10. These biopsies were taken immediately after euthanasia, following current legislation. All samples were stored at -80 °C in RNAlater-ICE (Ambion AM7030 - Life Technologies®/USA) until processing. The absence of infection in dogs serologically negative for CanL (controls) was confirmed in blood and lymph node samples by qPCR using primers described by Rodgers et al²⁴. DNA was extracted using the QIAamp DNA mini kit® (Qiagen, Hilden/Germany), according to the manufacturer's instructions. DNA was eluted in a final volume of 50 μ L for blood samples and 40 μ L for lymph node samples. These were evaluated in duplicate by qPCR according to using 40 ng of DNA. The reaction efficiency values, the coefficient of determination (r^2) and the slope (β) were assessed using a 10 fold serial dilution of *L. infantum* DNA (L579 MHOM/BR/1974/PP75).

Skin parasite load

DNA from skin samples was extracted using the Ear Tissue-ET kit (Chemagen cmg-1011, Perkin Elmer®), according to the manufacturer's instructions. The amplification of a 120 bp minicircle kinetoplast DNA (kDNA) fragment of *Leishmania spp.* was carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems®). Primers Leish1 (AACTTTTCTggTCCTCCgggTAG) and Leish2 (ACCCCAgTTTTCcGcC) described by Francino et al.²⁵ and modified by Calvo-Bado (unpublished) were used in a concentration of 900 nM each. Additionally, 250 nM of the probe 6FAM 5' AAAAATgggTgCAGAAACCCcGtTTC-3' – BBQ was used. The reaction was carried out in a total volume of 15 μ L containing 2x TaqMan master mix (Applied Biosystems®). Amplification conditions were 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Quantification of PL was performed by comparing the sample exponential phase threshold (Ct - cycle threshold) values with those obtained from a standard curve constructed from 10-fold serial dilutions (0.01 to 105 parasites/ml) of DNA extracted from cultured *L. infantum* (ITAMP DD8-263) promastigotes. All samples were assessed in triplicates.

Gene expression in the skin

Total RNA was extracted from skin samples and stored at -80 °C in RNAlater-ICE (Ambion AM7030-Life Technologies®/USA) using the RNeasy Mini Kit (74104-Qiagen®), according to the manufacturer's specifications. Total RNA was quantified by a NanoDrop® spectrophotometer ND-100 and used conditional on a 260/280 ratio between 2.0 and 2.3. For cDNA production, 200 ng of each RNA sample was subjected to reverse transcription using the QuantiTect Reverse Transcription commercial kit (205311-Qiagen®, Hilden/Germany), according to the manufacturer's specifications. Canine-specific primers for amplifying fragments of cDNA from TLR-2, iNOS, IL-10 and TNF- α , as well as from the reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT-1), were selected from the literature (Table 1). The qPCR assay was carried out using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories®-CA/USA), 320 nM of each primer and 1 μ L of cDNA in a total volume of 25 μ L. Duplicates were incubated at 95 °C for 30 seconds, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 10 seconds, when the fluorescence data were captured, followed by dissociation curve in

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2 137 65 °C to 95 °C, with an increase of 0.5 °C every 10 seconds. For each evaluated gene, values of the
3 138 reaction efficiency, r^2 and β were obtained from the amplification of a serial dilution of a cDNA pool
4 139 from both groups C and P (**Table 1**). Quantification of gene expression was performed using the $2^{-\Delta\Delta C_t}$
5 140 method²⁶, using HPRT-1 as a reference gene. The results were expressed as the relative gene
6 141 expression that indicated how many times (fold change) the cytokine gene expression was higher or
7 142 lower in the infected dogs in relation to the control group. The gene expression of TLR-2, IL-10 and
8 143 TNF- α was evaluated in 29 dogs and the expression of iNOS was evaluated only in 24 infected dogs,
9 144 apart from eight control dogs.
10 145

13 146 **Xenodiagnosis**

14 147 Adult sandflies were field-caught by manual aspiration in urban areas of Araçatuba. To facilitate
15 148 capture, collections were carried out in households sheltering at least one chicken, as the latter favors
16 149 the aggregation of phlebotomines². Sandflies were transferred to 20 x 20 cm fine-mesh cages and kept
17 150 at 86% humidity and 25.5 °C in a climate-controlled unit (BOD - Electro lab - 101 M). Sandflies were
18 151 maintained on a diet of honey solution (1:1 in distilled water) for 12 hours before being separated into
19 152 tested and control groups. At 12 hours, to enhance the chance of sandflies feeding during the
20 153 xenodiagnosis, only non-blood-fed and partially fed (maximum half of the midgut) female field-caught
21 154 sandflies were separated for blood feeding on a dog from group P (xenodiagnosis). Engorged field-
22 155 caught individuals were reserved as naturally infected specimens that would display only natural
23 156 infection (control group of sandflies), and were offered only honey solution. An equal number of males
24 157 was also separated into the cages in order to maximize normal behavior and survival. Following
25 158 xenodiagnosis, sandflies were recovered and both the tested and control groups were maintained for
26 159 five days under the aforementioned conditions. Each infected dog was restrained in a metal cage,
27 160 covered by another fine-mesh net for 12 hours (covering periods of crepuscular sandfly activity), with
28 161 an average of 30 *L. longipalpis* females and accompanying males. A total of 29 xenodigamoses were
29 162 performed between November 2012 and May 2014. No xenodiagnosis was performed on negative dogs
30 163 (n=8) due to the fact that female sandflies were field-caught and could transmit the parasite to healthy
31 164 dogs. Due to variations on density of sandflies during captures, we analyzed data of a subset of 18
32 165 xenodiagnosis, for which the average number of control and tested sandflies were 23.4 ± 15.2 (min: 2 /
33 166 max: 50) and 27.4 ± 9.4 (min: 13 / max: 47), respectively.
34 167

39 168 **Parasite load in sandflies**

40 169 DNA extraction was performed in individual sandflies using the protocol of Kato et al.²⁷ with
41 170 modifications: dissected sandflies were centrifuged for 2 minutes at 15871 g and macerated with a
42 171 pestle and glass beads (2 mm). After discarding the supernatant, 75 μ L of the lysis buffer solution (150
43 172 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.1% SDS and 20 μ L of proteinase K -
44 173 Ambion®-D10860413) were added, followed by incubation for 6 hours at 65°C. Samples were then
45 174 centrifuged for 2 minutes at 15871 g, and 35 μ L sterile water added and mixed thoroughly, followed by
46 175 further centrifugation (15871 g for 2 minutes). The supernatant containing DNA was transferred to
47 176 another tube. The qPCR procedure was performed as described for the dog skin. Sandflies were then
48 177 classified as “infected” if their PL were different from zero and as “non-infected” otherwise for part of
49 178 the downstream analyses. All samples were assessed in triplicates.
50 179

53 180 **Statistical analysis**

54 181 In order to assess the influence of increased skin parasitism on gene expression, dogs from group P
55 182 were further divided into two subgroups based on a median split on PL values. Therefore, animals were
56 183 allocated to one of the following three groups for statistical analysis: C = eight control uninfected dogs,
57 184 as defined earlier; MP = 11 dogs from group P with PL values between 2.19×10^2 and 2.27×10^3
58 185 parasites/mL ; and HP = 18 dogs from group P with PL values between 2.27×10^5 and 4.13×10^7
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 2 186 parasites/mL. Distribution of PL values according to group is displayed in **Supplementary Figure 1**.
 3 187 An analysis of variance (ANOVA) with a *post hoc* Tukey's Honest Significant Difference (HSD) test
 4 188 was used to assess differences in $-\Delta\text{Ct}$ between groups. For the genes exhibiting significant differences
 5 189 ($p < 0.05$) among groups, we merged MP and HP back to a single parasited group (P) in order to
 6 190 examine the fold change in expression relative to group C using the $2^{-\Delta\Delta\text{Ct}}$ method²⁶. We also applied
 7 191 logistic regression models to the xenodiagnosis data considering a binary response variable encoded as
 8 192 1 = "infected sandfly" and 0 = "non-infected sandfly", according to qPCR results. In the first model,
 9 193 we examined the contribution of exposure to infected dogs to the probability of sandfly infection by
 10 194 including an indicator variable assuming values 0 and 1 for control and tested sandflies, respectively.
 11 195 Additionally, we performed a permutation test for differences in prevalence between control and tested
 12 196 sandflies with 10^6 randomizations of the data. Then, we used data only on tested sandflies to fit skin
 13 197 PL as a covariate in the logistic model to assess the influence of the level of skin parasitism on the
 14 198 probability of sandflies being infected. Prior to this analysis, skin PL was transformed to a $\log_{10}(1 +$
 15 199 PL) scale. Finally, we compared the ranks of parasite load of sandflies fed on HP dogs with those that
 16 200 were fed on MP dogs with a Mann-Whitney U test to evaluate whether parasite load in phlebotomines
 17 201 was dependent on parasite load in dogs. All analyses were performed in R v3.3.0 (available at:
 18 202 <https://www.r-project.org/>).
 19 203

20 204 RESULTS

21 205 **Increased load of *Leishmania* leads to up-regulation of IL-10, TNF- α and TLR-2 in the dog skin**

22 206 We used ANOVA with Tukey's HSD to compare the skin relative expression of iNOS, IL-10, TNF- α
 23 207 and TLR-2 among three groups of dogs (see details in Material and Methods): uninfected controls
 24 208 (group C, $n = 8$), animals with low-to-moderate infection (group MP, $n = 11$) and animals with
 25 209 moderate-to-high infection (HP, $n = 18$). Expression levels differed between groups only for IL-10
 26 210 ($p_{\text{ANOVA}} = 0.008$), TNF- α ($p_{\text{ANOVA}} = 0.007$) and TLR-2 ($p_{\text{ANOVA}} = 5.8 \times 10^{-6}$), with up-regulation of
 27 211 these transcripts following increased PL (**Figure 1**). Tukey's HSD was only significant ($p_{\text{HSD}} < 0.05$) in
 28 212 the comparisons of groups MP-HP for IL-10 ($p_{\text{HSD}} = 0.008$), C-HP for TNF- α ($p_{\text{HSD}} = 0.009$), and C-
 29 213 HP ($p_{\text{HSD}} = 6.6 \times 10^{-5}$) and MP-HP ($p_{\text{HSD}} = 9.1 \times 10^{-5}$) for TLR-2. The occurrence of significant
 30 214 differences for only a subset of the between-groups comparisons for each gene suggested that
 31 215 expression varied non-linearly as PL increased. In particular, we noticed that the standard deviation in
 32 216 MP was greater than in HP and C for all the three significant transcripts, which indicates that increased
 33 217 parasitism could affect not only the mean but also the variance of gene expression, especially for the
 34 218 group of dogs with low-to-moderate infection. In this case, increased variance in MP could lead to loss
 35 219 of power to detect mean differences between groups at small sample sizes. Considering the whole
 36 220 group of infected animals ($P = \text{MP} + \text{HP}$), the $2^{-\Delta\Delta\text{Ct}}$ method revealed that expression of IL-10, TNF- α
 37 221 and TLR-2 was 1.43, 2.66 and 2.55 fold greater in infected dogs as compared to controls. Moreover,
 38 222 expression of these three genes was highly correlated, with pairwise values of $r_{\text{IL-10/TNF-}\alpha} = 0.744$ ($p_{\text{cor}} =$
 39 223 1.30×10^{-7}), $r_{\text{IL-10/TLR-2}} = 0.706$ ($p_{\text{cor}} = 1.05 \times 10^{-5}$) and $r_{\text{TNF-}\alpha/\text{TLR-2}} = 0.678$ ($p_{\text{cor}} = 3.97 \times 10^{-6}$).
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42 226 **Skin parasitism by *Leishmania* is positively associated with infectiousness to sandflies**

43 227 We fitted a logistic regression model to assess the contribution of exposure to infected dogs to the
 44 228 probability of sandflies being infected. This analysis was intended to evaluate whether the
 45 229 xenodiagnosis was capable of increasing prevalence of *Leishmania* in exposed sandflies in comparison
 46 230 to control sandflies. We found that the odds of sandfly infection were 4.63 times higher ($p_{\text{logit}} = 7.62 \times$
 47 231 10^{-22}) given exposure to infected dogs as compared to no exposure, with a 95% confidence interval of
 48 232 3.39 – 6.33. A permutation test for the difference in prevalence between control and tested sandflies
 49 233 was also significant (**Figure 2 – a**), with no larger than observed differences arising by chance in one
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2 234 million randomizations of the data ($p_{\text{perm}} < 10^{-6}$). Given that the xenodiagnosis was successful in
3 235 shifting the prevalence of *Leishmania* in sandflies, we sought to evaluate the contribution of skin PL to
4 236 the probability of a sandfly being infected after xenodiagnosis. This analysis revealed that sandflies
5 237 became significantly ($p_{\text{logit}} = 0.009$) more prone to infection as the dog skin PL increased (**Figure 2 – b**
6 238 **and c, Supplementary Figure 2**). Moreover, phlebotomines exposed to HP dogs had a suggestively
7 239 higher PL than those exposed to MP dogs ($p_{\text{MW}} = 0.099$), indicating a potential association between
8 240 skin and phlebotomine PL.

11 242 DISCUSSION

12 243
13 244 In the present study we found evidence for up-regulation of the immune response genes TLR-2, TNF- α
14 245 and IL-10 in the skin of dogs naturally affected by CanL. We also found that this up-regulation was
15 246 positively associated with parasite load. Furthermore, elevated skin parasitism significantly increased
16 247 infectiousness to phlebotomines, supporting the hypothesis that dogs with higher skin PL have a greater
17 248 potential to spread the disease.

18 249
19 250 Increased expression of TLR-2 during canine leishmaniosis has been previously found in several
20 251 tissues, including skin^{28,29}, blood³⁰, liver²⁹, brain, spleen and lymph node³¹. Here, in addition to a higher
21 252 relative expression of TLR-2 in affected dogs, we observed a significant difference in transcript
22 253 abundance between dogs with low-to-moderate and moderate-to-high parasite load. A similar result
23 254 was observed by Figueiredo et al.³² in colon samples of dogs naturally infected with *L. infantum*, which
24 255 suggests a correlation between TLR-2 expression and the progression of infection. Indeed,
25 256 Monteserrat-Sangrà et al.³⁰ showed a positive correlation between TLR-2 and PL in unstimulated blood
26 257 samples from dogs with CanL. When these dogs were treated and showed clinical improvement, TLR-2
27 258 transcription was reduced. This is in agreement with findings reported by Murray et al.²², which
28 259 showed that blockage of TLR-2 leads to induction of *Leishmania donovani* killing in mice. However,
29 260 these results are not in concordance with those found by Amorim et al.¹⁶, which reported a higher TLR-
30 261 2 expression in monocytes associated to a lower parasite load in the skin of naturally infected dogs.
31 262 Moreover, Turchetti et al.¹⁰ did not observe interference of constitutive transcription of toll-like
32 263 receptors in intracellular survival of *L. infantum* tested *in vitro*.

33 264
34 265 Although TLR-2 is deemed to alter production of NO^{17,18}, our data did not support a correlation with
35 266 changes in expression of iNOS, in agreement with observations made in TLR-2-deficient mice²². Of
36 267 note, TNF- α has also been previously suggested to modulate induction of iNOS^{20,33,34,35}, while IL-10
37 268 was reported to have inhibitory effects on the production of the enzyme^{19,33,36,37}. Since TNF- α (pro-
38 269 inflammatory) and IL-10 (anti-inflammatory) have putative antagonistic effects on iNOS production,
39 270 and both transcripts were up-regulated in infected dogs in this study, we speculate whether expression
40 271 of iNOS in infected dogs has not significantly deviated from control levels as a result of an interplay
41 272 between TNF- α and IL-10.

42 273
43 274 We found that TLR-2 expression was positively correlated with TNF- α and IL-10. Gatto et al.¹⁷
44 275 observed similar results in human patients with active visceral leishmaniosis. These results indicate that
45 276 TLR-2 could be regulating TNF- α and IL-10 in an attempt to combat infection. Chandra and Naik³⁸
46 277 also observed an increased level of IL-10 when cells of patients were stimulated with TLR-2 agonists.
47 278 These data sustain the involvement of TLR-2 in the skin adaptive response to the parasite and the
48 279 subsequent production of pro-inflammatory and anti-inflammatory cytokines. As a putative
49 280 downstream effect, the increase in TNF- α expression may help recruiting cells to the infection site.
50 281 Indeed, an inflammatory infiltrate with predominance of macrophages was observed in the histological

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2 282 examination of the skin samples (see **Supplementary Figure 3**).
3 283

4 284 Our xenodiagnosis analysis revealed an increased risk of sandflies becoming infected when exposed to
5 285 dogs with increased skin parasite load. An association of dog infectiousness to sandflies with high skin
6 286 parasite numbers tested by qPCR was also found by Courtenay et al.³⁹ and Borja et al.⁴⁰. Furthermore,
7 287 a positive correlation of skin parasite load of infected dogs with the number of infected sandflies was
8 288 also demonstrated by Verçosa et al.⁴¹ and Amorim et al.¹⁶ although parasite load on sandflies was
9 289 assessed by the presence of amastigotes in the midgut. *A priori*, our result differs from that reported by
10 290 Laurenti et al.⁴², where asymptomatic dogs were found to be more competent in transmitting *L.*
11 291 *infantum* to phlebotomines than symptomatic dogs. However, in their morphometric quantitative
12 292 analysis, they did not find significant differences in the amount of amastigotes/mm² of skin between
13 293 symptomatic and asymptomatic dogs, which imposes a challenge in the comparison of our data with
14 294 theirs. Nevertheless, our approach of assessing the direct contribution of skin parasite load, as
15 295 quantified by qPCR, to the probability of a sandfly becoming infected should provide a more robust
16 296 evaluation of the influence of skin parasitism on the infectiousness to phlebotomines.
17 297

18 298 Finally, some limitations of our study should be highlighted. First, we evaluated the expression of
19 299 genes and not the production of cytokines. Second, dogs were naturally infected and, therefore, were at
20 300 various stages of the disease. Whether or not our results are replicable when cytokines are directly
21 301 evaluated in dogs with well-characterized disease stages is yet to be clarified. Altogether, our study
22 302 contributes with further insights on the dynamics of parasite load and immune response in the tissue
23 303 compartment that serves as the feeding site to the vector and as the first inoculation point in the
24 304 reservoir of leishmaniasis.
25 305

26 306 ACKNOWLEDGEMENTS

27 307
28 308 To Professor Marcelo Vasconcelos Meireles for bioinformatic assistance; to the Biochemistry and
29 309 Molecular Biology and the Immunology and Applied Pathology Laboratories for their collaboration in
30 310 laboratory procedures; to the Araçatuba Center for Zoonosis Control for dog management; to the
31 311 Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to
32 312 the first author; and to the anonymous Reviewers for their suggestions.
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2 451 **FIGURE LEGENDS**
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4 453 **Figure 1.** Dotplots of IL-10, TNF- α and TLR-2 expression ($-\Delta\text{Ct}$) in the skin of control (C, 0
5 454 parasites/mL), moderately parasited (MP, 2.19×10^2 to 2.27×10^5 parasites/mL) and highly parasited
6 455 (HP, 2.27×10^5 to 4.13×10^7 parasites/mL) dogs naturally affected by leishmaniosis. Horizontal black
7 456 bars represent medians within groups. Significance values were assessed on the basis of a *post hoc*
8 457 Tukey's Honest Significant Difference (HSD) test.
9 458

10 459 **Figure 2.** Xenodiagnosis of field-caught phlebotomines. (a) Sandflies subjected to a xenodiagnosis
11 460 (tested) presented significantly higher prevalence of *Leishmania spp.* than those fed with honey
12 461 solution (control), as assessed by a permutation test with one million randomizations of the data. (b) A
13 462 logistic regression analysis revealed that prevalence in phlebotomines exposed to infected dogs was
14 463 significantly ($p = 0.009$) dependent on the parasite load of the skin. (c) Sandflies were more prone to
15 464 infection when exposed to dogs with increased skin parasite load.
16 465

17 466 **Supplementary Figure 1.** Distribution of parasite load values according to dog group. C = eight
18 467 control uninfected dogs; MP = 11 dogs with parasite load between 2.19×10^2 and 2.27×10^5
19 468 parasites/mL; and HP = 18 dogs with parasite load between 2.27×10^5 and 4.13×10^7 parasites/mL.
20 469 Horizontal black bars represent medians within groups.
21 470

22 471 **Supplementary Figure 2.** Locally weighed regression (LOESS) of percentage of infected
23 472 phlebotomines onto parasite load in the dog skin.
24 473

25 474 **Supplementary Figure 3.** Histological section of skin of a xenodiagnosed dog with leishmaniosis
26 475 (stained by HE). (a) Diffuse mononuclear infiltrate (lymphocytes and macrophages) expanding the
27 476 dermis, and presence of multinucleated giant cells (arrow). (b) Detail of marked area in (a) where it is
28 477 possible to observe rare neutrophils (arrow) and presence of amastigotes in the cytoplasm of
29 478 macrophages (*).
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Table 1. Primers for the evaluation of the expression of immune genes in the skin

Target	Nucleotides Sequence (5' → 3')	GenBank accession number	Product size (bp)	Reaction efficiency (E)	r ²	Slope	Reference
HPRT-1	F - CACTGGGAAAACAATGCAGA	AY283372	123	105.5	0.986	-3.191	Peters et al. ⁴⁰
	R - ACAAAGTCAGGTTTATAGCCAACA						
TLR-2	F - TCGAGAAGAGCCACAAAACC	NM_001005264.2	91	108.8	0.981	-3.127	Mercier et al. ⁴¹
	R - CGAAAATGGGAGAAGTCCAG						
iNOS	F - AGACACACTTCACCACAAGG	AF077821	285	107	0.848	-3.165	Kaim et al. ⁴²
	R - TGCTTGGTGGCGAAGATGAGC						
IL-10	F - CGACCCAGACATCAAGAACC	U33843	101	104.9	0.986	-3.209	Peters et al. ⁴³
	R - CACAGGGAAGAAATCGGTGA						
TNF-α	F - ACCCATGTGCTCCTCACC	Z70046	87	100.7	0.946	-3.305	Melo et al. ⁴⁴
	R - AGGGCTCTTGATGGCAGAGA						

Or Peer Review

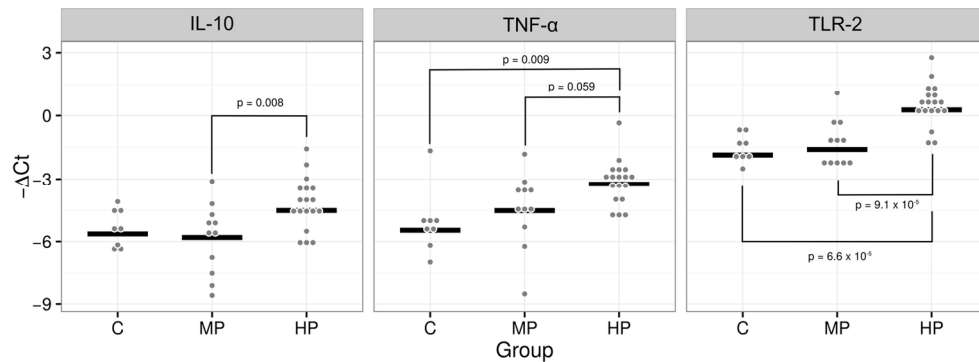
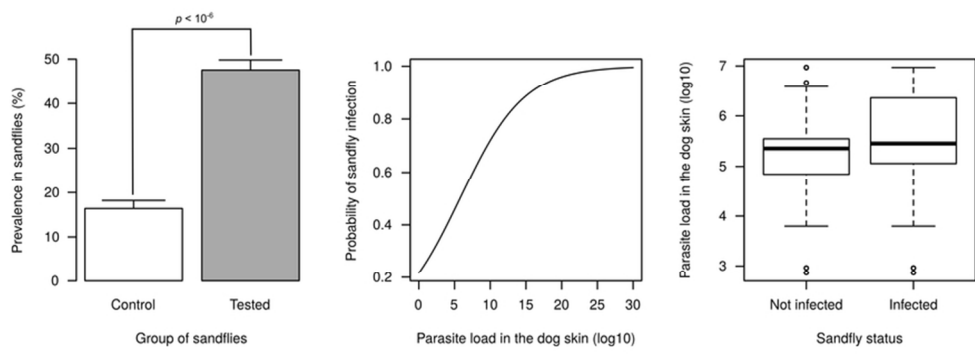


Figure 1. Dotplots of IL-10, TNF- α and TLR-2 expression ($-\Delta Ct$) in the skin of control (C, 0 parasites/mL), moderately parasited (MP, 2.19×10^2 to 2.27×10^5 parasites/mL) and highly parasited (HP, 2.27×10^5 to 4.13×10^7 parasites/mL) dogs naturally affected by leishmaniosis. Horizontal black bars represent medians within groups. Significance values were assessed on the basis of a post hoc Tukey's Honest Significant Difference (HSD) test.

76x28mm (600 x 600 DPI)

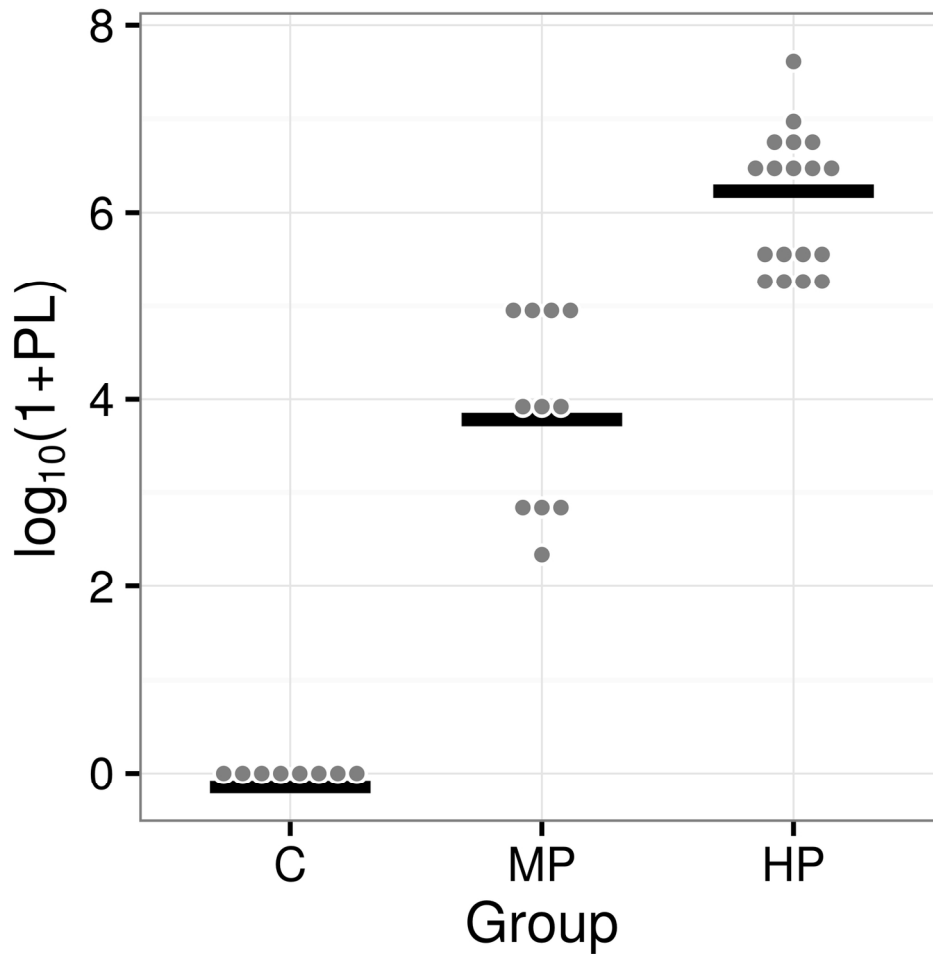
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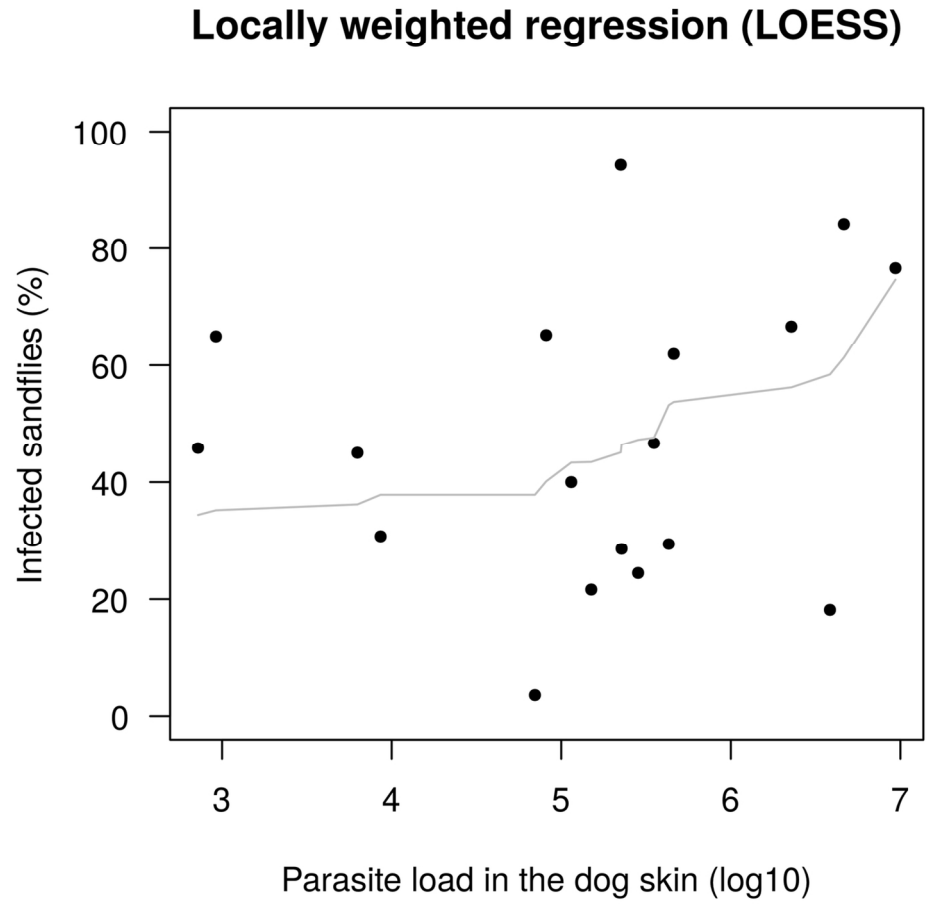
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Supplementary Figure 1. Distribution of parasite load values according to dog group. C = eight control uninfected dogs; MP = 11 dogs with parasite load between 2.19×10^2 and 2.27×10^5 parasites/mL; and HP = 18 dogs with parasite load between 2.27×10^5 and 4.13×10^7 parasites/mL. Horizontal black bars represent medians within groups.

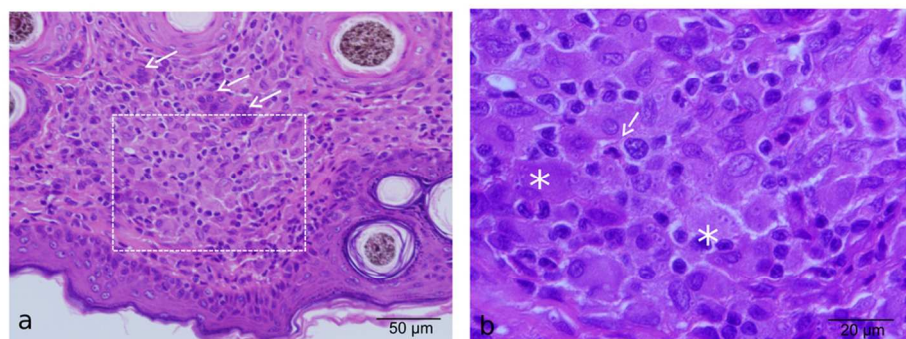
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Supplementary Figure 2. Locally weighed regression (LOESS) of percentage of infected phlebotomines onto parasite load in the dog skin.

127x127mm (300 x 300 DPI)



Supplementary Figure 23. Histological section of skin of a xenodiagnosed dog with leishmaniasis (stained by HE). (a) Diffuse mononuclear infiltrate (lymphocytes and macrophages) expanding the dermis, and presence of multinucleated giant cells (arrow). (b) Detail of marked area in (a) where it is possible to observe rare neutrophils (arrow) and presence of amastigotes in the cytoplasm of macrophages (*).

99x39mm (300 x 300 DPI)

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