



RESEARCH ARTICLE

Necropsies disclose a low helminth parasite diversity in periurban howler monkeys

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Abstract

Primate–parasite interactions are often investigated via coprological studies given ethical and conservation restrictions of collecting primate hosts. Yet, these studies are inadequate to recover adult helminths for taxonomic identification and to accurately assess their prevalence, intensity, abundance, and site of infection. Fresh carcasses found in anthropogenic landscapes come as informative and reliable alternatives. In this study, we identified the helminths of brown howler monkeys (*Alouatta guariba clamitans*) and their sites of infection, and measured their prevalence, intensity, and abundance of infection. We necropsied 18 adult males, 11 adult females, and 7 juvenile males that died in conflicts with the anthropogenic environment (domestic dog attacks, $n = 11$; electrocutions and road-kills, $n = 10$ each; unknown, $n = 5$) in periurban landscapes of southern Brazil between 2013 and 2019. We found three nematodes (*Trypanoxyuris minutus*, *Dipetalonema gracile*, and *Parabronema bonnei*) and one cestode (*Bertiella* cf. *studer*), a diversity estimated to account for a sampling completeness of 99%. Prevalence ranged from 3% for *P. bonnei* to 100% for *T. minutus*. Mean abundance ranged from 2 (*D. gracile* and *B. cf. studeri*) to 55,116 (*T. minutus*) and mean intensity of infection ranged from 4 (*B. cf. studeri*) to 55,116 (*T. minutus*). *Trypanoxyuris minutus* sex ratio was strongly male-biased. The intensity of infection with *T. minutus* was higher in juvenile males and adult females than in adult males. The low parasite diversity and the helminths' mode of transmission are compatible with howlers' arboreality and folivorous–frugivorous diet. The howlers were not infected with soil-transmitted helminth parasites of humans and domestic animals on the ground and probably did not eat invertebrates to complement the diet. Given the lack of evidence of howler health problems, we suggest that the causes of death of the necropsied howlers are the major threats to the long-term conservation of the species at the study periurban landscapes.

KEYWORDS

Alouatta guariba clamitans, *Bertiella* cf. *studer*, Brazil, *Dipetalonema gracile*, *Parabronema bonnei*, *Trypanoxyuris minutus*

1 | INTRODUCTION

Parasites are important sources of information on the ecology and behavior of their hosts (Combes, 2004). They are particularly informative for disclosing aspects that are difficult or impossible to record via behavioral observations, such as the identity of diminutive or concealed prey species (Calegario-Marques & Amato, 2010b, 2013) or the hosts' sex of monomorphic species (Silveira & Calegario-Marques, 2016) among other limitations inherent to field research.

Whereas parasitological studies on many free-ranging species often involve the necropsy of collected specimens (e.g., Calegario-Marques & Amato, 2010a; Kusmierik et al., 2020; Santos et al., 2013), ethical and conservation restrictions make unacceptable the collection of specimens belonging to rare, threatened and long-lived vertebrate taxa among others (e.g., Solórzano-García & Pérez-Ponce de León, 2018; Wolfe et al., 2001). Therefore, the interactions of these hosts with their parasites are often investigated via the examination of fecal samples (e.g., Aznar et al., 2012; Figueiredo et al., 2019; Paččo et al., 2018).

In the case of helminth parasites, coprological studies are inadequate to recover adult specimens for identification at lower taxonomic levels (Solórzano-García & Pérez-Ponce de León, 2017). They are also inappropriate for assessing the parasites' prevalence (Stuart et al., 1990), intensity, abundance, and site of infection. For instance, given that some parasites do not lay eggs on a daily basis, multiple fecal samples from the same individual may be necessary to detect an infection (Gillespie, 2006). Whereas modern techniques of metagenomics can fulfil the task of reliably classifying eggs and larvae of species found in feces that are available in parasite genetic libraries (Srivathsan et al., 2019), they cannot address the other aforementioned parameters. Given these limitations, the dissection of fresh host carcasses comes as an informative and reliable alternative.

Nonhuman primate populations inhabiting anthropogenic landscapes are particularly vulnerable to conflicts with humans, domestic animals, and the built environment. Roadkills, domestic dog attacks and electrocutions are major unintentional causes of primate death in these landscapes (Bicca-Marques, 2017; Chaves et al., in preparation; Gordo et al., 2013). In Latin America, the highly arboreal howler monkeys (*Alouatta* spp.) face these risks in anthropogenic landscapes while moving on the ground (Bicca-Marques & Calegario-Marques, 1995; Chaves et al., 2021, in preparation; Pozo-Montuy & Serio-Silva, 2007; Prates & Bicca-Marques, 2008) or on power lines (Jerusalinsky et al., 2010; Printes et al., 2010) to cross canopy gaps or the matrix between isolated habitat patches (Bicca-Marques et al., 2020; Serio-Silva et al., 2019).

Ground use and food provisioning by people in these landscapes (Back & Bicca-Marques, 2019) can also expose howlers to the infective stages of generalist parasites that infect humans and domestic animals (Cristóbal-Azkarate et al., 2015; Kowalewski & Gillespie, 2009; Nunn & Altizer, 2006; Werner & Nunn, 2020). This exposure may explain why about half of the parasites found in their fecal samples are shared with humans (Vitazkova, 2009). Although events of intentional consumption of animal matter have been reported (Bicca-Marques et al., 2009, 2014;

Queiroz, 1995), their often exclusive plant-based diet (Chaves & Bicca-Marques, 2013, 2016; Dias & Rangel-Negrín, 2015) reduces howlers' exposure to parasites transmitted via ingestion of intermediate hosts compared to insectivorous and omnivorous species.

The parasite fauna of howlers, the best studied among Platyrrhini primates (Solórzano-García & Pérez-Ponce de León, 2018), includes viruses, bacteria, fungi, protists, helminths and arthropods (Stuart et al., 1998). The known helminth parasites of howlers include 25 genera: the nematodes *Ancylostoma*, *Ascaris*, *Capillaria*, *Dipetalonema*, *Enterobius*, *Filaria*, *Filariopsis*, *Hassalstrongylus* (formerly *Longistriata*), *Mansonella*, *Necator*, *Oesophagostomum*, *Parabronema*, *Physaloptera*, *Strongyloides*, *Trichostrongylus*, *Trichuris*, *Trypanoxyuris*, and *Vianella*; the cestodes *Bertiella*, *Mathevotaenia*, *Moniezia* and *Raillietina*; the trematodes *Athesmia* and *Controrchis*; and the acantocephalan *Prosthenorchis* (revised by Solórzano-García & Pérez-Ponce de León, 2018; Stuart et al., 1998; Vitazkova, 2009). The infection with these helminths occurs via ingestion of eggs or larvae, often found in fecal remains or soil (nine genera), ingestion of intermediate hosts (eight), arthropod vector biting (three), active skin penetration by larvae found in the soil (three) or ingestion of eggs in the canopy (two). Most coprological studies have shown no differences in the prevalence of parasites between the sexes of adult howlers (e.g., *Alouatta palliata*, Cristóbal-Azkarate et al., 2010; Stoner, 1996; *Alouatta pigra*, Behie et al., 2014; Trejo-Macias & Estrada, 2012; Vitazkova & Wade, 2007), but there is some evidence of adult males with a greater parasite prevalence than adult females (*A. palliata*, Trejo-Macias & Estrada, 2012).

We studied the helminth fauna of brown howler monkeys (*Alouatta guariba clamitans*) that lived in forest fragments immersed in an anthropogenic matrix in south Brazil. Specifically, we assessed (i) the identity of the helminths to the species level and their site of infection, (ii) the helminth diversity and estimated the completeness of the sample, (iii) the prevalence, intensity and abundance of infection with each helminth, and (iv) we compared the intensity of infection among host age-sex classes. We expected that the peri-urban howler monkeys would host soil-transmitted helminths, possibly shared with humans or domestic animals, and that the prevalence of parasites would not differ between adult female and adult male hosts.

2 | METHODS

2.1 | Ethical statement

Studies using carcasses do not require a permit according to Brazilian Environmental Laws. However, we registered the study in the Brazilian Biodiversity Information System (SISBio) under the #71048 as recommended in the Normative Instruction #3 of the Chico Mendes Institute of Biodiversity Conservation (ICMBio) published on 1st September 2014 and in the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) protocol #A85A69B. We also adhered to the American Society of

Primatologists Code of Best Practices for Field Primatology (http://www.asp.org/resources/docs/Code%20of_Best_Practices%20Oct%202014.pdf).

2.2 | Carcass sample

We necropsied and inspected all organs and body cavities of 36 brown howler monkeys (18 adult males, 11 adult females, and 7 juvenile males) that died between 2013 and 2019. All individuals, except one adult male, died in periurban anthropogenic matrices of the metropolitan region of Porto Alegre and Viamão (30°01'23.9"–30°20'57.2"S, 50°59'44.5"–51°11'42.6"W), Rio Grande do Sul state, Brazil. The remaining adult male died in a road (BR-290, km 189; 30°09'17"S, 52°06'17"W) in Minas do Leão, about 100 km away from the other howler monkey casualties (Figure 1). The study region is near the southern limit of the distribution of the species and nonhuman primates in the Americas (Culot et al., 2019). We determined the age class of each specimen based on body size (length and mass) and pelage color. The main causes of death were domestic dog attacks ($n = 11$), electrocutions and road-kills ($n = 10$ each). The causes of death of the remaining five individuals were not reported and could not be determined, but they probably fell into one of the above categories. We deposited the carcasses in the Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul and the Museu de Ciências Naturais of the Fundação Zoobotânica do Rio Grande do Sul in Porto Alegre.

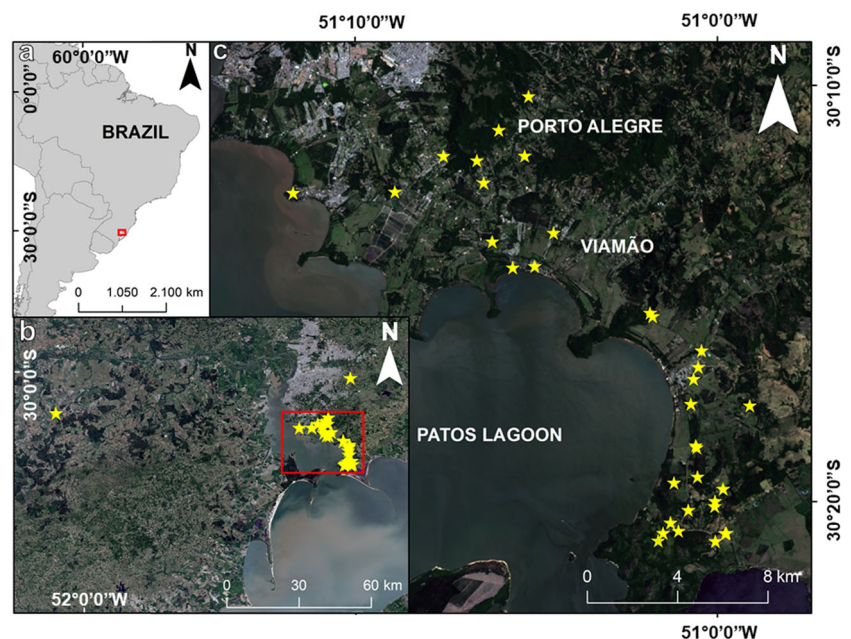
2.3 | Parasitological methods

We necropsied the corpses and processed the helminths following established protocols (Amato & Amato, 2010). These protocols

included the examination of nostrils and mouth with a brush and the washing of these cavities with saline solution 0.85% before dissection. Upon carcass dissection, we used the same technique to examine the abdominal cavity. We made a longitudinal incision in the tubular organs with scissors and we lacerated the massive organs with the help of tweezers, scissors, and teasing needles. After removing the contents of each organ, we thoroughly washed it with water and examined the water and the organ's mucosa under a stereomicroscope for collecting any specimen that was not freed during washing. Then, we inspected the submucosa against a light source. We also washed all fecal matter found in all organs in a 154- μ m-mesh sieve. This processing is adequate to retain minute helminths, such as *Trypanoxyuris minutus* and *Strongyloides cebus*. After this process, we carefully examined the material under a stereomicroscope using $\times 10$ and $\times 25$ magnifications. We stored the contents of organs with high parasitic loads in glasses containing AFA (ethanol 70°GL, formalin 37%–40%, and glacial acetic acid) for 48 h for fixation before transferring them to another recipient filled with ethanol 70°GL. We immersed cestode specimens in cleaning solution for 30 min before extending and compressing them between two microscope slides in petri dish with AFA for 30 min. Then, we immersed them in AFA for 48 h for fixation before storing them in ethanol 70°GL until staining. We stained cestodes with Delafield hematoxilin, cleared them in cedar oil, and mounted them in slides using Canada balsam. We also fixed nematode specimens in AFA for 48 h and stored them in ethanol 70°GL. We clarified nematodes in Amann's lactophenol and mounted them in temporary slides for analysis.

We calculated the prevalence (percentage of infected hosts in the sample), intensity of infection (number of individuals of a given parasite in infected hosts), and mean abundance of infection (mean number of parasites of a given species per infected or noninfected host) of each helminth parasite (Bush et al., 1997). We estimated the intensity of infection with *T. minutus* in 34 carcasses by homogenizing

FIGURE 1 Distribution of the brown howler monkeys that died in conflicts within the anthropogenic landscape. (a) Study region in Rio Grande do Sul state, Brazil (red rectangle). (b) Locations in Minas do Leão (single star to the left) and in Porto Alegre and Viamão (stars to the right). (c) Locations of the carcasses shown inside the red rectangle in (b)



the sieved gut content in 1 L of tap water, taking a subsample of 25 mL (2.5%), adding a volume of ethanol 70°GL sufficient to avoid missing individuals amidst plant fiber remains and, then, counting the numbers of females and males separately (females are ca. three times larger than males; Amato et al., 2002) under a stereomicroscope using $\times 10$ and $\times 25$ magnifications. We filtered the remaining sample in a 125- μm -mesh sieve to recover all individuals for storage in ethanol 70°GL.

2.4 | Data analysis

We assessed the helminth species diversity (i.e., Hill diversity order $q = 0$, which considers rare, common, and dominant species together; Jost, 2007) and the completeness of our sample (i.e., the likelihood that the sample was sufficiently large to detect all helminth species of the population) using the functions “iNEXT,” “ChaoRichness,” and “estimateD” of the R package iNEXT (Hsieh et al., 2016). We compared the helminth diversity of age-sex classes using rarefaction curves via the function “ggiNEXT” of the same R package. We analyzed the data in terms of occurrence (“incidence_freq,” Hsieh et al., 2016) because of marked differences in the intensity of infection with the helminth taxa. We excluded the adult male carcass from Minas do Leão from these analyses because the individual came from a distinct region.

Given the small sample size of each host's age-sex class, whenever appropriate we compared their helminth species prevalence by estimating the 95% confidence interval (CI) via bootstrap analysis (1000 simulations). We run this analysis using the functions “boot” and “boot.ci” of the R package.

We compared the intensity of infection of each age-sex class with each helminth species by running a zero-inflation General Linear Model (ZI-GLM) of 1000 bootstrapped carcass sample per host class to account for the asymmetry in the number of dissected carcasses, the large frequency of zeros (noninfected hosts with a given parasite) and the overdispersion typical of count data (Zuur et al., 2009). We performed the ZI-GLM with the function “zeroinfl” of the R package pscl (Zeileis et al., 2008). We compared the performance of the zero-inflated Poisson (ZIP) model and the zero-inflated negative binomial (ZINB) model to determine if the model dealt properly with the zero inflation and overdispersion (Zuur et al., 2009). Then, we selected the ZINB model because it showed the lowest Akaike's Information Criterion (AIC = 1334). We used a likelihood ratio test over the R function “lrtest” to test the significance of the ZINB model compared with the null model (i.e., the model including only the intercept). Finally, we run post-hoc Tukey contrasts between hosts' age-sex classes and helminth species using the function “glht” of the R package MuMIn (Barton, 2020) to identify the sources of the significant differences.

We compared the intensity of infection with female and male *T. minutus* by first generating 100 bootstrapped samples for each hosts' age-sex class to fix the asymmetry in their number of carcasses. Then, we run a generalized linear mixed model (GLMM; Zuur et al., 2009)

using these new samples and specifying the interaction *T. minutus* sex * host age-sex as fixed factor and carcass ID as a random factor to account for potential pseudo-replication resulting from the fact that some carcasses were collected in similar locations (and, thereby, could have belonged to the same social group) through time. We set the Poisson error family for the response variable as recommended for count data (Crawley, 2012) and used the function “lmer” of the R package lme4 (Bates et al., 2015) for this analysis. We assessed the significance of the GLMM model and the contrasts between hosts' age-sex classes following the procedures described above.

We run all statistical analyses in R v.4.0.5 (R Core Team, 2021). We set the statistical significance threshold at $p \leq 0.05$.

3 | RESULTS

We found three species of helminth parasites in the 35 howler monkeys from the same region, a diversity that reached an overall sampling completeness of 99% (95% CI = 3.0–3.1, Figure 2a). A fourth helminth species was only found in the remaining adult male from the different region. Sampling completeness for the age-sex classes ranged from 99% for adult males and adult females to 100% for juvenile males (Figure 2b). Whereas adult males (3 spp.; 95% CI = 3.0–3.1) and adult females (3 spp.; 95% CI = 3.0–3.5) hosted a similar helminth diversity as shown by the overlap of their rarefaction

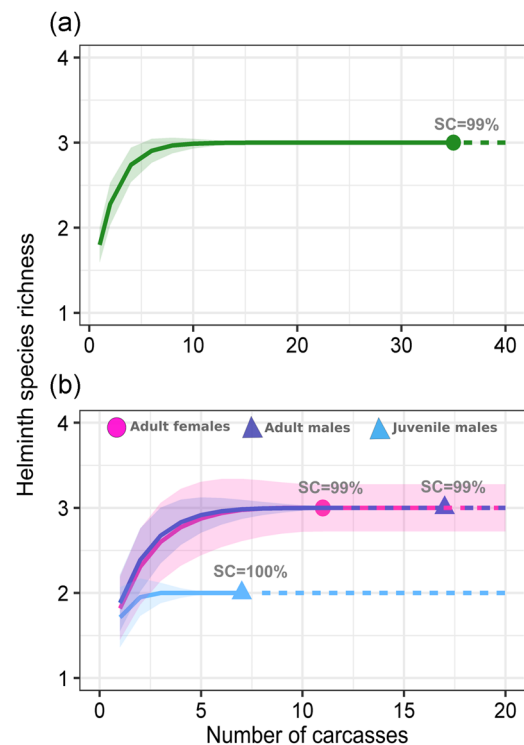


FIGURE 2 Individual-based rarefaction curves of sampling completeness of the helminth parasites of *Alouatta guariba clamitans* at the study region in south Brazil: (a) full sample ($N = 35$ carcasses), (b) adult male ($n = 17$), adult female ($n = 11$) and juvenile male ($n = 7$) subsamples. The sampling completeness (SC) of each curve is shown

TABLE 1 Helminths of brown howler monkeys (N = 36) from the State of Rio Grande do Sul, Brazil, their prevalence, intensity of infection, mean abundance, sites of infection, life cycle, and infection mode

Taxa	Number of infected hosts (prevalence)	Intensity of infection (mean ± SD)	Mean abundance	Sites of infection (number of hosts)	Life cycle	Infection mode
Nematoda Oxyuridae <i>Trypanoxyuris minutus</i>	36 (100%)	3200–229,040 (55,116 ± 52,915)	55,116	Cecum (35) Large intestine (29) Stomach (2) Small intestine (2) Rectum (2)	Monoxenous	Direct
Onchocercidae <i>Dipetalonema gracile</i>	12 (33%)	1–12 (5 ± 3)	2	Abdominal cavity (12)	Heteroxenous	Hematophagous arthropod (Diptera) vector
Habronematidae <i>Parabronema bonnei</i>	1 (3%) ^a	143 (143)	4	Stomach (1)	Heteroxenous	Oral ingestion of larvae carried by arthropod (Diptera)
Eucestoda Anoplocephalidae <i>Bertiella cf. studeri</i>	17 (47%)	1–21 (4 ± 4)	2	Small intestine (17)	Heteroxenous	Intermediate host (loribatid mites)

^aThis helminth was found in a single adult male carcass from a region distant from where all other howlers died.

curves, juvenile males (2 spp.; 95% CI = 2.0–2.2) hosted a less diverse helminth fauna than adults (Figure 2b). Helminth diversity per individual host ranged from 1 to 3 (mean = 2).

We found no difference in the prevalence of helminth species between the age-sex classes. All howlers were parasitized by *Trypanoxyuris minutus* (100% prevalence), about half by *Bertiella cf. studeri* (47%; juvenile males = 71%, 95% CI = 43%–100%; adult males = 50%, 95% CI = 28%–72%; adult females = 27%, 95% CI = 0%–55%), one-third by *Dipetalonema gracile* (33%; adult females = 55%, 95% CI = 27%–82%; adult males = 33%, 95% CI = 17%–56%) and the unique adult male found dead in a different region by *Parabronema bonnei* (3%; adult males = 6%) (Table 1 and Figure 3). We found *T. minutus* in five organs, mostly in the cecum and large intestine, *D. gracile* in the abdominal cavity, *P. bonnei* in the stomach and *B. cf. studeri* in the small intestine (Table 1).

The mean abundance of infection ranged from 2 (*D. gracile* and *B. cf. studeri*) to 55,116 (*T. minutus*), whereas the intensity of infection ranged from 1 (*D. gracile* and *B. cf. studeri*) to 229,040 specimens (*T. minutus*, Table 1). Male *T. minutus* were up to 24.7 times (mean = 5.3, SD = 6.4, median = 2.2, n = 31) more abundant than females in most (91%) hosts. In the three hosts in which females were more abundant, their numbers were, at most, 14% greater than those of males (Table S1).

The intensity of infection with the helminths differed between hosts' age-sex classes (ZINB-GLM, likelihood ratio test; $\chi^2 = 21,853$, df = 7, $p < 0.0001$). The intensity of infection with *T. minutus* was higher in juvenile males (mean ± SD of the bootstrap samples = 120,872 ± 62,794 pinworms) than in adult females (102,138 ± 60,846), which was higher than in adult males (59,677 ± 28,904; contrast test, $p < 0.001$ in all comparisons). Additionally, the striking dominance of male *T. minutus* over females was clear in the three age-sex classes (GLMM likelihood ratio test: $\chi^2 = 1225$, df = 5, $p < 0.0001$; Tukey contrast test; $p < 0.01$ in all cases, Figure 4). Whereas the infection with female *T. minutus* was similar between hosts' age-sex classes ($p > 0.05$ in all contrasts), the infection with male *T. minutus* was higher in adult female and juvenile male hosts than in adult male hosts ($p < 0.01$ in both cases, Figure 4). Adult males also showed a lower intensity of infection with *D. gracile* than adult females (Tukey contrast test; $p < 0.001$) and with *B. cf. studeri* than both adult females and juvenile males (contrast test, $p < 0.001$ in both comparisons). The intensity of infection of adult females and juvenile males with *B. cf. studeri* was similar (contrast test, $p > 0.05$).

4 | DISCUSSION

In this study, we assessed the helminth parasite community of wild brown howlers based on necropsies of individuals victimized in conflicts in anthropogenic landscapes in south Brazil. We found a low helminth diversity that was very close to sample completeness and a wide range of prevalence, intensity, and mean abundance of infection among helminth species. On one hand, the presence of the nematode *P. bonnei* in a single adult male from a more distant region from where all other carcasses were recovered is compatible with its absence in

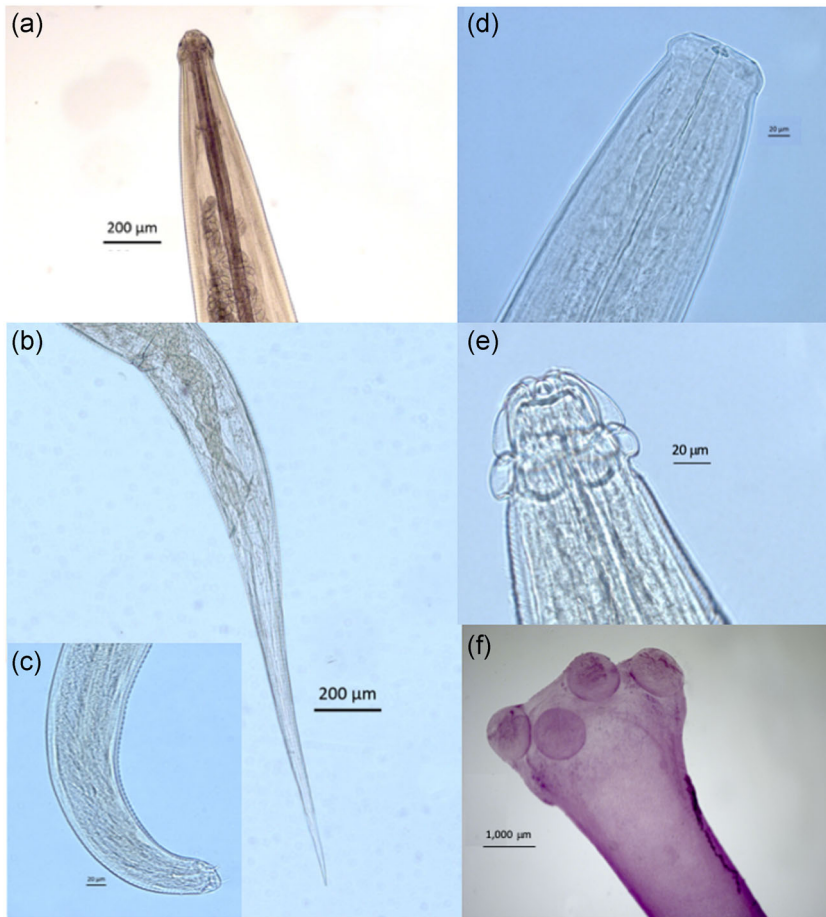


FIGURE 3 Helminth parasites of *Alouatta guariba clamitans* living in forest fragments in a periurban landscape in south Brazil: (a) anterior region of *Trypanoxyuris minutus* (scale: 200 µm), (b) posterior extremity of female *T. minutus* (200 µm), (c) posterior extremity of male *T. minutus* (20 µm), (d) anterior region of *Dipetalonema gracile* (20 µm), (e) anterior region of *Parabronema bonnei* (20 µm), and (f) scolex of *Bertiella cf. studeri* (1000 µm) (Photos by Stephanie Lopes)

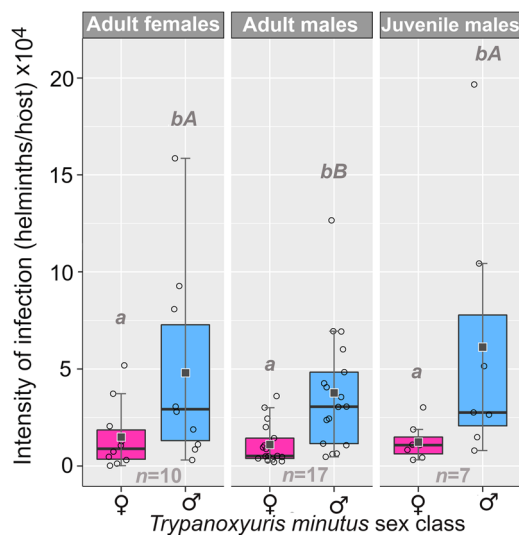


FIGURE 4 Intensity of infection of adult female, adult male, and juvenile male *Alouatta guariba clamitans* with female and male *Trypanoxyuris minutus*. Small letters indicate the result of the comparison of within-host class infection with female and male *T. minutus*. Capital letters indicate the result of the comparison of the infection with male *T. minutus* between host classes. Different letters highlight significant differences at $p < 0.05$

the other study regions. On the other hand, the nematode *T. minutus* was found in all howlers, often in tens of thousands of (mostly male) individuals.

Although some authors of coprological studies have classified *Bertiella* tapeworms found in howler monkeys as *B. mucronata* based on egg characteristics and host geographical occurrence (e.g., Kowalewski et al., 2017; Souza Júnior et al., 2008), we assigned the adult cestodes from our study to *B. cf. studeri*. We based our tentative taxonomic classification on the higher resemblance of the body measurements that we made on specimens (e.g., scolex, suckers, proglottids, and reproductive organs) with those cited for this taxon (Bhagwant, 2004; Galán-Puchades et al., 2000; Stunkard, 1940) compared with their lower resemblance with *B. mucronata*'s measurements (Costa et al., 1967; Gómez-Puerta et al., 2009). The occurrence of *B. studeri* in south Brazilian *A. g. clamitans* is possible because there is no correlation between *Bertiella* topologies and host or geography (Servián et al., 2020). Using molecular markers these authors found that the *Bertiella* species that parasitizes black-and-gold howler monkeys (*Alouatta caraya*) in Argentina is closer to *B. studeri* (18S rDNA and ITS1-5.8S rDNA) or to *Bertiella* sp. from chimpanzees (mitochondrial cox1 and 5.8S rDNA-ITS2). None of the four markers that they analyzed led to the assignment of the tapeworms as *B. mucronata*. However, we cannot reject the possibility

that the *Bertiella* sp. that we found in *A. g. clamitans* belongs to a new species, alike that suggested for the tapeworms of *A. caraya* (Servián et al., 2020).

The low diversity and the helminths' modes of infection via oral contamination or arthropod vector biting are compatible with howlers' arboreality and plant-based diet (Dias & Rangel-Negrín, 2015; Martínez-Mota et al., 2015) and with a decrease in the prevalence (Werner & Nunn, 2020) and diversity (Calegaro-Marques & Amato, 2014) of parasites in urban habitats. Contrary to our expectation, the composition of this species-poor helminth community is strong evidence that the study howlers' use of the ground (Bicca-Marques, 1992; Chaves & Bicca-Marques, 2017; Chaves et al., 2021; Corrêa et al., 2018) has not caused their infection with soil-transmitted helminths, as reported in other studies (see reviews in Kowalewski & Gillespie, 2009; Vitazkova, 2009).

It is likely that the common crop-feeding in orchards by howlers inhabiting periurban landscapes (Chaves & Bicca-Marques, 2017; Corrêa et al., 2018) has buffered them from the expected lower food availability in small habitat patches that could compromise their immune system and leave them more vulnerable to parasitic infections. In this respect, we found similar levels of physiological stress in groups inhabiting small unprotected periurban forest fragments and those inhabiting larger protected forests (Chaves et al., 2019). We have also only rarely seen individuals with morphological or behavioral signs of illness during our studies of many howler groups over the past 20 years in the region (JC Bicca-Marques and ÓM Chaves pers. comm.).

The lack of difference in the prevalence of *T. minutus*, *B. cf. studeri*, and *D. gracile* in adult female and adult male howler monkeys confirmed our expectation. In the absence of information on the timing (day or night) of *T. minutus*'s oviposition, two alternative explanations for its 100% prevalence can be offered to stimulate future research. Both explanations are related to this pinworm's direct cycle and the itching caused by the presence of gravid females that reach the perianal region of the host to lay eggs directly there (Kaur & Singh, 2009), as widely reported by humans infected with pinworms (Amiri et al., 2016; Royer & Berdnikoff, 1962; Sha-Mohammadi et al., 2014; Vose, 2012). On one hand, if oviposition occurs at daytime, tree trunks may get contaminated by the frequent habit of howlers of rubbing their perianal region after defecation (Braza et al., 1981; Hirano et al., 2008; Kowalewski & Zunino, 2005). This habit contrasts with the reported use of latrines (i.e., defecation sites in the canopy with no trees or understory beneath) as a strategy to avoid the contamination of food with fecal remains containing the infective stages of parasites (Gilbert, 1997). The presence of clusters of eggs in tree trunks would facilitate new infections and reinfections (Solórzano-García et al., 2017), thereby explaining the frequent high intensity of infection with this oxyurid. On the other hand, if oviposition occurs at nighttime, as in human pinworms, howlers may contaminate their fingers and nails with eggs while unconsciously scratching their perianal region during sleep. This pinworm has been recorded in the cecum, large and/or small intestine (Corrêa et al., 2016) of all howler monkey species so far studied (Solórzano-García & Pérez-Ponce de León, 2018).

The individual-level prevalence of *T. minutus* in the carcasses contrasts with the 43% group-level prevalence found in a coprological study of 60 howler groups in the same study region (Klain et al., 2021), a difference that supports the need of multiple fecal samples to detect infection (Gillespie, 2006). The results of these studies are compatible with the pattern found in Costa Rican mantled howler monkeys (*A. palliata*). Whereas the three necropsied howlers were infected with *T. minutus*, only 22% of the 200 individual fecal samples were positive (Stuart et al., 1990).

Given that both prevalence and intensity of infection with this haplodiploid oxyurid (haploid males are generated from unfertilized eggs, whereas diploid females hatch from fertilized ones; Adamson, 1989) were very high, it is not surprising that the sex ratio deviated markedly from the female-biased pattern expected when either one or both of these parameters are low (D'Ávila et al., 2012; Haukisalmi et al., 1996; Tingley & Anderson, 1986; but see Kloch et al., 2015; Poulin, 1997). The pervasive predominance of male *T. minutus* in the sample (see also Souza et al., 2010) is compatible with a higher intrasexual competition among females promoting their density-dependent regulation (Stien et al., 1996; Tingley & Anderson, 1986). The mechanisms involved in this regulation remain to be studied.

Unlike the aforementioned marked differences in prevalence of *T. minutus* between studies using distinct parasitological methods, the prevalence of *B. cf. studeri*, the second most common helminth in the carcasses, was quite similar (47% in the current study vs. 40% in the pooled fecal samples per group analyzed by Klain et al., 2021). Although this cestode releases gravid proglottids containing large numbers of eggs in the host's feces, false-negative fecal samples have been recorded (Oliveira et al., 2011), also compromising the reliability of analyses based on single individual fecal samples for detecting infection. Coprological studies involving the analysis of many samples per individual collected over several months from *A. caraya* belonging to groups of 12–14 and 9 individuals inhabiting 0.7-ha (Oliveira et al., 2011) and 2.0-ha (Jesus, 2013) orchards, respectively, have shown a *Bertiella* sp. prevalence of 100%. The likely absence of difference in this cestode's prevalence between adult males, adult females, and juvenile males agrees with the findings on *A. caraya* in Argentina (Kowalewski et al., 2017).

The host's rubbing of the perianal region after defecation mentioned above can also influence the prevalence of *B. cf. studeri* in howler monkey groups by contaminating the canopy with eggs. This hypothesis assumes that the oribatid mite intermediate hosts (Denegri, 1993) live on trees instead of exclusively in the soil (Schäffer et al., 2020). The single presence of this cestode as a helminth with an intermediate host is strong evidence that the inadvertent ingestion of invertebrates during the consumption of fruit (e.g., Urquiza-Haas et al., 2008) and other food items by the study howlers in their fragmented periurban habitats has not resulted in parasitic infections. This result is also interesting in light of the sporadic finding of invertebrate remains (e.g., ants, flies, spiders) in the gut (cecum) contents of, at least, nine carcasses (data recorded opportunistically and not shown) and an anecdotal observation of an

individual howler eating ants in the study region (JR Gonçalves, pers. comm.). The ingestion of these invertebrates could have exposed howlers to helminths, such as the trematode *Controrchis* spp. carried by *Azteca* ants (Kowalzik et al., 2010). However, the unique presence of this cestode is compatible with the lack of records of intentional consumption of invertebrates in all our other behavioral studies in the region (e.g., Back & Bicca-Marques, 2019; Chaves & Bicca-Marques, 2016; Corrêa et al., 2018; Koch & Bicca-Marques, 2007; Martins et al., 2015).

The sites of infection of *D. gracile* and *P. bonnei* in the abdominal cavity and the stomach, respectively, are in accordance with those described for *Dipetalonema* spp. in many Platyrrhini primates (Corrêa et al., 2016) and for *P. bonnei* in *A. caraya* (Vicente et al., 1992). We cannot eliminate the possibility that the absence of *D. gracile* in juvenile male hosts, responsible for the difference in their helminth richness compared to adults, resulted from the smaller sample of this age-sex class. However, given that over 40% of the adults were infected, the absence of this helminth in juvenile howlers may not be spurious. We speculate whether the less active behavior of adult howlers renders them more vulnerable to fly bites during their longer daytime resting (Bicca-Marques & Calegario-Marques, 1994; Koch & Bicca-Marques, 2007; Prates & Bicca-Marques, 2008).

The lower intensities of infection of adult male howlers with *T. minutus*, *B. cf. studeri*, and *D. gracile* contradict the expected pattern among mammals, including primates (Klein, 2000; Moore & Wilson, 2002; Nunn & Altizer, 2006; Roberts et al., 2001), and are unlikely to be explained by their larger bodies leading to a greater food intake or to a different diet or habitat use as suggested for mammals (Nunn & Altizer, 2006). In addition to the incompatibility of the life cycles of these helminths with the food intake hypothesis, howlers live in cohesive social groups whose non-infant members explore very similar diets (Koch & Bicca-Marques, 2007; see also Bicca-Marques & Calegario-Marques, 1994; Prates & Bicca-Marques, 2008).

Sexual or stress hormone levels may play some role in these differences (Klein, 2004). In a previous study we found that adult males and non-nursing females presented lower levels of fecal glucocorticoid metabolites (fGC) than nursing females (Chaves et al., 2019). However, higher fGC levels were correlated with lower parasite species diversity and nematode egg count in the fecal samples of a prosimian (*Eulemur fulvus rufus*; Clough et al., 2010). Although time in social contact is a stronger modulator of the gut microbiota of adult female and adult male howlers than kinship (Amato et al., 2017) and, probably, sex, the potential role of the microbiome (Aivelo & Norberg, 2018) on the prevalence and intensity of infection of howler parasites remains to be explored.

In sum, we found a low helminth parasite diversity in periurban brown howler monkeys. The proximity to humans and domestic animals does not seem to influence the helminths' prevalence and intensity of infection, which also do not seem to critically compromise the health of howlers. There is no theoretical basis to speculate that the helminth species parasitizing howler monkeys in our study promote behavioral changes that could lead to an increase in their

vulnerability to the threats of the anthropogenic environment. Therefore, except for viral diseases that cause significant losses in anthropogenic and wild habitats, such as yellow fever (Almeida et al., 2019; Bicca-Marques et al., 2020), we believe that the major threats to the long-term survival of periurban howler populations are the very causes of death of the individuals that we necropsied. Additionally, the identification of the sites of infection, the determination of the intensity of infection and the sex ratio of *T. minutus* are good examples of knowledge that could not be assessed based on fecal samples, either via traditional parasitological methods or modern metagenomics. We join other authors (e.g., Solórzano-García & Pérez-Ponce de León, 2018) in urging parasitologists, zoologists, geneticists, veterinarians, conservationists, and environmental authorities to collaborate to avoid wasting carcasses of wild animals that die in their periurban habitats. These valuable samples can enable us to access critical data to improve our understanding of host-parasite relationships and how the anthropogenic landscape affects them.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Stephanie Lopes: investigation (lead); methodology (supporting); writing original draft (supporting); writing review & editing (supporting). **Cláudia Calegario-Marques:** conceptualization (lead); investigation (lead); methodology (lead); resources (lead); supervision (lead); validation (lead); writing original draft (supporting); writing

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DATA AVAILABILITY STATEMENT

The data set is available online as Table S1.

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