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Prevalence and distribution of *Giardia intestinalis* genotypes in black and gold howler monkeys, *Alouatta caraya*, in relation to interspecies overlap and inter-annual variability in northern Argentina

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Advisor: Thomas Gillespie, PhD

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ABSTRACT

Prevalence and distribution of *Giardia intestinalis* genotypes in black and gold howler monkeys, *Alouatta caraya*, in relation to interspecies overlap and inter-annual variability in northern Argentina

By: Sahana Kuthyar

Increasing anthropogenic activities in Argentina are forcing howler monkeys to live in ecological overlap with humans and domestic animals. This ecological overlap among species at the human-wildlife interface presents the high potential for zoonotic disease transmission. This study investigated the prevalence of *Giardia intestinalis,* a zoonotic parasite, in black and gold howler monkeys, *Alouatta caraya*, across a gradient of inter-species overlap and terrestriality in northern Argentina. This study records the first genetic characterization of *G. intestinalis* in northern Argentina and examines the genotypic distribution and variation in *A. caraya*. Black and gold howler monkeys are sentinels of ecosystem health as they can advise on potential disease outbreaks in a variety of species. Thus, howler monkeys were sampled as a wildlife proxy for zoonotic transmission of *G. intestinalis* as they interact in varying degrees with other species, including humans, dogs, and livestock. From June to August 2016 and July to August 2017, fresh fecal samples (N=182) were non-invasively collected from groups of howler monkeys, in remote, rural, and village sites, all which differed in their degrees of overlap with humans and domesticated animals and terrestriality. Molecular methods were used to identify the prevalence and genotypic variability of *G. intestinalis* in all howler monkey samples. Prevalence of *G. intestinalis* in howler monkeys in Northern Argentina varied from 31-93% across the three types of inter-species overlap over 2016 and 2017. *Giardia* infection was highest in 2017 compared to 2016 and 2011, and village sites had the lowest prevalence during all three years. Genotype B was found in all types of inter-species overlap across both 2016 and 2017. Since host-adapted genotypes (C, D, and E) were not found in Northern Argentina, the source of *G. intestinalis* in howler monkeys does not seem to be cows or dogs. Furthermore, since *Giardia* and genotype B were found in howler monkeys regardless of type of inter-species overlap, howlers are thought to be a reservoir for *Giardia*, and potentially, for the zoonotic genotype B.

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1. INTRODUCTION

1.1. *Giardia* and Giardiasis

The protozoan flagellate *Giardia* is one of the most common zoonotic enteric parasites of domestic animals, wildlife, and humans (Feng and Xiao 2011; Johnston et al., 2010; Thompson 2000; Thompson 2004). *Giardia* is the etiological agent of giardiasis, a gastrointestinal disease with a variety of symptoms, including malabsorptive diarrhea, dehydration, abdominal pain, weight loss, nausea, and vomiting (Ryan and Caccio 2013; Savioli et al., 2006; Sprong et al., 2009; Thompson 2004). However, *Giardia* can often be asymptomatic (Sprong et al., 2009; Thompson 2000; Thompson 2004), which makes the disease burden in a population difficult to assess (Feng and Xiao 2011).

Although giardiasis leads to significant morbidity worldwide, the greatest impact is in developing countries in tropical and subtropical areas (Caccio et al., 2005; Feng and Xiao 2011; Minivielle et al., 2008; Traub et al., 2004). According to a 1996 study conducted by the World Health Organization (WHO 1996), 200 million people in Asia, Africa, and Latin America are symptomatic for *Giardia* (Ryan and Caccio 2013). Moreover, *Giardia* is included in the WHO Neglected Disease Initiative, as children living in poverty are most at risk for *Giardia* infection (Savioli et al., 2006; Sprong et al., 2009; Thompson and Ash 2016). The protozoan parasite is associated with poor cognitive function, nutritional disorders, and impediments to growth in young children (Caccio et al., 2005; Hunter and Thompson 2005; Thompson 2000). Although treatment for Giardiasis with nitroimidazoles (metronidazole and tinidazole) and benzimidazoles (albendazole) is effective, re-infection is likely if sources of environmental contamination are not extinguished and/or if frequency of transmission is high (Savioli et al., 2006; Thompson 2000; Thompson 2004).

There are six known species of *Giardia* (Ryan and Caccio 2013). *Giardia intestinalis* is the only *Giardia* species that infects humans, making it a public health priority. Furthermore, *G. intestinalis* has the broadest host range so zoonotic transmission is a potential issue (Feng and Xiao 2011). *G. intestinalis*, synonymous with *G. lamblia* and *G. duodenalis* (Feng and Xiao 2011; Traub et al., 2004), is found globally in humans and a majority of domestic and wild mammals (Feng and Xiao 2011; Thompson 2004) and is the most common cause of parasitic-associated diarrhea in the world (Molina et al., 2011).

1.2. *Giardia intestinalis*

G. intestinalis completes a two-stage lifecycle where infectious cysts are passed through feces and then ingested by a host, directly or indirectly, which catalyzes the release of trophozoites that replicate on the surface of the host intestinal tract. The infectious cyst stage is where cross-species transmission is possible (Monis and Thompson 2003; Ryan and Caccio 2013; Thompson 2000). Although it can remain in a cool, damp environment for months, *G. intestinalis* needs a living host to reproduce (Feng and Xiao 2011). *Giardia* cysts are more infectious longer in water than in soil and feces (Olson et al., 1999), and remain infectious the longest in river and sea water when compared to tap and lake water (Feng and Xiao 2011). In a four degrees Celsius environment, cysts can be infective in water for 8-11 weeks and in soil for 7 weeks (Olson et al., 1999; Feng and Xiao 2011).

As a multispecies complex, *G. intestinalis* has 8 distinct genotypes or assemblages (labeled A-H). The genotypes are distinguished by protein or DNA polymorphisms (Feng and Xiao 2011; Ryan and Caccio 2013). Genotypes A and B are host generalists, infecting a variety of species, including humans, nonhuman primates, domestic and wild ruminants, domestic and wild canines, cats, horses, and other mammals (Feng and Xiao 2011; Heyworth 2016). Only

genotypes A and B are known to infect humans (Xiao and Fayer 2008). Four genetic clusters within genotypes A and B have been identified: AI, AII, BIII, and BIV (Feng and Xiao 2011). Genotype AI has consistently been found in closely related human and animal isolates, including cows and dogs, with geographically widespread distribution. Humans are more commonly infected with genotype AII than AI (Feng and Xiao 2011; Ryan and Caccio 2013; Thompson 2003; Traub et al., 2004; Thompson 2004). Genotype AII has not yet been detected in other species, which leads to the theory that AI and AII differ in their host ranges (Minivielle et al., 2008; Xiao and Fayer 2008).

Genotype B is most prevalent in humans. In the United Kingdom, a study found 64% of people were infected with genotype B, 27% with genotype AII, and 9% with a mixed genotype infection (Amar et al., 2002). Studies from Australia and India found more humans were infected with genotype B (70% and 61%, respectively) than genotype A (30% and 39%, respectively) (Read et al., 2002; Traub et al., 2004).

Genotypes C-H have stronger host specificities (Table 1) and are not considered to be a human public health risk (Thompson 2000). In most cases of competitive interaction, host-adapted genotypes (C-H) are dominant over zoonotic genotypes A and B (Thompson 2004). There have been some exceptions where host-adapted genotypes have been found in other species (Feng and Xiao 2011).

All genotypes are genetically different, but morphologically similar; thus, molecular methods are required to distinguish them (Thompson 2004; Xiao and Fayer 2008). Variations in detection techniques and irregular reporting have led to inconsistent genotypic characterization and discrepancies in prevalence estimates (Thompson 2004; Thompson and Ash 2016; Xiao and Fayer 2008). A nested polymerase chain reaction (PCR) of fecal and environmental samples is

considered the best mechanism to identify genotypes (Ryan and Caccio 2013; Thompson 2004). Due to the heterogeneity of the organism, a multi-locus approach to genotyping is necessary, where targeted genes can include glutamate dehydrogenase (gdh), beta-giardin (bg), and triosephosphate isomerase (tpi) (Feng and Xiao 2011; Savioli et al., 2006). Multi-locus molecular methods are crucial to eliminate the risk of false negatives and imprecise amplification. Both gdh and tpi genes are housekeeping enzymes, whereas the bg gene is a structural protein uniquely associated with *G. intestinalis* (Ryan and Caccio 2013); additionally, the tpi gene can distinguish the variation in sub genotypes of A and B (Molina et al., 2007). However, *Giardia* has a high potential for mixed genotypic infections, so it is possible to assign different genotypes for the same isolate among the three different genes (Feng and Xiao 2011).

1.3. Potential for Zoonotic Transmission

G. intestinalis has four major cycles of transmission in mammalian hosts: humans, wildlife, companion animals (dogs and cats), and livestock (Thompson 2004). These cycles of transmission, where the parasite is passed from host to host, can be host-independent or zoonotic and can occur concurrently in given foci (Thompson 2000; Thompson 2004; Thompson and Ash 2016). Hosts can become infected directly through fecal-oral contact or indirectly through ingestion of contaminated food or water (Ryan and Caccio 2013; Sprong et al., 2009; Thompson 2004).

The possibilities for zoonotic *Giardia* transmission are numerous, including sources of environmental contamination, amplified infection in wildlife, and cross-transmission from companion animals and livestock (Thompson 2004; Thompson 2013). The frequency of transmission is currently unknown (Thompson 2004), making it difficult to map transmission dynamics and pathways in a given ecosystem. However, if host species are infected with a

zoonotic genotype of *Giardia* in defined foci, evidence for zoonotic transmission is viable (Thompson and Ash 2016).

Since genotypes A and B have the broadest host range, their prevalence among species in defined foci presents strong evidence for zoonotic transmission (Feng and Xiao 2011). Thus far, genotype A has been reported more frequently in livestock and companion animals when compared to genotype B (Feng and Xiao 2011; Xiao and Fayer 2008). The greatest risk for zoonotic infection is thought to be from AI as it has been detected in both humans and animals (Thompson 2000; Thompson 2004).

The role of wildlife in zoonotic *Giardia* is not clear, as there is very little research on *Giardia* genotypes in wildlife. Silent, or asymptomatic, infections in wildlife make it difficult to ascertain which individuals are infected within a population (Thompson 2013). However, natural *Giardia* infection in mammalian wildlife is possible so novel infection might not always be introduced from other sources (Appelbee et al., 2005). Genotypes A and B have been detected in humans, non-human primates, and other species of mammalian wildlife (Feng and Xiao 2011), so natural cycles and zoonotic cycles must be distinguished in defined foci.

In zoonotic cycles, the source of *Giardia* infection, whether wildlife, livestock, dog, cat, or human, can be difficult to determine. It is widely thought that wildlife species are most likely to become infected from either water or other modes of contact with human and domestic animal fecal material (Thompson 2004; Thompson 2013). *Giardia* cysts from humans can infect certain wildlife species, which then act as reservoirs and amplify the infection in the ecosystem (Heyworth 2016).

1.4. *Giardia* in non-human primates

Only genotypes A and B of *Giardia* have been found in both captive and wild non-human primates, and genotype B has been predominant (Ryan and Caccio 2013). Captive primates are usually in proximity with humans so their potential susceptibility and exposure to parasitic infections increases.

Different species of non-human primates were surveyed in a European zoo; genotype B was most prevalent (78.6%), but mixed infection of genotypes A and B was also detected in 32.7% of *Giardia*-positive individuals (Levecke et al., 2009). In captive non-human primates in two Spanish zoos, genotype A was most frequent (Martinez-Diaz et al., 2011). Similarly, genotype A, specifically subtype A1, was found in captive southern brown howler monkeys, *Alouatta guariba clamitans*, in Brazil (Volotao et al., 2008).

In wild populations of non-human primates, both genotypes A and B have been detected, (Table 2). However, a study in Uganda documented the first infection of genotype E in a non-human primate (Johnston et al., 2010). Here, the cycles of transmission existed between humans and red colobus (*Piliocolobus sp*.) for genotype BIV and livestock and red colobus for genotype E, demonstrating the potential for cross-species transmission.

Cross-species transmission is more likely to occur in disturbed habitats where ecological overlap exists among humans, domesticated animals, and wildlife (Johnston et al., 2010). Studies in defined foci in Africa have demonstrated that habitat disturbance affects zoonotic transmission of *Giardia* and other waterborne parasites (Johnston et al., 2010; Salzer et al., 2007). Furthermore, anthropogenic activities, such as human population expansion and land-use change, have the capacity to influence the dynamics and frequency of multi-directional *Giardia* transmission in ecosystems (Johnston et al., 2010; Thompson 2013). It is known that inter-species overlap in the form of human contact in Argentina affects the parasite prevalence in

howler monkeys (Kowalewski and Gillespie 2008). Therefore, it is highly possible that similar disease dynamics may be at play in Argentina where the ecological overlap among humans, livestock, and wildlife is high.

1.5. *Giardia* in Argentina

G. intestinalis is recognized as the national parasite of Argentina (Molina et al., 2011). Infection in humans varies from 6 to 36% and is related to sanitation practices, personal hygiene, and environmental disturbance (Molina et al., 2011). Prevalence in dogs and cats varies from 1.3 to 8.9%, based on geographic region (Feng and Xiao 2011). Genotypic characterization has show genotype B is predominant throughout Argentina in humans and dogs (Molina et al., 2011a; Molina et al., 2011b; Molina and Minvielle 2011).

Diarrheal disease poses a significant threat to public health in northeastern Argentina, one of the poorest regions in the country (Molina et al. 2011). Surveillance for specific pathogens causing such disease is rare in this region. As such, there has only been one study of *Giardia* in children in San Cayetano, Corrientes, where prevalence was 29% (Borda et al., 1995). Poor environmental hygiene and a lack of public water supply, sewage and waste removal services are important factors in disease management in the town (Borda et al., 1995). Furthermore, the region as a whole is prone to flooding, which often leads to fecal matter contamination in water sources.

1.6. *Giardia* in *Alouatta caraya*

The black and gold howler monkey, *Alouatta caraya*, is a habitat generalist and is able to adapt and survive in fragmented landscapes (Kowalewski et al., 2011). *A. caraya* is generally arboreal, but descend to the ground in order to move through forest patches. Howler monkeys also descend to drink water from creeks and rivers where other animals, including cows, drink

and defecate (Kowalewski et al., 2011). As the dominant primate species in northeastern Argentina, *A. caraya* is considered to be a sentinel species of ecosystem health, where they can advise about potential disease outbreaks in a variety of species (Kowalewski et al., 2011). These characteristics make them a model organism to study zoonotic disease transmission.

Giardia in non-human primates manifests in diarrhea and growth failure in young individuals (Levecke et al., 2009), but howler monkeys are mostly asymptomatic for many parasites, including *Giardia* (Martinez-Mota et al., 2015), which makes it difficult to assess the disease burden. Additionally, infections tend to be aggregated in any population, such that only a few individuals sustain infections at any given time, so a robust sample size is needed to adequately examine patterns of infection (Martinez-Mota et al., 2015).

The relationship between rainfall and *Giardia* prevalence is unclear. One study found rainfall to be associated with higher prevalence of *Giardia* in human and non-human primates, potentially due to high concentrations of cysts in water sources (Martinez-Mota et al., 2015). High humidity favors the survival of the infectious stages of *Giardia* cysts (Martinez-Mota et al., 2015), and parasite prevalence has been associated with high levels of precipitation in howler monkey habitats (Kowalewski and Gillespie 2008). Another study found a negative exponential correlation between precipitation and *Giardia* prevalence for different species of howler monkeys; in this case, a lower annual precipitation correlated with a higher *Giardia* prevalence in individuals (Martinez-Mota et al., 2015).

In a previous study in northern Argentina by the Estacion Biologica de Corrientes, *G. intestinalis* was prevalent across a range of inter-species overlap, which were characterized by variable levels of interaction with humans and domestic animals (Kowalewski et al., 2011). Howler monkeys live in groups of three to 21 individuals, and these groups fell within three

categories of variable interaction. Howlers in remote sites had little contact with humans and domestic animals, whereas howlers in rural and village sites had a high level of interaction with cattle and high level of interaction with humans and dogs, respectively. High infection prevalences in all sites, regardless of human-primate contact, suggest that howlers are a viable reservoir for *Giardia* (Kowalewski et al., 2011). However, prevalence was highest at rural sites (67%) when compared to remote (57%) and village (40%) sites (Kowalewski et al., 2011). These rural sites are where primate-livestock interaction is highest as cows also enter the forests; the reasoning behind this trend is unclear as multiple host-adapted genotypes could exist or zoonotic transmission could be occurring (Kowalewski et al., 2011).

2. Purpose and Hypotheses

Kowalewski and colleagues (2011) demonstrated the relationships among *Giardia* prevalence and inter-species overlap; however the study lacked genotypic analysis. Discovering the genetic diversity of *Giardia* in the primate species and sites of inter-species overlap may help understand and clarify different pathways and modes of transmission (zoonotic or host-adapted).

I investigated the prevalence of *G. intestinalis* in black and gold howler monkeys from three sites in Corrientes and Chaco, Argentina. These three sites follow categories for degrees of inter-species overlap and howler terrestriality defined by Kowalewski et al. (2011). Remote sites are characterized by low overlap with humans and domesticated animals and intermediate terrestriality. Rural sites are characterized by intermediate overlap with humans and dogs and high overlap with cows and high terrestriality. Village sites are characterized by high overlap with humans and dogs and intermediate overlap with cows and low terrestriality. My study also explored the inter-annual variation of *Giardia* infection prevalence in all three types of sites

between 2011 and 2016 as well as the inter-annual variation in rural and village sites between 2011, 2016, and 2017.

Thus far, *Giardia* genotypes in *A. caraya* are unknown. As a crucial step to further understand *Giardia* infectivity in the region, I attempt to complete the first genetic characterization of *G. intestinalis* in Corrientes, Argentina and examines the genotypic distribution and variation in howler monkeys.

My primary objectives were to determine for black and gold howler monkeys, *A. caraya*, in northern Argentina:

1. If the prevalence of *G. intestinalis* differs in relation to different types of human and domesticated animal overlap and howler terrestriality,
2. If the prevalence of *G. intestinalis* differs between years,
3. Which *Giardia* genotypes are present in the study system,
4. If the composition of *Giardia* genotypes differ between years or among sites varying in inter-species overlap and howler terrestriality.

I hypothesize that:

- Prevalence will be directly correlated with the degrees of inter-species overlap and terrestriality.
    - Since howlers interact with more domestic species at rural sites, where there is the highest degree of terrestriality, infection prevalence in rural sites will be the highest when compared to remote and village sites.

In this analysis, howler monkeys were sampled as a wildlife proxy for zoonotic transmission of *G. intestinalis* as they interact in varying degrees with other species, including humans, dogs, and livestock. Discerning the *Giardia* genotypes in howlers can reveal the

potential for zoonotic transmission amongst these species as well as the possible direction of zoonosis, depending with what genotypes the howler groups are infected.

Hypotheses and predictions for these objectives include:

- There will be a positive relationship between the degrees of species overlap and terrestriality and the diversity of *Giardia* genotypes.
    - Since genotype B has been detected in other non-human primates and since genotype B is predominant in Argentina, genotype B will be present in howler monkeys.
    - A greater diversity of *Giardia* genotypes (A, B, C, D, and E) will be present in howler monkeys in rural sites due to the howler monkeys' high terrestriality and interaction with a large variety of species.

3. MATERIALS AND METHODS

3.1. Study Site

My study was conducted in San Cayetano (27°34' S, 58°42' W), the Estacion Biologica de Corrientes (27°30' S, 58°41' W), and the surroundings of the Parque Provincial San Cayetano, Corrientes Province, Argentina as well as on Isla Brasilera (27°20' S, 58°40' W) and Cerrito (27°17' S, 58°37' W) in Chaco, Argentina (Figure 1). San Cayetano has 4,000 human inhabitants, and Cerrito has 2,000 human inhabitants. Both towns are village study sites where howler density is 3.24 howlers per hectare (M. Kowalewski). Isla Brasilera is an island where howler monkeys are mostly isolated in a flooded forest, so it is classified as remote study site, with a howler density of 3.25 howlers per hectare (M. Kowalewski). The Estacion Biologica de Corrientes and the surroundings of the Parque Provincial San Cayetano are characterized by a semi-deciduous forest in a matrix of grassland vulnerable to deforestation and are classified as

rural sites (Kowalewski et al., 2011). At the rural sites, the howler density is 1.04 howlers per hectare (M. Kowalewski).

The climate is subtropical with an average annual temperature of 21.6°C and an annual average rainfall of 1,200mm (Rumiz et al., 1986). Rainfall is higher during the spring and summer seasons (September to December). All three types of study sites are prone to flooding, and flash floods have been occurring more frequently. In April-May 2017, 600mm of rainfall was recorded, leading to severe flooding (M. Kowalewski, personal communication).

3.2. Sample Collection

To assess *Giardia* prevalence, fresh fecal samples were collected from groups of howler monkeys at each of the three study sites categories (remote, rural, and village) from June to August 2016 and July to August 2017. Fecal samples were collected from the following locations: Isla Brasilera (remote), Estacion Biologica de Corrientes (rural), and the towns of Cerrito and San Cayetano (village). A total of 130 and 52 samples were collected across all three types of sites in 2016 and 2017, respectively (Table 3). In 2016, 14 remote, 12 rural, and 8 village groups were sampled. In 2017, due to temporal and flooding constraints, a subset of the groups from 2016 were sampled – six rural and seven village groups.

All fecal samples were collected non-invasively immediately after defecation in the morning, following protocols of Gillespie (2006). Characteristics including sex and stage of maturity (infant, juvenile, sub-adult, or adult) of the individual sampled were noted. Each sample was considered independent across both years because infective *Giardia* cysts pass through individuals within a few months, so there was no sampling bias in resampling the same individuals across years (Gardner and Hill 2001). For each sample, one gram of fecal matter was

homogenized in one milliliter of RNAlater nucleic acid stabilizing buffer (Ambion, Life Technologies, Grand Island, NY) and stored at 4°C until transport to the USA for processing.

3.3. Molecular Analyses

Molecular methods were used to identify the presence and genotypes of *G. intestinalis* found in the howler monkey fecal samples.

DNA extractions were performed on the RNAlater-preserved fecal samples using the FastDNA Spin Kit for Soil (MP Biomedicals LLC), and multi-locus genetic regions (genes: glutamate dehydrogenase [gdh], triosephosphate isomerase [tpi], and beta-giardin [bg]) were amplified using a nested Polymerase Chain Reaction (PCR) protocol modified from Roellig et al. (2015). For the gdh gene, a 771bp fragment was first amplified using the primers GDH F3 and GDH R3, from which a 599bp fragment was then amplified with the primers GDH F4 and GDH R4. For the tpi gene, DNA was first amplified using the primers TPI F1 and TPI R1, followed by the amplification of a 530bp fragment using primers TPI F2 and TPI R2. For the bg gene, DNA was first amplified using the primers BG F1 and BG R1, from which a 511bp fragment was then amplified with the primers BG F2 and BG R2. All primers used are detailed in Table 4.

All PCR reactions were prepared in a final volume of 25uL containing 1x Taq PCR Master Mix (Qiagen), 400ng/uL BSA, 500nM of each primer, nuclease-free water and genomic DNA (2uL in first PCR reaction and 2uL of first reaction product in the second PCR reaction). Positive (DNA control provided by the Centers for Disease Control (CDC) in Atlanta, Georgia) and negative controls were included in each reaction, and all reactions were performed using a Mastercycler Pro thermal cycler (Eppendorf).

For the gdh gene, PCR reactions started with a first denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45

seconds, and extension at 72°C for 1 minute. All reactions concluded with a final extension at 72°C for 10 minutes.

For the tpi gene, PCR reactions started with a first denaturation step at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing for 45 seconds (54°C and 58°C for primary and nested reactions, respectively), and extension at 72°C for 1 minute. All reactions concluded with a final extension at 72°C for 10 minutes.

For the bg gene, PCR reactions started with a first denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing for 30 seconds (65°C and 64°C for primary and nested reactions, respectively), and extension at 72°C for 1 minute. All reactions concluded with a final extension at 72°C for 10 minutes.

Accurate amplification was verified by running 5uL of PCR products on a 1.5% agarose gel stained with Gel Red Nucleic Acid (Biotium).

3.4. Sequencing Analyses

One hundred and forty-eight PCR amplicons were sequenced in both directions using the secondary forward primers for all three genes (Macrogen, USA). Matching sequences were aligned using Muscle in Mega 7.0 (Pennsylvania State University) and compared to reference isolates per gene (Roellig et al., 2015). Samples that did not match reference isolates were compared in a BLAST NCBI nucleotide search.

3.5. Statistical Analyses

Infection prevalence per year was calculated as the proportion of individuals infected divided by the total number of individuals sampled in that year. Infection prevalence per type of site was calculated as the proportion of individuals infected divided by the number of individuals sampled per site.

Statistical analyses were performed in R Studio 1.1 (Boston, Massachusetts). Chi-square tests of independence were utilized to test if infection prevalence and genotype prevalence differed across sites and years. P-values less than 0.05 were considered statistically significant. Logistic regressions using a generalized linear mixed model (GLMM) were used to determine how Giardia prevalence differed in the three types of inter-species overlap (variable 1) across the years (variable 2), where year was treated as a random effect.

4. RESULTS

4.1. Sample Size

One hundred and eighty-two fecal samples from black and gold howler monkeys were screened for *G. intestinalis*, including 130 samples from 2016 and 52 samples from 2017. In 2016, 46 monkeys from rural sites, 32 monkeys from village sites, and 52 monkeys from remote sites were sampled. In 2017, 28 monkeys from rural sites and 24 monkeys from village sites were sampled. In 2011, 90 fecal samples were screened where 30 monkeys each were sampled from rural, village, and remote sites (Kowalewski et al., 2011).

4.2. 2016 Samples

In 2016, 39.23% (51/130) of monkeys sampled tested positive for *G. intestinalis*, where 54.34% (25/46) were positive in rural sites, 31.25% (10/32) were positive in village sites, and 30.77% (16/52) were positive in remote sites. Females comprised 68.63% (35/51) of positive monkeys whereas, 31.37% (16/51) were males; 15.69% (8/51) were juveniles, and 84.31% (43/51) were adults. Both sex and age were independent of *Giardia* infection ($\chi^2 = 3.0687$, p-value = 0.0798 and $\chi^2 = 0.0913$, p-value = 0.7625, respectively).

When determining if *Giardia* prevalence was dependent on sites as a proxy for the type of inter-species overlap, years 2016 and 2017 were analyzed separately, as remote sites were not

sampled in 2017. In 2016, *Giardia* prevalence was highest in rural sites ($\chi^2 = 6.826$, p-value = 0.0329) when compared to village and remote sites. When sampling sites were compared individually in 2016 (remote vs. rural, remote vs. village, and rural vs. village), differences were found between remote and rural sites ($\chi^2 = 4.6497$, p-value = 0.03106). No differences were found between remote and village sites or rural and village sites.

4.3. 2017 Samples

In 2017, 90.38% (47/52) of monkeys sampled tested positive for *G. intestinalis*, where 92.86% (26/28) were positive in rural sites and 87.50% (21/24) were positive in village sites. Infection prevalence for each year and type of site are summarized in Table 5. Females comprised 72.34% (34/47) of positive individuals, whereas 27.66% (13/47) were males; 21.28% (10/47) of positive individuals were juveniles, and 78.72% (37/47) were adults.

Both sex and age were independent of *Giardia* infection ($\chi^2 = 0.3353$, p-value = 0.563 and Fisher's exact p-value = 0.3248, respectively). In 2017, infection prevalence did not differ between rural and village sites (Fisher's exact test p-value = 0.6521).

4.4. Inter-annual variation

The overall infection prevalence was higher in 2017 (90.38%) when compared to 2016 and 2011 ($\chi^2 = 39.1416$, p-value <0.00001). When compared to 2011 (54.44%), infection prevalence in 2016 (39.23%) was lower ($\chi^2 = 4.3701$, p-value = 0.03658), and when compared to 2016, infection prevalence in 2017 was higher ($\chi^2 = 37.077$, p-value < 0.00001). Howler monkeys in 2017 were eight times as likely to be infected with *Giardia* when compared to previous years (OR=8.417392, CI 2.906646-24.37602, p-value < 0.00001).

Howler monkeys in rural sites in 2011 and 2016 had almost twice the odds of being infected with *G. intestinalis* when compared to howlers in remote sites (OR=1.93006, CI

1.016729-3.663831, p-value = 0.0444). However, when compared to rural sites, village sites have lower prevalence across all three years (p-value = 0.0244). Figure 2 exhibits the trends in infection prevalence over the three years across remote, rural, and village sites.

When comparing infection prevalence among groups, rural groups had the highest prevalence, and almost all groups in 2017 had higher prevalence of *Giardia* compared to groups in 2016 (Figures 3-5). Groups with zero prevalence were not included, and for six groups (Costanehra, Scheep, Cochelo Q, Vazquez, and Caravana Naranja), infection prevalence for 2017 was blank because those groups were not sampled.

4.5. Genotypic Analysis

All sequences (N=148) were compared to gene-specific reference isolates taken from Roellig et al. 2015 (Table 6). Some samples directly matched these reference isolates (*n*=90) and were characterized as genotype B. For samples that did not match the reference isolates, raw sequences were inputted into a BLAST NCBI search to find known highly similar nucleotide regions. Here, genotypic characterization was based on query cover and identity (Table 7). The sequences that matched were characterized as genotype B (*n*=14). The remaining samples did not match any known sequences from the reference isolates or from the BLAST search.

5. DISCUSSION

G. intestinalis is a ubiquitous zoonotic parasite that can exhibit cross-species transmission in localized foci. It has been reported that anthropogenic activities, such as human population expansion and land-use change, are the source for *Giardia* infection in wildlife species (Thompson 2013; Thompson 2016). However, these studies that suggest zooanthroponosis fail to consider natural parasite communities in wildlife (Appelbee et al., 2005). My study on the prevalence and distribution of *G. intestinalis* and its genotypes in black and gold howler

monkeys, *A. caraya*, in northern Argentina supports the theory that the howler monkeys are a natural reservoir of *Giardia*.

Prevalence of *G. intestinalis* in all (both male and female and all age groups) howler monkeys in northern Argentina ranged from 31-93% across sites of inter-species overlap and across years. *Giardia* infection was highest in 2017 compared to 2016 and 2011. Village sites had the lowest prevalence during all three years. My study provided the first characterization of *Giardia* genotypes in *A. caraya*. Genotype B was found in all sites of inter-species overlap across both 2016 and 2017.

Following the trends observed by Kowalewski et al. (2011), rural sites had the highest prevalence of *Giardia* infection when compared to remote and village sites in 2016 (54.3%) and 2017 (92.9%). Rural sites are where howler monkeys experience high overlap with cows and intermediate overlap with humans and dogs and where they have the highest degree of terrestriality. The matrix of grassland and fragmented forests forces howlers to travel terrestrially across the grasslands to get from one patch of forest to the next one. In Uganda, chimpanzees (*Pan troglodytes*) that spent more time on the ground had a higher parasite burden due to increased exposure (Zommers et al., 2012). Since terrestrial activity is high at rural sites, an increased interaction with both animals and infective parasite cysts on the ground and in water bodies could lead to higher frequency of infection and re-infection. Furthermore, multiple transmission cycles could be occurring among the howler monkeys, cows, and dogs that reinforce infection.

Across all three years, village sites had the lowest prevalence of *G. intestinalis*. At these sites, the density of dogs is relatively high (at least one per household), and there is more tree coverage, so howlers are more reluctant to come down to the ground. This behavior leads to less

exposure to infective *Giardia* cysts on the ground and in water bodies. Thus, ecological overlap without terrestriality may not lead to *Giardia* infection in howler monkeys even though they share the same habitats with humans, dogs, and cows in village sites.

Although remote sites are characterized by low ecological overlap with humans and domesticated animals, *Giardia* prevalence at 31% in this analysis was still relatively high. A study in Uganda with red colobus (*Piliocolobus tephrosceles*), black and white colobus (*Colobus guereza*), and red-tailed guenons (*Cercopithecus ascanius*) demonstrated no *Giardia* in undisturbed habitats and infection prevalences of 3.8% and 11.1% in 2007 and 2010, respectively, in disturbed habitats (Salzer et al., 2007; Johnston et al., 2010). The high prevalence presenting in my study may indicate that *A. caraya* are a natural reservoir for *G. intestinalis,* as first suggested by Kowalewski et al., 2011, where *Giardia* could be a natural component of the *A. caraya* parasite community.

However, the remote site is not entirely isolated as sometimes residents from the surrounding village introduce cows (5-10) into the north part of island, which then defecate in the forest. Additionally, fishermen enter the shoreline of the island and occasionally use the forest edges as a latrine. Since the water bodies are naturally connected, when flooding occurs, water contamination from multiple sources of feces is possible.

Vitazkova and Wade (2006) posited that a higher primate density would increase *Giardia* prevalence in black howler monkeys, *Alouatta pigra*, in Belize. In contrast to this hypothesis, the sites that had the lowest *A. caraya* density (1.04 howlers per hectare in rural sites compared to 3.24 and 3.25 howlers per hectare in village and remotes sites, respectively) had the highest *Giardia* prevalence in my study. However, primate density has been increasing in the village site

of San Cayetano due to deforestation and land-use change; this increase in howler monkeys per hectare may lead to higher possibilities for disease spillover to humans.

The prevalence of *Giardia* changed significantly in the study system from 2011 to 2016 to 2017. Infection prevalence was lower in 2016 compared to 2011 and higher in 2017 compared to 2016; additionally, *Giardia* prevalence was higher in 2017 when compared to 2011. In 2017, overall *Giardia* prevalence was unusually high (almost 91%). Both rural and village sites had very similar prevalence (93% and 87.5%, respectively), so inter-species overlap might not have contributed to *Giardia* transmission. The year was characterized by severe flooding as half (600mm) of the annual rainfall occurred in four days. All sampling sites were inundated. Since high infection prevalences have been associated with precipitation (Kowalewski and Gillespie 2008; Martinez-Mota et al., 2015), high *Giardia* prevalences across sites most likely resulted from flooding and water contamination. Flooding may have spread *Giardia* infective cysts to multiple howler groups, and water bodies used by howlers may have been contaminated.

Since *Giardia* cysts remain infective for longer in cold water (four degrees Celsius) (Olson et al., 1999; Feng and Xiao 2011), there is a higher chance of howlers becoming infected during the winter flooding, which is when the samples were collected. Infective cysts can travel downstream from floodwaters and rivers to multiple howler populations and to other species as well. When howlers come down to drink the contaminated water, re-infection cycles can occur.

Recently, due to changes in the landscape and forest fragmentation, cycles of flooding have changed from every 15 years to every two to three years. *Giardia* prevalence in 2017 may reflect this change in the flooding cycle and its implications for *Giardia* infectivity in the region.

Natural cycles of peaks and troughs in *Giardia* prevalence in howler monkeys in the system over the years could also exist. One study reviewed *Giardia*-positive cases in humans in

the United States and found seasonality trends in infection where peak incidence occurred during the summer at a stable annual rate (Mohamed et al., 2014). This trend does not appear to occur in howler monkeys in northern Argentina, but to substantiate this claim, annual data on *Giardia* prevalence would need to be collected for multiple continuous years.

I found genotype B of *G. intestinalis* to be predominant in *A. caraya* (70.3%) across all sites of inter-species overlap in both years. This pattern is consistent with previous studies in wild primates (Ye et al., 2012; Itagaki et al., 2005; Teichroeb et al. 2009). Genotype B has also been found in other species of *Alouatta*: the southern brown howler monkey (*A. guariba clamitans*), in Brazil and the black howler monkey (*A. pigra*), in Belize. Volotao et al. (2008) found genotype A, specifically subtype A1, in captive *A. guariba clamitans*; however, captivity might have caused unnatural infection due to more interactions with humans, and thus, higher exposure to infective pathogens when compared to wild populations. *A. guariba* was found living in sympatry with *A. caraya* in the wild (Cortes-Ortiz et al., 2015), so cross-species transmission of *Giardia* genotypes A and B may be likely to occur. In addition to genotype B, genotype A was detected in three individuals of *A. pigra*, the black howler monkey, in Belize (Vitazkova and Wade 2006). The Vitazkova and Wade (2006) study is considered anecdotal due to a very small sample size. My study, with a large sample size of wild individuals, is a crucial first step in determining the potential genotypes of *G. intestinalis* in *Alouatta* genus.

In the remaining monkeys sampled, genotypes are unknown as their sequences did not match reference isolates or BLAST sequences. It is possible that these unknown sequences could be characterized as genotype B due to the high heterogeneity and diversity of subtypes within genotype B (Nunes et al., 2018). Phylogenetic trees could be constructed to tease out the genetic

variation within the clades of genotype B to ascertain if these unknown sequences fall within the genotype.

Genotype A was not detected in any sampled howler monkeys. Flooding and precipitation events may have homogenized *Giardia* genotypes across all sites. Alternatively, genotype B could be the natural genotype for *A. caraya* because monkeys were infected with genotype B regardless of degree of inter-species overlap or terrestriality. Genotype B is predominant in Argentina, especially in humans and canines (Minvielle et al., 2008; Molina et al., 2011a; Molina et al., 2011b; Molina and Minvielle 2011), so further genotypic characterization of other species in the study system will illuminate a larger part of the transmission dynamics of *G. intestinalis* in Northern Argentina.

The lack of genotypes C, D, and E is interesting especially at rural sites where howlers experience high overlap with cows and have a high degree of terrestriality, as these sites are where *Giardia* prevalence is the highest. In Uganda, genotype E was found in the red colobus monkey, in addition to genotype B (Johnston et al., 2010). This first record of host-adapted genotype E in a non-human primate in an area of high ecological overlap provides strong evidence of cross-species transmission of *G. intestinalis*. Here, cows, which are conventional hosts of genotype E, seem to be the source of *Giardia* infection in red colobus monkeys. Since host-adapted genotypes (C, D, and E) were not found in Northern Argentina and since host-adapted genotypes usually out-compete genotypes A and B, the source of *G. intestinalis* in howler monkeys does not seem to be cows or dogs. Thus, it seems the ecological overlap does not equate the transmission of host-adapted genotypes. Furthermore, since *Giardia* was found in howler monkeys regardless of type of inter-species overlap, howlers are thought to be a reservoir for *Giardia*, and potentially, for the zoonotic genotype B.

To assess the potential for zoonotic transmission in the localized system, human, cows, and canine samples collected in July and August 2017 should be genotypically characterized for *G. intestinalis*. Unveiling the multiple pathways and the sources of zoonotic *Giardia* is crucial to understanding *Giardia* infectivity in the region.

A number of factors constrained the number of fecal samples collected and analyzed in my study. Due to flooding, no remote samples were collected in 2017. I was not able to see how flooding impacted howler monkeys groups which had zero to little inter-species overlap. Trends in infection prevalence cannot be determined when comparing samples from 2011, 2016, and 2017 since there was no data from 2012 to 2015.

Additionally, not all individuals were sampled in a group, and so total individual prevalence among groups was not analyzed. Sometimes, fecal material was stuck in the trees or a few monkeys did not defecate. However, more than 50% of all individuals in a given group were sampled, which can predict the relative infection prevalence of the group as whole.

It is important to study the black and gold howler monkeys as they are considered sentinels of ecosystem health. Increasing anthropogenic activities in Argentina are forcing them to live in ecological overlaps with humans, livestock, and companion animals. In 2010, *A. caraya* went from Least Concerned to Vulnerable status on the Argentinean Mammal Red List, so conservation challenges for the primate are very real. Currently, Argentina is in the process of re-introducing confiscated howler monkeys from illegal smuggling and pet trades. My study will help form the various quarantine screenings needed after confiscation before howlers are released into the wild. Additionally, all data will be shared in a report to the local community to increase awareness of potentially zoonotic *Giardia* and its risk factors. Through gaining a greater

understanding of the ecological and epidemiological drivers of Giardiasis, strategic and informed solutions will hopefully reduce the burden of diarrheal disease within the region.

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7. TABLES

Table 1 Known hosts of genotypes of *Giardia intestinalis*.

Genotype	Host range
A	Humans, non-human primates, domestic and wild ruminants, alpacas, pigs, horses, domestic and wild canines, cats, marsupials (and other mammals)
B	Humans, non-human primates, cattle, dogs, horses, rabbits, beavers, muskrats
C	Domestic and wild canines
D	Domestic and wild canines
E	Domestic ruminants and pigs
F	Cats
G	Mice and rats
H	Seals

Table 2 Genotypes of *Giardia intestinalis* detected in wild species of non-human primates

Primate species	Geographic area	Genotype detected	Reference
Macaca mulatta (rhesus macaque)	China	A and B	Ye et al., 2012
Gorilla gorilla beringei (mountain gorilla)	Uganda	A	Graczyk et al., 2002
Macaca fuscata (Japanese macaque)	Japan	B	Itagaki et al., 2005
Alouatta pigra (black howler monkey)	Belize	A and B	Vitazkova and Wade 2006
Colobus vellerosus (ursine colobus monkey)	Ghana	B	Teichroeb et al. 2009
Piliocolobus sp. (red colobus monkey)	Uganda	B(IV) and E	Johnston et al., 2010

Table 3 Number of fecal samples from black and gold howler monkeys (*Alouatta caraya*) screened for *Giardia intestinalis* per site per year in northern Argentina.

Year	Number of Groups	Total	Remote	Rural	Village
2016	35	130	52	46	32
2017	10	52	N/A	28	24

Table 4 List of primer sequences used in a nested polymerase chain reaction (PCR) for tpi, gdh, and bg genes for detection of *Giardia intestinalis* in black and gold howler monkeys (*Alouatta caraya*) in northern Argentina.

Primer	Sequence (5' – 3')
TPI F1	AATAAATIATGCCTGCTGGTCG
TPI R1	ATGGACITCCTCTGCCTGCTC
TPI F2	CCCTTCATCGGIGGTAACTTCAA
TPI R2	GTGGCCACCACICCCGTGCC
GDH F3	GAGGTCATGCGCTTCTGCCA
GDH R3	CGTCCACTGGAGCCTCACGGA
GDH F4	ATGACCGAGCTCCAGAGGCACGT
GDH R4	CCCTCGGCCACGAACTTGAG
BG F1	AAGCCCGACGACCTCACCCGCAGTGC
BG R1	GAGGCCGCCCTGGATCTTCGAGACGAC
BG F2	GAACGAACGAGATCGAGGTCCG
BG R2	CTCGACGAGCTTCGTGTT

Table 5 Infection prevalence of *Giardia intestinalis* in 2011, 2016, and 2017 in black and gold howler monkeys (*Alouatta caraya*) in northern Argentina.

Year	Total prevalence	Rural	Village	Remote
2011	54.44% (49/90)	67.66% (20/30)	40% (12/30)	56.67% (17/30)
2016	39.23% (51/130)	54.38% (25/46)	31.25% (10/32)	30.77% (16/52)
2017	90.38% (47/52)	92.86% (26/28)	87.50% (21/24)	N/A

Table 6 Genotypic characterization of black and gold howler monkeys, *Alouatta caraya*, per gene in the detection of *Giardia intestinalis* genotypes in northern Argentina.

Gene	Matched to reference isolates (N)	Matched in BLAST (N)	No match to known sequences (N)	Genotype
gdh	10	9	14	B
tpi	39	1	20	B
bg	41	4	6	B

Table 7 Search yield for *Giardia intestinalis*-positive sequences from black and gold howler monkeys, *Alouatta caraya*, matched in BLAST NCBI for genotype B.

Sample	Year	Gene	Query Cover	Identity
10	2016	BG	83%	89%
17	2016	TPI	71%	90%
20	2016	BG	95%	79%
22	2016	BG	62%	91%
43	2016	TPI	81%	100%
50	2016	TPI	96%	94%
68	2016	GDH	87%	99%
86	2016	GDH	82%	99%
106	2016	TPI	58%	83%
120	2016	GDH	29%	89%
123	2016	TPI	23%	92%
123	2016	GDH	96%	99%
124	2016	TPI	71%	90%
124	2016	GDH	29%	89%
127	2016	GDH	86%	89%
130	2016	TPI	84%	88%
3	2017	BG	63%	95%
3	2017	GDH	98%	99%
14	2017	BG	95%	88%
25	2017	TPI	88%	84%
36	2017	GDH	73%	97%
41	2017	GDH	70%	92%
42	2017	GDH	40%	83%
48	2017	GDH	73%	96%

8. FIGURES

Figure 1 Satellite map of the study site in San Cayetano (27°34' S, 58°42' W), the Estacion Biologica de Corrientes (27°30' S, 58°41' W), Isla Brasilera (27°20' S, 58°40' W) and Cerrito (27°17' S, 58°37' W) in northern Argentina.

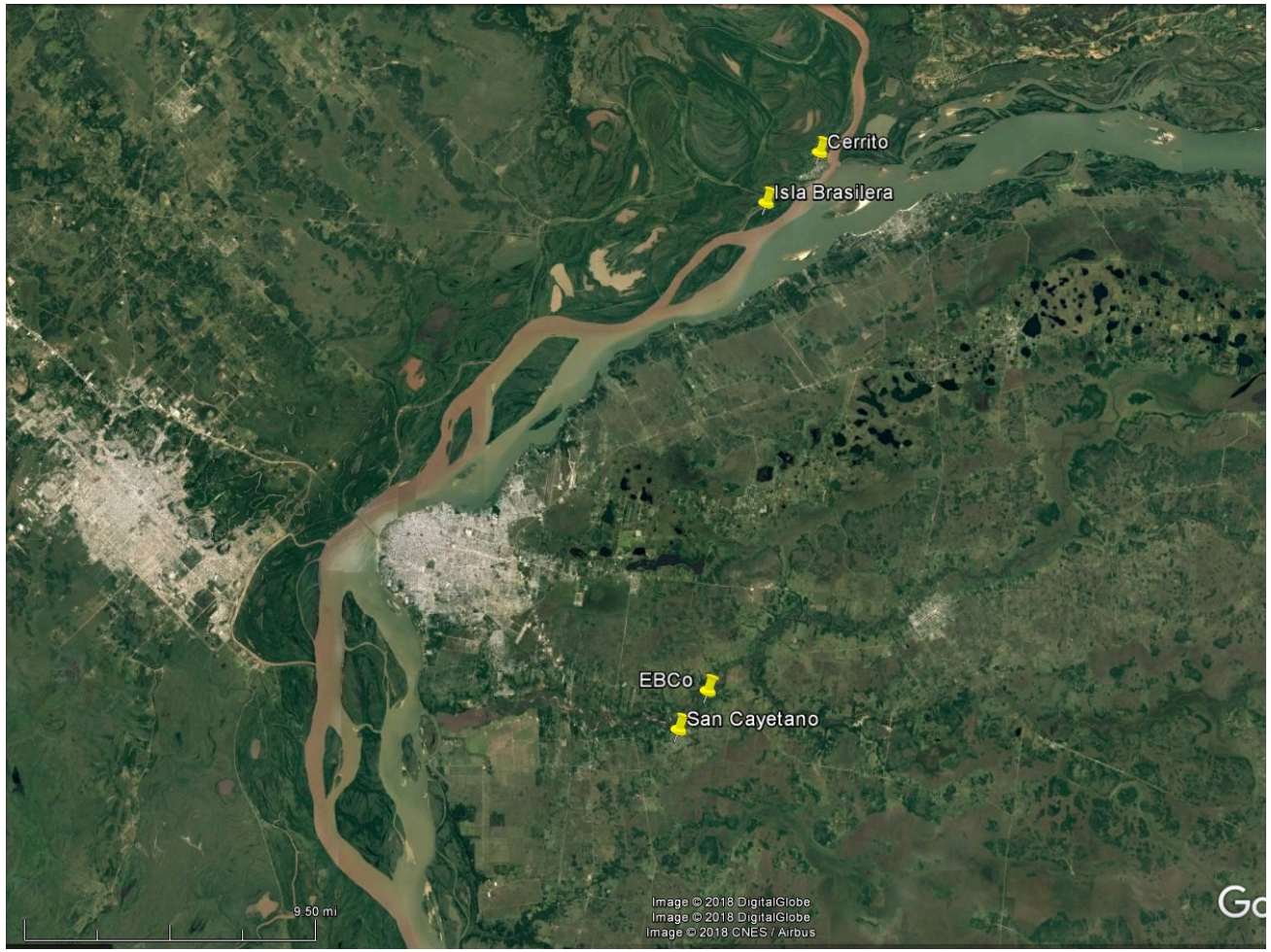

Figure 2 Bar chart of prevalence of *Giardia intestinalis* in black and gold howler monkeys (*Alouatta caraya*) over the three years in remote, rural, and village sites in northern Argentina.

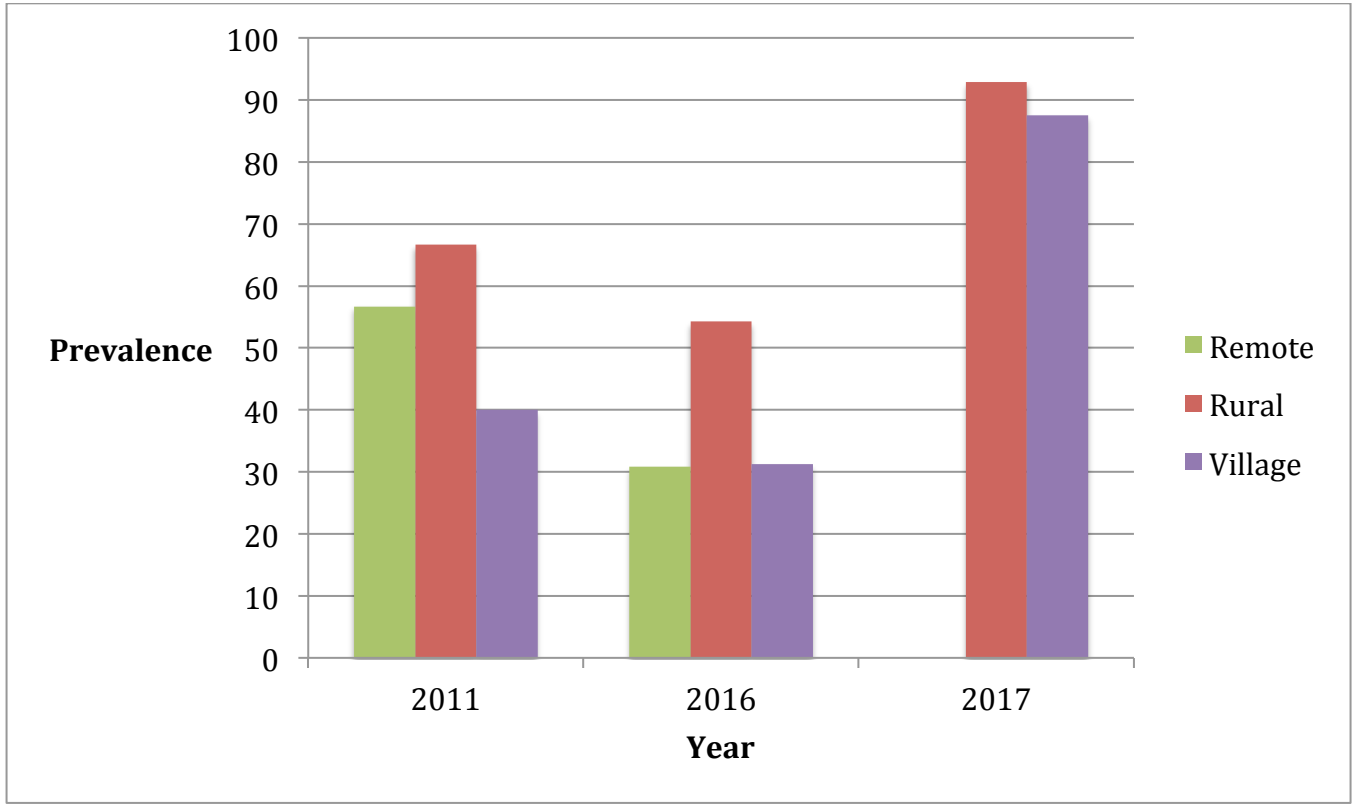

Figure 3 Bar chart of prevalence of *Giardia intestinalis* in village groups of black and gold howler monkeys (*Alouatta caraya*) in 2016 and 2017 in northern Argentina

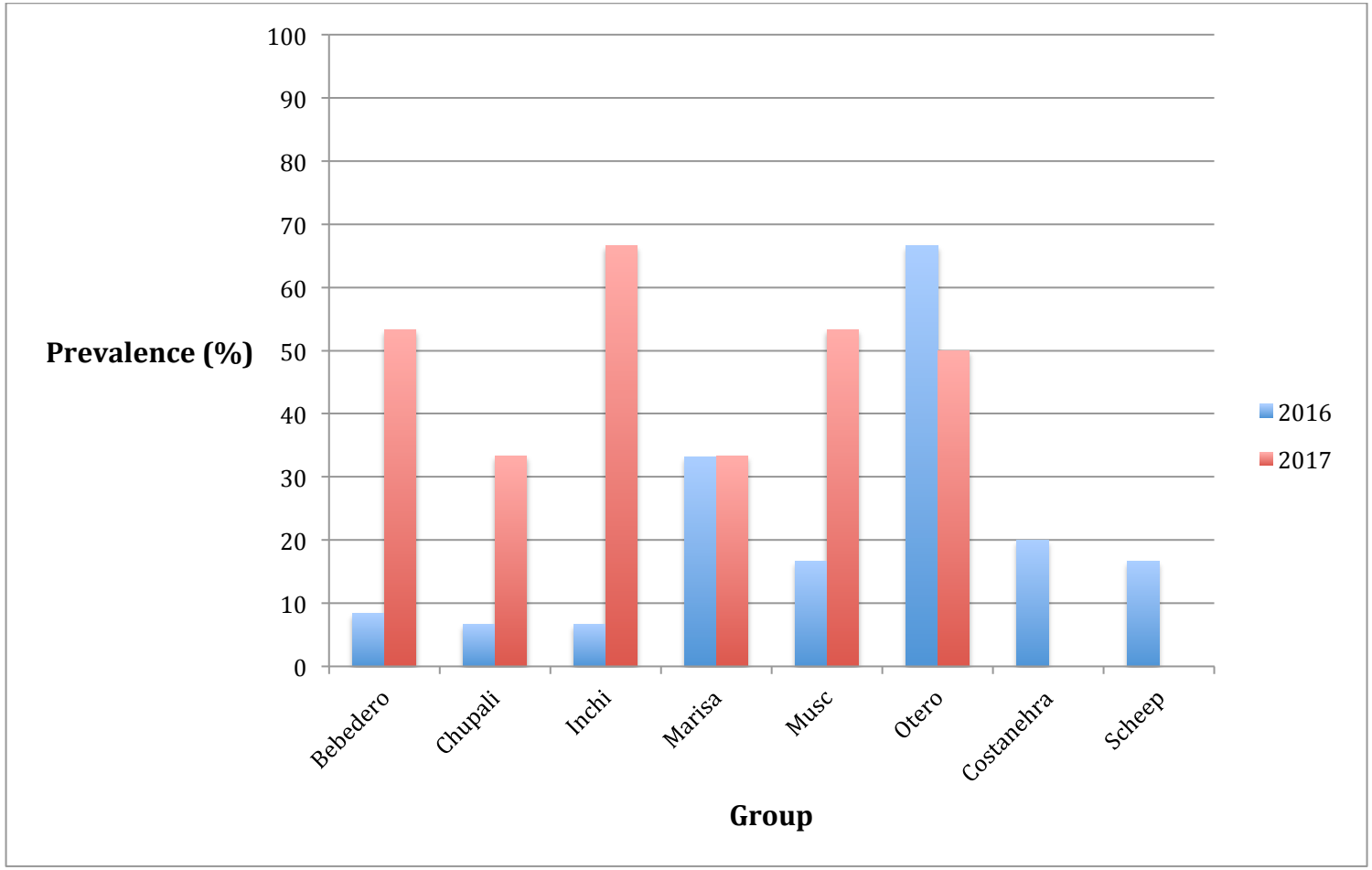

Figure 4 Bar chart of prevalence of *Giardia intestinalis* in rural groups of black and gold howler monkeys (*Alouatta caraya*) in 2016 and 2017 in northern Argentina

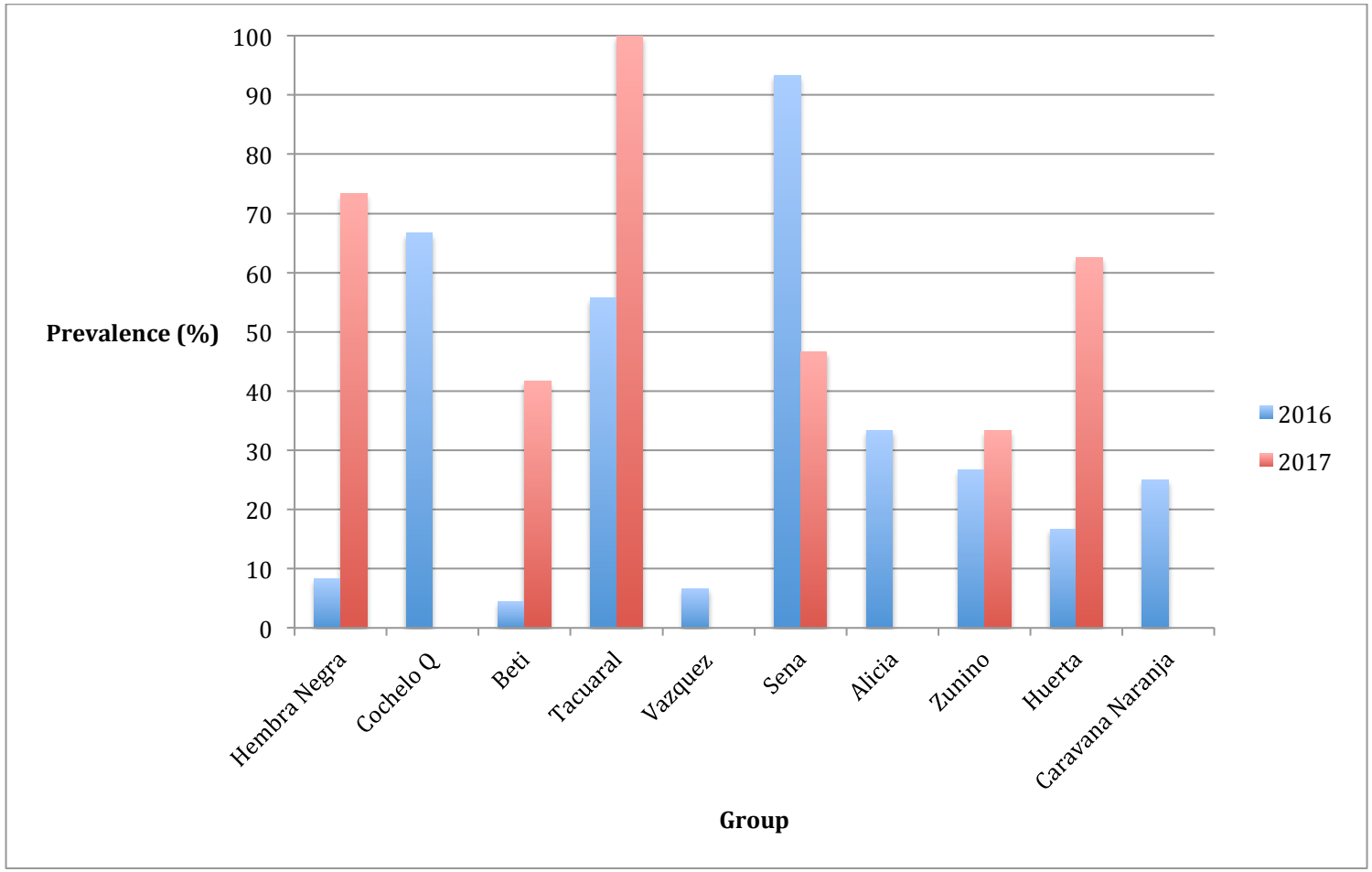

Figure 5 Bar chart of prevalence of *Giardia intestinalis* in remote groups of black and gold howler monkeys (*Alouatta caraya*) in 2016 in northern Argentina