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First Record of *Leucocytozoon* (Haemosporida: Leucocytozoidae) in Amazonia: Evidence for Rarity in Neotropical Lowlands or Lack of Sampling for This Parasite Genus?

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ABSTRACT: Birds harbor an astonishing diversity of haemosporidian parasites belonging to the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. Currently there are more than 250 morphologically described avian haemosporidian species and 2,828 unique lineages found in virtually all avian clades and zoogeographic regions, except for Antarctica. Our report is based on PCR and microscopic screening of 1,302 individual avian samples from Brazil to detect the underrepresented genus *Leucocytozoon*. This survey primarily focuses on passerine birds collected from Amazonia, the Atlantic Rain Forest, and Pantanal. We also summarize studies conducted in Brazil that report haemosporidian prevalence using both microscopy and molecular tools and present for the first time a record of *Leucocytozoon* infecting an avian host population in Amazonia. Based on our findings, we suggest that high average temperatures may be constraining both the distribution and diversity of *Leucocytozoon* in lowland tropical South America.

Avian haemosporidians are a widely distributed group of parasites in terms of both avian host associations and geography, infecting nearly all avian orders and inhabiting all continents except for Antarctica (Valkiunas, 2005). Currently there are more than 250 named species of avian haemosporidians from the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*, all characterized and differentiated morphologically by studying blood films (morphospecies) (see Valkiunas, 2005). However, the true diversity of this cosmopolitan parasite group is not well established, in part, because of insufficient host sampling across highly diverse regions such as the Neotropics. In the past 2 decades the use of polymerase chain reaction (PCR) for amplification of *cytochrome b* has revealed many new lineages that are known only from nucleic acid sequence (genetic lineage). Only a few of these genetic lineages have been matched to known morphospecies (Valkiunas et al., 2008a), with 10-fold more identified genetic lineages (2,828) of avian haemosporidians in the MalAvi database than described morphospecies (Bensch et al., 2009). Efforts to link these genetic lineages to described species are continuing (Valkiunas et al., 2008a, 2008b), indicating the high diversity of this group as well as the need for further studies using both microscopic examination and molecular analysis.

Leucocytozoon is the least studied haemosporidian genus (see Lutz et al., 2015 and Outlaw et al., 2017 for reviews). However, it is quite diverse in some Neotropical communities and reaches its highest prevalence and diversity in the Peruvian and Colombian highlands above 2,200 and 3,000 meters of altitude, respectively (Galen and Witt, 2014; Lotta et al., 2016), or in high latitudes of the Chilean lowlands (Merino et al., 2008). There are currently more than 50 named species (see Valkiunas, 2005) and 749 genetic lineages (MalAvi database). The genus *Leucocytozoon* has 2 subgenera, *Leucocytozoon* and *Akiba*, which are distinguished based on the cells in which exo-erythrocytic merogony occurs and by the vectors that transmit them. For the subgenus *Leucocytozoon*, merogony occurs in hepatocytes and the vectors are blackflies (Diptera: Simuliidae). In contrast, the subgenus *Akiba*, which includes only *Leucocytozoon caulleryi*, does not occur in hepatocytes but undergoes merogony in a variety of different host cells and is vectored by biting midges (Diptera: Ceratopogonidae) (Valkiunas, 2005).

A review by White et al. (1978), published 4 decades ago, summarized the prevalence of avian haemosporidians and other blood parasites in the Neotropics based on records from 35,555 birds (955 species). Remarkably, only 54 individual birds (0.15% prevalence; mostly from Mexico) were infected by *Leucocytozoon*, followed by *Plasmodium* (1.9%) and *Haemoproteus* (7.9%). Before that, Lainson et al. (1970) found no *Leucocytozoon* in 145 blood slides from 45 bird species from northeast Amazonia. A much larger survey of blood parasites in Brazil a decade later reported only 2 (0.06% prevalence) *Leucocytozoon* infections in 3,449 individual birds from 195 species sampled from 3 Atlantic Rain Forest sites in São Paulo State (Bennett and Lopes, 1980). The authors of this study concluded, based on their data from Brazil, and with a comparison among 3 regions (Neotropical, Nearctic, and Southeast Asia) that *Leucocytozoon* was virtually absent in Brazil and rare in the Neotropics (Bennett and Lopes, 1980). A more comprehensive sampling 9 years later in Brazil reported a prevalence of 0.8% for *Leucocytozoon* (it should be noted that the authors included in their calculation of prevalence *Atoxoplasma*, *Babesia*, *Hepatozoon*, *Lankesterella*) from 15,574 birds of 266 species collected from altitudes ranging from sea level to 800 meters in the Atlantic Rain Forest (Woodworth-Lynas et al., 1989). Since then, 6 studies and our own unpublished data (Table I) using microscopy failed to find *Leucocytozoon* in 2,659 birds sampled across 4 Brazilian biomes: Amazonia, the Atlantic Rain Forest, Cerrado, and Pantanal (Table I). Thus, *Leucocytozoon* has never been found in Brazilian biomes, other than the Atlantic Rain Forest in the 1980s. Although Brazil ranks among countries with the highest diversity of haemosporidian parasites, no DNA sequence data on *Leucocytozoon* from the Brazilian

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TABLE I. Summary of studies conducted in Brazil after 1989 using microscopy that failed to find *Leucocytozoon* and additional surveys using molecular and microscopic screening to detect all 3 haemosporidian genera in Brazilian birds: *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*.

Location	Biome	Individuals	Species	Diagnostic	Latitude South	Longitude West	Altitude (meters)	Reference
Brasília, Federal District	Cerrado	772	17	Microscopy	15	47	1,040	Fecchio et al., 2011
Brasília, Federal District	Cerrado	92	1	Microscopy	15	47	1,040	Fecchio et al., 2015
Three sites Tocantins State	Cerrado	676	122	Microscopy	10	48–49	Below 500	Belo et al., 2011
Twelve sites Minas Gerais State	Atlantic Rain Forest	925	109	Microscopy	16–20	40–43	Below 1,300	Sebaio et al., 2012
RDS Mamirauá, Amazonas State	Amazonia	99	1	Microscopy	2	65	Below 100	Roos et al., 2015
Corumbá, Mato Grosso do Sul State	Pantanal	54	1	Microscopy	19	57	93	Dias et al., 2016
Total		2,618						
Corumbá, Mato Grosso do Sul State	Pantanal	20	2	Microscopy	19	57	93	Unpublished
Nossa Senhora Livramento, Mato Grosso State	Pantanal	21	12	Microscopy	16	56	130	Unpublished
Purus-Madeira interfluvium, Amazonas State*	Amazonia	90	1	Molecular	3	60	30–50	Unpublished
Seven locations Tapajós River, Pará State	Amazonia	675	120	Molecular	4–5	56	10–146	Unpublished
REBIO Gurupi, Maranhão State	Amazonia	323	162	Molecular	3	46	177	Unpublished
Reserva Duke, Amazonas State	Amazonia	39	15	Molecular	2	59	50–110	Unpublished
PARNA Serra da Bocaina, Rio de Janeiro State	Atlantic Rain Forest	76	30	Molecular	23	44	700–1,500	Unpublished
PARNA São Joaquim, Santa Catarina State	Atlantic Rain Forest	58	22	Molecular	28	49	1,551–1,666	Unpublished
Total		1,302						

* Indicates the only record of *Leucocytozoon* in Brazil after 1989 and the first record for this parasite genus in Amazonia.

avifauna have been reported. Currently, the MalAvi database contains 414 lineages of *Haemoproteus* and *Plasmodium* recovered from more than 4,000 birds screened in large-scale surveys across the Atlantic Rain Forest and Cerrado (Lacorte et al., 2013) and Amazonia (Fecchio et al., 2017a). Neither of these studies, nor any other published reports using molecular methods (Belo et al., 2011; Fecchio et al., 2013, 2015, 2017b, 2017c; Ferreira Junior et al., 2017), has screened avian samples specifically for the presence of *Leucocytozoon*.

Blackflies, the primary vectors for *Leucocytozoon*, can be found in practically all environments, from altitudes ranging from sea level to over 5,000 meters, with the aquatic larvae found in water ranging from very cold (0 C) to rather warm (exceeding 25 C), and varying in salinity (Coscarón and Arias, 2007). The only necessary condition for larval development in this vector family is continuously running water. With this broad range of environments, blackflies are found in a variety of flowing water bodies across the Neotropics, from tiny streams to large rivers (Coscarón and Arias, 2007). This range of environmental conditions is found across all areas where bird communities have been surveyed in Brazil for haemosporidian parasites, as detailed above. In fact, there are more than 550 blackflies species recorded for the New World, 92 of which are known from Brazil (Adler and Crosskey, 2017).

Consequently, 3 questions arise from this short review of *Leucocytozoon* in Brazilian birds. First, is *Leucocytozoon* absent in avian communities outside the Atlantic Rain Forest? Second, is the prevalence of this parasite genus so low that even a large-scale survey using molecular screening techniques fails to detect them? Third, why is this parasite genus scarce if its potential vectors are present year-round, in virtually all Brazilian biomes?

To answer the first 2 questions, we molecularly screened 1,261 samples collected from the Brazilian Amazonia and Atlantic Rain Forest biomes for the presence of all 3 haemosporidian genera using either the protocol of Hellgren et al. (2004) or the new method published by Bell et al. (2015) that combines real-time PCR for haemosporidian detection and genus-specific nested PCR of positive samples for identification of haemosporidian taxa. This dataset includes a population of blue-crowned manakins (*Lepidothrix coronata*) that was previously screened for *Plasmodium* and *Haemoproteus*, with 34% of individuals (23 infected out of 67 analyzed) infected by *Plasmodium* (Bosholn et al., 2016). Although the other samples from Amazonia, the Atlantic Rain Forest, and Pantanal (using microscopic detection for haemosporidians) lacked any *Leucocytozoon* infections (Table I), we found that 5 of the 90 individuals analyzed from this same population of blue-crowned manakins in central Amazonia were infected with *Leucocytozoon*. These are the only 5 *Leucocytozoon* infections known outside of the Atlantic Rain Forest in Brazil. DNA sequencing revealed that all 5 positive samples were infected by a single unique genetic lineage that differs by over 5% from all other known *Leucocytozoon* morphospecies. Based on this level of genetic divergence this lineage may be a new species (Outlaw and Ricklefs, 2014). The lineage was named LEPCOR08 following standard naming practices (Bensch et al., 2009) and submitted to both GenBank (MG714922–MG714926) and the MalAvi database. Thus, although extremely rare, the genus *Leucocytozoon* does exist outside of the Atlantic Rain Forest, at least within the

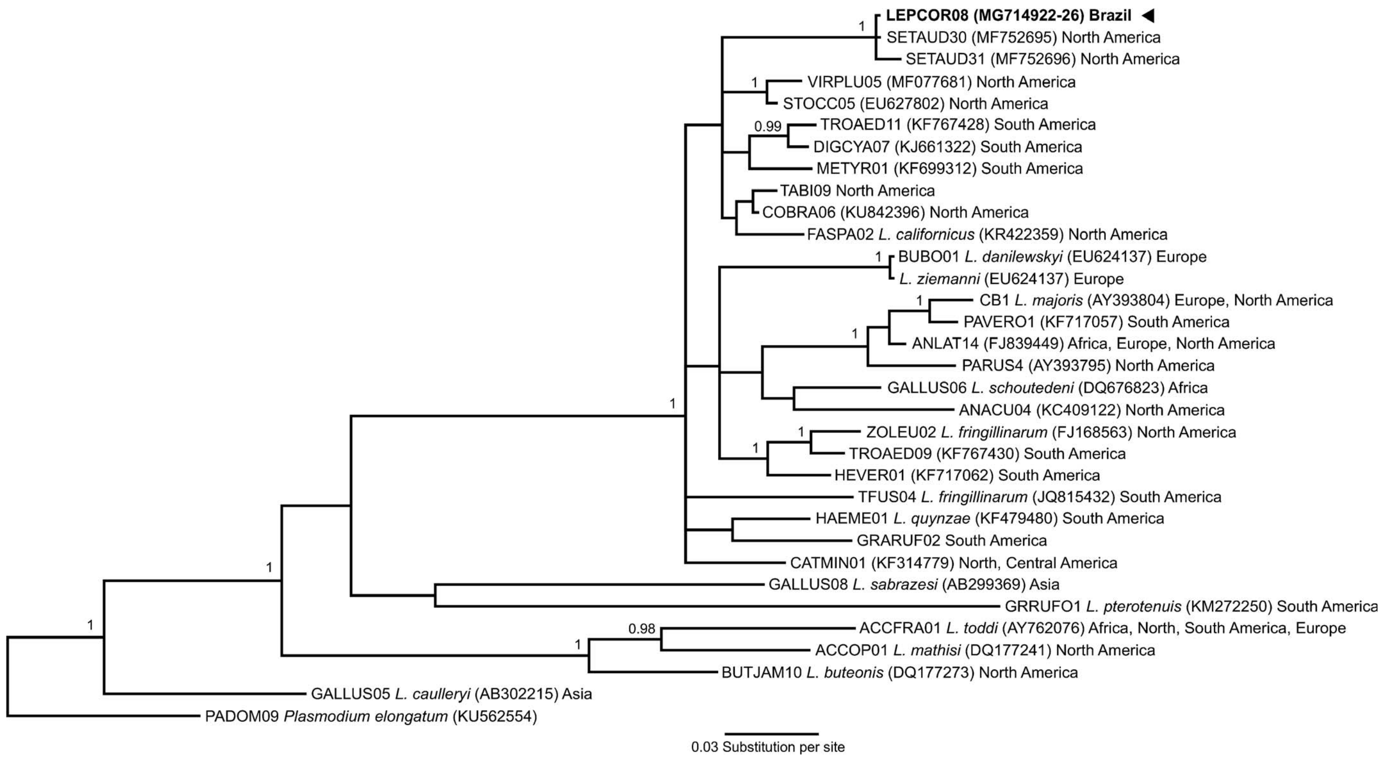

FIGURE 1. Bayesian inference phylogeny showing the position of the new Brazilian *Leucocytozoon* lineage LEPCOR08 among representative *Leucocytozoon* lineages. Geographic origin of each lineage is indicated, and GenBank accession numbers are given in parentheses. Node labels indicate posterior probability support exceeding 0.89.

Amazonian biome. However, the factors causing the scarcity of *Leucocytozoon* throughout Brazil are not well understood.

To determine the phylogenetic position of this new lineage within the genus *Leucocytozoon* a Bayesian inference (BI) phylogeny was reconstructed using Mr. Bayes v.3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Available sequences of known morphospecies and representative lineages from the Americas from GenBank and the MalAvi database were aligned using BioEdit v.7.2.5 (Hall, 1999) with *Plasmodium elongatum*, PADOM09 (KU562554) included as the outgroup. The GTR+I+G model of nucleotide substitution as determined by jModelTest (Guindon and Gascuel, 2003; Darriba et al., 2012) was used for phylogenetic reconstruction with the resulting consensus tree visualized in FigTree v.1.4.3 (Rambaut, 2009).

The new lineage, LEPCOR08, is most closely related to 2 lineages, SETAUD30 and SETAUD31, recovered from the yellow-rumped warbler (*Setophaga coronata*) in New Mexico, United States (Fig. 1), a species not known to winter in South America (Hunt and Flaspohler, 1998). These 3 lineages form a highly supported clade (BI posterior probability = 1.00) and are nested within a larger clade composed of lineages from both North and South America (Fig. 1), although the monophyly of this larger clade is not strongly supported. The new lineage is not closely related to any of the known morphospecies. The closest morphospecies, *Leucocytozoon californicus*, recently described by Walther et al. (2016) from American kestrels (*Falco sparverius sparverius*) in California has 6.4% uncorrected pairwise base pair difference from the new lineage (Fig. 1). The identification of additional Brazilian lineages will be beneficial in understanding the evolutionary history of this parasite genus throughout the Neotropics.

The third question, about the scarcity of *Leucocytozoon*, despite its potential vectors being widespread, may be answered by data on parasite development and not solely by data on vector distribution. *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* have different optimal temperature requirements for their development (Valkiunas, 2005). For example, Roller and Desser (1973) reported a decrease in time needed for exflagellation of *Leucocytozoon simondi* as the temperature increased from 15 to 26 C. LaPointe et al. (2010) showed that sporogonic development of *Plasmodium relictum* was completed at temperatures between 17 to 30 C, but development, prevalence, and intensity decreased significantly below 21 C, and complete sporogony was not observed at temperatures below 17 C. Lower temperature not only constrains the sporogonic development of these parasites, but also reduces the activity of potential *Leucocytozoon* vectors (Valkiunas, 2005; LaPointe et al., 2010). However, both Neotropical sites where *Leucocytozoon* was abundant and diverse are associated with low annual average temperature: highlands in Colombia (Lotta et al., 2016) and Peru (Galen and Witt, 2014) and high-latitude lowlands in Chile (Merino et al., 2008). In contrast, the only 2 regions in Brazil where *Leucocytozoon* has been found, in both cases with very low prevalence and diversity, were the Atlantic Rain Forest (Bennett and Lopes, 1980; Woodworth-Lynas et al., 1989) and Amazonia (this study). These 2 regions, especially Amazonia, have a higher annual average temperature than the highlands of Colombia and Peru and high-latitude lowlands of Chile. Thus, we suggest that higher rather than lower temperature is constraining the distribution of *Leucocytozoon* across lowland Brazil and perhaps lowland tropical South America. Further spatial analyses using environmental data such as temperature, altitude, latitude, and DNA sequences deposited in the MalAvi database could test whether these variables affect prevalence, diversity, and distribution of *Leucocytozoon* in birds across South America. However, this will be possible only if researchers studying avian haemosporidians in South America screen for *Leucocytozoon* as they currently do for *Plasmodium* and *Haemoproteus* by using methods that detect all 3 haemosporidian genera in a single assay, such as the restriction-enzyme digestion method described by Beadell and Fleischer (2005) or the real-time PCR method combined with the genus-specific nested-PCR used herein (Bell et al., 2015). Our detection of *Leucocytozoon* infecting a non-migratory host species in the tropical lowlands suggests that there is a vector capable of transmitting the parasite. This also highlights the importance of conducting studies on vector competence of Brazilian blackflies and sampling the whole avian community to confirm patterns of transmission and host specificity among coexisting vertebrate hosts.

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