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Original Article

Molecular and serological detection of arthropod-borne pathogens in carnivorous birds from Brazil

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ABSTRACT

Rickettsiales, Haemosporida and Rhizobiales agents can cause diseases that affect various animal species, including humans. Due to predation behaviour, carnivorous birds may play an important role in spreading these etiological agentes across geographically distant areas, specially if they are migratory. The aim of this study was to investigate the occurrence and to access the phylogenetic relations among Anaplasmataceae (*Ehrlichia, Anaplasma, Neorickettsia*), Bartonellaceae (*Bartonella* spp.), and Haemosporida (*Plasmodium, Haemoproteus* and *Leucocytozoon*) agents in blood samples from 121 carnivorous birds sampled in the states of São Paulo and Rio de Janeiro, Brazil. Inclusions resembling hemoparasites were not observed in Giemsa-stained preparations. While three animals were seropositive for *E. chaffeensis* (3.41% [3/88]; 95% CI:1.17–9.55%), five showed antibodies to *A. phagocytophilum* (5.68% [5/88]; 95% CI: 2.45–12.62%). Despite the detection of *rrs* gene fragments closely related to *E. chaffeensis* (4.13% [5/121]; 95% CI: 1.78–9.31%), no positivity was observed in the qPCR based on the genes *vlpt* for the organism. Similarly, 12 (9.91% [12/121]; 95% CI: 5.76–16.74%) samples were positive in the qPCR for *Anaplasma* spp. based on *groEL* gene, but negative in the qPCR for *A. phagocytophilum* based on *msp-2* gene. Three samples were positive in the nPCR for *E. canis* based on *rrs* gene. Three samples were positive for *Haemoproteus* spp. and one for *Plasmodium* spp. in the nPCR based on *cytB* gene. Four birds (3.3% [4/121]; 95% CI: 1.29–8.19%) presented co-positivity by *Ehrlichia* sp. and *Anaplasma* sp. in molecular assays. One (0.82% [1/121]; 95% CI:0.15–4.53%) bird showed to be seropositive for *E. chaffeensis* and and positive in PCR for *Haemoproteus* sp. All birds were negative in the qPCR assay for *Bartonella* spp. (*nuoG*). The present work showed the occurrence of Anaplasmataceae agents and hemosporidians in carnivorous birds from southeastern Brazil. The role of these animals in the dispersion of Anaplasmataceae agents should be further investigated.

1. Introduction

The role of avian populations as reservoirs and carriers of arthropod-born agents has been extensively investigated (Ogden et al., 2008; Machado et al., 2012; Lommano et al., 2014; Werther et al., 2017). Haemosporida (Krone et al., 2008; Chagas et al., 2017), Rhizobiales (Mascarelli et al., 2014) and Rickettsiales (Machado et al., 2012; Erwin et al., 2016) orders contain some of the pathogens that can be spread by avian species.

More than 200 hemosporidian species distributed among more than 100 bird species have been described so far. Haemosporidian protozoans (*Plasmodium* spp., *Leucocytozoon* spp. and *Haemoproteus* spp.) are dipterans-borne agents that can infect birds and lead to shortening of telomeres, which damage cells and tissues, reducing the avian life time and interfering in the reproductive success and in species maintenence (Asghar et al., 2015).

Anaplasmataceae agents (*Ehrlichia* spp. and *Anaplasma* spp.), belonging to Rickettsiales order, may cause diseases in animals and

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humans. The real role of avian species in the epidemiology of Anaplasmataceae agents remains unexplored. Nevertheless, *Anaplasma* spp. have already been detected in birds from Brazil (Machado et al., 2012; Werther et al., 2017) and Greece (Ioannou et al., 2009), and in ticks collected from avian hosts from the United States (Daniels et al., 2002), Italy (Mannelli et al., 2003), Canada (Ogden et al. 2008), Germany (Hildebrandt et al., 2010) and Greece (Hoffman et al., 2020). *Ehrlichia* spp. have already been detected in birds from Brazil (Machado et al., 2012; Werther et al., 2017) and in ticks collected from avian hosts from Italy (Toma et al., 2014).

Some *Bartonella* species (Rhizobiales: Bartonellaceae) are associated to human infections, causing four well known diseases: The Carrión disease, Trench fever, Cat scratch disease and bacilar angiomatosis (Dehio, 2004; Liu et al., 2012). *Bartonela henselae* and *B. quintana* are related to the major rate of morbidity and mortality in immunodeficient individuals, while *B. henselae* is the species most frequently associated with human disease (Koehler et al., 2003; Harms & Dehio, 2012). Studies suggest that, due the carriage of infected ticks, some species of wild birds may act in the maintenence and dissemination of *Bartonella* spp. For instance, *Bartonella* spp. have already been detected in ticks collected from migratory birds from Korea (Kang et al., 2013), and Mascarelli et al. (2014) detected two pathogenic species of *Bartonella* spp. (similar to *B. henselae* and *B. koehlerae*) in the blood of migratory birds, belonging to four different species in North Carolina, USA.

In this body of work we aimed to evaluate the occurrence of haemosporidians (*Leucocytozoon* spp., *Plasmodium* spp. and *Haemoproteus* spp.), Bartonellaceae (*Bartonella* spp.) and Anaplasmataceae agents (*Anaplasma* spp. and *Ehrlichia* spp.) in wild carnivorous birds from Brazil, in order to shed some light in the epidemiology of these arthropod-borne agents in avian hosts.

2. Material and methods

2.1. Animals and studied areas

During the years of 2012 to 2014, blood samples were obtained from 121 free-living carnivorous birds, belonging to the Orders Accipitriforme (eagles and hawks), Falconiformes (falcons and carcaras), Strigiformes (owls) and Cathartiformes (vultures) (Table 1). The birds were sampled in the cities of Jaboticabal (21°13′83"S; 48°17′06"W), Santa Ernestina (21°27′94"S; 48°23′81"W), Descalvado (21°54′60"S; 47°37′14"W) and Jundiaí (23°10′11"S; 46°56′33"W), in the state of São Paulo, and in the city of Rio de Janeiro (22°58′46"S; 43°27′25"W), in the state of Rio de Janeiro (Fig. 1). While eagles, hawks and owls were captured using a bal-chatri trap (de Carvalho-Filho et al., 2005), baited with isogenic black fur mice, vultures were captured in an old pigsty baited with viscera. After the blood collection, the captured birds were released in the same location where they were trapped. Additionally, blood samples were obtained from carnivorous birds attended at three wild animal rescue centers, located in the cities of Jaboticabal, Jundiaí and Rio de Janeiro. Blood samples were collected from the right jugular, ulnar, basilica or dorsal metatarsal veins, according to the bird species. Giemsa-stained smears were made from each sample and serum and EDTA-blood samples were stored at −20 °C for further analysis.

This work was approved by govermental license Instituto Chico Mendes para Conservação da Biodiversidade (ICMBio - SISBIO number 31743–1) and Animal Use Ethics Comitee (Comissão de Ética no Uso de Animais - CEUA - FCAV/UNESP number 4153/12).

2.2. Indirect Fluorescent Antibody Test (IFAT) for detection of antibodies to Ehrlichia sp. and Anaplasma sp.

The small quantity of blood obtained from 33 birds precluded performing both molecular and IFAT analyses. For those samples, only molecular analyses were performed. A total of 88 carnivourous bird serum samples were individually tested by Indirect Fluorescent Antibody Test (IFAT) in order to detect IgG antibodies to *E. chaffeensis*, *E. canis* and *A. phagocytophilum*. For this purpose, *E. canis* crude antigens were cultivated in DH82 cells infected with Jaboticabal strain of *E. canis* (Aguiar et al., 2007). Commercial slides (Focus Diagnostics, Cypress, CA, USA) coated with *E. chaffeensis*-infected DH82 cells and *A. phagocytophilum*- infected HL-60-infected were also used as antigens.

Briefly, antigen slides were removed from storage (−20 °C) and allowed to thaw at room temperature for 30 min. Ten microliters of two-fold dilutions of sera (1:40 and 1:80) were placed in wells on antigen slides. Known positive serum samples for the studied agents were obtained from naturally infected deer (Sacchi et al., 2012) and dogs (de Sousa et al., 2014) from Brazil. Negative serum samples were obtained from wild deer captured in Brazil (Sacchi et al., 2012), and dogs maintained in the kennel of the Department of Veterinary Pathology, UNESP, Jaboticabal, São Paulo, Brazil, that had not been exposed to these agents, according to negative PCR and IFAT results. Slides were incubated at 37 °C in a moist chamber for 45 min, washed 3 times in PBS (pH 7.2) for 5 min, and air dried at room temperature. FITC-labeled anti-dog, deer and chicken IgG conjugates (Sigma–Aldrich®, St. Louis, MO, USA) were diluted according to the manufacturer (dilution of 1:32 for anti-dog, 1:10 for anti-deer and 1:10 for anti-bird conjugates) and then added to each well. These slides were incubated again at 37 °C, washed 3 times in PBS, once more in distilled water, and air dried at room temperature. Next, slides were coversliped, and examined under a fluorescence microscope (Olympus®, Tokyo, Japan). The samples were considered positive when fluorescent morulae were observed inside the cells and negative when fluorescent morulae were absent. Positive samples indicated that the birds were exposed to Anaplasmataceae

Table 1
Species and number of birds sampled, including locality and presence or absence of ectoparasites, in the States of São Paulo and Rio de Janeiro, Brazil.

Species	Common name	Number of animals sampled	Locality	Ectoparasites
Speotyto cunicularia	Burrowing own	09	FCAV, AMC, Desc	No
Tyto alba	Western barn owl	07	FCAV; AMC	No
Polyborus plancus	Southern crested caracara	06	FCAV; RJ	No
Rupornis magnirostris	Roadside hawk	16	FCAV; AMC; RJ; Jab; Desc	1 nymph engorged and 3 larvae of *Amblyomma* spp. in one bird
Coragyps atratus	American black vulture	38	FCAV; SE	No
Asio clamator	Striped owl	09	FCAV; AMC; RJ	No
Ictinia plumbea	Plumbeous kite	01	FCAV	No
Megascops choliba	Tropical Screech-Owl	14	FCAV; AMC; RJ	No
Falco femoralis	Aplomado falcon	04	FCAV; Desc	No
Falco sparverius	American kestrel	10	RJ; Jab; Desc	No
Milvago chimachima	Yellow-headed caracara	03	AMC; Desc	No
Parabuteo unicinctus	Harris's hawk	02	RJ	No
Asio stygius	Stygian owl	01	RJ	No
Falco peregrinus	Peregrine falcon	01	RJ	No

FCAV: Ambulatório de Animais Selvagens FCAV – UNESP Jaboticabal-SP; Jab: Jaboticabal-SP; AMC:Associação Mata Ciliar – Jundiaí – SP; RJ: CRAS – Vargem Pequena/RJ; Desc: Descalvado – SP; SE: Santa Ernestina – SP.

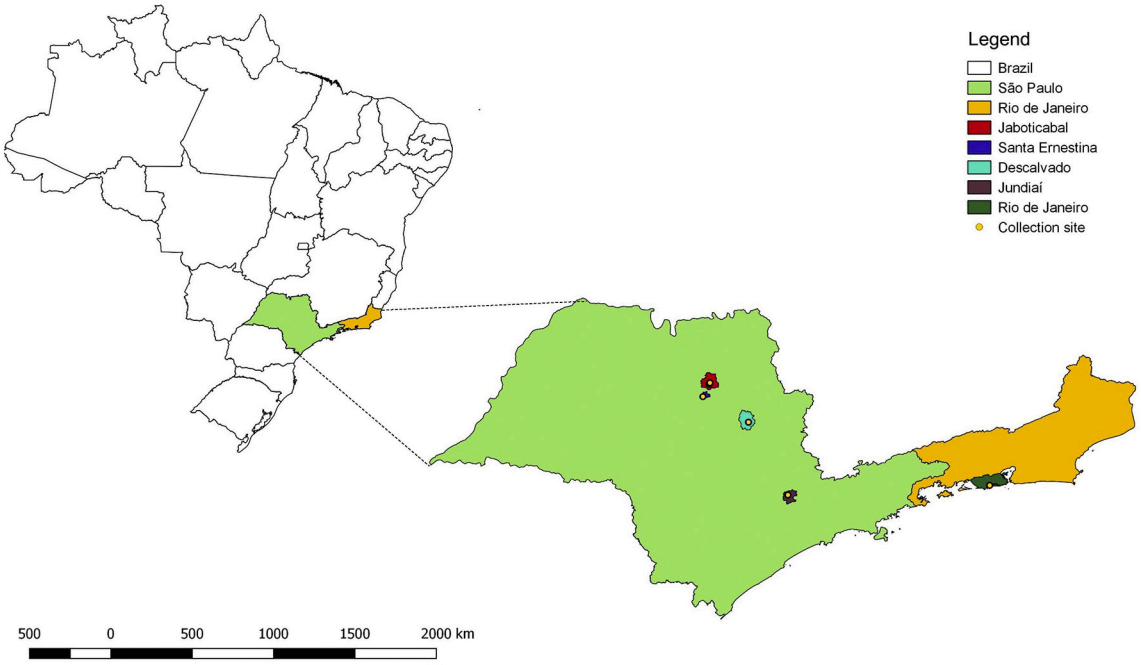

Fig. 1. Map of Brazil showing the states of São Paulo and Rio de Janeiro, on the cities where carnivorous blood were sampled.

agents.

2.3. Molecular analysis

2.3.1. DNA extraction

A total of 121 carnivourous bird blood samples were submitted to molecular analyses. The DNA was extracted from 10 μL of each blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. DNA concentration and quality were measured using absorbance ratio between 260/280 nm (Nanodrop, Term Scientific, USA). The presence of amplifiable DNA was verified by a conventional PCR (cPCR) assay targeting endogenous glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) (Birkenheuer et al., 2003). The positive samples were submitted to real time PCR (qPCR) assays for *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., *A. phagocytophilum* and *E. chaffeensis* cPCR assays for Anaplasmataceae agents and hemosporidians.

2.3.2. Real time PCR (qPCR) assays

Screening was performed for *Ehrlichia* spp. and *Anaplasma* spp. using a multiplex qPCR based on the gene *groEL* (Benevenute et al., 2017), and for *Bartonella* spp. using qPCR based on the gene *nuoG* (André et al., 2016) (Table 2). Positive samples were subsequently submitted to qPCR assays based on the *vlpt* and *msp-2* genes for *E. chaffeensis* and *A. phagocytophilum*, respectively (Table 2). The amplifications were performed with a final volume of 10 μL containing 1 μL of DNA samples, 0.2 μM of each primer and hydrolysis probe, GoTaq Probe qPCR Master Mix (Promega Corporation, Madison WI, USA), and sterilized ultrapure water (Nuclease-Free Water; Promega Corporation). PCR amplifications were performed in low-profile multiplate unskirted PCR plates (Bio-Rad, Hercules, CA USA) using a CFX96 Thermal Cycler (Bio-Rad). Serial dilutions were performed to construct standard curves with different plasmid DNA concentrations (2.0×10^7 to 2.0×10^0 copies/μL). Each qPCR assay was performed in duplicate for each DNA sample. All duplicates showing cycle quantification (Cq) values differing by >0.5 were re-tested. The reactions followed the standards established by the Minimum Information for Publication of Quantitative real-time PCR

Table 2
Description of primers, hydrolysis probes and thermal sequences used in qPCR assays for Ehrlichia spp., Anaplasma spp. and Bartonella spp.[a]

Agents (target-genes)	Primers	Hydrolysis probe (TaqMan)	Thermal sequences	References
Ehrlichia spp. (*groEL* gene)[a]	5′-GCGAGCATAATTACTCAGAG-3′ 5′-CAGTATGGAGCATGTAGTAG-3′	TET- 5′-CATTGGCTCTTGCTATTGCTAAT-3′[BHQ2a-Q]3′	95 °C for 3 min; 40 cycles: 95 °C for 10 min and 52,8 °C for 30 s	Benevenute et al., 2017
Anaplasma spp. (*groEL* gene)[a]	5′-TTATCGTTACATTGAGAAGC-3′ 5′GATATAAAGTTATTAAAAGTATAAAGC-3′	Cy-5- 5′-CCACCTTATCATTACACTGAGACG-3′[BHQ2a-Q]3′	95 °C for 3 min; 40 cycles: 95 °C for 10 min and 52,8 °C for 30 s	Benevenute et al., 2017
Bartonella (*dsb* gene)	5′-CAATCTTCTTTTGCTTCACC-3′ 5′-TCAGGGCTTTATGTGAATAC-3′	TexasRed-5′-TTYGTCATTTGAACACG-3′[BHQ2a-Q]3′	95 °C for 5 min, 40 cycles: 95 °C for 15 s and 60 °C for 1min	André et al., 2012
E. chaffeensis (*vlpt* gene)	5′- CTAATTCTGATTTACACGAGTCTTC-3′ 5′- GCATCATCTTCGAATTGAACTTC-3′	5′[TAMRA] (TTGAGTGTCC[BHQ2a-Q] 3′	95 °C for 3 min, 40 cycles: 95 °C for 10 min e 55 °C for 30 s	Reller and Dumler, 2018
A.phagocytophilum (*msp-2* gene)	5′- 5′-GAAGATGAW GCTGATACAGTA-3′ 5′- CAACHGCCTTAGCAAA CT-3′	5′[Cy-5](TTATCAGTC TGTCCAGTAACA[BHQ2a-Q] 3′	95 °C for 3 min, 40 cycles: 95 °C for 10 min and 55 °C for 30 s	Reller and Dumler, 2018

[a] qPCR multiplex.

experiments (Bustin et al., 2009).

2.3.3. cPCR and nPCR assays

Conventional (cPCR) and nested (nPCR) PCR based on the gene *rrs* for Anaplasmataceae agents were performed (Table 3). Positive samples for *Ehrlichia* spp. and *Anaplasma* spp. were submitted to additional molecular characterization using nPCR assays for *Ehrlichia* spp. and *Anaplasma* spp. based on the *omp*-1 and *groESL* genes, respectively, and by cPCR based on *dsb* gene for *Ehrlichia* spp. (Table 3). *Anaplasma phagocytophilum*, *E. chaffeensis*, *N. risticii*, and *N. helminthoeca* DNA samples were kindly supplied by Prof. Dr. John Stephen Dumler (Uniformed Services of Health Scientes, Bethesda, MD, USA) and used as positive controls. *Ehrlichia canis* (Jaboticabal strain) and *A. platys* (Dagnone et al., 2009) were also used as positive controls. Sterile ultrapure water (Nuclease-Free Water, Promega Corporation) was used as a negative control.

Additionally, the DNA samples were tested in cPCR and nPCR assays for *Leucocytozoon* spp., *Plasmodium* spp. and *Haemoproteus* spp. based on cytochrome B *(cytB)* gene. Positive and negative DNA controls for *Plasmodium* sp. and *Haemoproteus* sp. were used in the cPCR assays for hemosporidians (Werther et al., 2017) (Table 2).

All cPCR assays were performed using 5 μL of the DNA samples in a mixture containing 1.25 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA), PCR buffer (PCR buffer 10 X – 100 nM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM deoxynucleotides (dATP, dTTP, dCTP, and dGTP) (Invitrogen, Carlsbad, California, United States), 1.5 mM of Magnesium Chloride (Invitrogen, Carlsbad, CA, United States), 0.5 μM of each primer (Invitrogen), and sterile ultrapure water (Invitrogen) q.s.p. 25 μL. In nPCR assays, 3 μL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction.

The products were separated by 1% agarose gel electrophoresis at an electric current of 100 V/150 mA for 50 min. The gel was stained with 1% ethidium bromide (Life Technologies, Carlsbad, CA, USA) and examined under ultraviolet light illumination using the ChemiDoc MP Imaging System (Bio-Rad) and imaged using Image Lab Software version 4.1. The bands corresponding to each amplificon were cut from the agarose gel with a sterile scalpel blade, weighed and stored in a 2.0 mL polypropylene tube. Subsequently, the PCR product was extracted in agarose gel, using a DNA purification kit (Fermentas), according to the manufacturer's recommendations. For samples in which only one band was observed in the gel, DNA was purified directly from the nPCR product using the same kit. The purified products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems) (SANGER et al., 1977).

2.3.4. Phylogenetic analyses

The sequences obtained were submitted to a quality-screening test using Phred (EWING et al., 1998) to evaluate the quality of the electropherograms. Consensus sequences were obtained through the analysis of the sense and antisense sequences using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Comparisons with sequences deposited in GenBank were done using the basic local alignment search tool (BLAST) (Benson et al., 2002). The sequences were aligned with sequences published in GenBank using Clustal W in Bioedit v. 7.0.5.3 (HALL, 1999). The Maximum-likelihood (ML) analysis was inferred with the W-IQ-Tree tool available online (http://iqtree.cibiv.univie.ac.at/) (Nguyen et al., 2016; Trifinopoulos et al., 2016) using 1000 bootstrapping replicates. The best evolution model was selected by the program jModelTest2 (version 2.1.6) on XSEDE (Darriba et al., 2012) via CIPRES Science Gateway (Miller et al., 2010), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). The phylogenetic trees were edited using TreeGraph 2.0.56–381 beta software (Stover and Muller, 2010).

3. Results

3.1. Blood smears

No inclusions suggestive of Anaplasmataceae agents or hemosporidians (*Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp.) were found in blood smears from the sampled avian hosts.

3.2. Serological assays (IFAT)

Out of 88 serum or plasma samples analyzed, five (5.68% 95% CI: 2.45–12.62%) were seropositive for *A. phagocytophilum* (two *Rupornis magnirostris* and three *Coragyps atratus* sampled in São Paulo state) and three (3.41%; 95% CI:1.17–9.55%) were seropositive for *E. chaffeensis* (one *C. atratus*, one *Asio clamator* and one *Megascops choliba*), all sampled in São Paulo state) (Table 4). All the samples showed titers of 40. None was seropositive for *E. canis*.

3.3. PCR assays, BLAST and phylogenetic analyses

All 121 DNA samples were positive in the cPCR assay based on the *gapdh* endogenous gene.

3.3.1. Anaplasmataceae agents

Out of 121 DNA samples from carnivorous birds, 9.91% (12 = 1 *Megascops choliba,* 2 *Speotyto cunicularia,* 1 *Tyto alba*, 1 *Asio clamator,* 1 *Milvago chimachima*, 2 *Falco femoralis* and 4 *Rupornis magnirostris*; 95% CI: 5.76–16.74%) were positive in the qPCR for *Anaplasma* spp. based on the *groEL* gene (Table 4). None of the samples were positive for the other qPCR assays for *Ehrlichia* spp., *A. phagocytophilum* and *E. chaffeensis* based on *groEL, msp-2* and *vlpt* genes, respectively.

In nPCRs assays based on *rrs* gene, five (4.13% - 2 *S. cunicularia,* 1 *Megascops choliba*, 1 *R. magnirostris* and 1 *T. alba;* 95% CI: 1.78–9.31%) samples were positive for *E. chaffeensis* and three (2.47% - 2 *R. magnirostris* and 1 *A. clamator;* 95% CI: 0.85–7.04%) samples were positive *E. canis* (Table 4). The sequences obtained were deposited in GenBank under accession numbers MT752954 to MT752960.

BLASTn analysis performed on five sequences obtained in the nPCR for *E. chaffeensis rrs* gene showed 98.30–100% identity with *Ehrlichia* sp. detected in Orinoco goose (*Neochen jubata*) from Brazil. Two sequences obtained in the nPCR for *E. canis* based on *rrs* gene showed 99.23 and 99.48% identity with *Ehrlichia* sp. detected in ticks from Brazil (Table 4).

The phylogenetic analysis by ML showed that two *Ehrlichia* spp. sequences detected in *Asio clamator* and *Rupornis magnirostris* were positioned together with *Ehlichia* sp. detected in ticks from Brazil into the *E. canis* clade with 94% branch support. Five sequences (two detected in *S. cunicularia*; one each in *R. magnirostris*, *T. alba* and *M. choliba* specimens) formed a clade with other *Ehrlichia* sp. sequences previously detected in Orinoco goose in Brazil, with 71% branch support. This clade was positioned into the *E. chaffeensis* clade (Fig. 2).

None of the samples was positive for *A. phagocytophilum*, *A. platys*, *E. ewingii*, *N. risticii* and *N. helminthoeca* based on *rrs* gene, neither in the PCR assays targeting other genes for additional molecular characterization (*groEL, omp-1* and *dsb*).

3.3.2. Haemosporidian agents

Out of 121 DNA samples, 3.30% (4: 3 *M. choliba* and 1 *Ictinia plumbea*; 95% CI: 1.29–8.19%) were positive for hemosporidians based on *cytB*. In the BLASTn analysis, two sequences showed 96.42 and 97.13% identity with *Haemoproteus* sp. detected in a Barred owl (*Strix varia*) from USA, while one sequence showed 98.28% identity with *Haemoproteus syrnii* detected in a Tropical Screech-Owl (*M. choliba*) in Brazil. Addictionaly, the sequence detected in a *I. plumbea* specimen showed 97.39% identity with *Plasmodium paranucleophilum* detected in a Southern crested caracara (*Caracara planctus*) from Brazil (Table 4). The sequences obtained were deposited in GenBank under accession

Table 3
Description of primers, amplicon sizes and thermal sequences used in conventional PCR (cPCR) and nested PCR (nPCR) assays for Ehrlichia spp., Anaplasma spp. and hemosporidian.

Agents	Primers sequences	Size (bp)	Thermal sequences	References
Ehrlichia spp.	5'- GAACGAACGCTGGCGGCAAGC-3'	478	94 °C for 5 min	Murphy et al., 1998
- ECC			40 cycles: 94 °C for 1 min, 60 °C for 1 min	
- ECB	5'- CGTATTACCGCGGCTGCTGGCA −3'		and 72 °C for 1 min	
			72 °C for 5 min	
Nested *E. canis*	5'-CAATTATTTATAGCCTCTGGCTATAGGA −3'	358	94 °C for 5 min	Murphy et al., 1998
- ECAN-5	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'		40 cycles: 94 °C for 1 min, 55 °C for 1 min	
-HE3			and 72 °C for 1 min	
			72 °C for 5 min	
Nested *E. chaffeensis*- CHAF	5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'	410	94 °C for 5 min	Persing, 1996
- GAIUR	5'-GACTTTGCCGGGACTTC TCT - 3'		40 cycles: 94 °C for 1 min, 55 °C for 1 min	
			and 72 °C for 1 min	
			72 °C for 5 min	
Nested *E. ewingii*		412	94 °C for 5 min	Persing, 1996
- 18F	5'- AGTTTGATCATGGCTCAG-3'		40 cycles: 94 °C for 1 min, 45 °C for 2 min	
- 1448R	5'- CCATGGCGTGACGGGCAGTGTG-3'		and 72 °C for 2 min	
- Ewingii	5'-CAATTCCTAAATAGTCTCTGACTATT-3'		72 °C for 5 min	
- GAIUR	5'-GACTTTGCCGGGACTTCTTCT-3			
Anaplasma spp. (*rrs* gene)*		932	94 °C for 5 min	Massung et al., 1998
			40 cycles: 94 °C for 30s, 55 °C for 30s and	
External primers			72 °C for 1 min	
- gE3a	5'- CACATGCAAGTCGAACGGATTATTC-3'		72 °C for 5 min	
- gE10R	5'- TTCCGTTAAGAAGGATCTAATCTCC'-3'			
Internal primers		546		
- gE2	5'- GGCAGTATTAAAAGCAGCTCCAGG-3'			
- gE9f	5'-AACGGATTATTCTTTATAGCTTGCT-3'			
Anaplasma platys		504	94 °C for 5 min	Inokuma et al., 2001
- Platys F	5'- AAGTGCAACGGATTTTTGTC-3'		40 cycles: 95 °C for 30s, 60 °C for 30 s and	
- Platys-R	5'- CTTTAACTTACCGAACC-3		72 °C for 90s	
			72 °C for 5 min	
Neorickettsia risticii		529	94 °C for 5 min	Chae et al., 2003
- ER3-F	5'- ATTTGAGAGTTTGATCCTGG-3'		40 cycles: 94 °C for 1 min, 72 °C for 2 min	
- ER2-R	5'- GTTTTAAATGCAGTTCTTGG-3'		and 72 °C for 2 min	
- ER3a-F	5'- CTAGCGGTAGGCTTAAC-3'		72 °C for 5 min	
- ER2a-R	5'- CACACCTAACTTACGGG-3'			
N. helminthoeca		200	94 °C for 2 min	Headley et al., 2006
NeoSH – F	5'-TAGGCCCGCGTTAGATTAGCTTGT-3'		40 cycles: 94 °C for 1 min, 67 °C for 45 s	
NeoSH - R	5'-TACAACCCAAGGGCCTTCATCACT-3'		and 72 °C for 45 s	
			72 °C for 5 min	
Plasmodium spp. e		480	94 °C for 3 min	Hellgren et al., 2004
Haemoproteus spp.			20 cycles: 94 °C for 30s, 50 °C for 30s and	
HamNF-1	5' – CATATATTAAGAGAATATGGAG-3'		72 °C for 45 s	
HaemNR3	5' – ATAGAAAGATAAGAAATACCATTC-3'		72 °C for 10 min	
Haem F	5' – ATGGTGCTTTCGATATATGCATG-3'		94 °C for 3 min	Bensch et al., 2000
Haem R2	5' – GCATTATCTGGATGTGATAATGGT-3'		35 cycles: 94 °C for 30s, 55 °C for 30s and	
			72 °C for 45 s	
			72 °C for 10 min	
Leucocytozoon spp.		478	94 °C for 3 min	Hellgren et al., 2004
HaemFL	5' – ATGGTGTTTTAGATACTTACATT – 3'		35 cycles: 94 °C for 30s, 55 °C for 30s and	
HaemR2L	5' – CATTATCTGGATGAGATAATGG – 3'		72 °C for 45 s	
			72 °C for 10 min	
Ehrlichia spp. (*dsb* gene)		409	95 °C for 2 min;	Doyle et al., 2005
			50 cycles: 95 °C for 15 s, 58 °C for 30s and	
-dsb-330 (F)	5'- GATGATGTCTGAAGATATGAAACAAAT-3'		72 °C for 30s	
-dsb-728 (R)	5'- CTGCTCGTCTATTTTACTTCTTAAAGT-3'		72 °C for 5 min	
Ehrlichia spp. (*groEL* gene)		1297	3 cycles: 94 °c for 1 min, 48 °C for 2 min, 72 °C for 1 min	Sumner et al., 1997; Nicholson et al., 1999; Lotric-Furlan et al., 1998; Liz et al., 2000
- HS1a	5'-AITGGGCTGGTAITGAAAT-3'		37 cycles: 94 °C for 1 min, 48 °C for 2 min	
- EHR-CS778R	5'- CCICCIGGIACIAIACCTTC-3'		and 72 °C for 1 min	
- HS43	5'-AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC-3'		72 °C for 5 min	
- HSVR	5'-CTCAACAGCAGCTCTAGTAGC-3'		Annealing temp. in 2° round: 55 °C	
Ehrlichia spp. (*omp-1* gene)*		300	94 °C for 3 min, 35 cycles: 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min	Inayoshi et al., 2004
External primers			72 °C for 5 min	
-conP28-F1	5'AT(C/T)AGT(G/C)AAA(A/G)TA(T/C)(A/G)T(G/A)CCAA-3'			
-conP28-R1	5'TTA(G/A)AA(A/G)G(C/T)AAA(C/T)CT(T/G)CCTCC-3'			
Internal primers				
-conP28-F2	5'CAATGG(A/G)(T/A)GG(T/C)CC(A/C)AGA(*AG*)TAG-3'			
-conP29-R2	5'TTCC(T/C)TG(A/G)TA(A/G)G(A/C)AA(T/G)TTTAGG-3′			

Table 4
Results obtained from serological analyses, PCR assays and maximum identity provided by BLASTn for Anaplasmataceae agents and hemosporidian.

Identification / Locality	Species	IFAT - *E. chaffeensis*	IFAT - *A. phagocytophilum*	qPCR – *Anaplasma* spp. (*groEL* gene)	nPCR – *E. chaffeensis* (*rrs* gene)	nPCR – *E. canis* (*rrs* gene)	nPCR – *Plasmodium* spp. and *Haemoproteus* spp. (*cyt B* gene)
10/FCAV	*Ictinia plumbea*	–	–	–	–	–	97.39% - *Plasmodium paranucleophilum*, *Carcara planctos*, Brazil (KX159495)
21/JAB	*Rupornis magnirostris*	–	+	–	–	–	–
24/FCAV	*Asio clamator*	+	–	–	–	–	–
32/SE	*Coragyps atratus*	–	+	–	–	–	–
35/SE	*Coragyps atratus*	+	–	–	–	–	–
54/SE	*Coragyps atratus*	–	+	–	–	–	–
59/SE	*Coragyps atratus*	–	+	–	–	–	–
62/FCAV	*Speotyto cunicularia*	–	–	–	98.30% - *Ehrlichia* sp., *Neochen jubata*, Brazil (KX898561)	–	–
63/FCAV	*Megascops choliba*	–	–	–	98.79% - *Ehrlichia* sp., *Neochen jubata*, Brazil (KX898561)	–	–
68/FCAV	*Rupornis magnirostris*	–	–	–	–	99.23% - *Ehrlichia* sp., tick, Brazil (KY930382)	–
69/Desc	*Rupornis magnirostris*	–	+	–	–	–	–
71/AMC	*Rupornis magnirostris*	–	–	–	99.76% - *Ehrlichia* sp., *Neochen jubata*, Brazil (KX898561)	–	–
75/AMC	*Megascops choliba*	–	–	+	–	–	–
78 /AMC	*Speotyto cunicularia*	–	–	+	100% - *Ehrlichia* sp., *Neochen jubata*, Brazil (KX898561)	–	–
83/AMC	*Tyto alba*	–	–	+	99.76% - *Ehrlichia* sp., *Neochen jubata*, Brazil (KX898561)	–	–
87/AMC	*Asio clamator*	–	–	+	–	99.48% - *Ehrlichia* sp., tick, Brazil (KY930382)	–
89/AMC	*Megascops choliba*	+	–	–	–	–	97.13% - *Haemoproteus* sp., *Strix varia*, USA (AF465589)
90/AMC	*Megascops choliba*	–	–	–	–	–	98.28% - *Haemoproteus syrnii*, *Megascops choliba*, Brazil (KJ575554)
92/AMC	*Megascops choliba*	–	–	–	–	–	96.42% - *Haemoproteus* sp. *Strix varia*, USA (AF465589)
113/Desc	*Speotyto cunicularia*	–	–	+	–	–	–
115/Desc	*Milvago chimachima*	–	–	+	–	–	–
116/Desc	*Falco femoralis*	–	–	+	–	–	–
117/Des	*Falco femoralis*	–	–	+	–	–	–
118/Jab	*Rupornis magnirostris*	–	–	+	–	–	–
119/Desc	*Rupornis magnirostris*	–	–	+	–	–	–
120/Jab	*Rupornis magnirostris*	–	–	+	–	–	–
121/Desc	*Rupornis magnirostris*	–	–	+	–	NS	–

numbers MT762169 to MT762172.

The ML phylogenetic analysis for *Haemoproteus* spp. positioned the three sequences detected in *M. choliba* in a clade formed by *Haemoproteus syrni* and *Haemoproteus* sp. detected in Barred owl, with 95% branch support (Fig. 3). The phylogenetic analysis for *Plasmodium* spp. positioned the obtained sequence in the present study in a separate branch, close to the clade formed by *P. paranucleophilum*, *P. nucleophilum* and *P. ashfordi,* with 98% branch support (Fig. 4).

3.3.3. Bartonellaceae agents

None of the samples was positive in the qPCR assay based on the *nuoG* gene for *Bartonella* spp.

3.4. Co-positivity by arthropod-borne pathogens

Co-positivity for *Ehrlichia* spp. (obtained by nPCR based on the *rrs* gene) and *Anaplasma* spp. (obtained by qPCR based on the *groEL* gene)

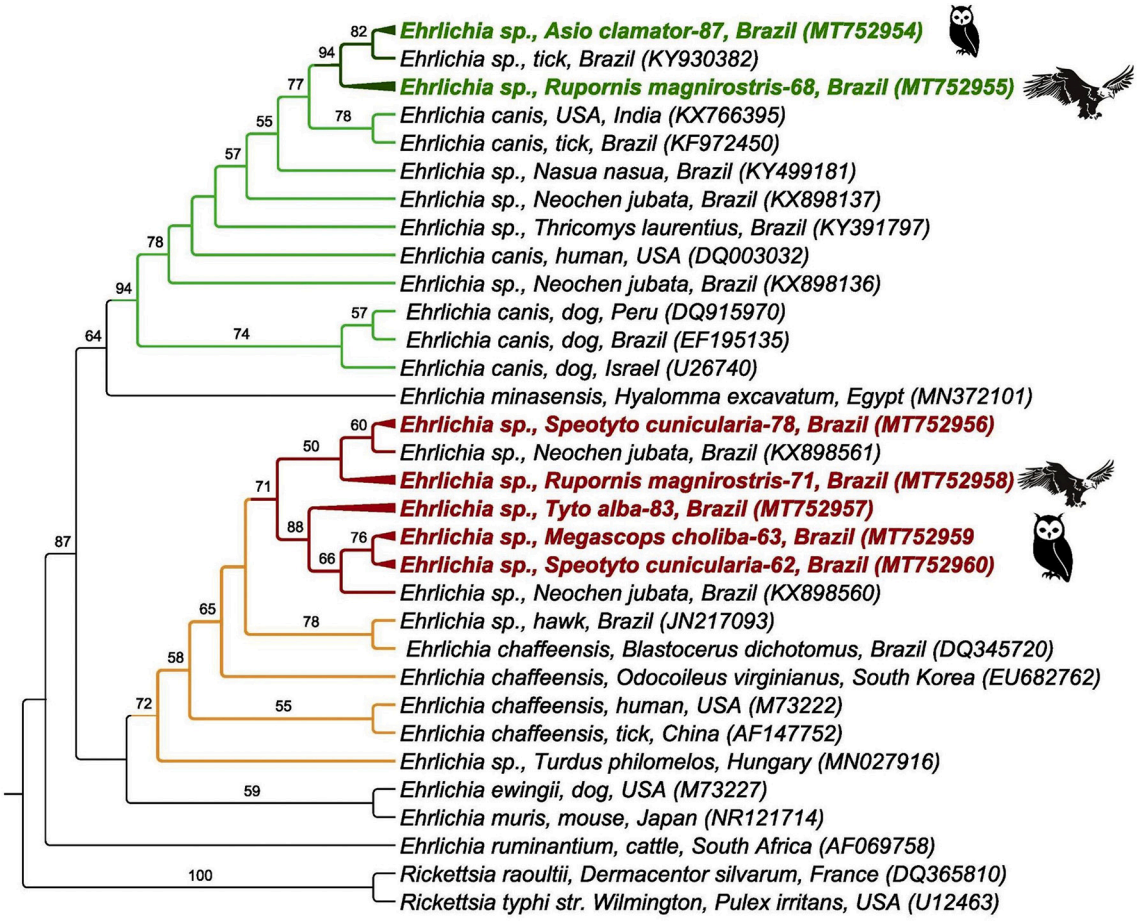

Fig. 2. Phylogenetic analysis of *Ehrlichia rrs* sequences based on the topology Maximum Likelihood, with *GTR + G* as evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1000 replicates *Rickettsia raoultii and R. typhi* were used as an external group.

was detected in 3.3% (4/121) of the sampled carnivourous birds (*T. alba*, *S. cunicularia*, *A. clamator* and *R. magnirostris*). Additionally, a Tropical Screech-Owl (*M. choliba)* was shown to be co-positive in IFAT for *E. chaffeensis* and for *Haemoproteus* spp. (cPCR based on the *cytB*).

4. Discussion

This study demonstrated by molecular and serological techniques the exposure of carnivorous birds to Anaplasmataceae agents and hemosporidians. In IFAT, 5.68% (4/88) and 3.41% (3/88) of the birds showed to be seropositive to *A. phagocytophilum* and *Ehrlichia chaffeensis*, respectively. While 9.91% (12/121) of the birds were positive for *Anaplasma* spp. (*groEL*) using a qPCR assay, 6.61% (8/121) were positive for *Ehrlichia* spp. (*rrs*), 2.48% (3/121) for *Haemoproteus* spp. (*cytB*) and 0.83% (1/121) for *Plasmodium* spp. (*cytB*) using nPCR and cPCR.

The results obtained for Anaplasmataceae agents showed that fewer birds were positive in IFAT for *E. chaffeensis* and *A. phagocytophilum* when compared to the number of animals positive in the PCR assays. This finding may be related to the genetic variability and antigenic diversity of this group of pathogens. A similar result was obtained by Sacchi et al. (2012) among free-ranging deer from Brazil: 20.2% of the animals were seropositive for *A. phagocytophilum*, whereas 50% were positive for *Anaplasma* sp. in the molecular assays. The obtained sequences were phylogenetically close to *A. platys*, suggesting that IFAT might have detected antibodies to this agent, which serologically cross-react with *A. phagocytophilum* antigen. Therefore, considering the low number of seropositive samples and the low antibody titers, the positivity in the serological assays detected in the present study might represent the exposure to an Anaplasmataceae agent that cross-reacted with *A. phagocytophilum* and *E. chaffeensis*. On the other hand, Werther et al. (2017) found a higher number of Orinoco geese seropositive in IFAT for *A. phagocytophilum* (10% [6/60]) when compared to the number of animals positive in PCR assays for *Anaplasma* spp. (3.33% [2/60]). Despite the positivity of 11.29% and 25.8% for *E. canis* and *E. chaffeensis* in *rrs*-based nPCR assays, Werther et al. (2017) did not find Orinoco geese seropositive for *E. canis* and *E. chaffeensis*.

Johnston et al. (2013), in an experimental infection-based study, reported that, even though *Turdus migratorius* and *Dumetella carolinensis* became infected with *A. phagocytophilum,* they were not very eficient to transmit this agent to ticks. Therefore, it seems that birds may not develop high bacteremia, specific antibodies or severe disease due to exposure, which indicates that serological surveys in birds may not represent a reliable method for investigating the exposure to *A. phagocytophilum*.

Eight birds were positive in nPCR assays for *Ehrlichia* spp. based on the *rrs* gene, and five of the sequences obtained were phylogenetically positioned in a clade containing sequences of *Ehrlichia* sp. detected in Orinoco geese and hawks from Brazil, near the *E. chaffeensis* clade. These samples were not positive in a specific qPCR for *E. chaffeensis* (*vlpt* gene), which indicates the occurrence of a probable new genotype of *Ehrlichia* sp. in birds from Brazil. Additionally, two sequences were positioned into *E. canis* clade, closer to a sequence of *Ehrlichia* sp. detected in a tick from Brazil.

Ehrlichia sp. phylogenetically related to *E. chaffeensis* has already been reported in American kestrel (*Falco sparverius*) (*rrs* gene) and Orinoco geese (*Neochen jubata*) (*rrs* gene) from Brazil, vultures in Florida (*groESL* gene), song thrushes (*Turdus philomelos*) from Hungary (*rrs* gene), and Common pheasants (*Phasianus colchicus*) from China (*rrs*

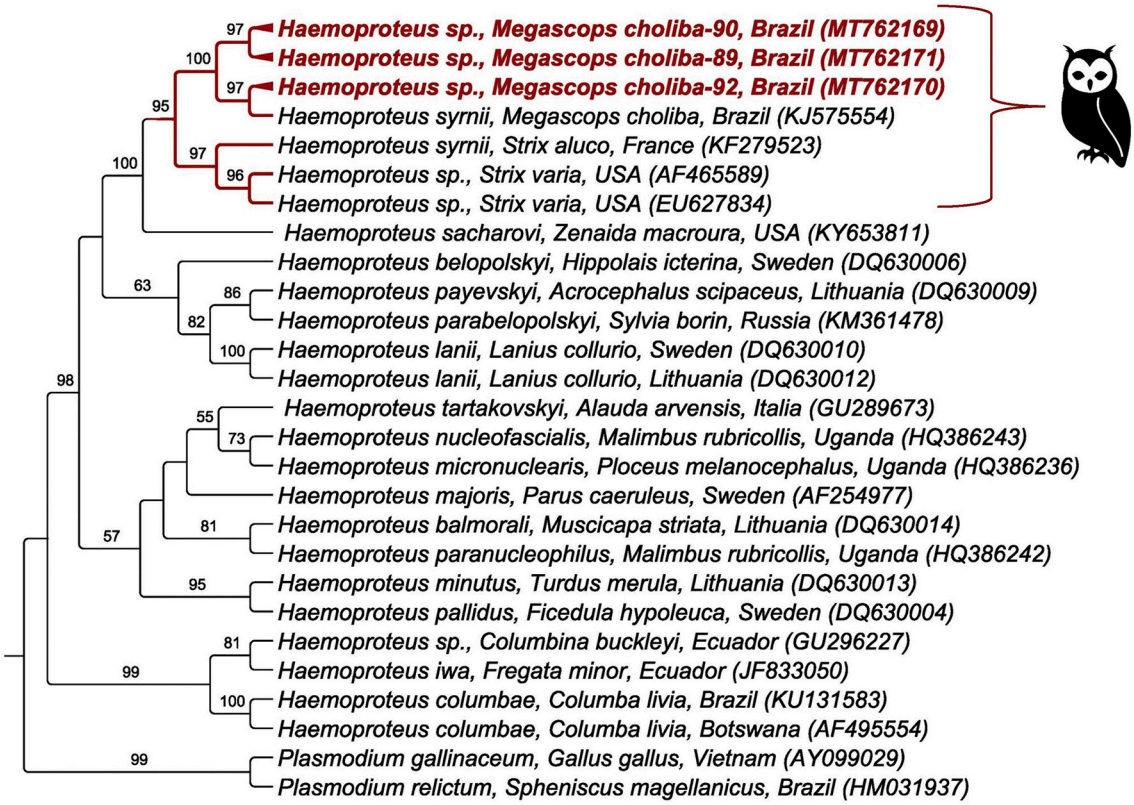

Fig. 3. Phylogenetic analysis of *Haemoproteus cytB* sequences based on the topology Maximum Likelihood, with *GTR + I + G* as evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1000 replicates *Plasmodium gallinaceum and P. relictum* were used as an external group.

gene) (Machado et al., 2012; Yang et al., 2015; Erwin et al., 2016; Werther et al., 2017; Hornok et al., 2020). Additionally, *rrs* genotypes phylogenetically close to *E. canis* were also reported in vultures and Orinoco geese from Brazil (Machado et al., 2012; Werther et al., 2017). A new genetic variant based on the *rrs*, *groEL* and *dsb* genes, called *Ehrlichia* sp. *Magellanica,* has been detected in penguins (*Spheniscus demersus*) from Chile (Muñoz-Leal et al., 2019). In Brazil, Machado et al. (2012) and Werther et al. (2017) detected a new *Ehrlichia omp-1* genotype in Orinoco goose, which showed to be related to genotypes previously detected in wild felids (André et al., 2010), indicating the occurrence of a probable new species not yet isolated in wild animals from Brazil. Additionally, genotypes related to *E. chaffeensis* and *E. canis* have been described in other wild animals in Brazil, such as deer (Machado et al., 2006; Silveira et al., 2012), wild carnivores (André et al., 2012; de Sousa et al., 2017), rodents (Benevenute et al., 2017; de Sousa et al., 2017; Braga et al., 2018), opossums (Guimarães et al., 2019), coatis (de Sousa et al., 2017), sloths and anteaters (Calchi et al., 2020). The isolation of these agents for Whole Genome Sequencing (WGS) and antigenic characterization is much needed in order to better understand the identity, epidemiology and zoonotic potential of the Anaplasmataceae strains that occur in Brazil.

Twelve birds were positive in the qPCR for *Anaplasma* spp. based on the *groEL* gene. Considering the short fragment obtained (75 bp), the samples were not sequenced since it would be impossible to obtain a robust phylogenetic analysis. Unfortunately, these *groEL* qPCR-positive samples were negative in the cPCR assays, precluding the molecular characterization of these positive samples. It is known that qPCR shows higher sensitivity when compared to cPCR. It is most likely that the number of *Anaplasma* DNA copies present in bird blood samples was very low, hampering the detection by cPCR. *Anaplasma rrs* genotypes related to *A. phagocytophilum* have already been detected in vultures, caracaras, Orinoco geese and dusky-legged guan (*Penelope obscura*) from Brazil, as well as in Eurasian collared doves (*Streptopelia decaocto*) and Eurasian eagle owls (*Bubo bubo*) from China (Yang et al., 2015; Machado et al., 2012; Mongruel et al., 2017; Werther et al., 2017). Vanstreels et al. (2018) detected the so-called '*Candidatus* Anaplasma sphenisci' in penguins from South Africa, which presented inclusions in erythrocytes. Based on *rrs* and *groEL* genes, this probable new species was positioned in a single clade sister to ruminant-associated *Anaplasma* spp. (*A. capra*, *A. marginale*, *A. ovis*). Recently, '*Candidatus* Anaplasma brasiliensis' and '*Candidatus* Anaplasma amazonensis' were detected in Xenarthra mammals from Brazil, highlighting the occurrence of new *Anaplasma* species in wild animals from Brazil (Calchi et al., 2020).

The present study did not identify the presence of *Bartonella* DNA in the sampled birds. Previously, 16S—23S intergenic spacer and *pap-31* genic regions of *B. henselae*, *B. koehlerae* and *B. vinsonii* subsp. *berkhoffii* were detected in Northern mockingbird (*Mimus polyglottos*), Red-winged blackbird (*Agelaius phoeniceus*), Red-bellied woodpecker (*Melanerpes carolinus*), Common loon (*Gavia immer*) and loggerhead sea turtles (*Caretta caretta*) in the USA (Valentine et al., 2007; Mascarelli et al., 2014). A new variant of *Bartonella* spp. was detected in Purple martin (*Progne subis*), Tree swallow (*Tachycineta bicolor*) and Eastern bluebird (*Sialia sialis*) also in the USA, using the *gltA* and *rrs* genes as target genes. In the same study, ectoparasites collected from the sampled birds (*Dermanyssus prognephilus*, *Ceratophyllus idius* and *Protocalliphora sialia*) were also positive for the same variants detected in the birds, which indicates a possible host-vector-*Bartonella* relationship in an avian system (Williams and Dittmar, 2020).

Even though the bird species studied herein do not have migratory habits and usually cover small territories, they may still be able to transport pathogens and possibly transfer them within their habitat. Despite the low prevalence of ectoparasites found in the sampled birds (only one parasitized hawk), the different hunting or feeding strategies of carnivorous birds may influence on the exposure to ectoparasites and, consequently, to vector-borne pathogens. Animals that commonly remain more on the ground for hunting and feeding, such as caracaras,

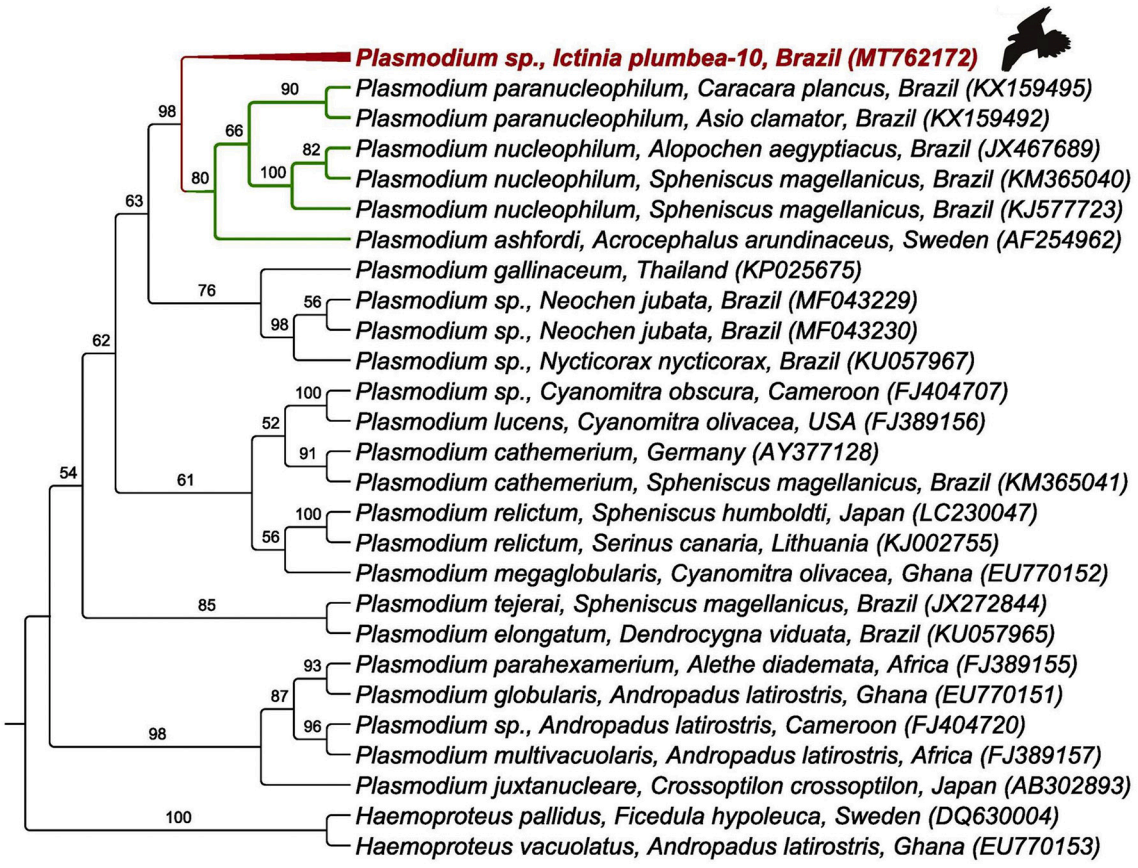

Fig. 4. Phylogenetic analysis of *Plasmodium cytB* sequences based on the topology Maximum Likelihood, with *TIM2* + *I* + *G* as evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1000 replicates *Haemoproteus pallidus and H. vacuolatus* were used as an external group.

vultures and seriemas, might be more susceptible to tick infestation (Teixeira et al., 2008). On the other hand, owls and hawks feed on insects, small mammals and even some bird species. It is worth pointing out that small wild rodents are incriminated as reservoirs for several pathogens, including Anaplasmataceae and Bartonellaceae (Wen et al., 2003; Cao et al., 2006; Kim et al., 2006; Buffet et al., 2013). Indeed, Anaplasmataceae agents have already been detected in small wild rodents (Wolf et al., 2016; Benevenute et al., 2017; de Sousa et al., 2017) in Brazil. Therefore, direct contact with the prey or with their ectoparasites may represent a source of Anaplasmataceae infection for carnivorous birds.

Four birds were positive in PCR assays for hemosporidians based on the *cyt B* gene. Three Tropical Screech-Owl and one Burrowing owl were positive for *Haemoproteus* spp. and *Plasmodium* spp., respectively. The phylogenetic analysis for *Haemoproteus* spp. positioned the obtained sequences in a clade composed of *H. syrnii* and *Haemoproteus* sp. detected in bird species of the order Strigiformes. The obtained *Plasmodium* sequence was positioned closely to *P. paranucleophilum* and *P. nucleophilum* detected in birds from Brazil, in the same clade containing *P. ashfordi* previously detected in Passeriformes. The high genetic variability of these parasites associated to the use of short conserved gene fragments (480 bp) contribute to the low robustness of the bootstrap values, precluding a reliable phylogenetic positioning. So far, 160 and 55 species of *Haemoproteus* spp. and *Plasmodium* spp. have been detected in birds, respectively (Fecchio et al., 2020). In addition, *Haemoproteus* spp. are more host-specific, at least at order level, when compared to *Plasmodium* spp. This may explain why the sequences obtained from owls in this study were positioned apart, in a clade with other sequences detected in owls, indicating a likely new variant circulating in these animals (Fecchio et al., 2020). A similar result was found in the study performed by Krone et al. (2008), in which the *Haemoproteus* sequence detected in an owl was positioned in a separate clade from the clade comprising sequences detected in Passeriformes.

Several studies on hemosporidian diversity in birds have been conducted in Brazil (Belo et al., 2011; Fecchio et al., 2013; Lacorte et al., 2013; Chagas et al., 2017; Fecchio et al., 2017; Ferreira Junior et al., 2017; Chahad-Ehlers et al., 2018; Taunde et al., 2019; Dutra et al., 2019; de Oliveira et al., 2019). In carnivorous birds, *Plasmodium* sp. phylogenetically related to *P. gallinaceum* was detected in Great Black-Hawk (*Buteogallus urubitinga*) and King vulture (*Sarcoramphus papa*) maintained in captivity in a zoo in São Paulo (Chagas et al., 2017). *Plasmodium paranucleophilum* were detected in Strigiformes, Accipitriformes and Falconiformes birds maintained in captivity in Rio de Janeiro (Tostes et al., 2017). In the present study, hemosporidian-positive birds were also in captivity, which indicates that stressful conditions for captive birds (change of environment and sharing of the same space with other birds) as well as the presence of infected birds and vectors in the same site may lead to exacerbation of the disease. Santos et al. (2008) also found high occurrence of hemosporidians in captive Strigiformes birds in Portugal. Although haemosporidian infections have been associated with low pathogenicity, reduced productivity and even high mortality has been reported. Probably, the most significant impact on host birds occurs in the long term on the reproductive system, leading to a decrease in population (ELAHI et al., 2014).

Four birds sampled in this study (3.3%) presented co-positivity with *Ehrlichia* sp. (nPCR) and *Anaplasma* sp. (qPCR). One bird (0.82%) showed to be seropositive to *E. chaffeensis* (IFAT) and contained *Haemoproteus* sp. DNA (nPCR). Strinkling, all the birds that presented co-positivity for *Ehrlichia* and hemosporidians were owls maintained in captivity, in which stress factors might have account to the recrudescence of infections. Interestingly, Werther et al. (2017) also found co-positivity for different agents in the sampled Orinoco goose (3.22%

[2/62] *Babesia* sp. +*Ehrlichia* sp.; 11.29% [7/62] *Haemoproteus* sp. + *Ehrlichia* sp.; 1.61% [1/62] *Plasmodium* sp. + *Ehrlichia* sp. and 1.61% *Anaplasma* sp. + *Ehrlichia* sp.). It is worth noting that the blood samples from Orinoco geese were collected during the mating period, which also might have favored the recrudescence of such agents, due to the distress period. The impact of these co-infections on bird health is still unknown and further investigations are needed.

5. Conclusion

The present study showed the occurrence of *Ehrlichia* spp., *Anaplasma* spp., *Plasmodium* spp. and *Haemoproteus* spp. in carnivorous birds sampled in southeastern Brazil. While owls maintained in captivity represented the majority of the animals that were positive for *Ehrlichia* spp. and *Haemoproteus* spp., free-living carnivorous birds represented the majority of the sampled animals positive for *Anaplasma* spp. Co-infections by Anaplasmataceae agents and hemosporidians should be monitored in captive owls.

Ethical statement

This work was approved by govermental license Instituto Chico Mendes para Conservação da Biodiversidade (ICMBio - SISBIO number 31743–1) and Animal Use Ethics Comitee (Comissão de Ética no Uso de Animais - CEUA - FCAV/UNESP number 4153/12).

Declaration of Competing Interest

None.

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