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PROTOZOOLOGY - ORIGINAL PAPER

An *Hepatozoon americanum*-like protozoan in crab-eating (*Cerdocyon thous*) and grey pampean (*Lycalopex gymnocercus*) foxes from Uruguay

Luis Carvalho[1,2] · María L. Félix[1] · Valentin Bazzano[1] · Anthony da Costa[1] · María T. Armúa-Fernández[1] · Sebastián Muñoz-Leal[3] · José Manuel Venzal[1]

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Abstract

In South America, apicomplexan parasites of the genus *Hepatozoon* have been sporadically detected in mammals. Previous studies in wild canids from Brazil and Argentina demonstrated infections by species genetically related to *Hepatozoon americanum*. The aim of the present work was to detect the presence of *Hepatozoon* in road-killed foxes encountered in Uruguayan highways. Blood samples from 45 crab-eating (*Cerdocyon thous*) and 32 grey pampean (*Lycalopex gymnocercus*) foxes were analyzed by PCR for *Hepatozoon* 18S rRNA gene. Eight foxes (10.4%) were found to be infected with an *H. americanum*-like protozoan, an *Hepatozoon* closely related to *H. americanum*. Bayesian and maximum-likelihood phylogenetic analyses revealed that the sequences obtained in this study cluster with *H. americanum* from the United States, and with an *H. americanum*-like species from dog and foxes from Brazil and Argentina. In the Unites States, *H. americanum* causes severe disease in dogs. In addition to this, an increasing habitat overlap between dogs and foxes makes the presence of *H. americanum*-like protozoan in foxes acquires veterinary relevance. This work represents the first report of *L. gymnocercus* infected with an *H. americanum*-like protozoan, and of wild canids infected with *Hepatozoon* in Uruguay.

Keywords *Hepatozoon* · Molecular detection · *Cerdocyon thous* · *Lycalopex gymnocercus* · Uruguay

Introduction

Hepatozoonosis is a parasitic infection caused by apicomplexan protozoans of the genus *Hepatozoon* (family Hepatozoidae). More than 340 species of this genus parasitize a wide range of vertebrate hosts, including amphibians, reptiles, birds, and mammals (Baneth 2011; Smith 1996). The life cycle of *Hepatozoon* spp. is heteroxenous. The sporogonic development and oocyst formation occur in a hematophagous definitive invertebrate host, whereas merogonic and gamontogonic development occur in intermediate vertebrate hosts. Ixodid and Argasid ticks, mites, mosquitoes, sandflies, flies, fleas, sucking lice, and triatomid bugs have been described as vectors and definitive hosts (Smith 1996). Vertebrates usually become infected through the ingestion of an infected arthropod, but transplacental transmission and predation are also described as alternative pathways of transmission (Baneth et al. 2013; Johnson et al. 2009).

Two species, *Hepatozoon canis* and *Hepatozoon americanum*, have been described infecting domestic and wild canids. *Hepatozoon canis* was first described in dogs from India in 1905, and *Rhipicephalus sanguineus* was identified as the main vector (Christophers 1907; James 1905). *Hepatozoon canis* is widespread in tropical, sub-tropical, and temperate climate regions of the globe (Baneth et al. 2003; Ewing et al. 2000). *Hepatozoon americanum* was initially found in southern United States and is transmitted by the Gulf coast tick *Amblyomma maculatum* (Ewing et al. 2002; Vincent-Johnson et al. 1997). *Hepatozoon canis* infection is often subclinical but may vary from asymptomatic to severe. Potentially fatal disease develops in dogs with high parasitemia, causing extreme lethargy, cachexia, and

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✉ Luis Carvalho
luis.carvalho@agresearch.co.nz

[1] Laboratorio de Vectores Y Enfermedades Transmitidas, Departamento de Ciencias Biológicas, CENUR Litoral Norte, Universidad de La República, Rivera 1350, CP 50000 Salto, Uruguay

[2] Present Address: AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand

[3] Departamento de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad de Concepción, Av. Vicente Méndez 595, Casilla 537, Chillán, Ñuble, Chile

anemia (Baneth et al. 2003). However, *H. americanum* infection causes a severe disease in dogs, leading to debilitation and death. The clinical signs are characterized by muscular pain induced by myositis, severe lameness, and subsequent atrophy (Baneth 2011; Vincent-Johnson et al. 1997). In wild animals, *Hepatozoon* infections are usually subclinical (André et al. 2010; East et al. 2008; Kocan et al. 2000; Metzger et al. 2008). However, coyotes (*Canis latrans*) experimentally infected with *H. americanum* developed disease with lesions like those seen in dogs (Garrett et al. 2005; Kocan et al. 2000). Moreover, infection by protozoans of the genus was associated with mortality in young spotted hyena (*Crocuta crocuta*) (East et al. 2008).

The presence of *Hepatozoon* spp. in wild canids has been reported worldwide. For instance, *H. canis* was found in red foxes (*Vulpes vulpes*) from Spain, Germany, Portugal, Hungary, and Israel (Cardoso et al. 2014; Criado-Fornelio et al. 2003; Criado-Fornelio et al. 2006; Farkas et al. 2014; Margalit Levi et al. 2018; Najm et al. 2014), in golden jackals (*Canis aureus*) from Hungary and Israel (Farkas et al. 2014; Margalit Levi et al. 2018), and in crab-eating foxes (*Cerdocyon thous*), grey pampean foxes (*Lycalopex gymnocercus*), and maned wolves (*Chrysocyon brachyurus*) from Brazil (Arrais et al. 2021; Criado-Fornelio et al. 2006). On the other hand, while *H. americanum* was reported in coyotes from the USA (Mercer et al. 1988), an *H. americanum*-like protozoan, an *Hepatozoon* closely related to *H. americanum*, was detected in the South American grey fox (*Lycalopex griseus*) from Argentina, and *C. thous* and *C. brachyurus* from Brazil (Almeida et al. 2013; Andre et al. 2010; Arrais et al. 2021; Criado-Fornelio et al. 2006; de Sousa et al. 2017; Millan et al. 2019). Furthermore, *Hepatozoon* spp. were also found in hyenas from Tanzania (East et al. 2008), African wild dogs (*Lycaon pictus*) from South Africa (Matjila et al. 2008), and in bush dogs (*Speothos venaticus*), *C. brachyurus*, and a fox of undetermined identity from Brazil (Andre et al. 2010; Perles et al. 2019). Moreover, an *Hepatozoon* sp. closely related to *Hepatozoon felis* was reported in *L. gymnocercus* and *L. griseus* from Argentina (Giannitti et al. 2012; Millan et al. 2019).

In Uruguay, although *Hepatozoon* spp. was previously confirmed in snakes, *Philodryas patagoniensis*, and in domestic cats (Bazzano et al. 2020, 2021), the occurrence of this protozoan in native mammals is still obscure. The aim of this work was to assess the presence of *Hepatozoon* spp. in wild canids from Uruguay.

Materials and methods

Sample collection

Between May 2015 and July 2020, we collected blood samples and ticks from fresh road-killed foxes encountered along Uruguayan highways. Blood samples were collected in EDTA-tubes, identified, and kept with cool packs until arrival to the laboratory. Ticks retrieved from foxes were stored in tubes with 95% ethanol and identified using keys provided by Nava et al. (2017). At the laboratory, samples were stored at − 20 °C until DNA extraction.

DNA extraction and PCR amplification

For molecular analysis, DNA was extracted from 200 µl of whole blood using the commercial kit PureLink™ Genomic DNA Mini Kit (Invitrogen, Germany), following the manufacturer's instructions. DNA of *Hepatozoon* was amplified using two conventional PCR protocols. First, we used primers HEMO1 (5′-TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG-3′) and HEMO2 (5′-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3′) that amplify a region of approximately 900 base pairs (bp) of the 18S rRNA gene (Perkins and Keller 2001). PCR thermal conditions followed Harris et al. (2011). A second PCR targeting approximately 670 bp of the same gene was performed using primers HEP1mod (5′-CGC GAA ATT ACC CAA TTC TA-3′) and HEP4 (5′-TAA GGT GCT GAA GGA GTC GTT TAT-3′) as described by Spolidorio et al. (2009). Both pairs of primers were selected because the retrieved sequences overlap into a fragment of near 1300 bp. DNA of *Hepatozoon* sp. obtained from *P. patagoniensis* and DNAse-free water were included in each reaction as positive and negative controls, respectively. PCR products were visualized under UV transillumination in 1.5% agarose gels stained with GoodView™ Nucleic Acid Stain (Beijing SBS Genetech Co., LTd). Positive PCR products were purified using the PureLink™ Quick PCR Purification kit (Invitrogen, Germany) and sent to a commercial sequencing company (Macrogen Inc., Seoul, Korea).

Analyses of sequences and phylogenies

Each sequence was carefully checked, and manual corrections were done, when necessary, with Geneious (Kearse et al. 2012). The two overlapping fragments of each sample were assembled into consensus sequences. Nucleotide identities of obtained sequences were calculated using the Sequence Identity and Similarity (SIAS) calculator (http://imed.med.ucm.es/Tools/sias.html). Alignments with herein obtained sequences and 27 homologues retrieved from GenBank were performed with MUSCLE algorithm (Edgar 2004) implemented in MEGA 7 (Kumar et al. 2016).

Two phylogenetic trees were constructed. A maximum-likelihood (ML) inference was employed to get one tree using PhyML (Guindon and Gascuel 2003). Best fitting evolutionary model for 18S rRNA gene was calculated with MEGA 7 and the Tamura 3 parameters with gamma distribution selected. The support of the internal branching was assessed using 1000 bootstrap replicates. A second phylogeny was inferred using Bayesian statistics (Ronquist et al.

2012), using the general time reversible model. Four independent Markov chains run for 1,000,000 metropolis-couples MCMC generations, begun with random seeds, and ran four times sampling a tree every 100th generations. The first 25% of the trees were considered as burn-in. The remaining trees were employed to calculate the Bayesian posterior probability. *Adelina grylli* (DQ096836) rooted each tree.

Results

Overall, we collected blood samples from 45 *C. thous* and 32 *L. gymnocercus* in roads from 14 departments of Uruguay. Although eight *Amblyomma tigrinum* (four females and four males) and 54 *Amblyomma aureolatum* (eight females and 46 males) were collected from nine foxes (seven *C. thous* and two *L. gymnocercus*) (Supplementary Table 1), no PCR analyses were performed with the specimens.

Out of 77 tested blood samples, eight (10.4%) were positive for *Hepatozoon* 18S rRNA gene PCR. Sequences were obtained from six (6/45, 13.3%) *C. thous* (four females and two males) and two (2/32, 6.25%) *L. gymnocercus* (2 males). Four of these samples, F2, F5, F7, and F72, were amplified with one primer pair (HEP1mod, HEP4) obtaining fragments of 618, 630, 642, and 659 bp, respectively. The remaining positive samples, F18, F33, F52, and F59, were amplified by both sets of primers, whose amplified fragments overlapped, obtaining larger sequences (1322, 1322,

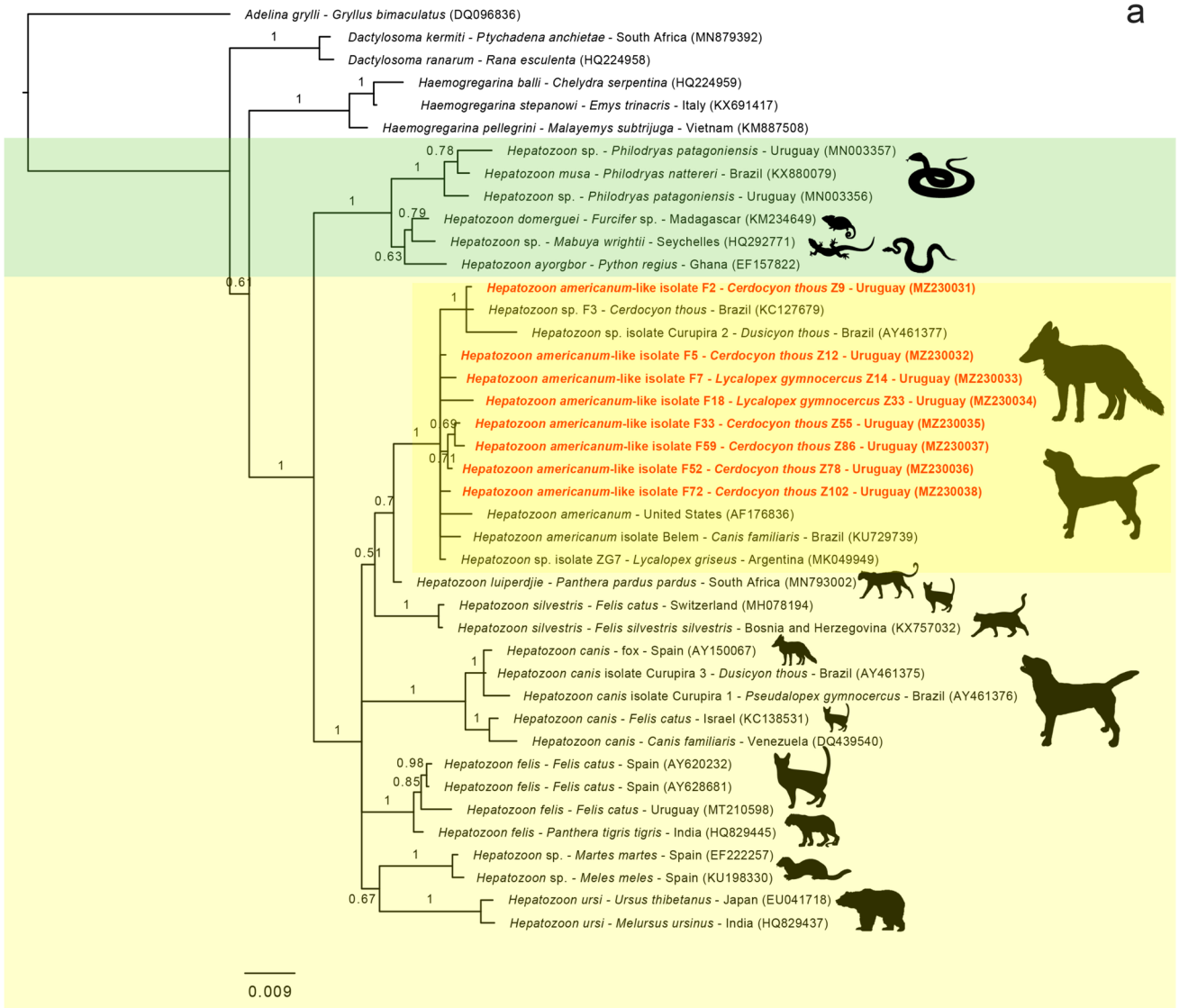

Fig. 1 Phylogenetic trees for a subset of 18S rDNA sequences of *Hepatozoon* spp. **a** Bayesian phylogeny. Bayesian posterior probabilities are indicated upon or arrowing each branch. **b** Maximum-likelihood tree. Numbers represent bootstrap supports. Sequences generated in this study are annotated in red bold letters. The clade of *Hepatozoon americanum* is highlighted within yellow box. GenBank accession numbers are indicated in brackets. Scale bars indicate the number of substitutions per nucleotide positions, respectively

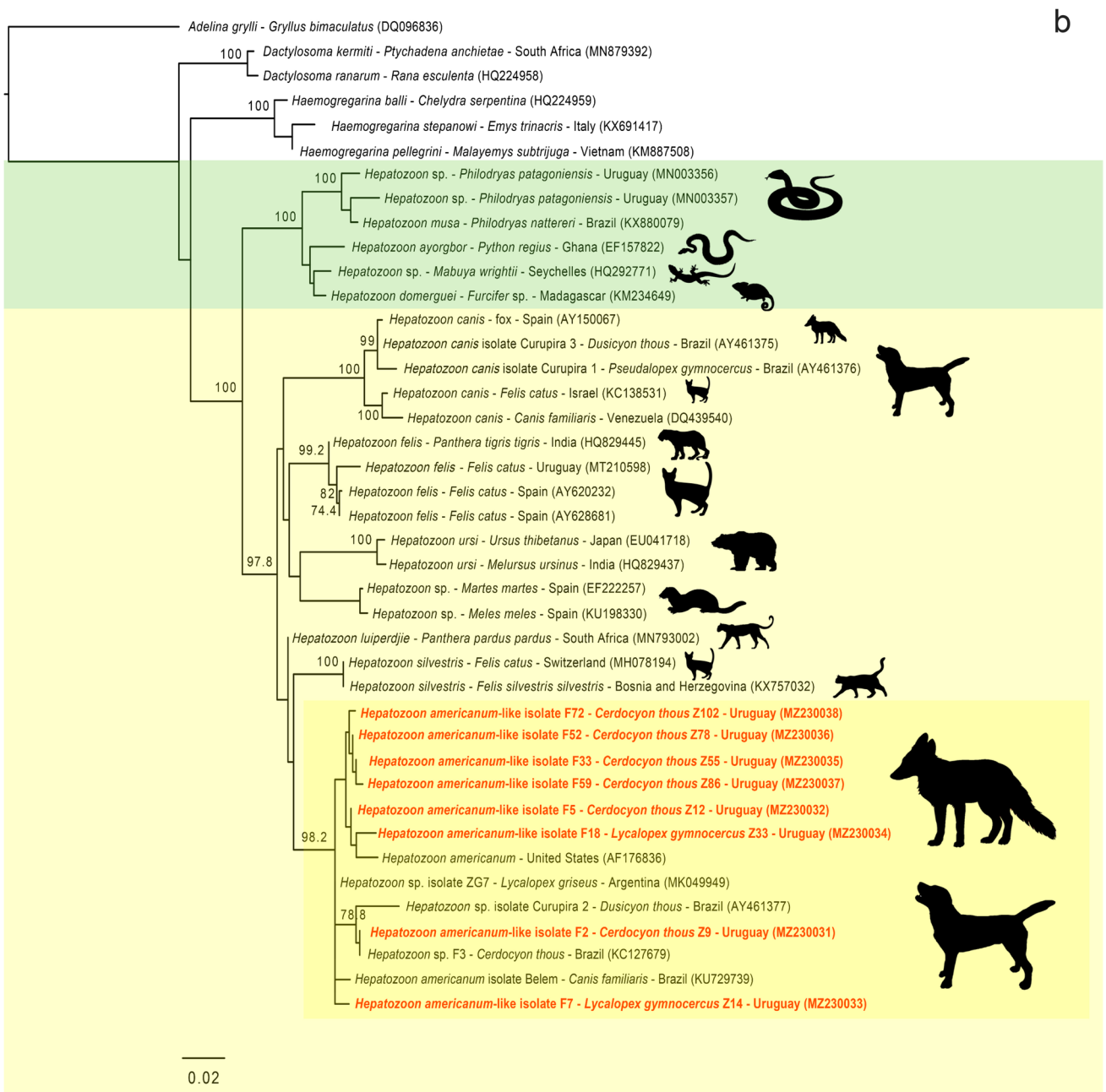

Fig. 1 (continued)

1318, and 1322 bp, respectively). The phylogenetic analyses using both ML and Bayesian methods showed the same topology for main branches (Fig. 1a, b). With high support values, *Hepatozoon* sequences obtained from *C. thous* and *L. gymnocercus* from Uruguay clustered with *H. americanum* from USA (AF176836), and with *H. americanum*-like species from dog and foxes from Brazil (KU729739, AY461377, KC127679) and Argentina (MK049949) (Fig. 1a, b). As 18S rRNA gene is conserved, we opted to retain the nomenclature *H. americanum*-like for the sequences retrieved in this study (GenBank accession numbers: MZ230031-038). Due to length differences between the sequences we obtained, pairwise comparisons were done considering a 571 bp overlapping fragment and showed 97.32–99.82% identity between them. Moreover, 96.63–100% of nucleotide identity was observed in comparisons with *H. americanum* and *H. americanum-like* included in the phylogenetic trees (Supplementary Table 2).

Discussion

The eight *Hepatozoon* spp. 18S rRNA gene partial sequences amplified in samples from *C. thous* and *L. gymnocercus* are closely related with *H. americanum* (Mathew et al. 2000), and *H. americanum*-like previously reported in Argentina and Brazil (Almeida et al. 2013; Criado-Fornelio et al. 2006; Gomes et al. 2016; Millan et al. 2019). Maximum-likelihood and Bayesian phylogenetic analyses yielded a highly similar tree topology, and a high support value suggests that sequences related to *H. americanum* conform a monophyletic group. Within this clade, the sample F2 grouped with *Hepatozoon* sp. F3 (KC127679) and *Hepatozoon* sp. Curupira 2 (AY461377) detected in *C. thous* from Brazil. Further phylogenetic analysis using less conserved genes will be necessary to clarify if *H. americanum* is one species or a species complex distributed along the American continent.

The prevalence of *Hepatozoon* sp. infection in wild canids from South America varies between studies. The 10.4% prevalence of *Hepatozoon* spp. infection found in foxes from Uruguay is much more lower than the 77.6% reported by Criado-Fornelio et al. (2006) in foxes from the State of Rio Grande do Sul (Southern Brazil). However, we found *C. thous* and *L. gymnocercus* infected with *H. americanum*-like protozoan, whereas Criado-Fornelio et al. (2006) detected an *H. americanum*-like species only in *C. thous* (7.6% prevalence), and both *C. thous* and *L. gymnocercus* infected with *H. canis* (69.2% prevalence). Our study represents the first report of *L. gymnocercus* infected with an *H. americanum*-like protozoan. Another study with wild canids from Brazil reported *C. thous* infected with *H. americanum*-like protozoan (2.5% prevalence) and *C. brachyurus* with *Hepatozoon* sp. closely related with *H. canis* genotype "Spain 1" (5% prevalence) (Andre et al. 2010). Almeida et al. (2013) reported 28 *C. thous* infected with *H. americanum*-like protozoan (48.3% prevalence) and 1.7% infected by an organism closely related to reptile associated *Hepatozoon*. Furthermore, *Hepatozoon* sp. closely related with *Hepatozoon felis* was found in a *L. gymnocercus* from the north-central Patagonia region of Argentina (Giannitti et al. 2012).

Although we found ticks only in 10 foxes, it is noteworthy that we worked with dead animals encountered in different levels of decay, so it is also possible that more animals were infested at the moment of death. The tick species collected from foxes in this study (i.e., *A. aureolatum* and *A. tigrinum*) have been reported parasitizing domestic dogs in Uruguay (Lado et al. 2014; Martins et al. 2014; Venzal et al. 2003). The presence of *A. aureolatum* in foxes was previously reported by Criado-Fornelio et al. (2006), Nava et al. (2017), and Labruna et al. (2005). While herein collected ticks were not analyzed, previous work in South America have suggested *A. tigrinum* as putative vector of *Hepatozoon* (Arrais et al. 2021; Giannitti et al. 2012; Millan et al. 2019).

The expansion of human activities has forced the coexistence of domestic and wild canids into the same habitat. Although *H. americanum* infection is of minor veterinary concern for wild canids, habitat overlap between dogs and foxes sharing the same tick species in Uruguay makes the presence of *H. americanum*-like protozoan in foxes a probable threat to domestic canids. This work represents the first report of wild canids infected with *Hepatozoon* in Uruguay and reaffirms the importance of further studies to elucidate the vectors of *Hepatozoon* spp. in South America.

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Author contribution Luis A. Carvalho: conceptualization, data curation, methodology, formal analysis, investigation, writing — original draft, and writing — review and editing. María L. Félix: formal analysis, investigation, methodology, and writing — review and editing. Valentin Bazzano: methodology, and review and editing. Anthony da Costa: methodology. Sebastián Muñoz-Leal: conceptualization, formal analysis, methodology, writing — original draft, and writing — review and editing. María T. Armúa-Fernández: conceptualization, data curation, methodology, formal analysis, and investigation. José M. Venzal: conceptualization, data curation, methodology, resources, formal analysis, investigation, project administration, writing — original draft, and writing — review and editing. All authors read and approved the final manuscript.

Declarations

Competing interests The authors declare no competing interests.

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